Frontiers in Ecology and the Environment

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Front Ecol Environ 2010; doi:10.1890/100053

Alteration of streamflow magnitudes and potential ecological consequences: a multiregional assessment

Daren M Carlisle[1*], David M Wolock[2], and Michael R Meador[1]

Human impacts on watershed hydrology are widespread in the US, but the prevalence and severity of streamflow alteration and its potential ecological consequences have not been quantified on a national scale. We assessed streamflow alteration at 2888 streamflow monitoring sites throughout the conterminous US. The magnitudes of mean annual (1980–2007) minimum and maximum streamflows were found to have been altered in 86% of assessed streams. The occurrence, type, and severity of streamflow alteration differed markedly between arid and wet climates. Biological assessments conducted on a subset of these streams showed that, relative to eight chemical and physical covariates, diminished flow magnitudes were the primary predictors of biological integrity for fish and macroinvertebrate communities. In addition, the likelihood of biological impairment doubled with increasing severity of diminished streamflows. Among streams with diminished flow magnitudes, increasingly common fish and macroinvertebrate taxa possessed traits characteristic of lake or pond habitats, including a preference for fine-grained substrates and slow-moving currents, as well as the ability to temporarily leave the aquatic environment.

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Human influence on watershed hydrology is extensive and may be a primary cause of ecological impairment in river and stream ecosystems. In the US, natural streamflow regimes are influenced by dams and diversion structures (Graf 1999; Poff *et al.* 2007), land uses that alter runoff to stream channels, groundwater withdrawals from contributing aquifers, and interbasin water transfers (Jackson *et al.* 2001). Because the natural timing, magnitude, and frequency of streamflows dictate the evolutionary adaptations of many river biota (Bunn and Arthington 2002) and control many physical and chemical processes (Poff *et al.* 2010), anthropogenic alterations of streamflows may have profound effects on ecosystem structure and function.

Major questions about streamflow alteration and its ecological consequences remain unresolved. First, although streamflow is continuously monitored at thousands of sites across the conterminous US, a basic accounting of the prevalence and severity of streamflow alteration is lacking because there has not been a systematic national assessment of these sites. Second, sound management requires an understanding of the relationship between ecological integrity and streamflow alteration, yet few quantitative relationships have been reported at spatial scales beyond specific stream segments (Poff *et al.* 2003; Arthington *et al.* 2006). A key hindrance to addressing these questions is the inconsistency with which streamflow alteration and various biological responses have been quantified (Poff and Zimmerman 2010).

[1]*National Water-Quality Assessment Program, US Geological Survey, Reston, VA* [*]*(dcarlisle@usgs.gov);* [2]*US Geological Survey, Lawrence, KS*

Using standardized indicators, we assessed streamflow magnitudes and associated biological communities across the conterminous US. We focused on streamflow magnitudes because this dimension of the flow regime is frequently linked to ecological impairment (reviewed by Poff and Zimmerman 2010) and has clear implications for water management (Postel and Richter 2003). Our first objective was to assess whether observed magnitudes of annual minimum and maximum flows differed from reference (ie estimated least disturbed) conditions at 2888 streamflow monitoring sites. Our second objective was to determine whether the integrity of two aquatic communities (ie fish and macroinvertebrates) was associated with the type and severity of streamflow alteration at a subset (~ 250) of these sites. At each monitoring site, alterations – in either streamflow or biological communities – were quantified as the ratio of observed conditions to expected reference conditions. This approach provides an intuitive indicator of the degree to which a stream exhibits the hydrological and biological characteristics that should naturally occur; data can therefore be aggregated and interpreted across diverse regions because they are standardized by each site's natural potential.

Methods

We quantified streamflow alteration as the ratio of observed magnitudes to those expected under reference conditions. We first identified a set of 1059 streamflow monitoring sites with perennial flows and with reference-quality (ie least disturbed) basins across the conterminous US (Carlisle *et al.* 2010; Falcone *et al.* 2010). We

developed random forest (Cutler *et al.* 2007) models that use 93 geospatial attributes (eg climate, topography, soils, geology) for a given watershed to predict its observed mean annual minimum (7-day moving average) flow and, separately, mean annual maximum (daily average) flow (Carlisle *et al.* 2010; WebPanel 1). These models were used to predict expected magnitudes at 2888 non-reference streamflow monitoring sites based on the geospatial attributes of their respective watersheds. We quantified streamflow alteration at each assessed site as the ratio of observed mean annual (1980–2007) minimum and maximum magnitudes to expected mean annual magnitudes. The ratio can be either < 1 or > 1, indicating that observed magnitudes are either diminished or inflated, respectively, relative to their respective expected reference conditions. We summarized streamflow alteration across the US by tabulating the number of sites that were inflated (ie observed/expected [O/E] values > 90% of those from reference sites), diminished (ie O/E values < 90% of those from reference sites), or unaltered (ie O/E values within the above limits) (WebTable 1). In addition, the severity of streamflow alteration was summarized by tabulating the number of sites with O/E values within quartiles > 1 or < 1.

Likewise, biological integrity was quantified as the ratio of observed community attributes to those expected under reference conditions (O/E value, sensu Hawkins 2006). Selected community-level attributes varied slightly because of inherent differences in aquatic communities. For macroinvertebrates nationwide and for fish in the eastern US, the O/E value was the fraction of the set of taxa (in most cases, genera or species) expected at a site that was actually observed there. Estimates of expected community attributes were generated from regional multivariate predictive models, which have previously been described and validated (Wright 2000; WebPanel 1). The O/E value of fish communities in the western US was derived from an index of biological integrity (ie based on observed attributes) normalized to expectations from regional reference sites (Meador *et al.* 2008). Our final definition of biological integrity was binary, in which the aquatic community at each site was considered "impaired" if its O/E value was less than that of 90% of reference sites within the same region, or "unimpaired" if its O/E value did not meet this condition (WebPanel 1).

Three hypotheses about the relationship between biological integrity and streamflow alteration were evaluated. First, we hypothesized that, relative to eight covariates, streamflow alteration would be a primary predictor of biological integrity (ie impaired versus unimpaired). These covariates included water temperature, specific conductance, pH, total nitrogen, total phosphorus, channel gradient, agricultural land cover, and urban land cover of the riparian buffer (WebPanel 1). We performed classification tree analysis (De'ath and Fabricius 2000) with all covariates and the O/E indices for minimum and maximum flow as predictors. Trees were grown to maximum size and then pruned to minimize tree complexity and classification error based on K-fold cross-validation (where K=10 subsamples of the original observations; Venables and Ripley 2002). Our second hypothesis was that the likelihood of biological impairment would increase with the severity of streamflow alteration. For each community, the proportion of impaired sites was tabulated within categories of streamflow alteration severity, which were defined by quartiles of O/E either > 1 (ie inflated) or < 1 (ie diminished). The Kruskal-Wallis test was applied to determine whether covariates varied significantly among these same categories. Few of the sites with biological data experienced inflated maximum flows, so this dimension of streamflow alteration was not considered in our analysis. Our third hypothesis was that functional traits of macroinvertebrate and fish taxa would indicate the presence of altered streamflow magnitudes. Sites with diminished (minimum and maximum) and inflated (minimum only) magnitudes were identified based on the distribution of O/E values at reference sites as described above (WebTable 1). We used predictions of expected community composition to identify taxa at each site that (1) were expected but not observed (hereafter "decreaser taxa") and (2) were observed but not expected (hereafter "increaser taxa"). In the absence of pre- and post-disturbance data, these designations approximate taxa that have been lost or gained as a result of all anthropogenic influences at each site (Knapp *et al.* 2005). We aggregated lists of decreaser and increaser taxa across sites within each class of streamflow alteration (n = 119, 84, and 110 for inflated minimum, diminished minimum, and diminished maximum, respectively) and evaluated (using Fisher's exact test) whether the two sets of taxa differed in the frequencies of functional traits associated with hydrological attributes, including reproductive strategy, mode of mobility, and geomorphic habitat and substrate preferences (WebPanel 1).

Results

Streamflow magnitudes were altered in most (86%) of the assessed streams (Figure 1a and b). Minimum flows were the most frequently altered, being inflated or diminished in 74% of streams. Maximum flows were altered in 54% of streams and diminished in most cases. The type and severity of streamflow alteration were associated with climate (Figure 1b). In arid climates, minimum and maximum flows were severely diminished, being less than half of expected magnitudes in most (~ 70%) monitored streams. Maximum flow magnitudes in wet climates were also commonly diminished, being less than three-fourths of expected magnitudes in most (> 60%) monitored streams. In contrast, minimum flows in wet climates were commonly inflated, being > 25% higher than expected magnitudes in about half of monitored sites.

Streamflow alteration was the primary predictor of biological integrity for both communities (Figure 2). Impaired fish communities (70% correct classification) were associated solely with streamflow alteration and prominent at sites (1) with diminished maximum or minimum flows or

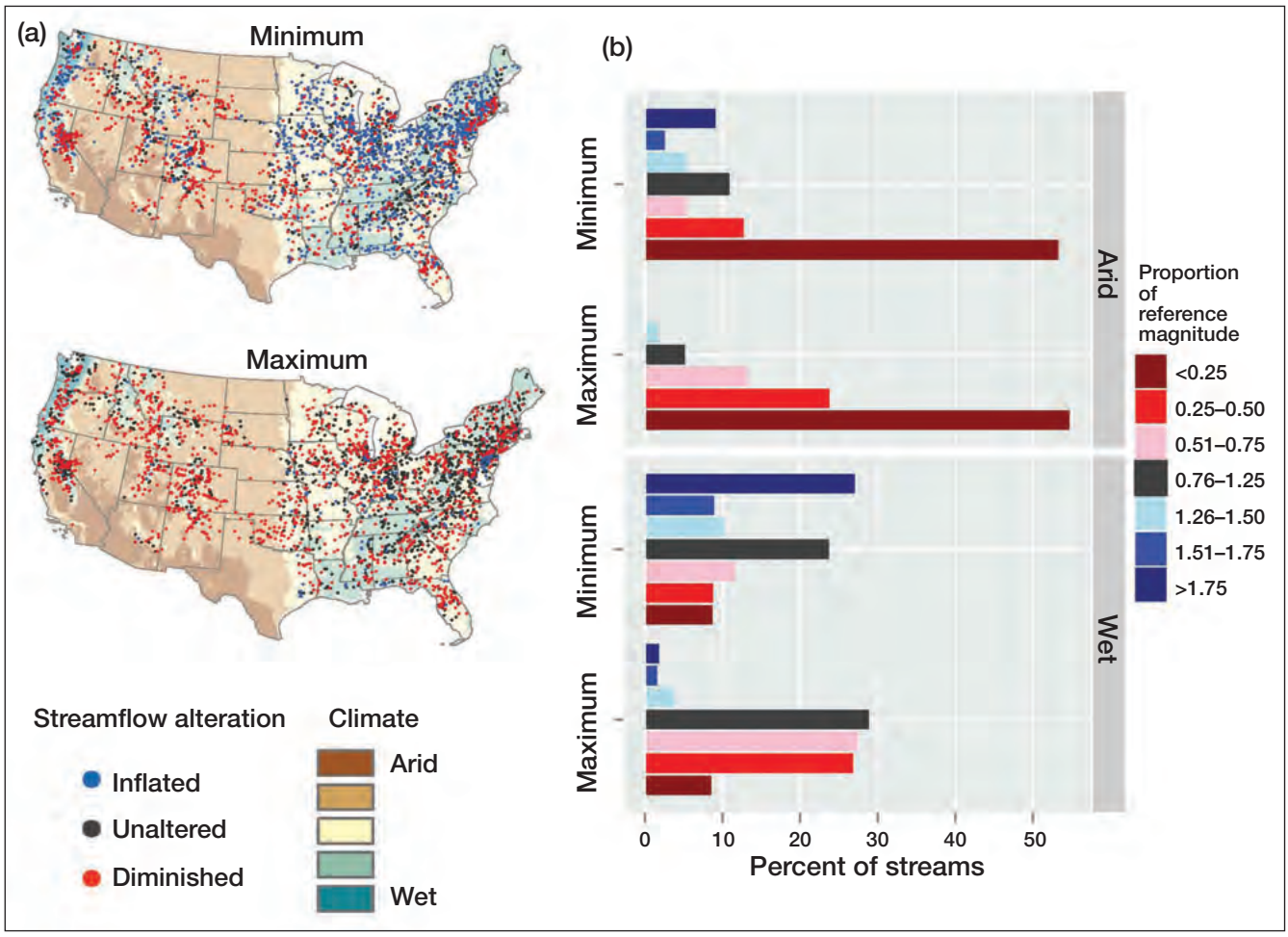

Figure 1. *Alteration of minimum and maximum annual streamflow magnitudes, (a) at 2888 sites monitored from 1980–2007. "Inflated" condition indicates that observed average magnitudes exceeded expected reference magnitudes; "diminished" condition indicates that observed average magnitudes were less than expected reference magnitudes. (b) Severity of streamflow alteration, as a proportion of expected reference magnitude, within two classes of climatic conditions, defined by the difference between mean annual precipitation and potential evapotranspiration (> 0 = "Wet", < 0 = "Arid").*

(2) with inflated minimum flows but unaltered maximum flows. Impaired macroinvertebrate communities (74% correct classification) were associated with diminished maximum flows, but this response was conditional on covariates such as stream gradient and land cover.

Biological impairment was associated with the severity of streamflow alteration (Figure 3). Increasing severity of diminished minimum and maximum flows was associated with a twofold increase in the likelihood that fish and macroinvertebrate communities were impaired. Two covariates (total phosphorus and specific conductance) were also associated with increased severity of diminished minimum and maximum flows, and sites in the highest severity classes were often diminished for both minimum and maximum flows. Severity of inflated minimum flow was less strongly associated with biological impairment than diminished streamflows, and appeared to be confounded with several covariates.

Differences between increaser and decreaser taxa suggested apparent shifts in functional traits of fish and macroinvertebrate taxa at sites with altered streamflows (Table 1). Fish reproduction generally shifted from simple nesting to nest-guarding or broadcast-spawning strategies in streams with either form of flow alteration. In streams with diminished minimum or maximum flows, active swimmers replaced benthic-oriented and streamlined fish species, whereas macroinvertebrate taxa with the ability to temporarily leave the aquatic environment or move quickly within it (eg strong swimmers, fast crawlers) replaced taxa lacking these traits; moreover, pool (ie relatively slow currents)-loving macroinvertebrate taxa that prefer fine substrates replaced riffle (ie turbulent flowing)-loving macroinvertebrate taxa that prefer coarse substrates. In streams with inflated minimum flows, there was also an apparent increase in macroinvertebrate taxa that prefer erosional (ie relatively high current velocity) habitats.

Discussion

Understanding the relationship between biological integrity and streamflow alteration is critical if society is to make decisions about tradeoffs between human and

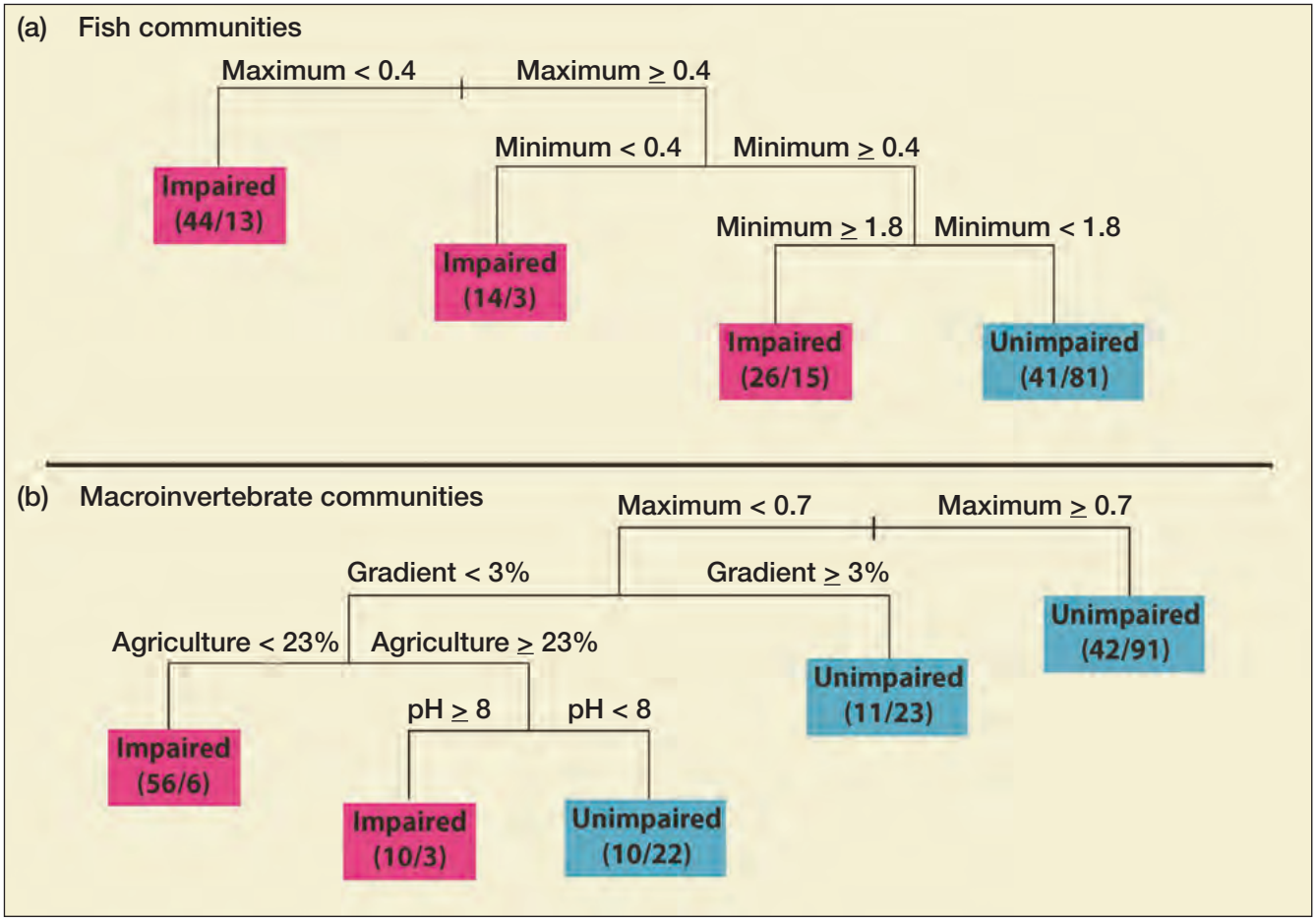

Figure 2. *Classification trees predicting impairment of (a) fish and (b) macroinvertebrate communities at 237 and 274 stream sites, respectively, through measures of streamflow alteration and eight covariates. Each split in the tree is annotated with the values of the primary predictor that defines each branch; for example, fish communities were impaired at sites where observed magnitudes of maximum flows were < 0.4 of expected natural magnitudes. Streamflow alteration is expressed as the proportion of expected reference magnitude. Agriculture is expressed as percent of riparian area within a 100-m buffer. Predicted class ("Impaired/Unimpaired") frequencies are given for each terminal node.*

ecosystem requirements for water (Postel and Richter 2003). This assessment quantifies, for the first time at a multiregional scale, the severity of streamflow alteration in a large portion of the current streamflow monitoring network, as well as the integrity of associated biological communities. Our work is also distinct from previous large-scale studies (eg Konrad *et al.* 2008) in that we examined biological and hydrological characteristics in terms of their deviations from reference conditions, seeking to understand the potential ecological consequences of anthropogenic changes to the natural flow regime (sensu Poff *et al.* 2010). Our primary findings are that (1) most of the monitored streams experience altered flow magnitudes and (2) there is a strong association between diminished streamflow magnitudes and impaired biological communities across the conterminous US.

Given the central influence of the flow regime on stream ecosystems, our finding that anthropogenic changes in streamflow magnitudes are pervasive and severe suggests this factor may be a ubiquitous constraint on biological integrity. Previous studies have drawn similar conclusions using indirect measures (Graf 1999; Nilsson *et al.* 2005) or at sites with known temporal changes in streamflow alteration (Poff *et al.* 2007). Despite finding a high percentage of altered sites, we probably underestimated the occurrence and severity of streamflow alteration for two reasons. First, our measures of deviation from expected magnitudes are conservative relative to pristine conditions or conditions prior to European settlement, because estimates of expected streamflow magnitudes were derived from many reference sites (particularly in the midwestern US) influenced by some anthropogenic disturbance. Second, we limited our assessment to a single dimension of the natural flow regime – magnitudes – but the timing, duration, and rate of change are also ecologically important (Bunn and Arthington 2002; Mathews and Richter 2007). Had these dimensions been included, our estimate of the pervasiveness and severity of streamflow alteration would likely have increased.

Pronounced differences in streamflow alteration between arid and wet climates are partly due to distinc-

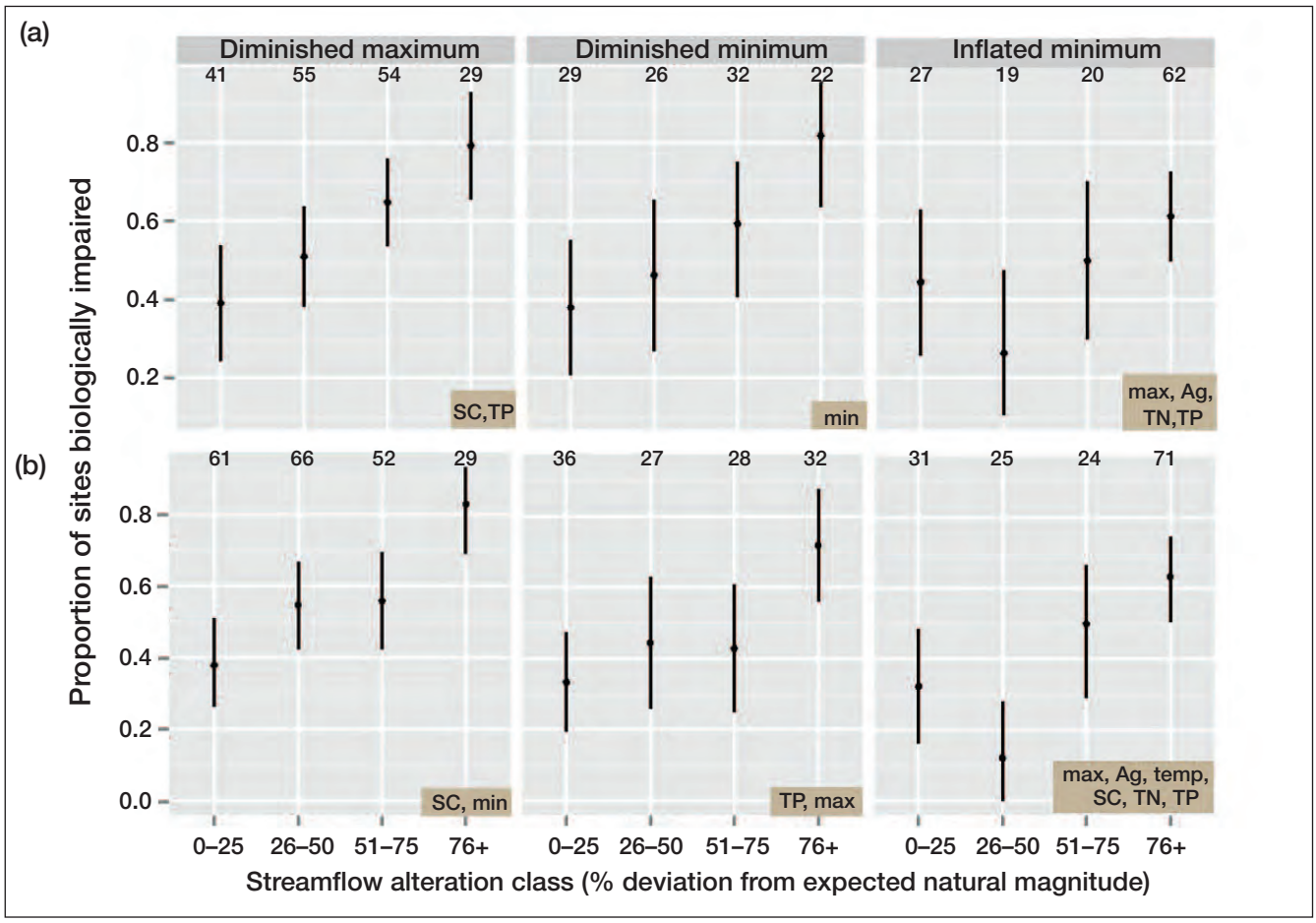

Figure 3. *Proportion of sites with impaired (a) fish and (b) macroinvertebrate communities within classes of severity of streamflow alteration (expressed as percent deviation from expected natural magnitudes). "Diminished" indicates observed magnitudes less than expected natural magnitudes; "inflated" indicates observed magnitudes greater than expected natural magnitudes. Vertical black lines indicate 95% confidence intervals generated with bootstrapping. Values above each vertical line indicate the number of sites with each severity class. Inset boxes display covariates that differed significantly ($P < 0.05$) among severity classes, where SC = specific conductance, TP = total phosphorus, TN = total nitrogen, temp = water temperature, Ag = riparian agriculture land cover, max = maximum flow observed/expected (O/E), and min = minimum flow O/E.*

tive management of watershed hydrology. The tendency for diminished flow magnitudes in arid climates is indicative of consumptive water uses causing net streamflow loss. The primary use of water in arid climates is for irrigated agriculture (Pimentel *et al.* 1997), but interbasin transfers and groundwater withdrawal for other uses also reduce streamflows (Jackson *et al.* 2001). Management of watershed hydrology in wet climates, in contrast, is often focused on flood control. This is most often achieved through small impoundments or large reservoirs that remove flood peaks and release the water later, during normally low flow periods; this management technique can result in inflated minimum flows and diminished maximum flows (Magilligan and Nislow 2005).

Streamflow alteration was the primary predictor of biological integrity, even after considering several covariates. Our set of anthropogenic covariates was not exhaustive, but some (eg riparian land cover) are potential surrogates for unmeasured factors, such as dissolved contaminants. Nevertheless, several covariates (eg nutrients and riparian land cover) that are recognized as influential to biological integrity were less important than streamflow alteration. Natural covariates were at least partially controlled for through the use of an O/E index for biological and streamflow measures, which predicts site-specific expectations based on natural factors such as climate and stream size (Hawkins 2006). Interactions of covariates and streamflow alteration in the macroinvertebrate model suggest that biological responses to diminished maximum flows depend on the environmental context. This phenomenon has not been explicitly studied, but may explain why a recent review (Poff and Zimmerman 2010) found that macroinvertebrate communities show a less consistent response to streamflow alteration than do fish communities.

The ecological importance of streamflow alteration is evident from our finding that the likelihood of biological impairment increased with the severity of diminished streamflow magnitudes. Some chemical covariates were also associated with increased severity of diminished

Table 1. Summary of trends in macroinvertebrate and fish traits at sites – with various forms of altered streamflow magnitudes – across the conterminous US

Trait	*Community*	*Diminished minimum*	*Diminished maximum*	*Inflated minimum*
Reproductive strategy	Fish	Nest guarders replace simple nesters	Broadcast spawners replace simple nesters	Broadcast spawners replace simple nesters
Morphology/ locomotion	Fish	Active swimmers replace benthic and streamlined forms	Active swimmers replace benthics	None observed
	Macro-invertebrates	Active swimmers replace taxa with slow crawling rates	Active swimmers replace taxa with slow crawling rates	None observed
Exit ability	Macro-invertebrates	Increased taxa with exit ability	Increased taxa with exit ability	None observed
Geomorphic and substrate preference	Fish and macro-invertebrates	Pool taxa preferring fine-grained substrates replace riffle taxa preferring coarse substrates	Pool taxa preferring fine-grained substrates replace riffle taxa preferring coarse substrates	Increased taxa preferring riffles (macro-invertebrates only)

Notes: See WebPanel 1 for detailed statistical results.

streamflow magnitudes, so we cannot rule out their influence on biological communities – although elevated concentrations of chemicals would also be an expected result of reduced streamflow magnitudes (Bunn and Arthington 2002). We also cannot distinguish the relative influences of minimum and maximum flows, because both tended to be diminished in streams with the most severe streamflow alteration. Nevertheless, our findings demonstrate that, across divergent natural and anthropogenic settings, the likelihood of biological impairment grows with increased reductions of maximum and minimum streamflow magnitudes.

Finally, biological communities in streams with altered flow magnitudes appeared to lose and gain taxa with traits indicative of specific flow regimes. Streams with diminished flows showed increases in taxa with preferences for low water velocities and fine sediments (eg absence of flushing flows), and with the ability to escape periodic environmental bottlenecks – possibly to avoid desiccation. Streams with inflated minimum flows showed increases in macroinvertebrate taxa with preferences for turbulent currents – a likely result of sustained high flows. Fish species that were favored in all hydrologically altered streams possess reproductive strategies that require either a high level of parental care or no care at all, whereas species that build simple nests appeared to be lost from the system. Simple nests generally require water circulation to maintain egg viability and would therefore be sensitive to desiccation under diminished flows or scouring under inflated flow regimes. In contrast, nest-guarding species protect nests from predators and can behaviorally provide circulation when necessary. Alternatively, species that broadcast spawn compensate for harsh environmental conditions with high reproductive output. Although these traits suggest a mechanistic link between biological impairment and altered streamflow magnitudes, some traits would be favored in any disturbed environment. Therefore, these traits are not themselves diagnostic of streamflow alteration, but are consistent with the hypothesis that altered streamflow magnitudes played a role in causing biological impairment.

Because the flow regime controls many physical, chemical, and biological processes, community responses to streamflow alteration are a product of direct and indirect pathways. We did not explore the mechanisms underlying the relationships between biological integrity and streamflow alteration, nor was the study design appropriate for evaluating thresholds of streamflow alteration that are protective of biological communities. Nevertheless, our study provides a multiregional-scale perspective on the importance of natural streamflow regimes to the maintenance of aquatic communities and ecosystems, and provides water-resource managers with a much-needed perspective on the pervasiveness and severity of anthropogenic alteration of streamflow magnitudes. The degree to which streamflows are controlled in many river systems and the pervasiveness of streamflow alteration across the US suggest that a national priority of restoring natural streamflow magnitudes could be broadly implemented and would produce widespread and measurable ecological dividends (Postel and Richter 2003).

Acknowledgements

We thank J Falcone for help in developing geographic information system (GIS) databases used in these assessments. Comments on early versions of this manuscript by S Postel, C Konrad, and W Wilber improved its quality and clarity.

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WebPanel 1. Assessing streamflow alteration and biological integrity

Predicting expected streamflow magnitudes

The procedure for predicting expected natural streamflow attributes is detailed elsewhere (Carlisle *et al.* 2010; Falcone *et al.* 2010), and briefly described here. Among ~7000 streamflow monitoring sites across the conterminous US, reference (ie least-disturbed) sites with perennial flow were selected through quantitative and qualitative criteria of human activity in the watersheds and local expert judgment. Minimum flow (annual minimum of the 7-day moving average of daily flow values) and maximum flow (annual maximum daily flow value) were averaged across all years of flow record available from 1950–2007 at reference sites (minimum of 20 years), and across at least 15 years of recent flow record (1980–2007) at assessed sites. Model performance was evaluated by computing the mean and standard deviation of the observed (O) to expected (E) ratio (from cross-validation) at reference sites. Predictive models exhibited 26–34% error, with relatively little bias (WebTable 1). Assessed sites were identified from stream gauges operated by the US Geological Survey (USGS) with at least 15 years of complete records (1980–2007) and whose watershed characteristics (eg terrain, soils, and climate) were within the multivariate distribution (sensu Bowman and Somers 2006) of those for reference sites. Drainage basins for the final set of 2888 assessed and 1059 reference sites included in this assessment encompass one-half of the total land area in the conterminous US and are typical of land use and water management across the country (WebTable 2).

Predicting expected biological communities

The details of model development and evaluation are documented elsewhere and briefly described here. The USGS National Water-Quality Assessment Program sampled macroinvertebrate (274 sites) and fish (237 sites) communities from 1993–2005 across the conterminous US where daily streamflow was also monitored. Field methods followed standard protocols and consistent quality assurance practices (Moulton *et al.* 2002) throughout the study period. Estimates of E were obtained from regional River Invertebrate Prediction and Classification System-type models (sensu Hawkins 2006) that predicted the probabilities of capturing at a site each taxon from the regional pool of native taxa. O for each site was calculated as the number of expected taxa that were actually collected in the sample. Separate predictive models for macroinvertebrates were developed with 338 reference sites in the eastern and central US (Carlisle and Meador 2007), 217 reference sites in the south–central US (Yuan *et al.* 2008), and 729 reference sites in the western US (Carlisle and Hawkins 2008). Predictive models for fish communities were developed with 266 reference sites in the eastern and central US (Meador and Carlisle 2009). Because fish communities in the western US are naturally species-poor, these sites were assessed with an index of biological integrity (IBI), which represents measures of community composition (eg proportion of exotic species) other than species richness. O for western fish was the observed value of the IBI calculated from the sample collected at each site (Meador *et al.* 2008), and E was estimated as the average IBI from reference sites within each ecoregion, which is conceptually similar to E derived from statistical models (Hawkins 2006). Thresholds for classifying communities as impaired were based on the uncertainty of each predictive model, as determined by the distribution of O/E values at reference sites within each modeled region. For this study, a consistent impairment threshold was applied across all sites for each community and was defined as the average of thresholds from each region (O/E < 0.80 for macroinvertebrates, O/E < 0.75 for fish).

Associations between streamflow alteration and biological condition

Because the lengths of antecedent (to biological sampling date) streamflow records varied among sites where biological communities were sampled, we evaluated whether streamflow indicators were influenced by the number of years used to compute O. For a set of 239 sites where 15 years of antecedent daily streamflow records existed, we found that O/E indicators for minimum and maximum flow computed with O averaged over 5, 10, or 15 years were highly correlated (Spearman rank > 0.90). We therefore used 5 years of antecedent streamflow records in order to maximize the number of sites where biology and hydrology were both assessed.

We compiled data for eight covariates in an attempt to evaluate whether these factors were confounded with streamflow alteration. Covariates were selected among available data to be broadly representative of natural and anthropogenic chemical and physical conditions at each site. Land-cover variables (percent of area within 100-m buffer of the stream network upstream of site) were included to represent the intensity of land use along the stream corridor. Details of sampling and calculation of covariates are given elsewhere (Carlisle *et al.* 2008). Spearman rank correlations between covariates and measures of streamflow alteration were generally weak (maximum |Spearman rho| = 0.57).

Trait analysis

Predictive models used to estimate expected community composition were used to identify taxa that were potentially lost ("increasers") or gained ("decreasers") in streams with altered streamflows. For each site with altered streamflow, taxa having a predicted probability occurrence > 0.50 (Carlisle and Hawkins 2008) but absent from the site were recorded as decreasers. Taxa having a predicted probability of occurrence < 0.50 but present at the site were recorded as increasers. In the western US, null models (Van Sickle *et al.* 2005) based on 158 reference sites for fish communities (Whittier *et al.* 2007) were used to predict the expected taxa at each site. All non-indigenous fish taxa were considered increasers in the western US. Lists of increaser and decreaser taxa were aggregated across all sites with each type of streamflow alteration, and those present in < 10% of sites were excluded. We analyzed select macroinvertebrate traits from Poff *et al.* (2006), enhanced with the database of Vieira *et al.* (2006), that we deemed would be responsive to hydrological characteristics or that were considered evolutionarily labile (Poff *et al.* 2006). We analyzed select fish species traits from Goldstein and Meador (2004). Fisher's exact test was used to determine whether the frequencies of decreaser and increaser taxa were statistically different for each trait category. Statistical summaries are presented in WebTables 3 and 4.

WebPanel 1. Assessing streamflow alteration and biological integrity – *continued*

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WebTable 1. Performance of models used to predict expected natural streamflow magnitudes for the conterminous US

Streamflow attribute	*Mean O/E*	*SD*	*10th percentile*	*90th percentile*
Minimum flow	1.00	0.26	0.71	1.26
Maximum flow	0.96	0.34	0.57	1.35

Notes: The 10th and 90th percentiles of observed/expected (predicted) values at reference sites were used as thresholds to classify streamflow alteration at assessed sites as diminished or inflated, respectively. SD = standard deviation.

WebTable 2. Characteristics of land- and water-use of river basins assessed in this study as compared with the entire conterminous US

Characteristic	*Conterminous US*	*This study (percent of US)*
Area (km^2)	8.08×10^6	4.07×10^6 (50%)
Total reservoir storage (acre ft)	1.38×10^9	0.82×10^9 (59%)
Agricultural land cover (%)	22	25
Urban land cover (%)	5	5

WebTable 3. Frequencies of functional trait states in fish taxa considered decreasers ("dec") or increasers ("inc") in streams with different types of streamflow alteration. *n* = number of taxa

	Diminished minimum	*Inflated minimum*	*Diminished maximum*
Trait	dec, inc	dec, inc	dec, inc
n	(22, 39)	(18, 47)	(17, 32)
Reproduction	***P = 0.010***	***P = 0.011***	***P = 0.045***
Bearer	***0.00, 0.03***	***0.00, 0.02***	***0.00, 0.03***
Complex nest	***0.23, 0.50***	***0.39, 0.33***	***0.35, 0.41***
Broadcast	***0.36, 0.39***	***0.22, 0.56***	***0.23, 0.47***
Simple nest	***0.41, 0.08***	***0.39, 0.09***	***0.41, 0.09***
Locomotion	***P = 0.010***	*P* = 0.278	***P = 0.019***
Accelerate	***0.04, 0.16***	0.05, 0.09	***0.00, 0.12***
Creeper	***0.18, 0.32***	0.22, 0.33	***0.23, 0.31***
Cruiser	***0.41, 0.24***	0.33, 0.41	***0.35, 0.37***
Hugger	***0.32, 0.05***	0.28, 0.06	***0.29, 0.00***
Maneuver	***0.04, 0.24***	0.11, 0.11	***0.12, 0.19***
Habitat preference	***P = 0.034***	*P* = 0.096	***P = 0.024***
Riffle/run	***0.18, 0.03***	0.28, 0.06	***0.23, 0.00***
Pool	***0.04, 0.23***	0.28, 0.25	***0.18, 0.25***
Backwater	***0.00, 0.03***	0.00, 0.02	***0.00, 0.03***
Variable	***0.77, 0.72***	0.44, 0.66	***0.59, 0.72***
Substrate preference	*P* = 0.492	***P = 0.059***	***P = 0.028***
Coarse	0.04, 0.03	***0.22, 0.04***	***0.18, 0.03***
Gravel	0.09, 0.03	***0.00, 0.04***	***0.00, 0.00***
Fines	0.04, 0.13	***0.05, 0.23***	***0.00, 0.22***
Vegetation	0.00, 0.00	***0.00, 0.00***	***0.00, 0.00***
Variable	0.81, 0.82	***0.72, 0.68***	***0.82, 0.75***

Notes: First row for each trait reports the *P* value from Fisher's exact test. Bold entries indicate *P* values <0.05. For each trait state, paired cells report the proportion of taxa possessing that trait for increasers and decreasers.

WebTable 4. Frequencies of functional trait states in macroinvertebrate taxa considered decreasers ("dec") or increasers ("inc") in streams with different types of streamflow alteration. *n* = number of taxa.

	Diminished minimum	*Inflated minimum*	*Diminished maximum*
Trait	dec, inc	dec, inc	dec, inc
n	(22, 31)	(23, 33)	(27, 29)
Exit ability	*P* = 0.161	*P* = 0.776	***P = 0.008***
Absent	0.68, 0.45	0.70, 0.64	***0.74, 0.38***
Present	0.32, 0.55	0.30, 0.36	***0.26, 0.62***
Desiccation resistance	*P* = 1.00	*P* = 1.00	*P* = 1.00
Absent	0.77, 0.81	0.78, 0.75	0.81, 0.82
Present	0.23, 0.19	0.22, 0.25	0.19, 0.18
Crawling rate	***P = 0.025***	*P* = 0.512	***P = 0.036***
Very low	***0.45, 0.64***	0.48, 0.42	***0.44, 0.66***
Low	***0.45, 0.13***	0.43, 0.36	***0.41, 0.10***
High	***0.09, 0.22***	0.09, 0.21	***0.15, 0.24***
Swimming ability	*P* = 0.804	*P* = 0.912	*P* = 0.646
None	0.77, 0.74	0.74, 0.67	0.70, 0.72
Weak	0.14, 0.10	0.17, 0.18	0.19, 0.10
Strong	0.09, 0.16	0.09, 0.15	0.11, 0.18
Flow preference	***P = 0.018***	***P = 0.040***	***P = 0.002***
Depositional	***0.09, 0.29***	***0.17, 0.15***	***0.11, 0.28***
Erosional	***0.50, 0.61***	***0.48, 0.76***	***0.48, 0.69***
Either	***0.41, 0.10***	***0.35, 0.09***	***0.41, 0.03***
Habit	*P* = 0.162	*P* = 0.912	***P = 0.014***
Burrower	0.27, 0.52	0.26, 0.33	***0.22, 0.59***
Sprawler	0.04, 0.06	0.13, 0.09	***0.07, 0.03***
Clinger	0.55, 0.26	0.48, 0.42	***0.55, 0.21***
Swimmer	0.14, 0.16	0.13, 0.15	***0.15, 0.17***

Notes: First row for each trait reports the *P* value from Fisher's exact test. Bold entries indicate *P* values <0.05. For each trait state, paired cells report the proportion of taxa possessing that trait for increasers and decreasers.

United States
Environmental Protection
Agency

Office of Research and
Development
Washington DC 20460

EPA/600/R-95/136
August 1995

EPA

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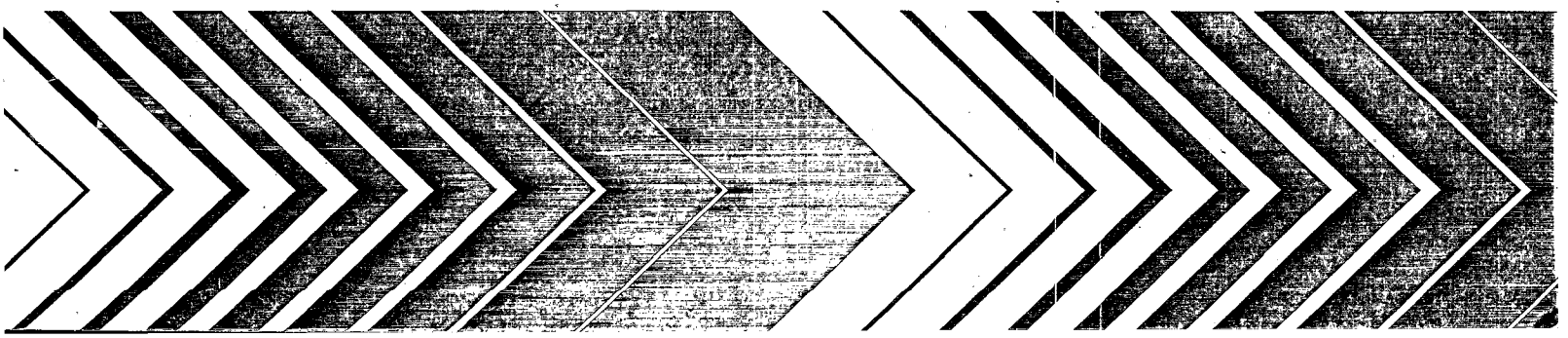

EPA/600/R-95-136
August 1995

SHORT-TERM METHODS FOR ESTIMATING THE CHRONIC TOXICITY OF EFFLUENTS AND RECEIVING WATERS TO WEST COAST MARINE AND ESTUARINE ORGANISMS

(First Edition)

Edited by

Gary A. Chapman[1], Debra L.Denton[2],
and James M. Lazorchak[3],

[1]National Health and Ecological Effects Research Laboratory, Newport, Oregon
[2]EPA Region IX, San Francisco, California
[3]National Exposure Research Laboratory, Cincinnati, Ohio

NATIONAL EXPOSURE RESEARCH LABORATORY - CINCINNATI
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
CINCINNATI, OHIO 45268

DISCLAIMER

This document has been reviewed by the National Exposure Research Laboratory-Cincinnati (NERL-Cincinnati), U. S. Environmental Protection Agency (USEPA), and approved for publication. The mention of trade names or commercial products does not constitute endorsement or recommendation for use. The results of data analyses by computer programs described in the section on data analysis were verified using data commonly obtained from effluent toxicity tests. However, these computer programs may not be applicable to all data, and the USEPA assumes no responsibility for their use.

FOREWORD

Environmental measurements are required to determine the quality of ambient waters and the character of waste effluent. The National Exposure Research Laboratory-Cincinnati (NERL-Cincinnati) conducts research to:

- Develop and evaluate analytical methods to identify and measure the concentration of chemical pollutants in drinking waters, surface waters, groundwaters, wastewaters, sediments, sludges, and solid wastes.

- Investigate methods for the identification and measurement of viruses, bacteria and other microbiological organisms in aqueous samples and to determine the responses of aquatic organisms to water quality.

- Develop and operate a quality assurance program to support the achievement of data quality objectives in measurements of pollutants in drinking water, surface water, groundwater, wastewater, sediment and solid waste.

- Develop methods and models to detect and quantify responses in aquatic and terrestrial organisms exposed to environmental stressors and to correlate the exposure with effects on chemical and biological indicators.

The Federal Water Pollution Control Act Amendments of 1972 (PL 92-500), the Clean Water Act (CWA) of 1977 (PL 95-217) and the Water Quality Act of 1987 (PL 100-4) explicitly state that it is the national policy that the discharge of toxic substances in toxic amounts be prohibited. Thus, the detection of chronically toxic effluents plays an important role in identifying and controlling toxic discharges to surface waters. This manual is the first edition of the west coast marine and estuarine chronic toxicity test manual for effluents. It provides standardized methods for estimating the chronic toxicity of effluents and receiving waters to estuarine and marine organisms for use by the USEPA regional programs, the state programs, and the National Pollutant Discharge Elimination System (NPDES) permittees.

PREFACE

This manual contains whole effluent toxicity (WET) test methods considered by USEPA's Office of Research and Development (ORD) to have the necessary characteristics for use in the NPDES program and other USEPA monitoring activities, in Pacific coastal waters, for estimating the chronic toxicity of effluents and receiving waters. All the species included in this report are currently specified in NPDES permits in one or more of the west coast states. The methods will likely be revised to some extent, especially if they are proposed in the Federal Register as 304(h) methods. Revisions would be made based upon comments received as a result of the proposed rule public comment period.

With one exception, other than changes necessary to identify the test species used in these methods and corrections of an editorial nature, the first ten sections of this document are identical to the first ten sections of the "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Estuarine and Marine Organisms, (Second Edition)." The exception occurs in chapter 7 where the use of synthetic (standard) dilution water for NPDES permit-related toxicity testing is not required. Validation and precision tests with natural seawater and HSB prepared from natural seawater (plus reagent water as necessary) have been acceptable, and synthetic waters have shown mixed results in limited testing.

The marine toxicity test procedures in this manual have been developed or refined by EPA and the states of California and Washington over a period of years. A significant number of organizations and individuals have contributed to this effort. A list of contributors is provided in the acknowledgements section. Among the major efforts that contributed critical data and critical analysis of the methods in this manual the following were vital:

1) The California Marine Bioassay Project (MBP). In 1984, the California State Water Resources Control Board initiated the MBP to develop sensitive methods for testing the toxicity of discharges to California marine waters. The MBP was funded wholly or in part by the USEPA using Section 205(j) grant funds. The MBP developed the tests with abalone (*Haliotis rufescens*),

topsmelt (*Atherinops affinis*), giant kelp (*Macrocystis pyrifera*), and mysid (*Holmesimysis costata*).

2) The EPA West Coast Marine Complex Effluent Program. Started in 1985, this program provided preliminary work for the topsmelt (*Atherinops affinis*), revision of methods for echinoid sperm with the purple sea urchin (*Strongylocentrotus purpuratus*) and the sand dollar (*Dendraster excentricus*), preparation of all methods into a standardized format, coordination of efforts among the various states and EPA regions 9 and 10, and development of yet unadopted test methods with the mysid (*Mysidopsis intii*) and the kelp (*Laminaria saccharina*).

3) The Protocol Review Committee (PRC) for the Triennial Review of the Marine Toxicity Test Protocols for the California Ocean Plan. In 1994 this committee reviewed a number of proposed test methods for inclusion in the California Ocean Plan. The methods included in this report are those recommended by the Protocol Review Committee. The *Mysidopsis intii* method developed by EPA was excluded from the recommended procedures because it was considered redundant with the *Holmesimysis costata* procedure. It was excluded from this report because its inclusion was also considered unneccesary by EPA region 10. The *Laminaria saccharina* test was excluded from the California recommendations because it was considered redundant with the *Macrocystis pyrifera* test. It was excluded from this report because the results from the West Coast Marine Species Chronic Protocol Variability Study indicated that more experience with the method was needed to produce acceptable precision.

4) West Coast Marine Species Chronic Protocol Variability Study. This study was a result of a 1991 settlement agreement among the Northwest Pulp and Paper Association, the Washington Dept. of Ecology, Puget Sound Water Quality Authority, and Tulalip Tribes of Washington. The year-long study in 1993-94 included monthly or quarterly interlaboratory toxicity test evaluation of tests with bivalve molluscs (*Crassostrea gigas*) and mussels (*Mytilus sp.*), echinoid sperm tests with purple sea urchins (*S. purpuratus*) and sand dollar (*D. excetricus*), sexual reproduction of kelp (*L. saccharina*), and the topsmelt (*A. affinis*).

Following review and recommendations by the PRC to the State of California for use of the procedures in this report, EPA (OR&D

and Region 9) modified the format for all methods to provide consistency among the methods as well as consistency with existing EPA Whole Effluent Toxicity Testing Manuals.

Review of the results from tests using the methods in this report indicated that they are analogous to, and as sensitive as, the methods previously proposed for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms (U.S. EPA 1994). The primary exception is the suite of invertebrate embryo-larval tests contained in this manual. These tests have been in regulatory and monitoring use on the west coast, some for many years. They tend to be more sensitive test organisms to many chemicals and the tests are more robust statistically. They have no analog in the previous EPA methods manuals, although a similar test has been proposed by the EPA laboratory in Narragansett for use in monitoring sediment-associated contaminants with the bivalve *Mulinia lateralis*.

ABSTRACT

This manual describes six short-term (forty minutes to seven days) estuarine and marine methods for measuring the chronic toxicity of effluents and receiving waters to eight species: the topsmelt, *Atherinops affinis*; the mysid, *Holmesimysis costata*; the sea urchin, *Strongylocentrotus purpuratus* and sand dollar *Dendraster excentricus*; the red abalone *Haliotis rufescens*; the bivalves *Crassostrea gigas* and mussel *Mytilus spp.* and the giant kelp, *Macrocystis pyrifera*. The methods include single and multiple concentration static renewal and static nonrenewal toxicity tests for effluents and receiving waters. Also included are guidelines on laboratory safety, quality assurance, facilities, and equipment and supplies; dilution water; effluent and receiving water sample collection, preservation, shipping, and holding; test conditions; toxicity test data analysis; report preparation; and organism culturing, holding, and handling. Examples of computer input and output for Dunnett's Procedure, Probit Analysis, Trimmed Speaman-Karber Method, and the Linear Interpolation Method are provided in the Appendices.

ACKNOWLEDGEMENTS

The principal authors of this document are: Gary A. Chapman, OR&D, Newport, Oregon; Debra L. Denton, Region 9, San Francisco, California; and James M. Lazorchak, OR&D, Cincinnati, Ohio.

Section 1 through 10 of this manual are only slightly modified from the same sections in the EPA Manual, "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms" (Second Edition) and are essentially the work of Klemm, D.J., G.E. Morrison, T.J. Norberg-King and W.H. Peltier. The numerous contributors to their manual are acknowledged therein.

Four of the seven methods in this manual were adapted from methods developed by the California State Water Resources Control Board's Marine Bioassay Project. These methods for red abalone, topsmelt, mysids, and kelp were prepared by the following staff from the University of California, Santa Cruz:

Brian A. Anderson
John W. Hunt
Matt Englund
Hilary McNulty
Sheila L. Turpen

The sea urchin embryo/larval development test was modified from a method prepared by staff from the Southern California Coastal Water Research Project:

Steven Bay
Darrin Greenstein

The sea urchin and sand dollar sperm tests and the bivalve mollusc embryo/larval development tests are ERL-N contributions 287 and 288, respectively, and were prepared by EPA staff:

Gary A. Chapman
Debra L. Denton

The data analysis and statistical sections and appendices were the work of Technology Applications, Inc. employee:

Laura Gast

Formal Peer-review comments from the following persons are gratefully acknowledged:

Amy Wagner, EPA Region 9 Richmond Laboratory, who reviewed all seven methods for technical detail and consistency (any existing errors sneaked in after her review).

Paul Dinnel, Dinnel Marine Research
Suzanne Lussier, EPA Narragansett
Doug Middaugh, EPA Gulf Breeze
George Morrison, EPA Narragansett
Diane Nacci, SAIC
Barry Snyder, Ogden Environmental and Energy Services
Glen Thursby, SAIC

Southern California Toxicity Assessment Group (SCTAG) and its members, especially chairmen Tim Mikel of ABC Labs, and Tom Dean of Coastal Resources Associates, Inc.

California Protocol Review Committee

Matthew Reeve, California State Water Resources Control Board (coordinator)

Business:

Steve Bay, Southern California Coastal Water Research Project
Tom Dean, Coastal Resources Associates, Inc.
Andrew Glickman, Chevron Research and Technology Company
Dave Gutoff, City of San Diego Marine Laboratory
Timothy Hall, National Council of the Paper Industry for Air and Stream Improvement

Government:

Gary Chapman, EPA
Debra Denton, EPA

Academia:

Gary Cherr, Bodega Marine Laboratory, University of California, Davis
Jo Ellen Hose, Occidental College
Donald Reish, California State University, Long Beach
Washington Dept. of Ecology Protocol Variability Study

Merley McCall, Washington Dept. of Ecology (coordinator)

Science Advisory Board:

Rick Cardwell, Parametrix, Inc.
Dick Caldwell, Northwest Aquatics
Peter Chapman, EVS Environment Consultants, Ltd.
Gary Cherr (chair), Bodega Marine Lab, UC Davis
Paul Dinnel, (vice-chair), Dinnel Marine Research

Some people have made continuing contributions to the development and evaluation of these and related marine toxicity test procedures. Special recognition need be given to: Paul Dinnel for extensive work with echinoid sperm and embryo/larval development tests; Susan Anderson of Lawrence Berkeley Laboratories (earlier with California State Water Resources Control Board) for echinoid sperm method modification and whole effluent testing implementation; Michael Ives, Telonichor Marine Laboratory, Humboldt State University, for providing his experience and insight into method miniaturization and streamlining; Gary Cherr and Jon Shenker at Bodega Marine Laboratory, UC Davis, for method development and improvement for most of these tests, especially for miniaturization of the bivalve embryo/larval development test; Sally Noack of AScI who contributed greatly to testing of the sea urchin sperm cell test; Robert Smith of EcoAnalysis performed much of the statistical work of determining the MSD for each of the test methods; Randall Marshall, Washington Department of Ecology for support and review

of the test method development and implementation; Kevin Brix, Parametrix, Inc. for providing information on sand-dollar embryo/larval development tests; Timothy Hall of NCASI for work with the echinoid sperm test; the Northern California Toxicity Assessment Group (NCTAG) for their review of the bivalve mollusc embryo/larval development test; and Phil Oshida, Steve Schimmel, and Steve Bugbee, EPA, for getting the EPA west coast methods program started and on track.

CONTENTS

SECTION 1

INTRODUCTION

1.1 This manual describes chronic toxicity tests for use in the National Pollutant Discharge Elimination System (NPDES) Permits Program to identify effluents and receiving waters containing toxic materials in chronically toxic concentrations. The test methods are also suitable for determining the toxicity of specific compounds contained in discharges. The tests may be conducted in a central laboratory or on-site, by the regulatory agency or the permittee.

1.2 The data are used for NPDES permits development and to determine compliance with permit toxicity limits. Data can also be used to predict potential acute and chronic toxicity in the receiving water, based on hypothesis testing or point estimate techniques (see Section 9, Chronic Toxicity Test Endpoints And Data Analysis) and appropriate dilution, application, and persistence factors. The tests are performed as a part of self-monitoring permit requirements, compliance biomonitoring inspections, toxics sampling inspections, and special investigations. Data from chronic toxicity tests performed as part of permit requirements are evaluated during compliance evaluation inspections and performance audit inspections.

1.3 Modifications of these tests are also used in toxicity reduction evaluations and toxicity identification evaluations to identify the toxic components of an effluent, to aid in the development and implementation of toxicity reduction plans, and to compare and control the effectiveness of various treatment technologies for a given type of industry, irrespective of the receiving water (USEPA, 1988c; USEPA, 1989b; USEPA, 1989c; USEPA, 1989d; USEPA, 1989e; USEPA, 1991a; USEPA, 1991b; USEPA, 1992).

1.4 This methods manual serves as a companion to the acute toxicity test methods for freshwater and marine organisms (USEPA, 1993a), the short-term chronic toxicity test methods for freshwater organisms (USEPA, 1993b), the short-term chronic toxicity test methods for east coast organisms (USEPA, 1994), and the manual for evaluation of laboratories performing aquatic toxicity tests (1991c).

1.5 Guidance for the implementation of toxicity tests in the NPDES program is provided in the Technical Support Document for Water Quality-Based Toxics Control (USEPA, 1991a).

similar to those developed for the freshwater organisms and east coast marine organisms to evaluate the toxicity of effluents discharged to estuarine and coastal marine waters under the NPDES permit program. Methods are presented in this manual for ten species from six phylogenetic groups. The red abalone larval development test method, the giant kelp germination and germ-tube length test method, the mysid survival and growth test method and the topsmelt survival and growth test method were developed and extensively field tested by University of California, Santa Cruz through the California State Water Resources Control Board's Marine Bioassay Project. The purple urchin and sand dollar fertilization test method was developed by U.S. Environmental Research Laboratory-Newport, Oregon. The purple urchin and sand dollar development test method was developed by the Southern California Coastal Water Research Project. The Pacific oyster and mussel survival and larval development test method was modified from ASTM 1989 by the Washington Department of Ecology and the USEPA. The methods vary in duration from 40 minutes to seven days.

1.7 The ten species for which toxicity test methods provided are: the topsmelt, *Atherinops affinis*, the red abalone, *Haliotis rufescens;* the Pacific oyster, *Crassostrea gigas*, mussel *Mytilus spp.;* the mysid, *Holmesimysis costata;* the sea urchin, *Strongylocentrotus purpuratus*, the sand dollar, *Dendraster excentricus;* and the giant kelp, *Macroystis pyrifera*.

1.7.1 Many of the tests included in this document are based on the following:

1. "Marine Bioassay Project Seventh Reports (Reports 1-7)" by Brian S. Anderson, John W. Hunt, and Hilary R. McNulty, University of California, Santa Cruz; Mark D. Stephenson, California Department of Fish and Game; and Francis H. Palmer, Debra L. Denton, and Matthew Reeve, State Water Resources Control Board.

2. "Procedures Manual for Conducting Toxicity Tests Developed by the Marine Bioassay Project by Brian S. Anderson, John W. Hunt, Shiela L. Turpen, A.R. Coulon, University of California, Santa Cruz; Mike Martin, California of Department of Fish and Game; Debra L. Denton and Frank H. Palmer, State Water Resources Control Board, 90-10WQ, 112 pp.

3. "Standard Practice for Conducting Static Acute Toxicity Tests with Larvae of Four Species of Bivalve Molluscs. ASTM 1989.

1.7.2 Three of the methods incorporate the chronic endpoints of growth or development (or both) in addition to lethality. The sea urchin sperm cell test uses fertilization as an endpoint and has the advantage of an extremely short exposure period (40 minutes).

1.8 The validity of similar marine/estuarine methods in predicting adverse ecological impacts of toxic discharges was demonstrated in field studies (USEPA, 1986d).

1.9 The use of any marine or estuarine test species or test conditions other than those described in the methods summary tables in this manual or in the east coast marine manual (USEPA/600/4-91/003) shall be subject to application and approval of alternate test procedures under 40 CFR 136.4 and 40 CFR 136.5.

1.10 These methods are restricted to use by or under the supervision of analysts experienced in the use or conduct of aquatic toxicity testing and the interpretation of data from aquatic toxicity testing. Each analyst must demonstrate the ability to generate acceptable test results with these methods using the procedures described in this methods manual.

1.11 The manual was prepared in the established NERL-Cincinnati format (USEPA, 1983).

SECTION 2

SHORT-TERM METHODS FOR ESTIMATING CHRONIC TOXICITY

2.1 INTRODUCTION

2.1.1 The objective of aquatic toxicity tests with effluents or pure compounds is to estimate the "safe" or "no-effect" concentration of these substances, which is defined as the concentration which will permit normal propagation of fish and other aquatic life in the receiving waters. The endpoints that have been considered in tests to determine the adverse effects of toxicants include death and survival, decreased reproduction and growth, locomotor activity, gill ventilation rate, heart rate, blood chemistry, histopathology, enzyme activity, olfactory function, and terata. Since it is not feasible to detect and/or measure all of these (and other possible) effects of toxic substances on a routine basis, observations in toxicity tests generally have been limited to only a few effects, such as mortality, growth, and reproduction.

2.1.2 Acute lethality is an obvious and easily observed effect which accounts for its wide use in the early period of evaluation of the toxicity of pure compounds and complex effluents. The results of these tests were usually expressed as the concentration lethal to 50% of the test organisms (LC50) over relatively short exposure periods (one-to-four days).

2.1.3 As exposure periods of acute tests were lengthened, the LC50 and lethal threshold concentration were observed to decline for many compounds. By lengthening the tests to include one or more complete life cycles and observing the more subtle effects of the toxicants, such as a reduction in growth and reproduction, more accurate, direct, estimates of the threshold or safe concentration of the toxicant could be obtained. However, laboratory life cycle tests may not accurately estimate the "safe" concentration of toxicants because they are conducted with a limited number of species under highly controlled, steady state conditions, and the results do not include the effects of the stresses to which the organisms would ordinarily be exposed in the natural environment.

2.1.4 An early published account of a full life cycle, fish toxicity test was that of Mount and Stephan (1967). In this study, fathead minnows, *Pimephales promelas*, were exposed to a graded series of pesticide concentrations throughout their life cycle, and the effects of the toxicant on survival, growth, and

reproduction were measured and evaluated. This work was soon followed by full life cycle tests using other toxicants and fish species.

2.1.5 McKim (1977) evaluated the data from 56 full life cycle tests, 32 of which used the fathead minnow, *Pimephales promelas*, and concluded that the embryo-larval and early juvenile life stages were the most sensitive stages. He proposed the use of partial life cycle toxicity tests with the early life stages (ELS) of fish to establish water quality criteria.

2.1.6 Macek and Sleight (1977) found that exposure of critical life stages of fish to toxicants provides estimates of chronically safe concentrations remarkably similar to those derived from full life cycle toxicity tests. They reported that "for a great majority of toxicants, the concentration which will not be acutely toxic to the most sensitive life stages is the chronically safe concentration for fish, and that the most sensitive life stages are the embryos and fry." Critical life stage exposure was considered to be exposure of the embryos during most, preferably all, of the embryogenic (incubation) period, and exposure of the fry for 30 days post-hatch for warm water fish with embryogenic periods ranging from one-to-fourteen days, and for 60 days post-hatch for fish with longer embryogenic periods. They concluded that in the majority of cases, the maximum acceptable toxicant concentration (MATC) could be estimated from the results of exposure of the embryos during incubation, and the larvae for 30 days post-hatch.

2.1.7 Because of the high cost of full life-cycle fish toxicity tests and the emerging consensus that the ELS test data usually would be adequate for estimating chronically safe concentrations, there was a rapid shift by aquatic toxicologists to 30- to 90-day ELS toxicity tests for estimating chronically safe concentrations in the late 1970s. In 1980, USEPA adopted the policy that ELS test data could be used in establishing water quality criteria if data from full life-cycle tests were not available (USEPA, 1980a).

2.1.8 Published reports of the results of ELS tests indicate that the relative sensitivity of growth and survival as endpoints may be species dependent, toxicant dependent, or both. Ward and Parrish (1980) examined the literature on ELS tests that used embryos and juveniles of the sheepshead minnow, *Cyprinodon variegatus*, and found that growth was not a statistically sensitive indicator of toxicity in 16 of 18 tests. They suggested that the ELS tests be shortened to 14 days posthatch and that growth be eliminated as an indicator of toxic effects.

2.1.9 In a review of the literature on 173 fish full life-cycle and ELS tests performed to determine the chronically safe concentrations of a wide variety of toxicants, such as metals, pesticides, organics, inorganics, detergents, and complex effluents, Woltering (1984) found that at the lowest effect concentration, significant reductions were observed in fry survival in 57%, fry growth in 36%, and egg hatchability in 19% of the tests. He also found that fry survival and growth were very often equally sensitive, and concluded that the growth response could be deleted from routine application of the ELS tests. The net result would be a significant reduction in the duration and cost of screening tests with no appreciable impact on estimating MATCs for chemical hazard assessments. Benoit et al. (1982), however, found larval growth to be the most significant measure of effect and survival to be equally or less sensitive than growth in early life-stage tests with four organic chemicals.

2.1.10 Efforts to further reduce the length of partial life-cycle toxicity tests for fish without compromising their predictive value have resulted in the development of an eight-day, embryo-larval survival and teratogenicity test for fish and other aquatic vertebrates (USEPA, 1981; Birge et al., 1985), and a seven-day larval survival and growth test (Norberg and Mount, 1985).

2.1.11 The similarity of estimates of chronically safe concentrations of toxicants derived from short-term, embryo-larval survival and teratogenicity tests to those derived from full life-cycle tests has been demonstrated by Birge et al. (1981), Birge and Cassidy (1983), and Birge et al. (1985).

2.1.12 Use of a seven-day, fathead minnow, *Pimephales promelas*, larval survival and growth test was first proposed by Norberg and Mount at the 1983 annual meeting of the Society for Environmental Toxicology and Chemistry (Norberg and Mount, 1983). This test was subsequently used by Mount and associates in field demonstrations at Lima, Ohio (USEPA, 1984), and at many other locations (USEPA, 1985c, USEPA, 1985d; USEPA, 1985e; USEPA, 1986a; USEPA, 1986b; USEPA, 1986c; USEPA, 1986d). Growth was frequently found to be more sensitive than survival in determining the effects of complex effluents.

2.1.13 Norberg and Mount (1985) performed three single toxicant fathead minnow larval growth tests with zinc, copper, and DURSBAN®, using dilution water from Lake Superior. The results

were comparable to, and had confidence intervals that overlapped with, chronic values reported in the literature for both ELS and full life-cycle tests.

2.1.14 USEPA (1987b) and USEPA (1987c) adapted the fathead minnow larval growth and survival test for use with the sheepshead minnow and the inland silverside, respectively. When daily renewal 7-day sheepshead minnow larval growth and survival tests and 28-day ELS tests were performed with industrial and municipal effluents, growth was more sensitive than survival in seven out of 12 larval growth and survival tests, equally sensitive in four tests, and less sensitive in only one test. In four cases, the ELS test may have been three to 10 times more sensitive to effluents than the larval growth and survival test. In tests using copper, the No Observable Effect Concentrations (NOECs) were the same for both types of test, and growth was the most sensitive endpoint for both. In a four laboratory comparison, six of seven tests produced identical NOECs for survival and growth (USEPA, 1987a). Data indicate that the inland silverside is at least equally sensitive or more sensitive to effluents and single compounds than the sheepshead minnow, and can be tested over a wider salinity range, 5-30‰ (USEPA, 1987a).

2.1.15 Lussier et al. (1985) and USEPA (1987e) determined that survival and growth are often as sensitive as reproduction in 28-day life-cycle tests with the mysid, *Mysidopsis bahia*.

2.1.16 Nacci and Jackim (1985) and USEPA (1987g) compared the results from the sea urchin fertilization test, using organic compounds, with results from acute toxicity tests using the freshwater organisms, fathead minnows, *Pimphales promelas*, and *Daphnia magna*. The test was also compared to acute toxicity tests using Atlantic silverside, *Menidia menidia*, and the mysid, *Mysidopsis bahia*, and five metals. For six of the eight organic compounds, the results of the fertilization test and the acute toxicity test correlated well ($r^2 = 0.85$). However, the results of the fertilization test with the five metals did not correlate well with the results from the acute tests.

2.1.17 USEPA (1987f) evaluated two industrial effluents containing heavy metals, five industrial effluents containing organic chemicals (including dyes and pesticides), and 15 domestic wastewaters using the two-day red macroalga, *Champia parvula*, sexual reproduction test. Nine single compounds were used to compare the effects on sexual reproduction using a

two-week exposure and a two-day exposure. For six of the nine compounds tested, the chronic values were the same for both tests.

2.1.18 The use of short-term toxicity tests in the NPDES Program is especially attractive because they provide a more direct estimate of the safe concentrations of effluents in receiving waters than was provided by acute toxicity tests, at an only slightly increased level of effort, compared to the fish full life-cycle chronic and 28-day ELS tests and the 28-day mysid life-cycle test.

2.2 TYPES OF TESTS

2.2.1 The selection of the test type will depend on the NPDES permit requirements, the objectives of the test, the available resources, the requirements of the test organisms, and effluent characteristics such as fluctuations in effluent toxicity.

2.2.2 Effluent chronic toxicity is generally measured using a multi-concentration, or definitive test, consisting of a control and a minimum of five effluent concentrations. The tests are designed to provide dose-response information, expressed as the percent effluent concentration that affects the survival, fertilization, growth, and/or development within the prescribed period of time (40 minutes to seven days). The results of the tests are expressed in terms of either the highest concentration that has no statistically significant observed effect on those responses when compared to the controls or the estimated concentration that causes a specified percent reduction in responses versus the controls.

2.2.3 Use of pass/fail tests consisting of a single effluent concentration (e.g., the receiving water concentration or RWC) and a control is not recommended. If the NPDES permit has a whole effluent toxicity limit for acute toxicity at the RWC, it is prudent to use that permit limit as the midpoint of a series of five effluent concentrations. This will ensure that there is sufficient information on the dose-response relationship. For example, if the RWC is >25% then, the effluent concentrations utilized in a test may be: (1) 100% effluent, (2) (RWC + 100)/2, (3) RWC, (4) RWC/2, and (5) RWC/4. More specifically, if the RWC = 50%, the effluent concentrations used in the toxicity test would be 100%, 75%, 50%, 25%, and 12.5%. If the RWC is <25% effluent the concentrations may be: (1) 4 times the RWC, (2) 2 times the RWC, (3) RWC, (4) RWC/2, and (5) RWC/4.

two treatments, a control and the undiluted receiving water, but may also consist of a series of receiving water dilutions.

2.2.5 A negative result from a chronic toxicity test does not preclude the presence of toxicity. Also, because of the potential temporal variability in the toxicity of effluents, a negative test result with a particular sample does not preclude the possibility that samples collected at some other time might exhibit chronic toxicity.

2.2.6 The frequency with which chronic toxicity tests are conducted under a given NPDES permit is determined by the regulatory agency on the basis of factors such as the variability and degree of toxicity of the waste, production schedules, and process changes.

2.2.7 Tests recommended for use in this methods manual may be static non-renewal or static renewal. Individual methods specify which type of test is to be conducted.

2.3 STATIC TESTS

2.3.1 Static non-renewal tests - The test organisms are exposed to the same test solution for the duration of the test.

2.3.2 Static-renewal tests - The test organisms are exposed to a fresh solution of the same concentration of sample every 24 h or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers.

2.4 ADVANTAGES AND DISADVANTAGES OF TOXICITY TEST TYPES

2.4.1 STATIC NON-RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Simple and inexpensive.
2. More cost effective in determining compliance with permit conditions.
3. Limited resources (space, manpower, equipment) required; would permit staff to perform more tests in the same amount of time.
4. Smaller volume of effluent required than for static renewal or flow-through tests.

Disadvantages:

1. Dissolved oxygen (DO) depletion may result from high chemical oxygen demand (COD), biological oxygen demand (BOD), or metabolic wastes.
2. Possible loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Generally less sensitive than renewal because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.4.2 STATIC RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Reduced possibility of DO depletion from high COD and/or BOD, or ill effects from metabolic wastes from organisms in the test solutions.
2. Reduced possibility of loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Test organisms that rapidly deplete energy reserves are fed when the test solutions are renewed, and are maintained in a healthier state.

Disadvantages:

1. Require greater volume of effluent than non-renewal tests.
2. Generally less chance of temporal variations in waste properties.

SECTION 3

HEALTH AND SAFETY

3.1 GENERAL PRECAUTIONS

3.1.1 Each laboratory should develop and maintain an effective health and safety program, requiring an ongoing commitment by the laboratory management and includes: (1) a safety officer with the responsibility and authority to develop and maintain a safety program; (2) the preparation of a formal, written, health and safety plan, which is provided to the laboratory staff; (3) an ongoing training program on laboratory safety; and (4) regularly scheduled, documented, safety inspections.

3.1.2 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to a lack of oxygen or the presence of noxious gases.

3.1.3 Prior to sample collection and laboratory work, personnel should determine that all necessary safety equipment and materials have been obtained and are in good condition.

3.1.4 Guidelines for the handling and disposal of hazardous materials must be strictly followed.

3.2 SAFETY EQUIPMENT

3.2.1 PERSONAL SAFETY GEAR

3.2.1.1 Personnel must use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes. Plastic netting on glass beakers, flasks and other glassware minimizes breakage and subsequent shattering of the glass.

3.2.2 LABORATORY SAFETY EQUIPMENT

3.2.2.1 Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, chemical spill clean-up kits, and eye fountains.

3.2.2.2 Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

3.3 GENERAL LABORATORY AND FIELD OPERATIONS

3.3.1 Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see safety manuals listed in Section 3, Health and Safety, Subsection 3.5). It is recommended that personnel collecting samples and performing toxicity tests should not work alone.

3.3.2 Because the chemical composition of effluents is usually only poorly known, they should be considered as potential health hazards, and exposure to them should be minimized. Fume and canopy hoods over the toxicity test areas must be used whenever possible.

3.3.3 It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

3.3.4 All containers should be adequately labeled to indicate their contents.

3.3.5 Staff should be familiar with safety guidelines on Material Safety Data Sheets for reagents and other chemicals purchased from suppliers. Incompatible materials should not be stored together. Good housekeeping contributes to safety and reliable results.

3.3.6 Strong acids and volatile organic solvents employed in glassware cleaning must be used in a fume hood or under an exhaust canopy over the work area.

3.3.7 Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.8 Mobile laboratories should be properly grounded to protect against electrical shock.

3.4 DISEASE PREVENTION

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, polio, and hepatitis B.

3.5 SAFETY MANUALS

3.5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general safety manuals, including USEPA (1986e), and Walters and Jameson (1984).

3.6 WASTE DISPOSAL

3.6.1 Wastes generated during toxicity testing must be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, state and Federal rules and regulations. It is extremely important that these rules and regulations be known, understood, and complied with by all persons responsible for, or otherwise involved in, performing toxicity testing activities. Local fire officials should be notified of any potentially hazardous conditions.

SECTION 4

QUALITY ASSURANCE

4.1 INTRODUCTION

4.1.1 Development and maintenance of a toxicity test laboratory quality assurance (QA) program (USEPA, 1991b) requires an ongoing commitment by laboratory management. Each toxicity test laboratory should (1) appoint a quality assurance officer with the responsibility and authority to develop and maintain a QA program, (2) prepare a quality assurance plan with stated data quality objectives (DQOs), (3) prepare written descriptions of laboratory standard operating procedures (SOPs) for culturing, toxicity testing, instrument calibration, sample chain-of-custody procedures, laboratory sample tracking system, glassware cleaning, etc., and (4) provide an adequate, qualified technical staff for culturing and toxicity testing the organisms, and suitable space and equipment to assure reliable data.

4.1.2 QA practices for toxicity testing laboratories must address all activities that affect the quality of the final effluent toxicity data, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation.

4.1.3 Quality control practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance and general guidance on good laboratory practices and laboratory evaluation related to toxicity testing, see FDA (1978); USEPA (1979d); USEPA (1980b); USEPA (1980c); USEPA (1991c); DeWoskin (1984); and Taylor (1987).

4.1.4 Guidelines for the evaluation of laboratory performing toxicity tests and laboratory evaluation criteria are found in USEPA (1991c).

4.2 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.2.1 Separate test organism culturing and toxicity testing areas should be provided to avoid possible loss of cultures due to cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation

areas into organism culturing or testing areas, and from testing and sample preparation areas into culture rooms.

4.2.2 Laboratory and toxicity test temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 5, Facilities, Equipment, and Supplies; and specific toxicity test method).

4.3 TEST ORGANISMS

4.3.1 The test organisms used in the procedures described in this manual are the red abalone, *Haliotis rufescens*; the Pacific oyster, *Crassostrea gigas*, and mussel, *Mytilus spp.*; the topsmelt, *Atherinops affinis*; the mysid, *Holmesimysis costata*; the sea urchin, *Strongylocentrotus purpuratus*, and the sand dollar *Denstraster excentricus*; and the giant kelp, *Macrocystis pyrifera*. The organisms used should be disease-free and appear healthy, behave normally, feed well, and have low mortality in cultures, during holding, and in test control. Test organisms should be positively identified to species (see Section 6, Test Organisms).

4.4 LABORATORY WATER USED FOR CULTURING AND TEST DILUTION WATER

4.4.1 The quality of water used for test organism culturing and for dilution water used in toxicity tests is extremely important. Water for these two uses should come from the same source. The dilution water used in effluent toxicity tests will depend on the objectives of the study and logistical constraints, as discussed in Section 7, Dilution Water. The dilution water used in the toxicity tests may be natural seawater, hypersaline brine (100‰) prepared from natural seawater, or artificial seawater prepared from commercial sea salts, such as FORTY FATHOMS® or HW MARINEMIX®, if recommended in the method. GP2 synthetic seawater, made from reagent grade chemical salts in conjunction with natural seawater, may also be used if recommended. Types of water are discussed in Section 5, Facilities, Equipment, and Supplies. Water used for culturing and test dilution water should be analyzed for toxic metals and organics at least annually or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. The concentration of the metals, Al, As, Cr, Co, Cu, Fe, Pb, Ni, Zn, expressed as total metal, should not exceed 1 µg/L each, and Cd, Hg, and Ag, expressed as total metal, should not exceed 100 ng/L each. Total organochlorine pesticides plus PCBs should be less than 50 ng/L (APHA, 1992). Pesticide

concentrations should not exceed USEPA's National Ambient Water Quality chronic criteria values where available.

4.5 EFFLUENT AND RECEIVING WATER SAMPLING AND HANDLING

4.5.1 Sample holding times and temperatures of effluent samples collected for on-site and off-site testing must conform to conditions described in Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

4.6 TEST CONDITIONS

4.6.1 Water temperature and salinity must be maintained within the limits specified for each test. The temperature of test solutions must be measured by placing the thermometer or probe directly into the test solutions, or by placing the thermometer in equivalent volumes of water in surrogate vessels positioned at appropriate locations among the test vessels. Temperature should be recorded continuously in at least one vessel during the duration of each test. Test solution temperatures must be maintained within the limits specified for each test. DO concentrations and pH should be checked as specified in each test method.

4.7 QUALITY OF TEST ORGANISMS

4.7.1 If the laboratory performs short-term chronic toxicity tests routinely but does not have an ongoing test organism culturing program and must obtain the test organisms from an outside source, the sensitivity of a batch of test organisms must be determined with a reference toxicant in a short-term chronic toxicity test performed monthly (see Section 4, Quality Assurance, Subsections 4.14, 4.15, 4.16, and 4.17). Where acute or short-term chronic toxicity tests are performed with effluents or receiving waters using test organisms obtained from outside the test laboratory, concurrent toxicity tests of the same type must be performed with a reference toxicant, unless the test organism supplier provides control chart data from at least the last five monthly short-term chronic toxicity tests using the same reference toxicants and test conditions (see Section 6, Test Organisms).

4.7.2 The supplier should certify the species identification of the test organisms, and provide the taxonomic reference (citation and page) or name(s) of the taxonomic expert(s) consulted.

4.7.3 If the laboratory maintains breeding cultures, the sensitivity of the offspring should be determined in a short-term chronic toxicity test performed with a reference toxicant at least once each month (see Section 4, Quality Assurance, Subsection 4.14, 4.15, 4.16, and 4.17). If preferred, this reference toxicant test may be performed concurrently with an effluent toxicity test. However, if a given species of test organism produced by inhouse cultures is used only monthly, or less frequently in toxicity tests, a reference toxicant test must be performed concurrently with each short-term chronic effluent and/or receiving water toxicity test.

4.7.4 If a routine reference toxicant test fails to meet acceptability criteria, the test must be immediately repeated. If the failed reference toxicant test was being performed concurrently with an effluent or receiving water toxicity test, both tests must be repeated (For exception, see Section 4, Quality Assurance, Subsection 4.16.5).

4.8 **FOOD QUALITY**

4.8.1 The nutritional quality of the food used in culturing and testing fish and invertebrates is an important factor in the quality of the toxicity test data. This is especially true for the unsaturated fatty acid content of brine shrimp nauplii, *Artemia*. Problems with the nutritional suitability of the food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. *Artemia* cysts and other foods must be obtained as described in Section 5, Facilities, Equipment, and Supplies.

4.8.2 Problems with the nutritional suitability of food will be reflected in the survival, growth, development and reproduction of the test organisms in cultures and toxicity tests. If a batch of food is suspected to be defective, the performance of organisms fed with the new food can be compared with the performance of organisms fed with a food of known quality in side-by-side tests. If the food is used for culturing, its suitability should be determined using a short-term chronic test which will determine the affect of food quality on growth or reproduction of each of the relevant test species in culture, using four replicates with each food source. Where applicable, foods used only in chronic toxicity tests can be compared with a food of known quality in side-by-side, multi-concentration chronic tests, using the reference toxicant regularly employed in the laboratory QA program. For list of commercial sources of *Artemia* cysts, see Table 2 of Section 5, Facilities, Equipment, and Supplies.

4.17 REFERENCE TOXICANTS

4.17.1 Reference toxicants such as zinc sulfate ($ZnSO_4$), cadmium chloride ($CdCl_2$), copper sulfate ($CuSO_4$), and copper chloride ($CuCl_2$), are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests. NERL-Cincinnati plans to release USEPA-certified solutions of cadmium and copper for use as reference toxicants, through cooperative research and development agreements with commercial suppliers, and will continue to develop additional reference toxicants for future release. Interested parties can determine the availability of "EPA Certified" reference toxicants by checking the NERL-Cincinnati electronic bulletin board, using a modem to access the following telephone number: 513-569-7610. Standard reference materials also can be obtained from commercial supply houses, or can be prepared inhouse using reagent grade chemicals. The regulatory agency should be consulted before reference toxicant(s) are selected and used.

4.18 RECORD KEEPING

4.18.1 Proper record keeping is important. A complete file must be maintained for each individual toxicity test or group of tests on closely related samples. This file must contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the toxicity test(s); chemical analysis data on the sample(s); detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions employed; and results of reference toxicant tests. Laboratory data should be recorded on a real-time basis to prevent the loss of information or inadvertent introduction of errors into the record. Original data sheets should be signed and dated by the laboratory personnel performing the tests.

4.18.2 The regulatory authority should retain records pertaining to discharge permits. Permittees are required to retain records pertaining to permit applications and compliance for a minimum of 3 years [40 CFR 122.41(j)(2)].

SECTION 5

FACILITIES, EQUIPMENT, AND SUPPLIES

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities must include equipment for rearing and/or holding organisms. Culturing facilities for test organisms may be desirable in fixed laboratories which perform large numbers of tests. Temperature control can be achieved using circulating water baths, heat exchangers, or environmental chambers. Water used for rearing, holding, acclimating, and testing organisms may be natural seawater or water made up from hypersaline brine derived from natural seawater, or water made up from reagent grade chemicals (GP2) or commercial (FORTY FATHOMS® or HW MARINEMIX®) artificial sea salts when specifically recommended in the method. Air used for aeration must be free of oil and toxic vapors. Oil-free air pumps should be used where possible. Particulates can be removed from the air using BALSTON® Grade BX or equivalent filters (Balston, Inc., Lexington, Massachusetts), and oil and other organic vapors can be removed using activated carbon filters (BALSTON®, C-1 filter, or equivalent).

5.1.2 The facilities must be well ventilated and free of fumes. Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories and/or sample handling areas is not circulated to test organism culture rooms or toxicity test rooms, or that air from toxicity test rooms does not contaminate culture areas. Sample preparation, culturing, and toxicity testing areas should be separated to avoid cross-contamination of cultures or toxicity test solutions with toxic fumes. Air pressure differentials between such rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely-fitting doors. Organisms should be shielded from external disturbances.

5.1.3 Materials used for exposure chambers, tubing, etc., which come in contact with the effluent and dilution water, should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON®) should be used whenever possible to minimize sorption and leaching of toxic substances. These materials may be reused following decontamination. Containers made of plastics, such as polyethylene, polypropylene, polyvinyl chloride, TYGON®, etc., may be used as test chambers or to ship, store, and transfer effluents and receiving waters, but they should not be reused unless absolutely necessary, because they might carry over

1. Sensitive species may not be present in the receiving water because of previous exposure to the effluent or other pollutants.
2. It is often difficult to collect organisms of the required age and quality from the receiving water.
3. Most states require collection permits, which may be difficult to obtain. Therefore, it is usually more cost effective to culture the organisms in the laboratory or obtain them from private, state, or Federal sources.
4. The required QA/QC records, such as the single-laboratory precision data, would not be available for non standardized test species.
5. Since it is mandatory that the identity of test organisms is known to the species level, it would be necessary to examine each organism caught in the wild to confirm its identity, which would usually be impractical or, at the least, very stressful to the organisms.
6. Test organisms obtained from the wild must be observed in the laboratory for a minimum of one week prior to use, to ensure that they are free of signs of parasitic or bacterial infections and other adverse effects. Fish captured by electroshocking must not be used in toxicity testing.

6.2.5.2 Guidelines for collection of naturally occurring organisms are provided in USEPA, (1973); USEPA, (1990a) and USEPA, (1993a).

6.2.5.3 Regardless of their source, test organisms and broodstock should be carefully observed to ensure that they are free of signs of stress and disease, and in good physical condition. Some species of test organisms, such as trout, can be obtained from stocks certified as "disease-free."

6.3 LIFE STAGE

6.3.1 Young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as juvenile mysids and larval fish, is required for all tests. There may be special cases, however, where the limited availability of organisms will require some deviation from the recommended life stage. In a given test, all organisms should be approximately the same age and should be taken from the same source. Since age may affect the results of the tests, it would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility.

6.4 LABORATORY CULTURING

6.4.1 Instructions for culturing, holding and/or handling the recommended test organisms and broodstock are included in specified test methods.

6.5 HOLDING AND HANDLING TEST ORGANISMS

6.5.1 Test organisms should not be subjected to changes of more than 3°C in water temperature or 3‰ in salinity in any 12 h period.

6.5.2 Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that are dropped or touch dry surfaces or are injured during handling must be discarded. Dipnets are best for handling larger organisms. These nets are commercially available or can be made from small-mesh nylon netting, silk bolting cloth, plankton netting, or similar material. Wide-bore, smooth glass tubes (4 to 8 mm ID) with rubber bulbs or pipettors (such as a PROPIPETTE® or other pipettor) should be used for transferring smaller organisms such as mysids, and larval fish.

6.5.3 Holding tanks for broodstock are usually supplied with a good quality water (see Section 5, Facilities, Equipment, and Supplies) with a flow-through rate of at least two tank-volumes per day. Otherwise, use a recirculation system where the water flows through an activated carbon or undergravel filter to remove dissolved metabolites. Culture water can also be piped through high intensity ultraviolet light sources for disinfection, and to photo-degrade dissolved organics.

6.5.4 Crowding should be avoided because it will stress the organisms and lower the DO concentrations to unacceptable levels. The DO must be maintained at a minimum of 4.0 mg/L. The solubility of oxygen depends on temperature, salinity, and altitude. Aerate gently if necessary.

6.5.5 The organisms should be observed carefully each day for signs of disease, stress, physical damage, or mortality. Dead and abnormal organisms should be removed as soon as observed. It is not uncommon for some larval fish and mysid mortality (5-10%) to occur during the first 48 h in a holding tank because of individuals that failed to feed and die of starvation.

6.5.6 Organisms in the holding tanks should generally be fed as in the cultures (see culturing methods in the respective methods).

6.5.7 Broodstock and test organisms should be observed carefully each day for signs of disease, stress, physical damage, and mortality. Dead and abnormal specimens should be removed as soon as observed.

6.5.8 A daily record of feeding, behavioral observations, and mortality should be maintained.

6.6 TRANSPORTATION TO THE TEST SITE

6.6.1 Test organisms and broodstock are transported from the base or supply laboratory to a remote test site (see the appropriate test method). Adequate DO is maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags. Another method commonly used to maintain sufficient DO during shipment is to aerate with an airstone which is supplied from a portable pump. The DO concentration must not fall below 4.0 mg/L.

6.6.2 Upon arrival at the test site, organisms are transferred to receiving water if receiving water is to be used as the test dilution water. All but a small volume of the holding water (approximately 5%) is removed by siphoning, and replaced slowly over a 10 to 15 minute period with dilution water. If receiving water is used as dilution water, caution must be exercised in exposing the test organisms to it, because of the possibility that it might be toxic. For this reason, it is recommended that only approximately 10% of the test organisms be exposed initially to the dilution water. If this group does not show excessive mortality or obvious signs of stress in a few hours, the remainder of the test organisms are transferred to the dilution water.

6.6.3 A group of organisms must not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed, or if mortality appears to exceed 10% preceding the test. If the organisms fail to meet these criteria, the entire group must be discarded and a new group obtained. The mortality may be due to the presence of toxicity, if receiving water is used as dilution water, rather than a diseased condition of the test organisms. If the acclimation process is repeated with a new group of test organisms and excessive mortality occurs, it is recommended that an alternative source of dilution water be used.

6.6.4 The marine organisms may be used at all concentrations of effluent by adjusting the salinity of the effluent to salinities specified for the appropriate species test condition or to the salinity approximating that of the receiving water, by adding sufficient dry ocean salts, such as FORTY FATHOMS®, or equivalent, GP2, or hypersaline brine.

6.6.5 Saline dilution water can be prepared with deionized water or a freshwater such as well water or a suitable surface water. If dry ocean salts are used, care must be taken to ensure that the added salts are completely dissolved and the solution is aerated 24 h before the test organisms are placed in the solutions. The test organisms should be acclimated in synthetic saline water prepared with the dry salts. **Caution:** addition of dry ocean salts to dilution water may result in an increase in pH. (The pH of estuarine and coastal saline waters is normally 7.5-8.3).

6.6.6 All effluent concentrations and the control(s) used in a test should have the same salinity. The change in salinity upon acclimation at the desired test dilution should not exceed 6‰. The required salinities for culturing and toxicity tests with estuarine and marine species are listed in the test method sections.

6.7 **TEST ORGANISM DISPOSAL**

6.7.1 When the toxicity test(s) is concluded, all test organisms (including controls) should be humanely destroyed and disposed of in an appropriate manner.

SECTION 7

DILUTION WATER

7.1 TYPES OF DILUTION WATER

7.1.1 The type of dilution water used in effluent toxicity tests will depend largely on the objectives of the study.

7.1.1.1 If the objective of the test is to estimate the chronic toxicity of the effluent, which is a primary objective of NPDES permit-related toxicity testing, a standard dilution water defined in each test method is used. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.2 If the objective of the test is to estimate the chronic toxicity of the effluent in uncontaminated natural seawater (receiving water), or with other uncontaminated natural seawater. Seasonal variations in the quality of receiving waters may affect effluent toxicity. Therefore, the salinity of saline receiving water samples should be determined before each use. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.3 If the objective of the test is to determine the additive or mitigating effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected outside the influence of the outfall. A second set of controls, using culture water, should be included in the test.

7.2 STANDARD, SYNTHETIC DILUTION WATER

7.2.1 Standard, synthetic, dilution water is prepared with reagent water and reagent grade chemicals (GP2) or commercial sea salts (FORTY FATHOMS®, HW MARINEMIX®) (Table 3). The source water for the deionizer can be ground water or tap water. This synthetic water should be used only if specified in the test method. These salts may be directly added to effluents to achieve appropriate salinities for testing high effluent concentration (e.g., greater than 60% effluent) where the use of hypersaline brine is insufficient to obtain test salinities.

7.2.2 REAGENT WATER USED TO PREPARE STANDARD, SYNTHETIC, DILUTION WATER

7.2.2.1 Reagent water is defined as distilled or deionized water that does not contain substances which are toxic to the test organisms. Deionized water is obtained from a MILLIPORE MILLI-Q®, MILLIPORE® QPAK™$_2$ or equivalent system. It is advisable to provide a preconditioned (deionized) feed water by using a Culligan®, Continental®, or equivalent system in front of the MILLI-Q® System to extend the life of the MILLI-Q® cartridges (see Section 5, Facilities, Equipment, and Supplies).

7.2.2.2 The recommended order of the cartridges in a four-cartridge deionizer (i.e., MILLI-Q® System or equivalent) is: (1) ion exchange, (2) ion exchange, (3) carbon, and (4) organic cleanup (such as ORGANEX-Q®, or equivalent), followed by a final bacteria filter. The QPAK™$_2$ water system is a sealed system which does not allow for the rearranging of the cartridges. However, the final cartridge is an ORGANEX-Q® filter, followed by a final bacteria filter. Commercial laboratories using this system have not experienced any difficulty in using the water for culturing or testing. Reference to the MILLI-Q® systems throughout the remainder of the manual includes all MILLIPORE® or equivalent systems.

7.2.3 STANDARD, SYNTHETIC SEAWATER

7.2.3.1 To prepare 20 L of a standard, synthetic, reconstituted seawater (modified GP2), using reagent grade chemicals (Table 2), with a salinity of 31‰, follow the instructions below. Other salinities can be prepared by making the appropriate dilutions. Larger or smaller volumes of modified GP2 can be prepared by using proportionately larger or smaller amounts of salts and dilution water.

1. Place 20 L of MILLI-Q® or equivalent deionized water in a properly cleaned plastic carboy.
2. Weigh reagent grade salts listed in Table 2 and add, one at a time, to the deionized water. Stir well after adding each salt.
3. Aerate the final solution at a rate of 1 L/h for 24 h.
4. Check the pH and salinity.

7.2.3.2 Synthetic seawater can also be prepared by adding commercial sea salts, such as FORTY FATHOMS®, HW MARINEMIX®, or equivalent, to deionized water. For example, thirty-one parts per thousand (31‰) FORTY FATHOMS® can be prepared by dissolving 31 g of sea salts per liter of deionized water. The salinity of the resulting solutions should be checked with a refractometer.

TABLE 2. PREPARATION OF GP2 ARTIFICIAL SEAWATER USING REAGENT GRADE CHEMICALS[1,2,3]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
KCl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

[1] Modified GP2 from Spotte et al. (1984).
[2] The constituent salts and concentrations were taken from USEPA (1993a). The salinity is 30.89 g/L.
[3] GP2 can be diluted with deionized (DI) water to the desired test salinity.

7.2.4 Artificial seawater is to be used only if specified in the method. The suitability of GP2 as a medium for culturing organisms has not been determined.

7.3 USE OF RECEIVING WATER AS DILUTION WATER

7.3.1 If the objectives of the test require the use of uncontaminated receiving water as dilution water, and the receiving water is uncontaminated, it may be possible to collect a sample of the receiving water close to the outfall, but away

from or beyond the influence of the effluent. However, if the receiving water is contaminated, it may be necessary to collect the sample in an area "remote" from the discharge site, matching as closely as possible the physical and chemical characteristics of the receiving water near the outfall.

7.3.2 The sample should be collected immediately prior to the test, but never more than 96 h before the test begins. Except where it is used within 24 h, or in the case where large volumes are required for flow through tests, the sample should be chilled to 4°C during or immediately following collection, and maintained at that temperature prior to use in the test.

7.3.3 The investigator should collect uncontaminated water having a salinity as near as possible to the salinity of the receiving water at the discharge site. Water should be collected at slack high tide, or within one hour after high tide. If there is reason to suspect contamination of the water in the estuary, it is advisable to collect uncontaminated water from an adjacent estuary. At times it may be necessary to collect water at a location closer to the open sea, where the salinity is relatively high. In such cases, deionized water or uncontaminated freshwater is added to the saline water to dilute it to the required test salinity. Where necessary, the salinity of a surface water can be increased by the addition of artificial sea salts, such as FORTY FATHOMS®, HW MARINEMIX®, or equivalent, GP2, a natural seawater of higher salinity, or hypersaline brine. Instructions for the preparation of hypersaline brine by concentrating natural seawater are provided below.

7.3.4 Receiving water containing debris or indigenous organisms, that may be confused with or attack the test organisms, should be filtered through a sieve having 60 µm mesh openings prior to use.

7.3.5 HYPERSALINE BRINE

7.3.5.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

7.3.5.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity.

7.3.5.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

7.3.5.4 Freeze Preparation of Brine

7.3.5.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20°C until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

7.3.5.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

7.3.5.4.3 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

7.3.5.5 Heat Preparation of Brine

7.3.5.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic

materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

7.3.5.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

7.3.5.5.3 Seawater should be filtered to at least 10 µm before being put into the brine generator. The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

7.3.5.5.4 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

7.3.5.6 Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. Thus, the proportion is one part brine plus 1.94 reagent water.

7.3.5.8 To make 1 L of seawater at 34‰ salinity from a hypersaline brine of 100‰, 340 mL of brine and 660 mL of reagent water are required.

7.4 USE OF TAP WATER AS DILUTION WATER

7.4.1 The use of tap water in the reconstituting of synthetic (artificial) seawater as dilution water is discouraged unless it is dechlorinated and fully treated. Tap water can be dechlorinated by deionization, carbon filtration, or the use of

sodium thiosulfate. Use of 3.6 mg/L (anhydrous) sodium thiosulfate will reduce 1.0 mg chlorine/L (APHA, 1992). Following dechlorination, total residual chlorine should not exceed 0.01 mg/L. Because of the possible toxicity of thiosulfate to test organisms, a control lacking thiosulfate should be included in toxicity tests utilizing thiosulfate-dechlorinated water.

7.4.2 To be adequate for general laboratory use following dechlorination, the tap water is passed through a deionizer and carbon filter to remove toxic metals and organics, and to control hardness and alkalinity.

7.5 DILUTION WATER HOLDING

7.5.1 A given batch of dilution water should not be used for more than 14 days following preparation because of the possible build up of bacterial, fungal, or algal slime growth and the problems associated with it. The container should be kept covered and the contents should be protected from light.

SECTION 8

EFFLUENT AND RECEIVING WATER SAMPLING, SAMPLE HANDLING, AND SAMPLE PREPARATION FOR TOXICITY TESTS

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point should be the same as that specified in the NPDES discharge permit (USEPA, 1988b). Conditions for exception would be: (1) better access to a sampling point between the final treatment and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to municipal waste treatment plants or separate wastewater streams in industrial facilities prior to their being combined with other wastewater streams or non-contact cooling water, additional sampling points may be chosen.

8.1.2 The decision on whether to collect grab or composite samples is based on the objectives of the test and an understanding of the short and long-term operations and schedules of the discharger. If the effluent quality varies considerably with time, which can occur where holding times are short, grab samples may seem preferable because of the ease of collection and the potential of observing peaks (spikes) in toxicity. However, the sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitively large number of separate samples and tests. Collection of a 24-h composite sample, however, may dilute toxicity spikes, and average the quality of the effluent over the sampling period. Sampling recommendations are provided below (also see USEPA, 1993a).

8.1.3 Aeration during collection and transfer of effluents should be minimized to reduce the loss of volatile chemicals.

8.1.4 Details of date, time, location, duration, and procedures used for effluent sample and dilution water collection should be recorded.

8.2 EFFLUENT SAMPLE TYPES

8.2.1 The advantages and disadvantages of effluent grab and composite samples are listed below:

8.2.1.1 GRAB SAMPLES

Advantages:

1. Easy to collect; require a minimum of equipment and on-site time.
2. Provide a measure of instantaneous toxicity. Toxicity spikes are not masked by dilution.

Disadvantages:

1. Samples are collected over a very short period of time and on a relatively infrequent basis. The chances of detecting a spike in toxicity would depend on the frequency of sampling, and the probability of missing spikes is high.

8.2.1.2 COMPOSITE SAMPLES:

Advantages:

1. A single effluent sample is collected over a 24-h period.
2. The sample is collected over a much longer period of time than grab samples and contains all toxicity spikes.

Disadvantages:

1. Sampling equipment is more sophisticated and expensive, and must be placed on-site for at least 24 h.
2. Toxicity spikes may not be detected because they are masked by dilution with less toxic wastes.

8.3 EFFLUENT SAMPLING RECOMMENDATIONS

8.3.1 When tests are conducted on-site, test solutions can be renewed daily with freshly collected samples.

8.3.2 When 7-day tests are conducted off-site, a minimum of three samples are collected. If these samples are collected on Test Days 1, 3, and 5, the first sample would be used for test initiation, and for test solution renewal on Day 2. The second sample would be used for test solution renewal on Days 3 and 4. The third sample would be used for test solution renewal on Days 5, 6, and 7.

8.3.3 Sufficient sample must be collected to perform the required toxicity and chemical tests. A 4-L (1-gal) CUBITAINER® will provide sufficient sample volume for most tests.

8.3.4 THE FOLLOWING EFFLUENT SAMPLING METHODS ARE RECOMMENDED:

8.3.4.1 Continuous Discharges

1. If the facility discharge is continuous, but the calculated retention time of the continuously discharged effluent is less than 14 days and the variability of the effluent toxicity is unknown, at a minimum, four grab samples or four composite samples are collected over a 24-h period. For example, a grab sample is taken every 6 h (total of four samples) and each sample is used for a separate toxicity test, or four successive 6-h composite samples are taken and each is used in a separate test.
2. If the calculated retention time of a continuously discharged effluent is greater than 14 days, or if it can be demonstrated that the wastewater does not vary more than 10% in toxicity over a 24-h period, regardless of retention time, a single grab sample is collected for a single toxicity test.
3. The retention time of the effluent in the wastewater treatment facility may be estimated from calculations based on the volume of the retention basin and rate of wastewater inflow. However, the calculated retention time may be much greater than the actual time because of short-circuiting in the holding basin. Where short-circuiting is suspected, or sedimentation may have reduced holding basin capacity, a more accurate estimate of the retention time can be obtained by carrying out a dye study.

8.3.4.2 Intermittent Discharges

8.3.4.2.1 If the facility discharge is intermittent, a grab sample is collected midway during each discharge period. Examples of intermittent discharges are:

1. When the effluent is continuously discharged during a single 8-h work shift (one sample is collected), or two successive 8-h work shifts (two samples are collected).
2. When the facility retains the wastewater during an 8-h work shift, and then treats and releases the wastewater as a batch discharge (one sample is collected).
3. When the facility discharges wastewater to an estuary only during an outgoing tide, usually during the 4 h following slack high tide (one sample is collected).
4. At the end of a shift, clean up activities may result in the discharge of a slug of toxic waste (one sample is collected).

8.4 RECEIVING WATER SAMPLING

8.4.1 Logistical problems and difficulty in securing sampling equipment generally preclude the collection of composite receiving water samples for toxicity tests. Therefore, based on the requirements of the test, a single grab sample or series of daily grab samples of receiving water is collected for use in the test.

8.4.2 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples should be collected at mid-depth.

8.4.3 To determine the extent of the zone of toxicity in the receiving water at estuarine and marine effluent sites, receiving water samples are collected at several distances away from the discharge. The time required for the effluent-receiving-water mixture to travel to sampling points away from the point of discharge, and the rate and degree of mixing, may be difficult to ascertain. Therefore, it may not be possible to correlate receiving water toxicity with effluent toxicity at the discharge point unless a dye study is performed. The toxicity of receiving water samples from five stations in the discharge plume can be evaluated using the same number of test vessels and test organisms as used in one effluent toxicity test with five effluent dilutions.

8.5 EFFLUENT AND RECEIVING WATER SAMPLE HANDLING, PRESERVATION, AND SHIPPING

8.5.1 Unless the samples are used in an on-site toxicity test the day of collection, it is recommended that they be held at approximately 4°C until used to inhibit microbial degradation, chemical transformations, and loss of highly volatile toxic substances.

8.5.2 Composite samples should be chilled as they are collected. Grab samples should be chilled immediately following collection.

8.5.3 If the effluent has been chlorinated, total residual chlorine must be measured immediately following sample collection.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the elapsed time (holding time) from sample collection to first use of the sample in test initiation must not exceed 36 h. EPA believes that 36 h

is adequate time to deliver the sample to the laboratories performing the test in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the USEPA Regional Administrator under 40 CFR 136.3(e), must include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond 36 h. However, in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, the original sample may also be used to prepare test solutions for renewal at 24 h and 48 h after test initiation, if stored at 4°C, with minimum head space, as described in Paragraph 8.5. Guidance for determining the persistence of the sample is provided in Subsection 8.7.

8.5.5 To minimize the loss of toxicity due to volatilization of toxic constituents, all sample containers should be "completely" filled, leaving no air space between the contents and the lid.

8.5.6 SAMPLES USED IN ON-SITE TESTS

8.5.6.1 Samples collected for on-site tests should be used within 24 h.

8.5.7 SAMPLES SHIPPED TO OFF SITE FACILITIES

8.5.7.1 Samples collected for off site toxicity testing are to be chilled to 4°C during or immediately after collection, and shipped iced to the performing laboratory. Sufficient ice should be placed with the sample in the shipping container to ensure that ice will still be present when the sample arrives at the laboratory and is unpacked. Insulating material must not be placed between the ice and the sample in the shipping container.

8.5.7.2 Samples may be shipped in one or more 4-L (1-gal) CUBITAINERS® or new plastic "milk" jugs. All sample containers should be rinsed with dilution water before being filled with sample. After use with receiving water or effluents, CUBITAINERS® and plastic jugs are punctured to prevent reuse.

8.5.7.3 Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. Saturday and Sunday shipping and receiving schedules of private carriers vary with the carrier.

8.6 SAMPLE RECEIVING

8.6.1 Upon arrival at the laboratory, samples are logged in and the temperature is measured and recorded. If the samples are not immediately prepared for testing, they are stored at approximately 4°C until used.

8.6.2 Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory, and the sample holding time should not exceed 36 h unless a variance has been granted by the NPDES permitting authority.

8.7 PERSISTENCE OF EFFLUENT TOXICITY DURING SAMPLE SHIPMENT AND HOLDING

8.7.1 The persistence of the toxicity of an effluent prior to its use in a toxicity test is of interest in assessing the validity of toxicity test data, and in determining the possible effects of allowing an extension of the holding time. Where a variance in holding time (>36 h, but ≤72 h) is requested by a permittee (See subsection 8.5.4), information on the effects of the extension in holding time on the toxicity of the samples must be obtained by comparing the results of multi-concentration chronic toxicity tests performed on effluent samples held 36 h with toxicity test results using the same samples after they were held for the requested, longer period. The portion of the sample set aside for the second test must be held under the same conditions as during shipment and holding.

8.8 PREPARATION OF EFFLUENT AND RECEIVING WATER SAMPLES FOR TOXICITY TESTS

8.8.1 Adjust the sample salinity to the level appropriate for objectives of the study using hypersaline brine or artificial sea salts.

8.8.2 When aliquots are removed from the sample container, the head space above the remaining sample should be held to a minimum. Air which enters a container upon removal of sample should be expelled by compressing the container before reclosing, if possible (i.e., where a CUBITAINER® used), or by using an appropriate discharge valve (spigot).

8.8.3 It may be necessary to first coarse-filter samples through a NYLON® sieve having 2 to 4 mm mesh openings to remove debris and/or break up large floating or suspended solids. If samples contain indigenous organisms that may attack or be confused with the test organisms, the samples must be filtered through a sieve with 60 µm mesh openings. Since filtering may increase the dissolved oxygen (DO) in an effluent, the DO should be determined prior to

filtering. Low dissolved oxygen concentrations will indicate a potential problem in performing the test. **Caution:** filtration may remove some toxicity.

8.8.4 If the samples must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved oxygen and nitrogen may become a problem. To avoid this problem, the effluent and dilution water are checked with a DO probe after reaching test temperature and, if the DO is greater than 100% saturation or lower than 4.0 mg/L, based on temperature and salinity, the solutions are aerated moderately (approximately 500 mL/min) for a few minutes, using an airstone, until the DO is lowered to 100% saturation (Table 3) or until the DO is within the prescribed range (≥4.0 mg/L). **Caution:** avoid excessive aeration.

8.8.4.1 Aeration during the test may alter the results and should be used only as a last resort to maintain the required DO. Aeration can reduce the apparent toxicity of the test solutions by stripping them of highly volatile toxic substances, or change the toxicity by altering the pH. However, the DO in the test solution must not be permitted to fall below 4.0 mg/L.

8.8.4.2 In static tests (non-renewal or renewal) low DOs may commonly occur in the higher concentrations of wastewater. Aeration is accomplished by bubbling air through a pipet at the rate of 100 bubbles/min. If aeration is necessary, all test solutions must be aerated. It is advisable to monitor the DO closely during the first few hours of the test. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8 h after the test is started. Unless aeration is initiated during the first 8 h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test.

8.8.5 At a minimum, pH, or salinity, and total residual chlorine are measured in the undiluted effluent or receiving water, and pH and salinity are measured in the dilution water.

8.8.6 Total ammonia is measured in effluent and receiving water samples where toxicity may be contributed by unionized ammonia (i.e., where total ammonia ≥5 mg/L). The concentration (mg/L) of unionized (free) ammonia in a sample is a function of temperature and pH, and is calculated using the percentage value obtained from Table 4,

TABLE 3. OXYGEN SOLUBILITY (MG/L) IN WATER AT EQUILIBRIUM WITH AIR AT 760 MM HG (AFTER RICHARDS AND CORWIN, 1956)

TEMP	SALINITY (‰)									
(C°)	0	5	10	15	20	25	30	35	40	43
0	14.2	13.8	13.4	12.9	12.5	12.1	11.7	11.2	10.8	10.6
1	13.8	13.4	13.0	12.6	12.2	11.8	11.4	11.0	10.6	10.3
2	13.4	13.0	12.6	12.2	11.9	11.5	11.1	10.7	10.3	10.0
3	13.1	12.7	12.3	11.9	11.6	11.2	10.8	10.4	10.0	9.8
4	12.7	12.3	12.0	11.6	11.3	10.9	10.5	10.1	9.8	9.5
5	12.4	12.0	11.7	11.3	11.0	10.6	10.2	9.8	9.5	9.3
6	12.1	11.7	11.4	11.0	10.7	10.3	10.0	9.6	9.3	9.1
8	11.5	11.2	10.8	10.5	10.2	9.8	9.5	9.2	8.9	8.7
10	10.9	10.7	10.3	10.0	9.7	9.4	9.1	8.8	8.5	8.3
12	10.5	10.2	9.9	9.6	9.3	9.0	8.7	8.4	8.1	7.9
14	10.0	9.7	9.5	9.2	8.9	8.6	8.3	8.1	7.8	7.6
16	9.6	9.3	9.1	8.8	8.5	8.3	8.0	7.7	7.5	7.3
18	9.2	9.0	8.7	8.5	8.2	8.0	7.7	7.5	7.2	7.1
20	8.9	8.6	8.4	8.1	7.9	7.7	7.4	7.2	6.9	6.8
22	8.6	8.4	8.1	7.9	7.6	7.4	7.2	6.9	6.7	6.6
24	8.3	8.1	7.8	7.6	7.4	7.2	6.9	6.7	6.5	6.4
26	8.1	7.8	7.6	7.4	7.2	7.0	6.7	6.5	6.3	6.1
28	7.8	7.6	7.4	7.2	7.0	6.8	6.5	6.3	6.1	6.0
30	7.6	7.4	7.1	6.9	6.7	6.5	6.3	6.1	5.9	5.8
32	7.3	7.1	6.9	6.7	6.5	6.3	6.1	5.9	5.7	5.6

TABLE 4. PERCENT UNIONIZED NH_3 IN AQUEOUS AMMONIA SOLUTIONS: TEMPERATURE 15-26°C AND pH 6.0-8.9[1]

pH	TEMPERATURE (°C)											
	15	16	17	18	19	20	21	22	23	24	25	26
6.0	0.0274	0.0295	0.0318	0.0343	0.0369	0.0397	0.0427	0.0459	0.0493	0.0530	0.0568	0.0610
6.1	0.0345	0.0372	0.0400	0.0431	0.0464	0.0500	0.0537	0.0578	0.0621	0.0667	0.0716	0.0768
6.2	0.0434	0.0468	0.0504	0.0543	0.0584	0.0629	0.0676	0.0727	0.0781	0.0901	0.0901	0.0966
6.3	0.0546	0.0589	0.0634	0.0683	0.0736	0.0792	0.0851	0.0915	0.0983	0.1134	0.1134	0.1216
6.4	0.0687	0.0741	0.0799	0.0860	0.0926	0.0996	0.107	0.115	0.124	0.133	0.143	0.153
6.5	0.0865	0.0933	0.1005	0.1083	0.1166	0.1254	0.135	0.145	0.156	0.167	0.180	0.193
6.6	0.109	0.117	0.127	0.136	0.147	0.158	0.170	0.182	0.196	0.210	0.226	0.242
6.7	0.137	0.148	0.159	0.171	0.185	0.199	0.214	0.230	0.247	0.265	0.284	0.305
6.8	0.172	0.186	0.200	0.216	0.232	0.250	0.269	0.289	0.310	0.333	0.358	0.384
6.9	0.217	0.234	0.252	0.271	0.292	0.314	0.338	0.363	0.390	0.419	0.450	0.482
7.0	0.273	0.294	0.317	0.342	0.368	0.396	0.425	0.457	0.491	0.527	0.566	0.607
7.1	0.343	0.370	0.399	0.430	0.462	0.497	0.535	0.575	0.617	0.663	0.711	0.762
7.2	0.432	0.466	0.502	0.540	0.581	0.625	0.672	0.722	0.776	0.833	0.893	0.958
7.3	0.543	0.586	0.631	0.679	0.731	0.786	0.845	0.908	0.975	1.05	1.12	1.20
7.4	0.683	0.736	0.793	0.854	0.918	0.988	1.061	1.140	1.224	1.31	1.41	1.51
7.5	0.858	0.925	0.996	1.07	1.15	1.24	1.33	1.43	1.54	1.65	1.77	1.89
7.6	1.08	1.16	1.25	1.35	1.45	1.56	1.67	1.80	1.93	2.07	2.21	2.37
7.7	1.35	1.46	1.57	1.69	1.82	1.95	2.10	2.25	2.41	2.59	2.77	2.97
7.8	1.70	1.83	1.97	2.12	2.28	2.44	2.62	2.82	3.02	3.24	3.46	3.71
7.9	2.13	2.29	2.46	2.65	2.85	3.06	3.28	3.52	3.77	4.04	4.32	4.62
8.0	2.66	2.87	3.08	3.31	3.56	3.82	4.10	4.39	4.70	5.03	5.38	5.75
8.1	3.33	3.58	3.85	4.14	4.44	4.76	5.10	5.46	5.85	6.25	6.68	7.14
8.2	4.16	4.47	4.80	5.15	5.52	5.92	6.34	6.78	7.25	7.75	8.27	8.82
8.3	5.18	5.56	5.97	6.40	6.86	7.34	7.85	8.39	8.96	9.56	10.2	10.9
8.4	6.43	6.90	7.40	7.93	8.48	9.07	9.69	10.3	11.0	11.7	12.5	13.3
8.5	7.97	8.54	9.14	9.78	10.45	11.16	11.90	12.7	13.5	14.4	15.2	16.2
8.6	9.83	10.5	11.2	12.0	12.8	13.6	14.5	15.5	16.4	17.4	18.5	19.5
8.7	12.07	12.9	13.8	14.7	15.6	16.6	17.6	18.7	19.8	21.0	22.2	23.4
8.8	14.7	15.7	16.7	17.8	18.9	20.0	21.2	22.5	23.7	25.1	26.4	27.8
8.9	17.9	19.0	20.2	21.4	22.7	24.0	25.3	26.7	28.2	29.6	31.1	32.6

[1]Table provided by Teresa Norberg-King, Environmental Research Laboratory, Duluth, Minnesota. Also see Emerson et al. (1975), Thurston et al. (1974), and USEPA (1985a).

under the appropriate pH and temperature, and multiplying it by the concentration (mg/L) of total ammonia in the sample.

8.8.7 Effluents and receiving waters can be dechlorinated using 6.7 mg/L anhydrous sodium thiosulfate to reduce 1 mg/L chlorine (APHA, 1992). Note that the amount of thiosulfate required to dechlorinate effluents is greater than the amount needed to dechlorinate tap water, (see Section 7, Dilution Water). Since thiosulfate may contribute to sample toxicity, a thiosulfate control should be used in the test in addition to the normal dilution water control.

8.8.8 The DO concentration in the samples should be near saturation prior to use. Aeration will bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH. However, aeration during collection, transfer, and preparation of samples should be minimized to reduce the loss of volatile chemicals.

8.8.9 Mortality or impairment of growth or reproduction due to pH alone may occur if the pH of the receiving water sample falls outside the range of 7.5 - 8.5 for marine. Thus, the presence of other forms of toxicity (metals and organics) in the sample may be masked by the toxic effects of low or high pH. The question about the presence of other toxicants can be answered only by performing two parallel tests, one with an adjusted pH, and one without an adjusted pH. Freshwater samples are adjusted to pH 7.0, and marine samples are adjusted to pH 8.0, by adding 1N NaOH or 1N HCl dropwise, as required, being careful to avoid overadjustment.

8.9 PRELIMINARY TOXICITY RANGE-FINDING TESTS

8.9.1 USEPA Regional and State personnel generally have observed that it is not necessary to conduct a toxicity range-finding test prior to initiating a static, chronic, definitive toxicity test. However, when preparing to perform a static test with a sample of completely unknown quality, or before initiating a flow-through test, it is advisable to conduct a preliminary toxicity range-finding test.

8.9.2 A toxicity range-finding test ordinarily consists of a down-scaled, abbreviated static acute test in which groups of five organisms are exposed to several widely-spaced sample dilutions in a logarithmic series, such as 100%, 10.0%, 1.00%, and 0.100%, and a control, for 8-24 h. **Caution:** if the sample must also be used for the full-scale definitive test, the 36-h limit on holding time (see Subsection 8.5.4) must not be exceeded before the definitive test is initiated.

8.9.3 It should be noted that the toxicity of a sample observed in a range-finding test may be significantly different from the toxicity observed in the follow-up, chronic, definitive test because: (1) the definitive test may be longer; and (2) the test may be performed with a sample collected at a different time, and possibly differing significantly in the level of toxicity.

8.10 MULTICONCENTRATION (DEFINITIVE) EFFLUENT TOXICITY TESTS

8.10.1 The tests recommended for use in determining discharge permit compliance in the NPDES program are multiconcentration or definitive tests. These tests provide a statistical measure of effluent toxicity, defined as mortality, fertilization, growth, and/or development. The tests may be static-renewal or static non-renewal.

8.10.2 The tests consist of a control and a minimum of five effluent concentrations commonly selected to approximate a geometric series, such as 60%, 30%, 15%, 7.5%, and 3.75%, using a ≥0.5 dilution series.

8.10.3 These tests are also to be used in determining compliance with permit limits on the mortality of the receiving water concentration (RWC) of effluents by bracketing the RWC with effluent concentrations in the following manner. For example, if the RWC is >25% then, the effluent concentrations utilized in a test may be: (1) 100% effluent, (2) (RWC + 100)/2, (3) RWC, (4) RWC/2, and (5) RWC/4. More specifically, if the RWC = 50%, the effluent concentrations used in the toxicity test would be 100%, 75%, 50%, 25%, and 12.5%. If the RWC is <25% effluent the concentrations may be: (1) 4 times the RWC, (2) 2 times the RWC, (3) RWC/2, and (4) RWC/4.

8.10.4 If acute/chronic ratios are to be determined by simultaneous acute and short-term chronic tests with a single species, using the same sample, both types of tests must use the same test conditions, i.e., pH, temperature, salinity, etc.

8.11 RECEIVING WATER TESTS

8.11.1 Receiving water toxicity tests generally consist of 100% receiving water and a control. The salinity of the control should be comparable to the receiving water.

8.11.2 The data from the two treatments are analyzed by hypothesis testing to determine if test organism survival, fertilization, growth or development in the receiving water differs significantly from the control. Four replicates and 10 organisms per replicate are required for each treatment (see Summary of Test Conditions and Test Acceptability Criteria in the specific test method).

8.11.3 In cases where the objective of the test is to estimate the degree of toxicity of the receiving water, a definitive, multiconcentration test is performed by preparing dilutions of the receiving water, using a ≥ 0.5 dilution series, with a suitable control water.

SECTION 9

CHRONIC TOXICITY TEST ENDPOINTS AND DATA ANALYSIS

9.1 ENDPOINTS

9.1.1 The objective of chronic aquatic toxicity tests with effluents and pure compounds is to estimate the highest "safe" or "no-effect concentration" of these substances. For practical reasons, the responses observed in these tests are usually limited to survival, fertilization, germination, growth and larval development and the results of the tests are usually expressed in terms of the highest toxicant concentration that has no statistically significant observed effect on these responses, when compared to the controls. The terms currently used to define the endpoints employed in the rapid, chronic and sub-chronic toxicity tests have been derived from the terms previously used for full life-cycle tests. As shorter chronic tests were developed, it became common practice to apply the same terminology to the endpoints. The terms used in this manual are as follows:

9.1.1.1 Safe Concentration - The highest concentration of toxicant that will ermit normal propagation of fish and other aquatic life in receiving waters. The concept of a "safe concentration" is a biological concept, whereas the "no-observed-effect concentration" (below) is a statistically defined concentration.

9.1.1.2 No-Observed-Effect-Concentration (NOEC) - The highest concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle (short-term) test, that causes no observable adverse effects on the test organisms (i.e., the highest concentration of toxicant in which the values for the observed responses are not statistically significantly different from the controls). This value is used, along with other factors, to determine toxicity limits in permits.

9.1.1.3 Lowest-Observed-Effect-Concentration (LOEC) - The lowest concentration of toxicant to which organisms are exposed in a life-cycle or
partial life-cycle (short-term) test, which causes adverse effects on the test organisms (i.e., where the values for the observed responses are statistically significantly different from the controls).

9.1.1.4 Effective Concentration (EC) - A point estimate of the toxicant concentration that would cause an observable adverse affect on a quantal, "all or nothing," response (such as death, fertilization, germination or, development) in a given percent of the test organisms, calculated by point estimation techniques. If

analysis alone, unless (1) the assumptions of a strict threshold model are accepted, and (2) it is assumed that the amount of adverse effect present at the threshold is statistically detectable by hypothesis testing. In this case, estimates obtained from a statistical analysis are indeed estimates of a "no-effect" concentration. If the assumptions are not deemed tenable, then estimates from a statistical analysis can only be used in conjunction with an assessment from a biological standpoint of what magnitude of adverse effect constitutes a "safe" concentration. In this instance, a "safe" concentration is not necessarily a truly "no-effect" concentration, but rather a concentration at which the effects are judged to be of no biological significance.

9.2.5 A better understanding of the relationship between endpoints derived by hypothesis testing (NOECs) and point estimation techniques (LCs, ICs, and ECs) would be very helpful in choosing methods of data analysis. Norberg-King (1991) reported that the IC25s were comparable to the NOECs for 23 effluent and reference toxicant data sets analyzed. The data sets included short-term chronic toxicity tests for the sea urchin, *Arbacia punctulata*, the sheepshead minnow, *Cyprinodon variegatus*, and the red macroalga, *Champia parvula*. Birge et al. (1985) reported that LC1s derived from Probit Analyses of data from short-term embryo-larval tests with reference toxicants were comparable to NOECs for several organisms. Similarly, USEPA (1988d) reported that the IC25s were comparable to the NOECs for a set of daphnia, *Ceriodaphnia dubia* chronic tests with a single reference toxicant. However, the scope of these comparisons was very limited, and sufficient information is not yet available to establish an overall relationship between these two types of endpoints, especially when derived from effluent toxicity test data.

9.3 PRECISION

9.3.1 HYPOTHESIS TESTS

9.3.1.1 When hypothesis tests are used to analyze toxicity test data, it is not possible to express precision in terms of a commonly used statistic. The results of the test are given in terms of two endpoints, the No-Observed-Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC). The NOEC and LOEC are limited to the concentrations selected for the test. The width of the NOEC-LOEC interval is a function of the dilution series, and differs greatly depending on whether a dilution factor of 0.3 or 0.5 is used in the test design. Therefore, **USEPA recommends the use of the ≥0.5 dilution factor** (see Section 4, Quality Assurance). It is not possible to place confidence limits on the NOEC and LOEC derived from a given test, and it is difficult to quantify the precision of the NOEC-LOEC endpoints between tests. If the data from a series of tests performed with the same

toxicant, toxicant concentrations, and test species, were analyzed with hypothesis tests, precision could only be assessed by a qualitative comparison of the NOEC-LOEC intervals, with the understanding that maximum precision would be attained if all tests yielded the same NOEC-LOEC interval. In practice, the precision of results of repetitive chronic tests is considered acceptable if the NOECs vary by no more than one concentration interval above or below a central tendency. Using these guidelines, the "normal" range of NOECs from toxicity tests using a 0.5 dilution factor (two-fold difference between adjacent concentrations), would be four-fold.

9.3.2 POINT ESTIMATION TECHNIQUES

9.3.2.1 Point estimation techniques have the advantage of providing a point estimate of the toxicant concentration causing a given amount of adverse (inhibiting) effect, the precision of which can be quantitatively assessed (1) within tests by calculation of 95% confidence limits, and (2) across tests by calculating a standard deviation and coefficient of variation.

9.4 DATA ANALYSIS

9.4.1 ROLE OF THE STATISTICIAN

9.4.1.1 The use of the statistical methods described in this manual for routine data analysis does not require the assistance of a statistician. However, the interpretation of the results of the analysis of the data from any of the toxicity tests described in this manual can become problematic because of the inherent variability and sometimes unavoidable anomalies in biological data. If the data appear unusual in any way, or fail to meet the necessary assumptions, a statistician should be consulted. Analysts who are not proficient in statistics are strongly advised to seek the assistance of a statistician before selecting the method of analysis and using any of the results.

9.4.1.2 The statistical methods recommended in this manual are not the only possible methods of statistical analysis. Many other methods have been proposed and considered. Certainly there are other reasonable and defensible methods of statistical analysis for this kind of toxicity data. Among alternative hypothesis tests some, like Williams' Test, require additional assumptions, while others, like the bootstrap methods, require computer-intensive computations. Alternative point estimation approaches most probably would require the services of a statistician to determine the appropriateness of the model (goodness of fit), higher order linear or nonlinear models, confidence intervals for estimates generated by inverse regression, etc. In addition, point estimation or regression approaches would require the specification

by biologists or toxicologists of some low level of adverse effect that would be deemed acceptable or safe. The statistical methods contained in this manual have been chosen because they are (1) applicable to most of the different toxicity test data sets for which they are recommended, (2) powerful statistical tests, (3) hopefully "easily" understood by nonstatisticians, and (4) amenable to use without a computer, if necessary.

9.4.2 PLOTTING THE DATA

9.4.2.1 The data should be plotted, both as a preliminary step to help detect problems and unsuspected trends or patterns in the responses, and as an aid in interpretation of the results. Further discussion and plotted sets of data are included in the methods and the Appendices.

9.4.3 DATA TRANSFORMATIONS

9.4.3.1 Transformations of the data, (e.g., arc sine square root and logs), are used where necessary to meet assumptions of the proposed analyses, such as the requirement for normally distributed data.

9.4.4 INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

9.4.4.1 Statistical independence among observations is a critical assumption in all statistical analysis of toxicity data. One of the best ways to ensure independence is to properly follow rigorous randomization procedures. Randomization techniques should be employed at the start of the test, including the randomization of the placement of test organisms in the test chambers and randomization of the test chamber location within the array of chambers. Discussions of statistical independence, outliers and randomization, and a sample randomization scheme, are included in Appendix A.

9.4.5 REPLICATION AND SENSITIVITY

9.4.5.1 The number of replicates employed for each toxicant concentration is an important factor in determining the sensitivity of chronic toxicity tests. Test sensitivity generally increases as the number of replicates is increased, but the point of diminishing returns in sensitivity may be reached rather quickly. The level of sensitivity required by a hypothesis test or the confidence interval for a point estimate will determine the number of replicates, and should be based on the objectives for obtaining the toxicity data.

9.4.5.2 In a statistical analysis of toxicity data, the choice of a particular analysis and the ability to detect departures from the assumptions of the analysis, such as the normal distribution of the

data and homogeneity of variance, is also dependent on the number of replicates. More than the minimum number of replicates may be required in situations where it is imperative to obtain optimal statistical results, such as with tests used in enforcement cases or when it is not possible to repeat the tests. For example, when the data are analyzed by hypothesis testing, the nonparametric alternatives cannot be used unless there are at least four replicates at each toxicant concentration.

9.4.6 RECOMMENDED ALPHA LEVELS

9.4.6.1 The data analysis examples included in the manual specify an alpha level of 0.01 for testing the assumptions of hypothesis tests and an alpha level of 0.05 for the hypothesis tests themselves. These levels are common and well accepted levels for this type of analysis and are presented as a recommended minimum significance level for toxicity data analysis.

9.5 **CHOICE OF ANALYSIS**

9.5.1 The recommended statistical analysis of most data from chronic toxicity tests with aquatic organisms follows a decision process illustrated in the flowchart in Figure 2. An initial decision is made to use point estimation techniques (Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber, the Graphical Method or Linear Interpolation Method) and/or to use hypothesis testing (Dunnett's Test, the t test with the Bonferroni adjustment, Steel's Many-one Rank Test, or Wilcoxon Rank Sum Test). If hypothesis testing is chosen, subsequent decisions are made on the appropriate procedure for a given set of data, depending on the results of tests of assumptions, as illustrated in the flowchart. A specific flow chart is included in the analysis section for each test.

9.5.2 Since a single chronic toxicity test might yield information on more than one parameter (such as survival, growth, and development), the lowest estimate of a "no-observed-effect concentration" from any of the responses would be used as the "no-observed-effect concentration" for each test. It follows logically that in the statistical analysis of the data, concentrations that had a significant toxic effect on one of the observed responses would not be subsequently tested for an effect on some other response. This is one reason for excluding concentrations that have shown a statistically significant reduction in survival from a subsequent hypothesis test for effects on another parameter such as growth. A second reason is that the exclusion of such concentrations usually results in a more powerful and appropriate statistical analysis. In performing the point estimation techniques recommended in this manual, an all-data

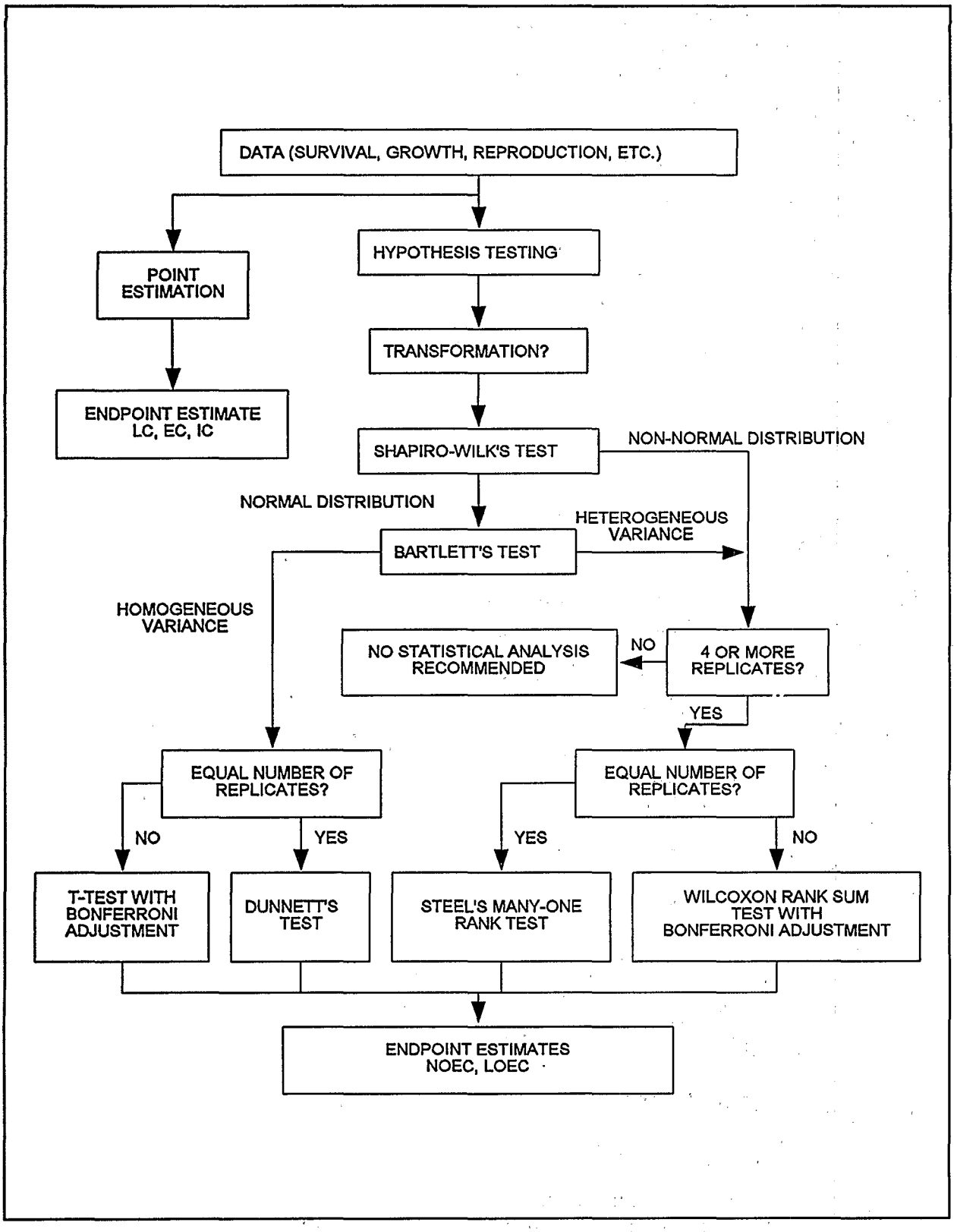

Figure 2. Flowchart for statistical analysis of test data.

approach is used. For example, data from concentrations above the NOEC for survival are included in determining ICp estimates usingthe Linear Interpolation Method.

9.5.3 ANALYSIS OF GROWTH DATA

9.5.3.1 Growth data from the topsmelt, *Atherinops affinis*, mysid, *Holmesimysis costata*, survival and growth tests, and the giant kelp, *Macrocystis pyriferia*, germination and germ-tube length test, are analyzed using hypothesis testing according to the flowchart in Figure 2. The above mentioned growth data may also be analyzed by generating a point estimate with the Linear Interpolation Method. Data from effluent concentrations that have tested significantly different from the control for survival are excluded from further hypothesis tests concerning growth effects. Growth is defined as the change in dry weight of the orginal number of test organisms when group weights are obtained. When analyzing the data using point estimating techniques, data from all concentrations are included in the analysis.

9.5.4 ANALYSIS OF FERTILIZATION, GERMINATION AND DEVELOPMENT DATA

9.5.4.1 Data from the purple urchin, *Strongylocentrotus purpuratus* and the sand dollar, *Denstraster excentricus*, fertilization test and development test; the red abalone *Haliotis rufescens*, the Pacific oyster, *Crassostrea gigas*, and mussel, *Mytilus spp.*, larval development tests; and the giant kelp, *Macrocystis pyrifera*, germination test may be analyzed by hypothesis testing after an arc sine transformation according to the flowchart in Figure 2. The fertilization, larval development or germination data may also be analyzed by generating a point estimate with the Linear Interpolation Method.

9.5.5 ANALYSIS OF MORTALITY DATA

9.5.5.1 Mortality data are analyzed by Probit Analysis, if appropriate, or other point estimation techniques, (i.e., the Spearman-Karber Method, the Trimmed Spearman-Karber Method, or the Graphical Method) (see Appendices G-I) (see discussion below). The mortality data can also be analyzed by hypothesis testing, after an arc sine square root transformation (see Appendices B-F), according to the flowchart in Figure 2.

9.6 **HYPOTHESIS TESTS**

9.6.1 DUNNETT'S PROCEDURE

9.6.1.1 Dunnett's Procedure is used to determine the NOEC. The procedure consists of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison

procedure for comparing each of the treatment means with the control mean, in a series of paired tests (see Appendix C). Use of Dunnett's Procedure requires at least three replicates per treatment to check the assumptions of the test. In cases where the numbers of data points (replicates) for each concentration are not equal, a t test may be performed with Bonferroni's adjustment for multiple comparisons (see Appendix D), instead of using Dunnett's Procedure.

9.6.1.2 The assumptions upon which the use of Dunnett's Procedure is contingent are that the observations within treatments are normally distributed, with homogeneity of variance. Before analyzing the data, these assumptions must be tested using the procedures provided in Appendix B.

9.6.1.3 If, after suitable transformations have been carried out, the normality assumptions have not been met, Steel's Many-one Rank Test should be used if there are four or more data points (replicates) per toxicant concentration. If the numbers of data points for each toxicant concentration are not equal, the Wilcoxon Rank Sum Test with Bonferroni's adjustment should be used (see Appendix F).

9.6.1.4 Some indication of the sensitivity of the analysis should be provided by calculating (1) the minimum difference between means that can be detected as statistically significant, and (2) the percent change from the control mean that this minimum difference represents for a given test.

9.6.1.5 A step-by-step example of the use of Dunnett's Procedure is provided in Appendix C.

9.6.2 T TEST WITH THE BONFERRONI ADJUSTMENT

9.6.2.1 The t test with the Bonferroni adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus, Dunnett's Procedure is a more powerful test.

9.6.2.2 The assumptions upon which the use of the t test with the Bonferroni adjustment is contingent are that the observations within treatments are normally distributed, with homogeneity of variance. These assumptions must be tested using the procedures provided in Appendix B.

9.6.2.3 The estimate of the safe concentration derived from this test is reported in terms of the NOEC. A step-by-step example of the use of a t-test with the Bonferroni adjustment is provided in Appendix D.

9.6.3 STEEL'S MANY-ONE RANK TEST

9.6.3.1 Steel's Many-one Rank Test is a multiple comparison procedure for comparing several treatments with a control. This method is similar to Dunnett's procedure, except that it is not necessary to meet the assumption of normality. The data are ranked, and the analysis is performed on the ranks rather than on the data themselves. If the data are normally or nearly normally distributed, Dunnett's Procedure would be more sensitive (would detect smaller differences between the treatments and control). For data that are not normally distributed, Steel's Many-one Rank Test can be much more efficient (Hodges and Lehmann, 1956).

9.6.3.2 It is necessary to have at least four replicates per toxicant concentration to use Steel's test. Unlike Dunnett's procedure, the sensitivity of this test cannot be stated in terms of the minimum difference between treatment means and the control mean that can be detected as statistically significant.

9.6.3.3 The estimate of the safe concentration is reported as the NOEC. A step-by-step example of the use of Steel's Many-One Rank Test is provided in Appendix E.

9.6.4 WILCOXON RANK SUM TEST

9.6.4.1 The Wilcoxon Rank Sum Test is a nonparametric test for comparing a treatment with a control. The data are ranked and the analysis proceeds exactly as in Steel's Test except that Bonferroni's adjustment for multiple comparisons is used instead of Steel's tables. When Steel's test can be used (i.e., when there are equal numbers of data points per toxicant concentration), it will be more powerful (able to detect smaller differences as statistically significant) than the Wilcoxon Rank Sum Test with Bonferroni's adjustment.

9.6.4.2 The estimate of the safe concentration is reported as the NOEC. A step-by-step example of the use of the Wilcoxon Rank Sum Test is provided in Appendix F.

9.6.5 A CAUTION IN THE USE OF HYPOTHESIS TESTING

9.6.5.1 If in the calculation of an NOEC by hypothesis testing, two tested concentrations cause statistically significant adverse effects, but an intermediate concentration did not cause statistically significant effects, the results should be used with extreme caution.

9.7 POINT ESTIMATION TECHNIQUES

9.7.1 PROBIT ANALYSIS

9.7.1.1 Probit Analysis is used to estimate an LC or EC value and the associated 95% confidence interval. The analysis consists of adjusting the data for mortality in the control, and then using a maximum likelihood technique to estimate the parameters of the underlying log tolerance distribution, which is assumed to have a particular shape.

9.7.1.2 The assumption upon which the use of Probit Analysis is contingent is a normal distribution of log tolerances. If the normality assumption is not met, and at least two partial mortalities are not obtained, Probit Analysis should not be used. It is important to check the results of Probit Analysis to determine if use of the analysis is appropriate. The chi-square test for heterogeneity provides a good test of appropriateness of the analysis. The computer program (see discussion, Appendix H) checks the chi-square statistic calculated for the data set against the tabular value, and provides an error message if the calculated value exceeds the tabular value.

9.7.1.3 A discussion of Probit Analysis, and examples of computer program input and output, are found in Appendix H.

9.7.1.4 In cases where Probit Analysis is not appropriate, the LC50 and confidence interval may be estimated by the Spearman-Karber Method (Appendix I) or the trimmed Spearman-Karber Method (Appendix J). If a test results in 100% survival and 100% mortality in adjacent treatments (all or nothing effect), the LC50 may be estimated using the Graphical Method (Appendix K).

9.7.2 LINEAR INTERPOLATION METHOD

9.7.2.1 The Linear Interpolation Method (see Appendix L) is a procedure to calculate a point estimate of the effluent or other toxicant concentration [Inhibition Concentration, (IC)] that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms. The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests.

9.7.2.2 Use of the Linear Interpolation Method is based on the assumptions that the responses (1) are monotonically non-increasing (the mean response for each higher concentration is less than or equal to the mean response for the previous concentration), (2) follow a piece-wise linear response function, and (3) are from a random, independent, and representative sample of test data. The assumption for piece-wise linear response cannot be tested

statistically, and no defined statistical procedure is provided to test the assumption for monotonicity. Where the observed means are not strictly monotonic by examination, they are adjusted by smoothing. In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean.

9.7.2.3 The inability to test the monotonicity and piece wise linear response assumptions for this method makes it difficult to assess when the method is, or is not, producing reliable results. Therefore, the method should be used with caution when the results of a toxicity test approach an "all or nothing" response from one concentration to the next in the concentration series, and when it appears that there is a large deviation from monotonicity. See Appendix L for a more detailed discussion of the use of this method and a computer program available for performing calculations.

SECTION 10

REPORT PREPARATION

The toxicity data are reported, together with other appropriate data. The following general format and content are recommended for the report:

10.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contract Laboratory (if the test was performed under contract)
   a. Name of firm
   b. Phone number
   c. Address

10.2 PLANT OPERATIONS

1. Product(s)
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Volume of waste flow (MGD, CFS, GPM)
8. Design flow of treatment facility at time of sampling

10.3 SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER

1. Effluent Samples
   a. Sampling point
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Mean daily discharge on sample collection date
   f. Elapsed time from sample collection to delivery
   g. Sample temperature when received at the laboratory

2. Receiving Water Samples
   a. Sampling point
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Tide stages
   f. Sample temperature when received at the laboratory
   g. Elapsed time from sample collection to delivery

3. Dilution Water Samples
   a. Source
   b. Collection date and time
   c. Pretreatment
   d. Physical and chemical characteristics

10.4 **TEST METHODS**

1. Toxicity test method used (title, number, source)
2. Endpoint(s) of test
3. Deviation(s) from reference method, if any, and the reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type of volume and test chambers
7. Volume of solution used per chamber
8. Number of organisms used per test chamber
9. Number of replicate test chambers per treatment
10. Acclimation of test organisms (temperature and salinity mean and range)
11. Test temperature (mean and range)
12. Specify if aeration was needed
13. Feeding frequency, and amount and type of food
14. Test salinity (mean and range)

10.5 **TEST ORGANISMS**

1. Scientific name and how determined
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)
7. Taxonomic key used for species identification

10.6 QUALITY ASSURANCE

1. Reference toxicant used routinely; source
2. Date and time of most recent reference toxicant test; test results and current control (cusum) chart
3. Dilution water used in reference toxicant test
4. Results (NOEC or, where applicable, LOEC, LC50, IC or EC value)
5. Physical and chemical methods used

10.7 RESULTS

1. Provide raw toxicity data in tabular form, including daily records of affected organisms in each concentration (including controls), and plots of toxicity data
2. Provide table of the statistical endpoints; LC50s, NOECs, EC or IC value, etc.
3. Indicate statistical methods used to calculate endpoints
4. Provide summary table of physical and chemical data
5. Tabulate QA data

10.8 CONCLUSIONS AND RECOMMENDATIONS

1. Relationship between test endpoints and permit limits.
2. Action to be taken.

SECTION 11

TOPSMELT, *Atherinops affinis* 7-DAY LARVAL GROWTH AND SURVIVAL TEST METHOD

Adapted from a method developed by
Brian S. Anderson, John W. Hunt, Sheila Turpen,
Hilary R. McNulty, and Matt A. Englund
Institute of Marine Sciences, University of California
Santa Cruz, California

(in association with)
California Department of Fish and Game
Marine Pollution Studies Laboratory
34500 Coast Route 1, Monterey, CA 93940

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SECTION 11

TOPSMELT, *ATHERINOPS AFFINIS* LARVAL SURVIVAL AND GROWTH TEST

11.1 SCOPE AND APPLICATION

11.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the topsmelt, *Atherinops affinis*, using nine-to-fifteen day old larvae in a seven-day, static-renewal exposure test. The effects include the synergistic, antagonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological an biochemical functions of the test organisms.

11.1.2 Daily observations of mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

11.1.3 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

11.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

11.1.5 This method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

11.1.6 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

11.2 SUMMARY OF METHOD

11.2.1 This method provides step-by-step instructions for performing a 7-day static-renewal toxicity test using survival and growth of topsmelt larval fish to determine the toxicity of substances in marine and estuarine waters. The test endpoints are survival and growth.

1.3 **INTERFERENCES**

11.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

11.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sample Preparation for Toxicity Tests).

11.3.3 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

11.3.4 Food added during the test may sequester metals and other toxic substances and confound test results.

11.4 **SAFETY**

11.4.1 See Section 3, Health and Safety.

11.5 **APPARATUS AND EQUIPMENT**

11.5.1 Tanks, trays, or aquaria -- for holding and acclimating topsmelt, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20°C), with appropriate filtration and aeration system. (See Anderson et al., 1994, Middaugh and Anderson, 1993).

11.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

11.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, and larvae at test temperature (20°C) prior to the test.

11.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

11.5.5 Refractometer -- for determining salinity.

11.5.6 Hydrometer(s) -- for calibrating refractometer.

11.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

11.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD 170.1, USEPA, 1979) -- to calibrate laboratory thermometers.

11.5.9 pH and DO meters -- for routine physical and chemical measurements.

11.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

11.5.11 Winkler bottles -- for dissolved oxygen determinations.

11.5.12 Balance -- Analytical, capable of accurately weighing to 0.00001 g.

11.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

11.5.14 Glass stirring rods -- for mixing test solutions.

11.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-1000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

11.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

11.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

11.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

11.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

11.5.20 Wash bottles -- for dilution water.

11.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

11.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

11.5.23 Beakers -- six Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

11.5.24 Brine shrimp, *Artemia*, culture unit -- see Subsection 11.6.25 and Section 4, Quality Assurance.

11.5.25 Separatory funnels, 2-L -- two-four for culturing *Artemia*.

11.5.26 Siphon tubes (fire polished glass) -- for solution renewals and handling larval fish.

11.5.27 Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring larvae.

11.5.28 Siphon with bulb and clamp -- for cleaning test chambers.

11.5.29 Light box -- for counting and observing larvae.

11.5.30 White plastic tray -- for collecting larvae during cleaning of the test chambers.

11.5.31 Forceps -- for transferring dried larvae to weighing pans.

11.5.32 Desiccator -- for holding dried larvae.

11.5.33 Drying oven -- 50-105°C range, for drying larvae.

11.5.34 NITEX® mesh screen tubes - (≤150 µm, 500 µm, 3 to 5 mm) -- for collecting *Artemia* nauplii and fish larvae. (NITEX® is available from Sterling Marine Products, 18 Label Street, Montclair, NJ 07042; 201-783-9800).

11.5.35 60 µm Nitex® filter -- for filtering receiving water.

11.6 **REAGENTS AND SUPPLIES**

11.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

11.6.2 Data sheets (one set per test) -- for data recording (Figures 1 and 2).

11.6.3 Tape, colored -- for labelling test chambers and containers.

11.6.4 Markers, water-proof -- for marking containers, etc.

11.6.5 Parafilm -- to cover graduated cylinders and vessels.

11.6.6 Gloves, disposable -- for personal protection from contamination.

11.6.7 Pipets, serological -- 1-10 mL, graduated.

11.6.8 Pipet tips -- for automatic pipets.

11.6.9 Coverslips -- for microscope slides.

11.6.10 Lens paper -- for cleaning microscope optics.

11.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

11.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

11.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

11.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

11.6.15 Laboratory quality assurance samples and standards -- for the above methods.

11.6.16 Test chambers -- 600 mL, five chambers per concentration. The chambers should be borosilicate glass (for effluents) or nontoxic disposable plastic labware (for reference toxicants). To avoid contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered during the test with safety glass plates or a plastic sheet (6 mm thick).

11.6.17 Ethanol (70%) or formalin (4%) -- for preserving the larvae.

11.6.18 *Artemia* nauplii -- for feeding test organisms.

11.6.19 Weigh boats or weighing paper -- for weighing reference toxicants.

11.6.20 Reference toxicant solutions (see Subsection 11.10.2.4 and see Section 4, Quality Assurance).

11.6.21 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies and Section 7, Dilution Water).

11.6.22 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

11.6.23 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 11.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-μm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

11.6.24 HYPERSALINE BRINES

11.6.24.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

11.6.24.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

11.6.24.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 μm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

11.6.24.4 Freeze Preparation of Brine

11.6.24.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20°C until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

11.6.24.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

11.6.24.4.3 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

11.6.24.5 Heat Preparation of Brine

11.6.24.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

11.6.24.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used,

followed by several (at least three) thorough reagent water rinses.

11.6.24.5.3 Seawater should be filtered to at least 10 μm before being put into the brine generator. The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

11.6.24.5.4 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water

cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

11.6.24.6 Artificial Sea Salts

11.6.24.6.1 No data from topsmelt larval tests using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

11.6.24.7 Dilution Water Preparation from Brine

11.6.24.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

11.6.24.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. The proportion of brine is 1 part plus 1.94 reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

11.6.24.8 Test Solution Salinity Adjustment

11.6.24.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent,

HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

11.6.24.8.2 Check the pH of all brine mixtures and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide (see subsection 8.8.9, Effluent and Receiving Water Sampling, Sampling Handling, and Sample Preparation for Toxicity Tests).

11.6.24.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

11.6.24.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

11.6.24.9 Preparing Test Solutions

11.6.24.9.1 Two hundred mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 10 mL of effluent to a 1-liter volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 1-liter mark with dilution water, stopper it, and shake to mix. Distribute equal volumes into the replicate test chambers.

11.6.24.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (x‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION

Effluent Conc. (%)	Effluent x‰	Brine(68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Pour into a (100-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

11.6.24.10 Brine Controls

11.6.24.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See SubSection, 11.6.24.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

11.6.25 BRINE SHRIMP, *ARTEMIA SP.*, NAUPLII -- for feeding cultures and test organisms.

11.6.25.1 Newly hatched *Artemia* sp. nauplii are used for food for the test organisms. Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are preferred because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size. (One source that has been found to be acceptable is Aquarium Products, 180L Penrod Ct., Glen Burnie, Maryland 21061). For commercial sources of brine shrimp, *Artemia*, cysts, see Table 2 of Section 5, Facilities, Equipment, and Supplies); and Section 4, Quality Assurance.

11.6.25.2 Each new batch of *Artemia* cysts must be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (Leger, et al., 1985, Leger, et al., 1986) against known suitable reference cysts by performing a side-by-side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be a previously tested and acceptable batch of cysts, or may be obtained from the Quality Assurance Research Division, EMSL,

Cincinnati, OH 45268, 513-569-7325. A sample of newly-hatched *Artemia* nauplii from each new batch of cysts should be chemically analyzed. The *Artemia* cysts should not be used if the concentration of total organochlorine pesticides 0.15 ug/g wet weight or that the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight (For analytical methods see USEPA, 1982).

11.6.25.3 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or an aqueous unionized salt (NaCl) solution prepared with 35 g salt or artificial sea salts per liter, to a 2-L separatory funnel, or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24 h at 27°C. Hatching time varies with incubation temperature and the geographic strain of *Artemia* used (see USEPA, 1985a; USEPA, 1993a; ASTM, 1993).
3. After 24 h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic, and will concentrate at the bottom of the funnel if it is covered for 5-10 minutes with a dark cloth or paper towel. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 min without aeration.
4. Drain the nauplii into a funnel fitted with a ≤150 µm NITEX® or stainless steel screen, and rinse with seawater or equivalent before use.

11.6.25.4 Testing *Artemia* nauplii as food for toxicity test organisms.

11.6.25.4.1 The primary criteria for acceptability of each new supply of brine shrimp cysts is adequate survival, and growth of the larvae. The larvae used to evaluate the acceptability of the brine shrimp nauplii must be the same geographical origin and stage of development (9 to 15 days old) as those used routinely in the toxicity tests. Two 7-day chronic tests are performed side-by-side, each consisting of five replicate test vessels containing five larvae (25 organisms per test, total of 50 organisms). The juveniles in one set of test chambers is fed

reference (acceptable) nauplii and the other set is fed nauplii from the "new" source of *Artemia* cysts.

11.6.25.4.2 The feeding rate and frequency, test vessels, volume of control water, duration of the tests, and age of the *Artemia* nauplii at the start of the test, should be the same as used for the routine toxicity tests.

11.6.25.4.3 Results of the brine shrimp, *Artemia*, nauplii nutrition assay, where there are only two treatments, can be evaluated statistically by use of a t test. The "new" food is acceptable if there are no statistically significant differences in the survival or growth of the mysids fed the two sources of nauplii.

11.6.26 TEST ORGANISMS

11.6.26.1 The test organisms for test method are larvae of the topsmelt, *Atherinops affinis*. Topsmelt occur from the Gulf of California to Vancouver Island, British Columbia (Miller and Lea, 1972). It is often among the most abundant fish species in central and southern California estuaries (Allen and Horn, 1975; Horn, 1979; Allen, 1982). Topsmelt reproduce from May through August, depositing eggs on benthic algae in the upper ends of estuaries and bays (Croaker, 1934; Fronk, 1969). Off-season spawning of *Atherinops affinis* has been successful in a laboratory-held population (Anderson et al., 1994). Their embryonic development is similar to that of other atherinids used widely in toxicity testing (eg, *Menidia* species, Borthwick et al.,1985; Middaugh et al., 1987; Middaugh and Shenker, 1988), and methods to assess sublethal effects with these species have proven to be adaptable for topsmelt (Anderson et al., 1991, Middaugh and Anderson, 1993, McNulty et al., 1994).

11.6.26.2 Species Identification

11.6.26.2.1 Topsmelt often co-occur with jacksmelt, *Atherinopsis californiensis*. The two species can be distinguished based on several key characteristics. Jacksmelt have 10-12 scales between their two dorsal fins; topsmelt have 5-8 scales between the two fins. Jacksmelt teeth are arranged in several bands on each jaw and the teeth are not forked; topsmelt teeth are arranged in one band and the teeth are forked. In jacksmelt, the insertion of

the first dorsal fin occurs well in advance of the origin of the anal fin. In topsmelt, the origin of the anal fin is under the insertion of the first dorsal fin. Consult Miller and Lea (1972) for a guide to the taxonomy of these two fishes.

11.6.26.3 Obtaining Broodstock

11.6.26.3.1 In California, adult topsmelt can be seined from sandy beaches in sloughs and estuaries from April through August. The size of the seine used depends on the number of people deploying it and the habitat being sampled. Larger seines can be used in open sandy areas, smaller seines are used in smaller areas with rocky outcroppings. Five or six people are an adequate number to set and haul a 100-ft beach seine. The seine is set on an ebbing tide using a small motor skiff with one person driving and a second deploying the net from the bow. The net is set parallel to shore then hauled in evenly from the wings. The net mesh diameter should be small enough to prevent the fish from damaging themselves; a one-centimeter diameter mesh in the middle panel and one-and-a-half-centimeter diameter mesh in the wing panel is adequate. As the net is pulled onto the shore, the adult topsmelt are sorted into five-liter plastic buckets, then immediately transferred to 100-liter transport tanks.

11.6.26.3.2 State collection permits are usually required for collection of topsmelt. Collection is prohibited or restricted in some areas. Collection of topsmelt is regulated by California law. Collectors must obtain a scientific collector's permit from the California Department of Fish and Game and observe any regulations regarding collection, transfer, and maintenance of fish broodstock.

11.6.26.3.3 Various containers can be used to transport fish; 100-liter covered plastic trash cans have been used successfully to transport topsmelt. New plastic containers should be leached in seawater for 96 hours prior to transporting fish. Each container can maintain approximately 20 adult fish for six to eight hours if adequate aeration is provided. Use compressed oxygen or air to supply aeration to the tanks during transport.

11.6.26.4 Broodstock Culture and Handling

11.6.26.4.1 Once in the laboratory the fish should be treated for 2 days with a general antibiotic in a separate tank (eg., Prefuran® as per label instructions), then divided among 1000-liter holding tanks. No more than 30 adult fish should be placed in each tank. Tank temperature should be maintained at 18°C using a 1500-watt immersion heater. To conserve heated seawater, the seawater in the tanks can be recirculated using the system similar to that described by Middaugh and Hemmer (1984). A one-thirtieth (1/30)-hp electric pump is used to circulate water (10 liters/minute) from the tanks through vertical, biologically activated nylon filter elements located in a separate reservoir, then back into the tanks. Fresh seawater should be constantly provided to the system at 0.5 liters/minute to supplement the recirculated seawater. The tanks are insulated with one inch thick closed cell foam to conserve heat. Dissolved oxygen levels should be maintained at greater than 6.0 mg/liter using aeration. Salinity should be checked periodically using a refractometer accurate to the nearest 0.5‰; tank salinity should be 34 ± 2‰.

11.6.26.4.2 Adult topsmelt in each tank are fed twice daily (at 0900 and 1500 hrs) approximately 0.3g of Tetramin™ flake food. Supplemental feedings of krill or chopped squid are recommended. Tanks are siphoned clean once weekly.

11.6.26.4.3 Dyeless yarn spawning substrates are attached to the surface of plastic grids cut from light diffuser panel (7 cm x 10 cm x 1 cm) and weighted to the bottom of each tank. Substrates are checked daily for the presence of eggs.

11.6.26.4.4 Spawning is induced by a combination of three environmental cues: lighting, 'tidal' cycle, and temperature. The photoperiod is 14 hours of light followed by 10 hours of darkness (14L:10D) with lights on at 0600 and off at 2000 hours. Use two cool white 40-watt fluorescent lamps suspended 1.25 meters above the surface of each tank to provide illumination. Light levels at the surface of the tanks should be 12 to 21 $\mu E/m^2/s$.

11.6.26.4.5 A 'tidal signal' of reduced current velocity is produced once daily in each tank, from 2400 to 0200 hrs, by turning off the circulating pump (Middaugh and Hemmer, 1984). A 1500-watt immersion heater is used to maintain constant temperature at 18°C and to provide temperature spikes. For

spiking, the temperature is raised from 18°C to 21°C over a 12 h period, then allowed to return to 18°C overnight. The temperature should be checked to the nearest 0.1°C at 1 to 4 hour intervals on days when the temperature spikes are introduced. It is common for the fish to appear stressed during the temperature increase and one or two fish may die. If significant mortality begins to occur, the temperature should be lowered immediately. Significant egg production usually begins within five days of the temperature spike (Middaugh, et al., 1992).

11.6.26.5 Culture Materials

11.6.26.5.1 See Section 5, Facilities and Equipment, for a discussion of suitable materials to be used in laboratory culture of topsmelt. Be sure all new materials are properly leached in seawater before use. After use, all culture materials should be washed in soap and water, then rinsed with seawater before re-use.

11.6.26.6 Test Organisms

11.6.26.6.1. Newly fertilized embryos should be placed in screen tubes set in aquaria and equipped with gently flowing seawater at 20 ± 1°C. The embryos can be left attached to the spawning substrates but care should be taken to ensure the substrates are relatively clean and free of food; strands of embryos should not overlap each other on the substrates, and gentle aeration must be provided. Beginning about day 9, check the screen tubes daily for the presence of larvae. Isolate newly-hatched larvae into a separate screen-tube at 21°C by slow siphoning. Provide larvae with newly-hatched *Artemia* nauplii (in excess) at 24-h post-hatch; supply gently flowing seawater, and aeration. Larvae aged 9 to 15 days are used in toxicity tests (McNulty et al., 1994). For information regarding topsmelt larva suppliers call the Marine Pollution Studies Laboratory (408) 624-0947.

11.6.26.6.2 Larvae can be transported in 1-liter ziplock plastic bags (double-bagged). No more than approximately 100 larvae should be transported in any one bag; do not include food. The seawater in the bags should be aerated with pure oxygen for 30 seconds prior to introduction of the larvae. The bag should be packed in an ice chest with one or two blue ice blocks (insulated by newspaper) for transport. The temperature during transport

should be held between 15 and 18°C. Larvae should be shipped via air-express overnight couriers.

11.6.26.6.3 Topsmelt larvae can tolerate a relatively wide range of salinities (5 to ≥35‰) if adequate acclimation is provided (Anderson, et al., In Press). In situations where the test salinity is significantly lower than the salinity at which the larvae were cultured, it may be necessary to acclimate the larvae to the test salinity.

11.7 EFFLUENTS AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

11.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

11.8 CALIBRATION AND STANDARDIZATION

11.8.1 See Section 4, Quality Assurance

11.9 QUALITY CONTROL

11.9.1 See Section 4, Quality Assurance

11.10 TEST PROCEDURES

11.10.1 TEST DESIGN

11.10.1.1 The test consists of at least five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain five replicates of a brine control.

11.10.1.2 Effluent concentrations are expressed as percent effluent.

11.10.2 TEST SOLUTIONS

11.10.2.1 Receiving waters

11.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined

with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using five replicate chambers per test, each containing 200 mL would require approximately 1 L of sample per test per day.

11.10.2.2 Effluents

11.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100% HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34% salinity.

11.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

11.10.2.2.3 The volume in each test chamber is 200 mL.

11.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

11.10.2.3 Dilution Water

11.10.2.3.1 Dilution water should be uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

11.10.2.4 Reference Toxicant Test

11.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

11.10.2.4.2 The preferred reference toxicant for topsmelt is copper chloride ($CuCl_2 \cdot 2H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a 10,000 µg/L copper stock solution by adding 0.0268 g of copper chloride ($CuCl_2 \cdot 2H_2O$) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

11.10.2.4.3 Reference toxicant solutions should be five replicates each of 0 (control), 56, 100, 180, and 320 µg/L total copper. Prepare one liter of each concentration by adding 0, 5.6, 10.0, 18.0, and 32.0 mL of stock solution, respectively, to one-liter volumetric flasks and fill with dilution water. Start with control solutions and progress to the highest concentration to minimize contamination.

11.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

11.10.3 START OF THE TEST

11.10.3.1 Prior to Beginning the Test

11.10.3.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see Section, 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

11.10.3.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (20 ± 1°C) and maintained at that temperature during the addition of dilution water.

11.10.3.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (20 ± 1°C).

11.10.3.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the larvae have been examined at the end of the test.

11.10.3.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

11.10.3.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

11.10.3.2 Randomized Placement of Larvae into Test Chambers

11.10.3.2.1 Larvae must be randomized before placing them into the test chambers. Pool all of the test larvae into a 1-liter beaker by slow siphoning from the screen-tube. The larvae in the screen-tube can be concentrated into the bottom by lifting the tube during siphoning. Using a fire-polished glass tube, place one larva into as many plastic cups as there are test chambers (including reference toxicant chambers). These cups should

contain enough reference seawater to maintain water quality and temperature during the transfer process (approx. 50 mL). When each of the cups contains one larva, repeat the process, adding one larva at a time until each cup contains 5 animals.

11.10.3.2.2 Carefully pour or pipet off excess water in the cups, leaving less than 5 mL with the test larvae. If more than 5 mLs of water are added to the test solution with the juveniles, report the amount on the data sheet. Carefully transfer the larvae into the test chambers immediately after reducing the water volume. Again, make note of any excess dilution of the test solution. Because of the small volumes involved in the transfer process, this is best accomplished in a constant temperature room. Be sure that all water used in culture, transfer, and test solutions is within 1°C of the test temperature.

11.10.3.2.3 Verify that all five animals are transferred by counting the number in each chamber after transfer. This initial count is important because larvae unaccounted for at the end of the test are assumed to be dead.

11.10.4 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

11.10.4.1 The light quality and intensity should be at ambient laboratory conditions are generally adequate. Light intensity should be 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle.

11.10.4.2 The water temperature in the test chambers should be maintained at 20 ± 1°C. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

15.10.4.3 The test salinity should be in the range of 5 to 34‰, and the salinity should not vary by more than ± 2‰ among the chambers on a given day. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.10.4.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the

test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

11.10.5 DISSOLVED OXYGEN (DO) CONCENTRATION

11.10.5.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue stress to the fish.

11.10.6 FEEDING

11.10.6.1 *Artemia* nauplii are prepared as described below.

11.10.6.2 The test larvae are fed newly-hatched (less than 24-h-old) *Artemia* nauplii once a day from Day 0 through Day 6; larvae are not fed on Day 7. Equal amounts of *Artemia* nauplii must be fed to each replicate test chamber to minimize the variability of larval weight. Add 40 newly hatched *Artemia* nauplii per larva twice daily: once in the morning and once in the afternoon. The density of *Artemia* may be determined by pipetting a known volume of nauplii onto a piece of filter paper and counting the number using a dissecting microscope. Feeding excessive amounts of *Artemia* nauplii will result in a depletion in DO to below an acceptable level. Siphon as much of the uneaten *Artemia* nauplii as possible from each chamber daily to ensure that the larvae principally eat newly hatched nauplii.

11.10.7 DAILY CLEANING OF TEST CHAMBERS

11.10.7.1 Before the daily renewal of test solutions, uneaten and dead brine shrimp, dead larvae, and other debris are removed

from the bottom of the test chambers with a siphon hose. Because of their small size during the first few days of the test, larvae are easily drawn into a siphon tube when cleaning the test chambers. By placing the test chambers on a light box, inadvertent removal of larvae can be greatly reduced because they can be more easily seen. If the water siphoned from the test chambers is collected in a white plastic tray, the live larvae caught up in the siphon can be retrieved, and returned by pipette to the appropriate test chamber and noted on the data sheet.

11.10.8 OBSERVATIONS DURING THE TEST

11.10.8.1 Routine Chemical and Physical Observations

11.10.8.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

11.10.8.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

11.10.8.1.3 Record all the measurements on the data sheet.

11.10.8.2 Routine Biological Observations

11.10.8.2.1 The number of live larvae in each test chamber are recorded daily and the dead larvae are discarded. These data provide daily mortality rates which may be used to calculate 24, 48, and 96-h LC50s.

11.10.8.2.2 Protect the larvae from unnecessary disturbances during the test by carrying out the daily test observations, solution renewals, and removal of dead larvae, carefully. Make sure the larvae remain immersed at all times during the performance of the above operations.

11.10.9 TEST SOLUTION RENEWAL

11.10.9.1 The test solutions are renewed daily using freshly prepared solutions, immediately after cleaning the test chambers. The old solution is carefully siphoned out, leaving enough water so that all of the larvae can still swim freely (approximately 50 mL). Siphon from the bottom of the test chambers so that dead *Artemia* nauplii are removed with the old test solution. It is convenient to siphon old solutions into a small (~500 mL) container in order to ensure that no larvae have been inadvertently removed during solution renewals. If a larva is siphoned, return it to the test chamber and note it on the data sheet.

11.10.9.2 New solution is siphoned into the test chambers using a U-shaped glass tube attached to plastic tubing to minimize disturbance to the larvae.

11.10.9.3 The effluent or receiving water used in the test is stored in an incubator or refrigerator at 4°C. Plastic containers such as 8-20 L cubitainers have proven suitable for effluent collection and storage. For on-site toxicity studies no more than 24 h should elapse between collection of the effluent and use in a toxicity test (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.10.9.4 Approximately 1 h before test initiation, a sufficient quantity of effluent or receiving water sample is warmed to 20 ± 1°C to prepare the test solutions. A sufficient quantity of effluent should be warmed to make daily test solutions.

11.10.10 TERMINATION OF THE TEST

11.10.10.1 Ending the Test

11.10.10.1.1 Record the time the test is terminated.

11.10.10.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control.

11.10.10.2 Sample Preservation

11.10.10.2.1 The surviving larvae in each test chamber (replicate) are counted, and immediately prepared as a group for dry weight determination, or are preserved in 4% formalin then 70% ethanol. Preserved organisms are dried and weighed within 7 d. For safety, formalin should be used under a hood. Note: Death is defined as lack of response to stimulus such as prodding with a glass rod; dead larvae are generally opaque and curled.

11.10.10.3 Weighing

11.10.10.3.1 For immediate drying and weighing, siphon or pour live larvae onto a 500 μm mesh screen in a large beaker to retain the larvae and allow *Artemia* to be rinsed away. Rinse the larvae with reagent water to remove salts that might contribute to the dry weight. Sacrifice the larvae in an ice bath of reagent water.

11.10.10.3.2 Small aluminum weighing pans can be used to dry and weigh larvae. An appropriate number of aluminum weigh pans (one per replicate) are marked for identification and weighed to 0.01 mg, and the weights are recorded on the data sheets.

11.10.10.3.3 Immediately prior to drying, the preserved larvae are in reagent water. The rinsed larvae from each test chamber are transferred, using forceps, to a tared weighing pans and dried at 60°C for 24 h, or at 105°C for a minimum of 6 h. Immediately upon removal from the drying oven, the weighing pans are placed in a desiccator to cool and to prevent the adsorption of moisture from the air until weighed. Weigh all weighing pans containing the dried larvae to 0.01 mg, subtract the tare weight to determine dry weight of larvae in each replicate. Record the weights.

11.10.10.4 Endpoints

11.10.10.4.1 Divide the dry weight by the number of original larvae (5) per replicate to determine the average dry weight, and record on the data sheets. For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptability criteria (see Subsection 11.11). Complete the summary data sheet after calculating the average measurements and statistically analyzing the dry weights and

percent survival for the entire test. Average weights should be expressed to the nearest 0.01 mg.

11.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

11.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE TOPSMELT, *ATHERINOPS AFFINIS*, LARVAL SURVIVAL AND GROWTH TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static-renewal
2. Salinity:	5 to 34‰ (± 2‰ of the selected test salinity)
3. Temperature:	20 ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m^2/s$ (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	600 mL
8. Test solution volume:	200 mL/replicate
9. Renewal of test solutions:	Daily
10. Age of test organisms:	9-15 days post-hatch
11. No. larvae per test chamber:	5
12. No. replicate chambers per concentration:	5
13. Source of food:	Newly hatched *Artemia* nauplii
14. Feeding regime:	Feed 40 nauplii per larvae twice daily (morning and night)

15. Cleaning:	Siphon daily, immediately before test solution renewal and feeding
16. Aeration:	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/min.
17. Dilution water:	Uncontaminated 1-μm-filtered natural seawater or hypersaline brine prepared from natural seawater
18. Test concentrations:	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
19. Dilution factor:	Effluents: ≥0.5 Receiving waters: None, or ≥0.5
20. Test duration:	7 days
21. Endpoints:	Survival and growth (weight)
22. Test acceptability criteria:	≥80% survival in controls, 0.85 mg average weight of control larvae (9 day old), LC50 with copper must be ≤205 μg/L, <25% MSD for survival and <50% MSD for growth

23. Sampling requirement:	For on-site tests, samples collected daily, and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples are collected on days one, three, and five with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
24. Sample volume required:	2 L per day

11.12 ACCEPTABILITY OF TEST RESULTS

11.12.1 Tests results are acceptable only if all the following requirements are met:

(1) The mean survival of larvae must be at least 80% in the controls.

(2) If the test starts with 9 day old larvae, the mean weight per larva must exceed 0.85 mg in the reference and brine controls; the mean weight of preserved larvae must exceed 0.72 mg.

(3) The LC50 for survival must be within two standard deviations of the control chart mean for the laboratory. The LC50 for survival with copper must be <205 μg/L.

(4) The minimum significant difference (%MSD) of <25% relative to the control for survival for the reference toxicant test. The (%MSD) of <50% relative to the control for growth for the reference toxicant test.

11.13 DATA ANALYSIS

11.13.1 GENERAL

11.13.1.1 Tabulate and summarize the data. A sample set of survival and growth response data is listed in Table 4.

11.13.1.2 The endpoints of toxicity tests using the topsmelt larvae are based on the adverse effects on survival and growth. The LC50 and the IC25 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values, for survival and growth, are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981)(see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50 and IC25. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for survival and growth, but included in the estimation of the LC50 and IC25. See the Appendices for examples of the manual computations and examples of data input and program output.

11.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

11.13.2 EXAMPLE OF ANALYSIS OF TOPSMELT, *ATHERINOPS AFFINIS* SURVIVAL DATA

11.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 1 and 2. The response used in the analysis is the proportion of animals surviving in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the LC50 endpoint. Concentrations at which there is no survival in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC, EC, and LC endpoints.

11.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's

Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

11.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

11.13.2.4 Probit Analysis (Finney, 1971; see Appendix H) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, or the Graphical Method may be used to estimate the LC50 (see Appendices H-K).

11.13.2.5 Example of Analysis of Survival Data

11.13.2.5.1 This example uses the survival data from the Topsmelt Larval Survival and Growth Test. The proportion surviving in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix . The raw and transformed data, means and variances of the transformed observations at each copper concentration and control are listed in Table 5. A plot of the survival proportions is provided in Figure 5. Since there was 100% mortality in all five replicates for the 100 µg/L and 180 µg/L concentrations, they are not included in the statistical analysis and are considered qualitative mortality effects.

TABLE 4. SUMMARY OF SURVIVAL AND GROWTH DATA FOR TOPSMELT, *ATHERINOPS AFFINIS*, LARVAE EXPOSED TO COPPER FOR SEVEN DAYS[1]

Copper Conc. (µg/L)	Replicate Survival Proportions					Mean Proportion Survival
	A	B	C	D	E	
0.0	1.0	0.8	1.0	1.0	1.0	0.96
32.0	1.0	1.0	1.0	1.0	1.0	1.00
56.0	0.0	0.6	0.2	1.0	0.6	0.48
100.0	0.0	0.0	0.0	0.0	0.0	0.00
180.0	0.0	0.0	0.0	0.0	0.0	0.00

Conc. (µg/L)	Replicate Average Dry Weights (mg)					Mean Dry $\overline{Wgt}$ (mg)
	A	B	C	D	E	
0.0	0.00134	0.00153	0.00134	0.00146	0.00144	0.00142
32.0	0.00146	0.00142	0.00150	0.00138	0.00128	0.00141
56.0	--	0.00147	0.00170	0.00124	0.00130	0.00114
100.0	--	--	--	--	--	--
180.0	--	--	--	--	--	--

[1]Five replicates of 5 larvae each.

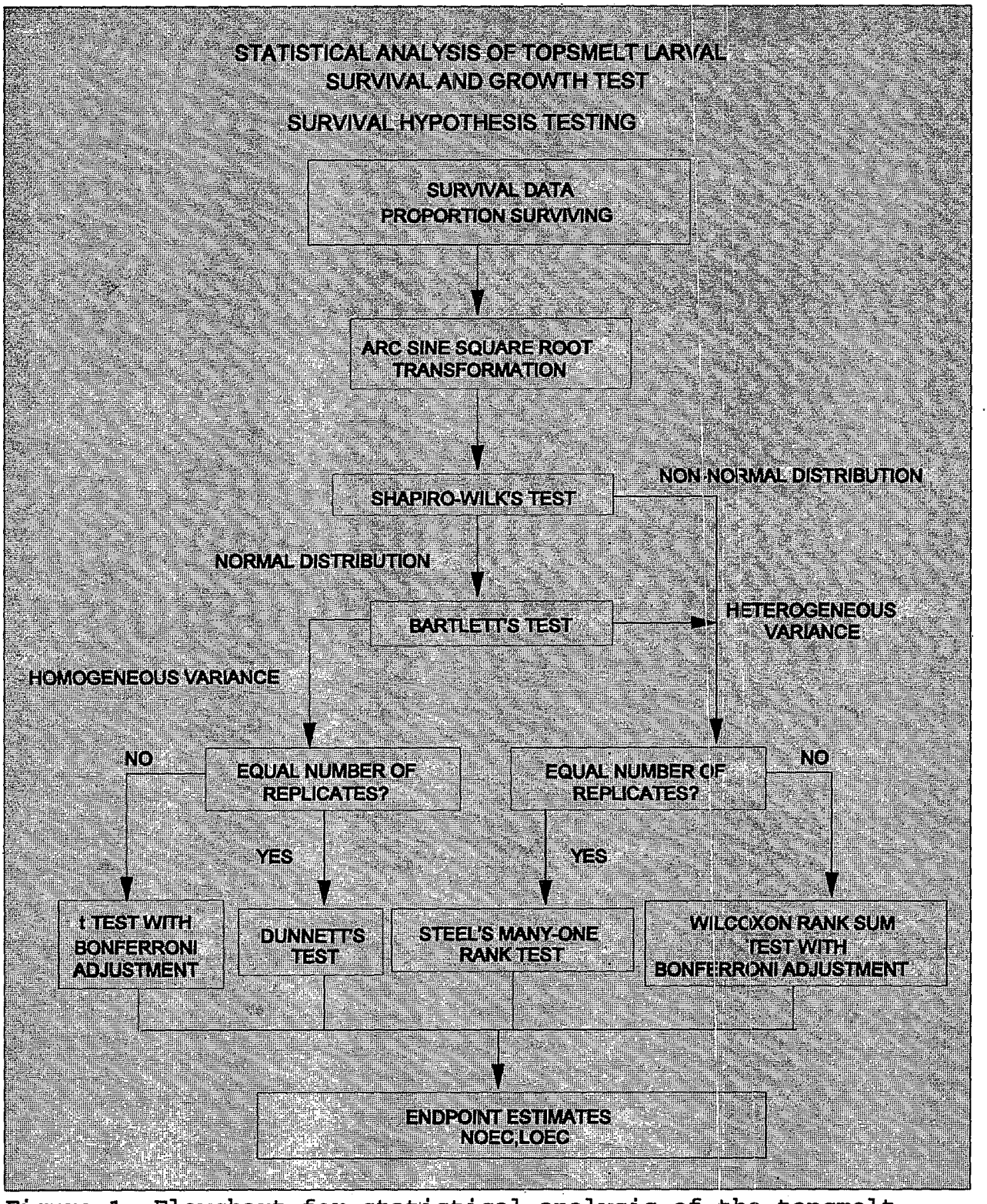

Figure 1. Flowchart for statistical analysis of the topsmelt, *Atherinis affinis*, larval survival data by hypothesis testing.

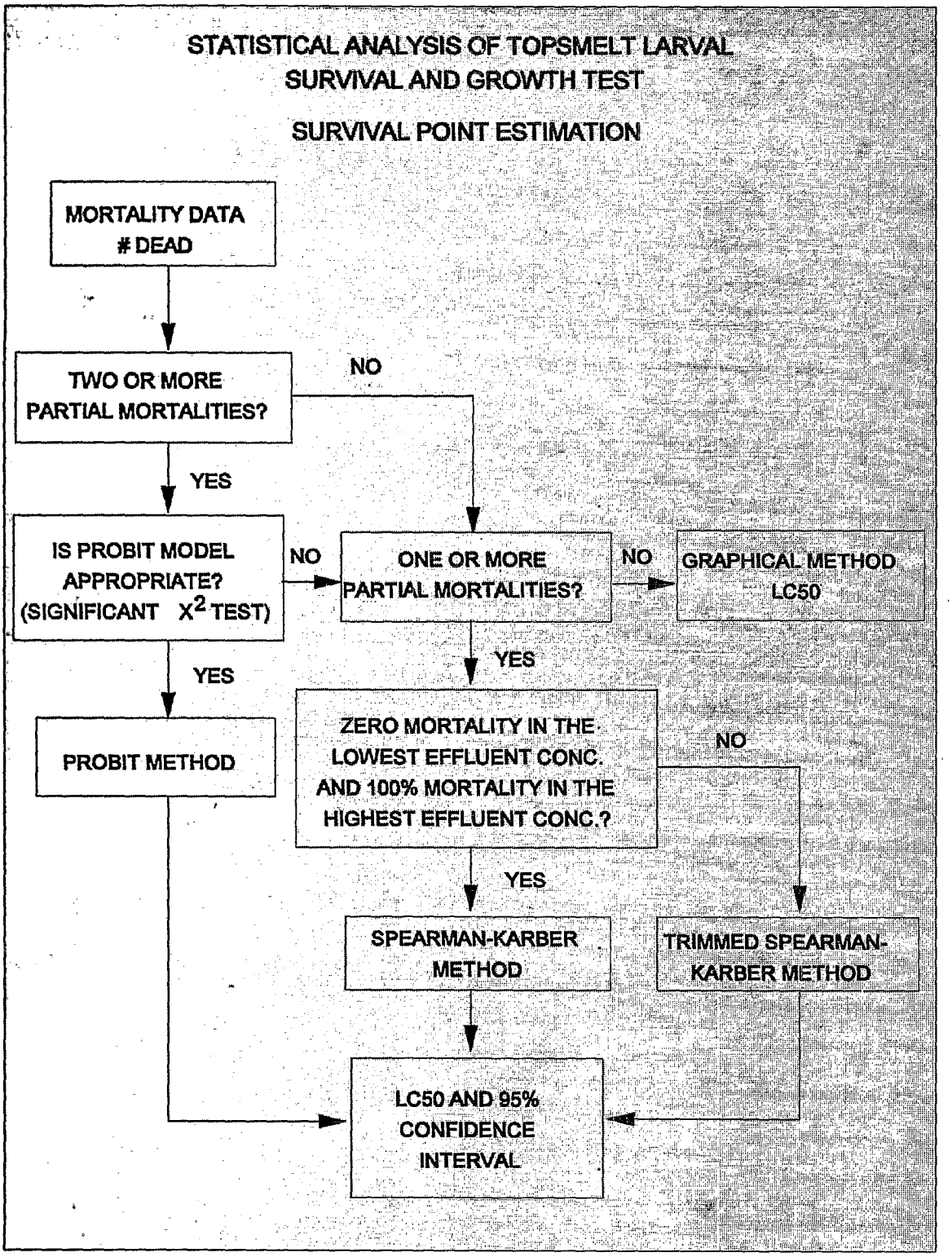

Figure 2. Flowchart for statistical analysis of the topsmelt, *Atherinis affinis*, larval survival data by point estimation.

TABLE 5. TOPSMELT, *ATHERINOPS AFFINIS*, SURVIVAL DATA

			Copper Concentration (µg/L)	
	Replicate	Control	32.0	56.0
RAW	A	1.0	1.0	0.0
	B	0.8	1.0	0.6
	C	1.0	1.0	0.2
	D	1.0	1.0	1.0
	E	1.0	1.0	0.6
ARC SINE SQUARE ROOT TRANSFORMED	A	1.345	1.345	0.225
	B	1.107	1.345	0.886
	C	1.345	1.345	0.464
	D	1.345	1.345	1.345
	E	1.345	1.345	0.886
Mean ($\bar{Y}_i$)		1.297	1.345	0.761
S^2		0.0113	0.000	0.187
i^i		1	2	3

11.13.2.6 Test for Normality

11.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

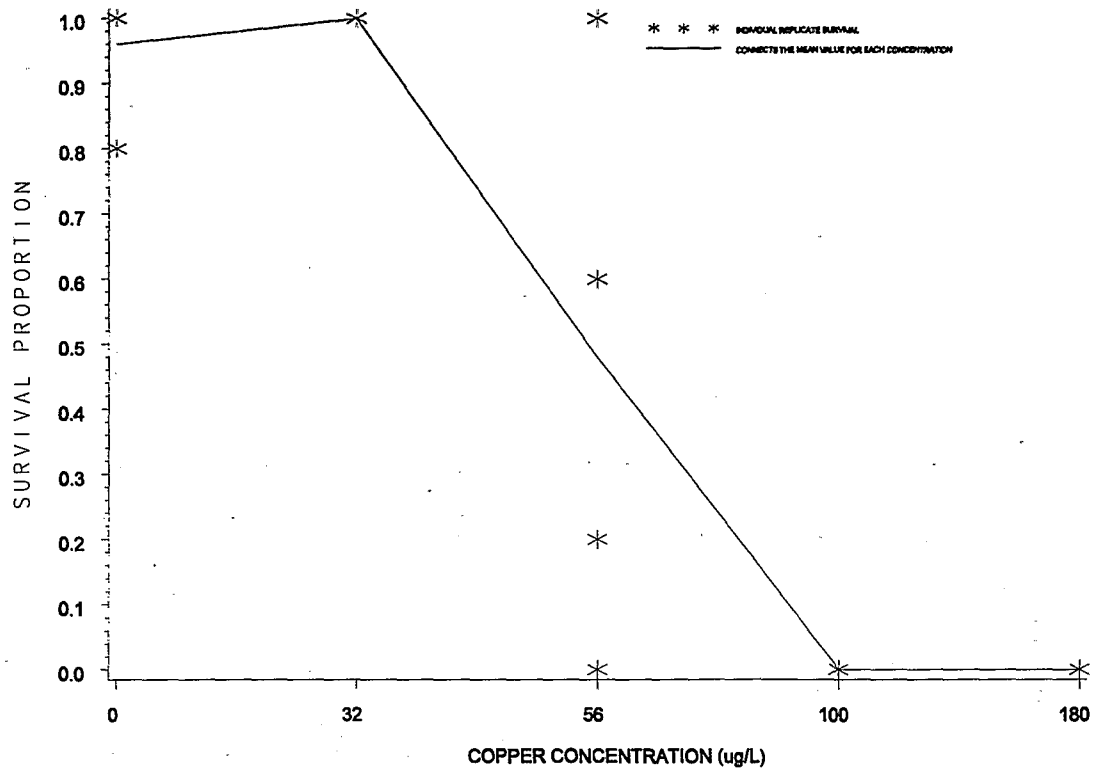

Figure 3 Plot of mean survival proportion data in Table 5.

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	Copper Concentration (µg/L)	
		32.0	56.0
A	0.048	0.000	-0.536
B	-0.190	0.000	0.125
C	0.048	0.000	-0.297
D	0.048	0.000	0.584
E	0.048	0.000	0.125

11.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

Where: X_i = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations

11.13.2.6.3 For this set of data,

$$n = 15$$

$$\overline{X} = \frac{1}{15} (0.003) = 0.000$$

$$D = 0.793$$

11.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 7.

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR THE SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.536	9	0.048
2	-0.297	10	0.048
3	-0.190	11	0.048
4	0.000	12	0.048
5	0.000	13	0.125
6	0.000	14	0.125
7	0.000	15	0.584
8	0.000		

11.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 15 and k = 7. The a_i values are listed in Table 8.

11.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 7. For the data in this example,

$$W = \frac{1}{0.793}(0.817)^2 = 0.842$$

11.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 11.13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 15 observations is 0.835. Since W = 0.842 is greater than the critical value, conclude that the data are normally distributed.

11.13.2.6.8 Since the variance of the lowest copper concentration group is zero, Bartlett's test statistic can not be calculated. Therefore, the survival data variances are considered to be heterogeneous.

11.13.2.6.9 Since the data do not meet the assumption of homogeneity of variance, Steel's Many-one Rank Test will be used

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.5150	1.120	$X^{(15)} - X^{(1)}$
2	0.3306	0.422	$X^{(14)} - X^{(2)}$
3	0.2495	0.315	$X^{(13)} - X^{(3)}$
4	0.1878	0.048	$X^{(12)} - X^{(4)}$
5	0.1353	0.048	$X^{(11)} - X^{(5)}$
6	0.0880	0.048	$X^{(10)} - X^{(6)}$
7	0.0433	0.048	$X^{(9)} - X^{(7)}$

to analyze the survival data.

11.13.2.7 Steel's Many-one Rank Test

11.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size

from smallest to largest. Assign the ranks (1, 2, ..., 10) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

11.13.2.7.2 An example of assigning ranks to the combined data for the control and 32.0 µg/L copper concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are next summed for each copper concentration, as shown in Table 11.

11.13.2.7.3 For this example, determine if the survival in any of the copper concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for the survival at each of the various copper concentrations with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with two concentrations (excluding the control) and five replicates is 18 (see Table 5, Appendix E).

11.13.2.7.4 Since the rank sum for the 56.0 µg/L copper concentration is equal to the critical value, the proportion surviving in the 56.0 µg/L concentration is considered significantly less than that in the control. Since the other rank sum is not less than or equal to the critical value, it is not considered to have a significantly lower proportion surviving than the control. Hence, the NOEC and the LOEC are the 32.0 µg/L and 56.0 µg/L concentrations, respectively.

11.13.2.8 Calculation of the LC50

11.13.2.8.1 The data used for the calculation of the LC50 is summarized in Table 12. For estimating the LC50, the data for the 100 µg/L and 180 µg/L copper concentrations with 100% mortality are included.

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 32.0 µg/L COPPER CONCENTRATION FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Surviving	Copper Concentration (µg/L)
1	1.107	Control
6	1.345	32.0
6	1.345	32.0
6	1.345	32.0
6	1.345	32.0
6	1.345	32.0
6	1.345	Control

TABLE 10. TABLE OF RANKS

Replicate	Copper Concentration (µg/L) Control	32.0	56.0
A	1.345 (6, 8)	1.345 (6)	0.225 (1)
B	1.107 (1, 5)	1.345 (6)	0.886 (3.5)
C	1.345 (6, 8)	1.345 (6)	0.464 (2)
D	1.345 (6, 8)	1.345 (6)	1.345 (8)
E	1.345 (6, 8)	1.345 (6)	0.886 (3.5)

TABLE 11. RANK SUMS

Copper Concentration (µg/L)	Rank Sum
32.0	30
56.0	18

11.13.2.8.2 Because there are is only one partial mortality in the set of copper concentration responses, Probit Analysis is not appropriate to calculate the LC50 and 95% confidence interval for this set of test data. Inspection of the data reveals that, once the data is smoothed and adjusted, the proportion mortality in the lowest effluent concentration will be zero and the proportion mortality in the highest effluent concentration will be one. Therefore, the Spearman-Karber Method is appropriate for this data.

11.13.2.8.3 Before the LC50 can be calculated the data must be smoothed and adjusted. For the data in this example, because the observed proportion mortality for the 32.0 µg/L copper concentration is less than the observed response proportion for the control, the observed responses for the control and this group must be averaged:

$$p_0^s = p_1^s = \frac{0.040+0.000}{2} = 0.020$$

Where: p_i^s = the smoothed observed mortality proportion for effluent concentration i.

11.13.2.8.3.1 Because the rest of the responses are monotonic, additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table 12.

11.13.2.8.4 Because the smoothed observed proportion mortality for the control is now greater than zero, the data in each effluent concentration must be adjusted using Abbott's formula

(Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i

11.13.2.8.4.1 For the data in this example, the data for each effluent concentration must be adjusted for control mortality using Abbott's formula, as follows:

$$p_0^a = p_1^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.020 - 0.020}{1 - 0.020} = \frac{0.000}{0.980} = 0.0$$

$$p_2^a = \frac{p_2^s - p_0^s}{1 - p_0^s} = \frac{0.520 - 0.020}{1 - 0.020} = \frac{0.500}{0.980} = 0.510$$

$$p_3^a = p_4^a = \frac{p_3^s - p_0^s}{1 - p_0^s} = \frac{1.000 - 0.020}{1 - 0.020} = \frac{0.980}{0.980} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table 12.

11.13.2.8.5 Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k-1} \frac{(p_{i+1}^a - p_i)(X_i + X_{i+1})}{2}$$

Where: p_i^a = the smoothed adjusted proportion mortality at concentration i

X_i = the $\log_{10}$ of concentration i

k = the number of effluent concentrations tested, not including the control

TABLE 12. DATA FOR EXAMPLE OF SPEARMAN-KARBER ANALYSIS

Copper Concentration %	Number of Deaths	Number of Organisms Exposed	Smoothed Mortality Proportion	Adjusted Mortality Proportion	Mortality Proportion
Control	1	25	0.040	0.020	0.000
32.0	0	25	0.000	0.020	0.000
56.0	13	25	0.520	0.520	0.510
100.0	25	25	1.000	1.000	1.000
180.0	25	25	1.000	1.000	1.000

11.13.2.8.5.1 For this example, the $\log_{10}$ of the estimated LC50, m, is calculated as follows:

$$m = [(0.510 - 0.000)\ (1.5051 + 1.7482)]/2 + [(1.000 - 0.510)\ (1.7482 + 2.0000)]/2 + [(1.000 - 1.000)\ (2.0000 + 2.2553)]/2 +$$

$$= 1.7479$$

11.13.2.8.6 Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k-1} \frac{p_i^a (1-p_i^a)\ (X_{i+1}+X_{i-1})^2}{4(n_i-1)}$$

Where: X_i = the $\log_{10}$ of concentration i

n_i = the number of organisms tested at effluent concentration i

p_i^a = the smoothed adjusted observed proportion mortality at effluent concentration i

k = the number of effluent concentrations tested, not including the control

11.13.2.8.6.1 For this example, the estimated variance of m, V(m), is calculated as follows:

$$V(m) = (0.510)(0.490)(2.0000 - 1.5051)^2/4(24) + (1.000)(0.000)(2.2553 - 1.7482)^2/4(24)$$

$$= 0.0006376$$

11.13.2.8.7 Calculate the 95% confidence interval for m: $m \pm 2.0 \sqrt{V(m)}$

11.13.2.8.7.1 For this example, the 95% confidence interval for m is calculated as follows:

$$1.7479 \pm 2 \sqrt{0.0006376} = (1.6974, 1.7984)$$

11.13.2.8.8 The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking $base_{10}$ antilogs of the above values.

11.13.2.8.8.1 For this example, the estimated LC50 is calculated as follows:

$$LC50 = antilog(m) = antilog(1.7479) = 56.0\ \mu g/L.$$

11.13.2.8.8.2 The limits of the 95% confidence interval for the estimated LC50 are calculated by taking the antilogs of the upper and lower limits of the 95% confidence interval for m as follows:

lower limit: $antilog(1.6974) = 49.8\ \mu g/L$

upper limit: $antilog(1.7984) = 62.9\ \mu g/L$

11.13.3 EXAMPLE OF ANALYSIS OF TOPSMELT, *ATHERINOPS AFFINIS*, GROWTH DATA

11.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 4.

The response used in the statistical analysis is mean weight per surviving organism for each replicate. The IC25 can be calculated for the growth data via a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC and LOEC for growth. Concentrations above the NOEC for survival are excluded from the hypothesis test for growth effects.

11.13.3.2 The statistical analysis using hypothesis testing consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steels' Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

11.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative. For detailed information on the Bonferroni adjustment, see Appendix D.

11.13.3.4 The data, mean and variance of the observations at each concentration including the control are listed in Table 13. A plot of the mean weights for each treatment is provided in Figure 5. Since there is no survival in the 100 µg/L and 180 µg/L copper concentrations, they are not considered in the growth analysis. Additionally, since there is significant mortality in the 56.0 µg/L concentration, its effect on growth is not considered.

11.13.3.5 Test for Normality

11.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 14.

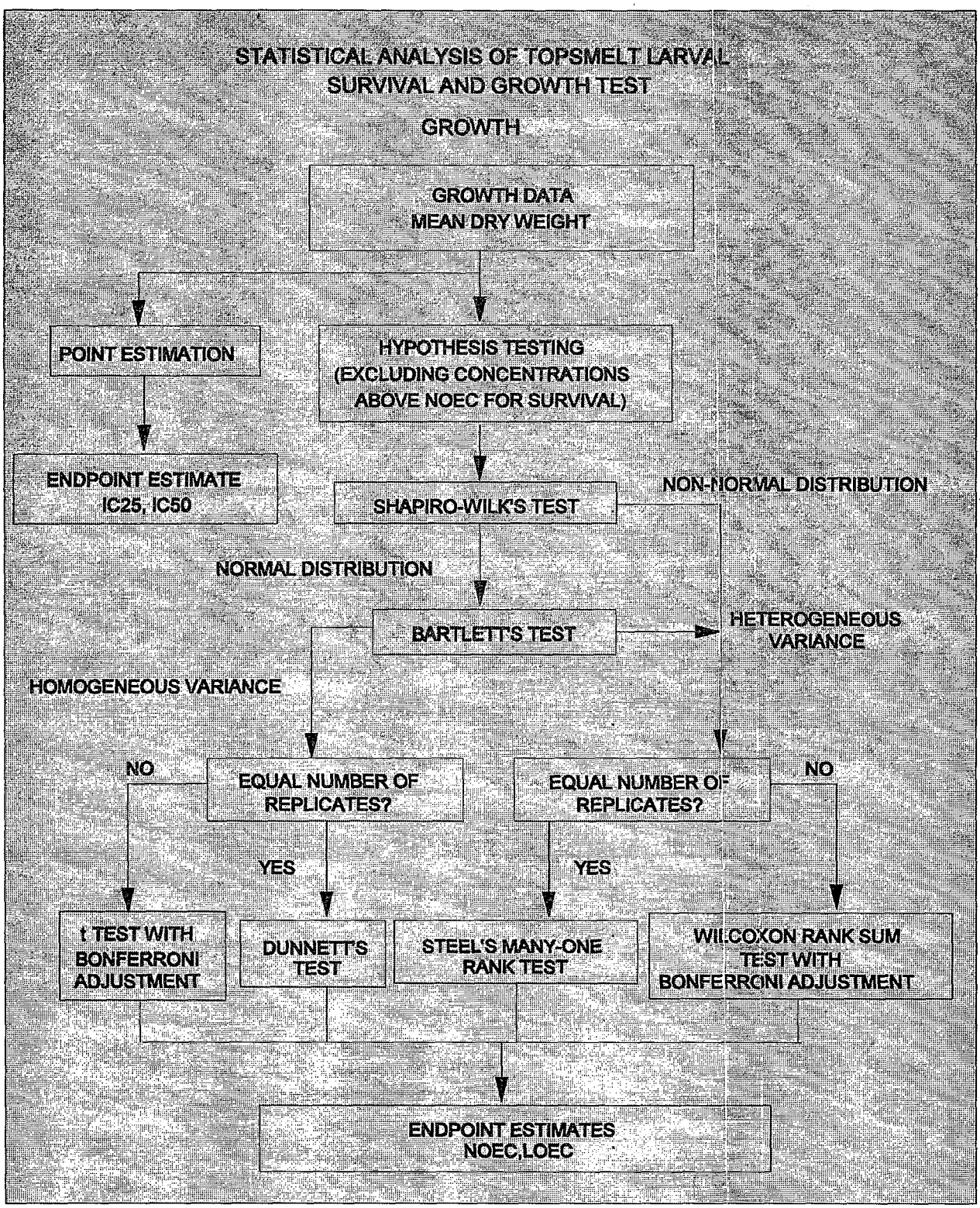

Figure 4. Flowchart for statistical analysis of the topsmelt, *Atherinops affinis*, larval growth data.

TABLE 13. TOPSMELT, *ATHERINOPS AFFINIS*, GROWTH DATA

		Copper Concentration (µg/L)			
Replicate	Control	32.0	56.0	100.0	180.0
A	0.00134	0.00146	-	-	-
B	0.00153	0.00142	-	-	-
C	0.00134	0.00150	-	-	-
D	0.00146	0.00128	-	-	-
E	0.00144	0.00141	-	-	-
Mean ($\bar{Y}_i$)	0.00142	0.00141	-	-	-
S_i^2	0.000000006	0.000000007	-	-	-
i	1	2	3	4	5

TABLE 14. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	32.0 µg/L Copper
A	-0.00008	0.00005
B	0.00011	0.00001
C	-0.00008	0.00009
D	0.00004	-0.00003
E	0.00002	-0.00013

11.13.3.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations.

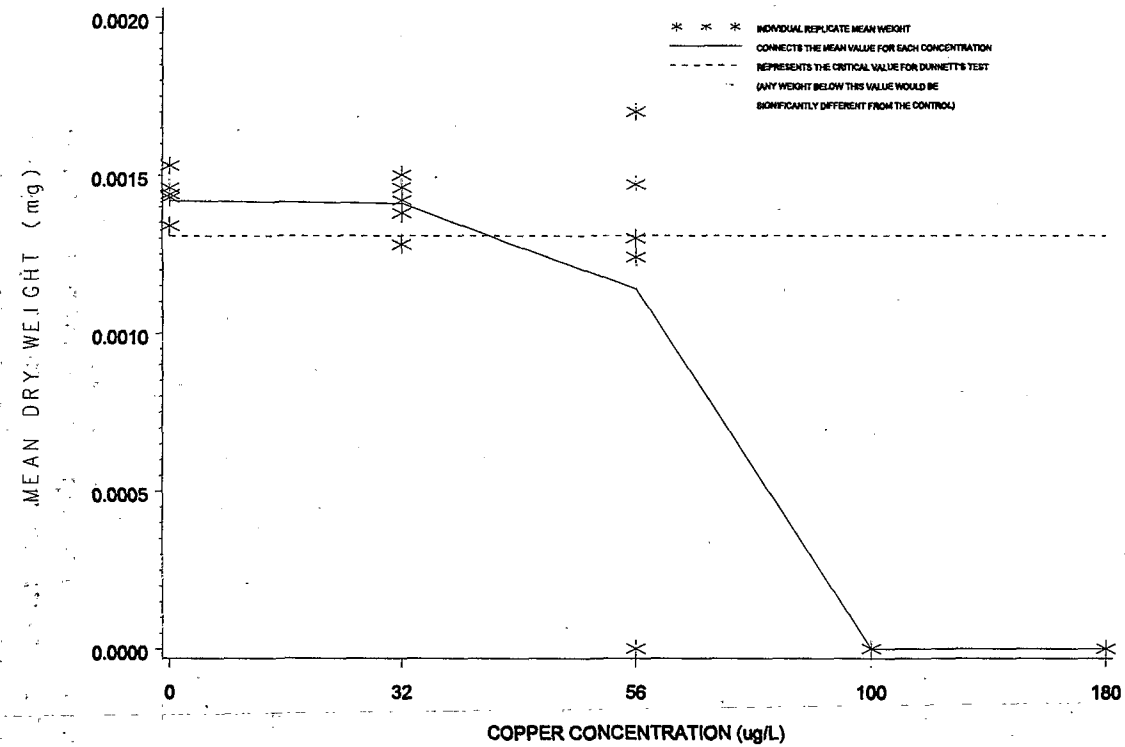

Figure 5. Plot of weight data from topsmelt, *Atherinis affinis*, larval survival and growth test.

For this set of data, n = 10

$$\overline{X} = \frac{1}{10}(0.00) = 0.00$$

$$D = 0.000000055$$

11.13.3.5.3 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 15.

11.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ..., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 10 and k = 5. The a_i values are listed in Table 16.

TABLE 15. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.00013	6	0.00002
2	-0.00008	7	0.00004
3	-0.00008	8	0.00005
4	-0.00003	9	0.00009
5	0.00001	10	0.00011

TABLE 16. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.5739	0.00024	$X^{(10)} - X^{(1)}$
2	0.3291	0.00017	$X^{(9)} - X^{(2)}$
3	0.2141	0.00013	$X^{(8)} - X^{(3)}$
4	0.1224	0.00007	$X^{(7)} - X^{(4)}$
5	0.0399	0.00001	$X^{(6)} - X^{(5)}$

11.13.3.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 16. For this set of data:

$$W = \frac{1}{0.000000055}(0.0002305)^2 = 0.966$$

11.13.3.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 10 observations (n) is 0.781. Since W = 0.966 is greater than the critical value, the conclude that the data are normally distributed.

11.13.3.6 Test for Homogeneity of Variance

11.13.3.6.1 The test used to examine whether the variation in mean dry weight is the same across all effluent concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{\left[\left(\sum_{i=1}^{P} V_i\right) \ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2\right]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, $V_i = (n_i - 1)$

n_i = the number of replicates for concentration i

p = number of levels of effluent concentration including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i^2)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{p} 1/V_i - (\sum_{i=1}^{p} V_i)^{-1}]$$

11.13.3.6.2 For the data in this example (see Table 14), all effluent concentrations including the control have the same number of replicates (n_i = 5 for all i). Thus, V_i = 4 for all i.

11.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(8)\ln(6.5 \times 10^{-9}) - 4\sum_{i=1}^{p}\ln(S_i^2)] / 1.125$$

$$= [8(-18.851) - 4(-37.709)]/1.125$$

$$= 0.028/1.125$$

$$= 0.0249$$

11.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with one degree of freedom, is 6.635. Since B = 0.0249 is less than the critical value of 6.635, conclude that the variances are not different.

11.13.3.7 Dunnett's Procedure

11.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 17.

TABLE 17. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$SSW = SST-SSB$ Within Sum of Squares

G = the grand total of all sample observations,

$$G = \sum_{i=1}^{P} T_i$$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean dry weight of the mysids for concentration i in test chamber j)

11.13.3.7.2 For the data in this example:

$n_1 = n_2 = 5$

$N = 10$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 0.00711$

$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 0.00704$

$G = T_1 + T_2 = 0.01415$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$

$$= \frac{1}{5}(1.001137 \times 10^{-4}) - \frac{(0.01415)^2}{10} = 4.90 \times 10^{-10}$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 0.0000201 - \frac{(0.01415)^2}{10} = 7.775 \times 10^{-8}$$

$SSW = SST-SSB = 7.775 \times 10^{-8} - (4.9 \times 10^{-10}) = 7.726 \times 10^{-8}$

$S_B = SSB/(p-1) = (4.9 \times 10^{-10})/(2-1) = 4.9 \times 10^{-10}$

$S_W = SSW/(N-p) = 7.726 \times 10^{-8}/(10-2) = 9.658 \times 10^{-9}$

11.13.3.7.3 Summarize these calculations in the ANOVA table (Table 18).

TABLE 18. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	1	4.90×10^{-10}	4.9×10^{-10}
Within	8	7.726×10^{-8}	9.658×10^{-9}
Total	9	7.775×10^{-8}	

11.13.3.7.4 To perform the individual comparisons, calculate the *t* statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean dry weight for effluent concentration i

$\bar{Y}_1$ = mean dry weight for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

11.13.3.7.5 Table 19 includes the calculated *t* values for each concentration and control combination. In this example there is only one comparison, of the 32.0 µg/L copper concentration with the control. The calculation is as follows:

$$t_2 = \frac{(0.00142 - 0.00141)}{[\,9.828 \times 10^{-5}\sqrt{(1/5) + (1/5)}\,]} = 0.161$$

TABLE 19. CALCULATED t VALUES

Copper Concentration (µg/L)	i	t_i
32.0	2	0.161

11.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean weight, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 8 degrees of freedom for error and one concentration (excluding the control) the critical value is 1.86. The mean weight for concentration i is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Since t_2 is less than 1.86, the 32.0 µg/L concentration does not have significantly lower growth than the control. Hence the NOEC and the LOEC for growth cannot be calculated.

11.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\ S_w\sqrt{(1/n_1) + (1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration
(this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

11.13.3.7.8 In this example:

$$MSD = 1.86\,(9.828 \times 10^{-5})\sqrt{(1/4) + (1/4)}$$

$$= 1.86\ (9.828 \times 10^{-5})\,(0.632)$$

$$= 0.000116$$

11.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.000116 mg.

11.13.3.7.10 This represents a 8.2% reduction in mean weight from the control.

11.13.3.8 Calculation of the ICp

11.13.3.8.1 The growth data from Table 4 are utilized in this example. As seen from Table 4 and Figure 6, the observed means are monotonically non-increasing with respect to concentration (mean response for each higher concentration is less than or equal to the mean response for the previous concentration and the responses between concentrations follow a linear trend). Therefore, the means do not require smoothing prior to calculating the IC. In the following discussion, the observed means are represented by $\overline{Y}_i$ and the smoothed means by M_i.

11.13.3.8.2 Since $\overline{Y}_5 = 0 < \overline{Y}_4 = 0 < \overline{Y}_3 = 0.00114 < \overline{Y}_2 = 0.00141 < \overline{Y}_1 = 0.00142$, set $M_1 = 0.00142$, $M_2 = 0.00141$, $M_3 = 0.00114$, $M_4 = 0$ and $M_5 = 0$.

11.13.3.8.3 Table 20 contains the response means and smoothed means and Figure 8 gives a plot of the smoothed response curve.

11.13.3.8.4 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in weight, compared to the controls, would result in a mean dry weight of 0.001065 mg, where $M_1(1-p/100) = 0.00142(1-25/100)$. Examining the smoothed means and their associated concentrations (Table 20), the response, 0.001065 mg, is bracketed by $C_3 = 56.0$ µg/L copper and $C_4 = 100.0$ µg/L copper.

11.13.3.8.5 Using the equation from Section 4.2 of Appendix M, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_i(1-p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 56.0 + [0.00142(1 - 25/100) - 0.00114]\ \frac{(100.0 - 56.0)}{(0.0 - 0.00114)}$$

$$= 58.9\ \mu g/L.$$

11.13.3.8.6 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 58.9089 µg/L. The empirical 95% confidence interval for the true mean was 44.2778 µg/L to 67.0000 µg/L. The computer program output for the IC25 for this data set is shown in Figure 7.

TABLE 20. TOPSMELT, *ATHERINOPS AFFINIS*, MEAN GROWTH RESPONSE AFTER SMOOTHING

Copper Conc. (µg/L)	i	Response Means (mg) $\bar{Y}_i$	Smoothed Means (mg) M_i
Control	1	0.00142	0.00142
32.0	2	0.00141	0.00141
56.0	3	0.00114	0.00114
100.0	4	0.0	0.0
180.0	5	0.0	0.0

11.14.1 PRECISION

11.14.1.1 Single-Laboratory Precision

11.14.1.1.1 Data on the single-laboratory precision of the topsmelt larval survival and growth test using copper chloride as the reference toxicant are provided in Tables 21 and 22. In the five copper tests presented here, the NOECs for survival were 100 µg/L for all tests but one; this test had a NOEC of 180 µg/L. The coefficient of variation for copper based on the LC25 is 17.3% for survival; the coefficient of variation for copper based

on the LC50 is 9.7% for survival. The weight endpoint was less sensitive than survival in all but one test. An IC25 could be calculated for three of five tests and the coefficient of variation for these three tests was 60.69%, the coefficient of variation based on the IC50 for these three tests was 4.75%.

11.14.1.2 Multilaboratory Precision

14.11.1.2.1 Data on the interlaboratory precision of the topsmelt larval survival and growth test are provided in Table 23. Three separate interlaboratory tests were conducted. In the first comparison both laboratories derived identical NOECs for copper (100µg/L). The coefficient of variation, based on LC50s for survival was 36%. In the second comparison the NOEC for effluent was 20% at both laboratories. The coefficient of variation, based on the LC50s for survival was 19%. In the third comparison the NOEC for copper was 32 µg/L at both laboratories. The coefficient of variation, based on the LC50s for survival was 3%.

11.11.2 ACCURACY

11.11.2.1 The accuracy of toxicity tests cannot be determined.

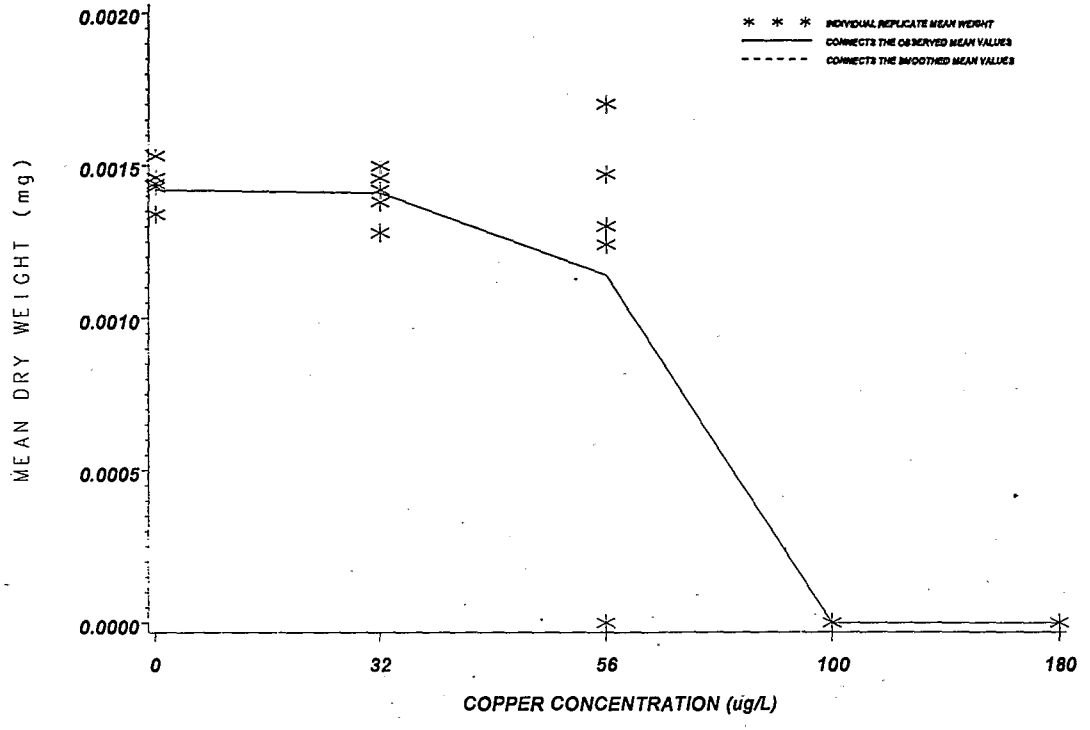

Figure 6. Plot of raw data, observed means, and smoothed means for topsmelt, *Atherinis affinis*, growth data from Tables 4 and 21.

Conc. ID	1	2	3	4	5
Conc. Tested	0	32	56	100	180
Response 1	.00134	.00146	0	0	0
Response 2	.00153	.00142	.00147	0	0
Response 3	.00134	.00150	.00170	0	0
Response 4	.00146	.00138	.00124	0	0
Response 5	.00144	.00128	.00130	0	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date: Test Ending Date:
Test Species: Atherinops affinis
Test Duration: 7 days
DATA FILE: wc_aa.icp
OUTPUT FILE: wc_aa.i25

Conc. ID	Number Replicates	Concentration ug/L	Response Means	Std. Dev.	Pooled Response Means
1	5	0.000	0.001	0.000	0.001
2	5	32.000	0.001	0.000	0.001
3	5	56.000	0.001	0.001	0.001
4	5	100.000	0.000	0.000	0.000
5	5	180.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 58.9089 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 58.1571 Standard Deviation: 7.9299
Original Confidence Limits: Lower: 44.2778 Upper: 67.0000
Expanded Confidence Limits: Lower: 36.9622 Upper: 71.0455
Resampling time in Seconds: 0.11 Random_Seed: -498847050

Figure 7. ICPIN program output for the IC25

TABLE 21. SINGLE LABORATORY PRECISION OF THE TOPSMELT, *ATHERINOPS AFFINIS* SURVIVAL ENDPOINT WITH COPPER (CU µG/L) CHLORIDE AS A REFERENCE TOXICANT

Test Number	NOEC	LC25	LC50
1	100	142.1	187.4
2	100	NC[3]	162.4
3	100	151.7	165.6
4	180	181.0	190.6
5	100	119.2	204.0

# of Tests	Statistic	LC25	LC50
5	Mean SD CV (%)	148.5 25.6 17.3	182.0 17.6 9.7%

TABLE 22. SINGLE LABORATORY PRECISION OF THE TOPSMELT, *ATHERINOPS AFFINIS* GROWTH ENDPOINT WITH COPPER (CU µG/L) CHLORIDE AS A REFERENCE TOXICANT

Test Number	NOEC	LC25	LC50
1	180	222.1	264.2
2	180	NC[4]	NC[4]
3	>180	NC[4]	NC[4]
4	56	47.6	NC[4]
5	>180	NC[4]	NC[4]

# of Tests	Statistic	LC25	LC50
5	Mean SD CV (%)	156.8 95.2 60.7%	

[1]Data from Anderson et al. 1994; point estimates calculated using probit analysis, except where noted.
[2]Five replicate exposure chambers with five larvae per chamber were used for each treatment.
[3]LC50 calculated using Spearman-Karber method, this method does not calculate an LC25.
[4]Point estimate not calculated because the response was less than either 25 or 50%.

TABLE 23. MULTI-LABORATORY PRECISION OF THE TOPSMELT, *ATHERINOPS AFFINIS*, GROWTH AND SURVIVAL TEST CONDUCTED WITH COPPER (CU μG/L) CHLORIDE AS A REFERENCE TOXICANT

Test Number	Toxicant	Laboratory	Survival NOEC	Survival LC50	Growth
1	Copper[a]	1[b]	100	162.0	NS[c]
	Copper[a]	2[d]	100	274.0	NS
	CV			36%	
2	Effluent	1[b]	20	31.4	NS
	Effluent	2[e]	20	23.9	10
	CV			19%	
3	Copper[a]	1[b]	32	55.7	NS
	Copper[a]	1[e]	32	58.4	NS
	CV			3%	

Two separate interlaboratory comparisons were conducted, in August 1990 and August 1991.

[a]The August 1990 copper test was conducted at 34‰ salinity; the August 1991 copper test was conducted at 20‰ salinity.
[b]Marine Pollution Studies Laboratory, Monterey County, California.
[c]Not Significant.
[d]Vantuna Research Group, Occidental College, California.
[e]Chevron Research and Technology Co., Environmental Research Group.

APPENDIX I. TOPSMELT TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution (10,000 µg/L) by adding 0.0268 g of copper chloride ($CuCl_2$°$2H_2O$) to 1 liter of reagent water.

D. Prepare zinc reference toxicant solution of 0 (control) 56, 100, 180, and 180 µg/L by adding 0, 5.6, 10.0, 18.0, and 32.0 mL of stock solution, respectively, to a 1-L volumetric flask and filling to 1-L with dilution water.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 20°C and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Obtain 9-15 day old larvae from a commerical supplier or in-house cultures.

B. Larvae must be randomized before placing them into the test chambers. Be sure that all water used in culture, transfer, and test solutions is within 1°C of the test temperature.

C. Remove all dead larvae daily, and add 40 newly hatched *Artemia* nauplii per larva twice daily; once in the morning and once in the afternoon. Adjust feeding to account for larva mortality.

D. Renew test solutions daily using freshly prepared solutions, immediately after cleaning the test chambers.

E. After 7 days, count and record the number of live and dead larvae in each chamber. After counting, use the randomization sheet to assign the correct test concentration to each chamber. Remove all dead larvae.

F. The surviving larvae in each test chamber are immediately prepared as a group for dry weight determination, or preserved in 4% formalin then 70% ethanol. Preserved organisms are dried and weighed with 7 days.

G. Carefully transfer the larvae to a prenumbered, preweighed micro-weigh boat using fine-tipped forceps. Dry for 24 hours at 60°C or at 105°C for a minimum of 6 hours. Weigh each weigh boat on a microbalance (accurate to 1 μg). Record the chamber number, larvae weight, weigh boat weight (recorded previously), and number of larvae per weigh boat (replicate) on the data sheet.

H. Analyze the data.

I. Include standard reference toxicant point estimate values in the standard quality control charts.

Data Sheet for Larval Fish Toxicity Test

Test Start Date: Start Time:

Fish Species:

Test End Date: End Time:

Collection/Arrival Date:

Reference Toxicant:

Broodstock Source:

Fish Age at Start:

Test Cont. #	Concentration	Numer Alive							Total Number Alive	Total Number at Start	Notes
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			

Computer Data Storage
Disk
File

Note: See larval weight data on separate sheet.

Data Sheet for Weighing Larval Fish

Test Start Date: Start Time: Fish Species :

Test End Date: End Time: Collection/Arrival Date:

Toxicant: Fish Age at Start:

Sample Source:

Sample Type: Sediment Elutriate Porewater Water

Test Container Number	Site Code or Concentration	Foil Number	Foil Weight (mg)	Total Weight (mg)	Weight of Larval Fish (mg)	Number of Fish Larvae	Weight per Larval Fish (mg)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							
21							
22							
23							
24							
25							
26							
27							
28							
29							
30							
31							
32							
33							
34							
35							

Computer Data Storage
Disk:
File:

Notes

Note: See larval mortality data on separate sheet.

SECTION 12

MYSID, *Holmesimysis costata* SURVIVAL AND GROWTH TEST METHOD

Adapted from a method developed by
John W. Hunt, Brian S. Anderson and Sheila L. Turpen
Institute of Marine Sciences, University of California
Santa Cruz, California

(in association with)
California Department of Fish and Game
Marine Pollution Studies Laboratory
34500 Coast Route 1, Monterey, CA 93940

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SECTION 12

MYSID, *HOLMESIMYSIS COSTATA* SURVIVAL AND GROWTH TEST

12.1 SCOPE AND APPLICATION

12.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the mysid, *Holmesimysis costata*, using three-to-four day old juveniles in a seven-day, static-renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all chemical, physical, and additive components which adversely affect the physiological and biochemical functions of the test organisms.

12.1.2 Daily observations of mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

12.1.3 Detection limits of the toxicity of an effluent or a pure substance are organism dependent.

12.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because test chambers are not sealed, highly volatile and highly degradable toxicants present in the source may not be detected in the test.

12.1.5 This method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

12.1.6 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

12.2 SUMMARY OF METHOD

12.2.1 This method provides step-by-step instructions for

performing a 7-day static-renewal toxicity test using growth and survival juvenile mysids to determine the toxicity of substances in marine waters. The test endpoints are survival and growth.

12.3 INTERFERENCES

12.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

12.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.3.3 The test results can be confounded by (1) the presence of pathogenic and/or predatory organisms in the dilution water, effluent, and receiving water, (2) the condition of the brood stock from which the test animals were taken, (3) the amount and type of natural food in the effluent, receiving water, or dilution water, (4) nutritional value of the brine shrimp, *Artemia* nauplii, fed during the test, and (5) the quality of the brine shrimp, *Artemia* nauplii, or other food added during the test, which may sequester metals and other toxic substances, and lower the DO.

12.4 SAFETY

12.4.1 See Section 3, Health and Safety.

12.5 APPARATUS AND EQUIPMENT

12.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult mysids, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20°C), with appropriate filtration and aeration system.

12.5.2 Air pump, air lines, and air stones -- for aerating water containing mysids for supplying air to test solutions with low dissolved oxygen.

12.5.3 Constant temperature chambers or water baths -- for maintaing test solution temperature and keeping dilution water

supply, juvenile mysids, and stock suspensions at test temperature (13 or 15°C) prior to the test.

12.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

12.5.5 Refractometer -- for determining salinity.

12.5.6 Hydrometer(s) -- for calibrating refractometer.

12.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

12.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD 170.1, USEPA, 1979) -- to calibrate laboratory thermometers.

12.5.9 pH and DO meters -- for routine physical and chemical measurements.

12.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

12.5.11 Winkler bottles -- for dissolved oxygen determinations.

12.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g (for weighing reference toxicants).

12.5.13 Microbalance -- Analytical, capable of accurately weighing to 0.000001 g (for weighing mysids).

12.5.14 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

12.5.15 Glass stirring rods -- for mixing test solutions.

12.5.16 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-1000 mL for making test solutions.

12.5.17 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

12.5.18 Pipets, automatic -- adjustable, to cover a range of

delivery volumes from 0.010 to 100 mL.

12.5.19 Pipet bulbs and fillers -- PROPIPET® or equivalent.

12.5.20 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

12.5.21 Wash bottles -- for dilution water.

12.5.22 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

12.5.23 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

12.5.24 Pipets, volumetric: 1, 10, 25, 50, and 100 mL -- for dilutions.

12.5.25 Plastic randomization cups (approximately 100 mL, one for each test chamber).

12.5.26 Brine shrimp, *Artemia*, culture unit -- see Subsection 12.6.24 and Section 4, Quality Assurance.

12.5.27 Separatory funnels, 2-L -- two to four for culturing *Artemia*.

12.5.28 Mysid culture apparatus (see Section 12.6.25.5). This test requires 400 three- to four-day-old juvenile mysids.

12.5.29 Gear for collecting adult mysids, including a small boat, 0.5 mm-mesh hand nets, plastic buckets, and portable air supply (mysids may also be obtained from commercial suppliers;).

12.5.30 Pipet bulbs and glass tubes (4 mm diameter, with fire-polished edges) for handling adult mysids.

12.5.31 Siphon tubes (fire polished glass with attached silicone tubing) -- for test solution renewals.

12.5.32 Fire-polished wide-bore 10 mL pipet -- for handling juveniles.

12.5.33 Forceps with fine points -- for transferring juveniles to weighing pans.

12.5.34 Light box -- for examining organisms.

12.5.35 Drying oven, 50-105°C range -- for drying organisms.

12.5.36 Desiccator -- for holding dried organisms.

12.5.37 Clean NITEX® mesh sieves (≤ 150 μm, 500-1000μm) -- for concentrating organisms. (NITEX® is available from Sterling Marine Products, 18 Label Street, Montclair, NJ 07042; 201-783-9800).

12.5.38 60 μm NITEX® filter - for filtering receiving water.

12.6 **REAGENTS AND SUPPLIES**

12.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

12.6.2 Data sheets (one set per test) -- for data recording (Figures 1 and 2).

12.6.3 Tape, colored -- for labelling test chambers and containers.

12.6.4 Markers, water-proof -- for marking containers, etc.

12.6.5 Parafilm -- to cover graduated cylinders and vessels.

12.6.6 Gloves, disposable -- for personal protection from contamination.

12.6.7 Pipets, serological -- 1-10 mL, graduated.

12.6.8 Pipet tips -- for automatic pipets.

12.6.9 Coverslips -- for microscope slides.

12.6.10 Lens paper -- for cleaning microscope optics.

12.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

12.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

12.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

12.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

12.6.15 Laboratory quality assurance samples and standards -- for the above methods.

12.6.16 Test chambers -- 1000 mL, five chambers per concentration. The chambers should be borosilicate glass (for effluents) or nontoxic disposable plastic labware (for reference toxicants). To avoid contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered during the test with safety glass plates or a plastic sheet (6 mm thick).

12.6.17 Micro-weighing pans, aluminum -- to determine the dry weight of organisms. Weighting pan should be about 5 mg or less to minimize noise in measurement of the small mysids.

12.6.18 Fronds of kelp (*Macrocystis*) for habitat in culture.

12.6.19 Reference toxicant solutions (see Subsection 12.10.2.4 and see Section 4, Quality Assurance).

12.6.20 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies and Section 7, Dilution Water).

12.6.21 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

12.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 12.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-μm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

12.6.23 HYPERSALINE BRINES

12.6.23.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

12.6.23.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

12.6.23.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 μm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

12.6.23.4 Freeze Preparation of Brine

12.6.23.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20°C until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

12.6.23.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100%. It is advisable not to exceed about 70% brine salinity unless it is necessary to test effluent concentrations greater than 50%.

12.6.23.4.3 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

12.6.23.5 Heat Preparation of Brine

12.6.23.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

12.6.23.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and

any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

12.6.23.5.3 Seawater should be filtered to at least 10 μm before being put into the brine generator. The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

12.6.23.5.4 After the required salinity is attained, the HSB

should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

12.6.23.6 Artificial Sea Salts

12.6.23.6.1 No data from mysids using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

12.6.23.7 Dilution Water Preparation from Brine

12.6.23.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

12.6.23.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. The proportion of brine is 1 part, plus 1.94 parts reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

12.6.23.8 Test Solution Salinity Adjustment

12.6.23.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

12.6.23.8.2 Check the pH of all brine mixtures and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide(see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.6.23.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

12.6.23.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

12.6.23.9 Preparing Test Solutions

12.6.23.9.1 Two hundred mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 10 mL of effluent to a 1-liter volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 1-liter mark with dilution water, stopper it, and shake to mix. Distribute equal volumes into the replicate test chambers.

12.6.23.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see

equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Distribute equal volumes into the replicate test chambers.

12.6.23.10 Brine Controls

12.6.23.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 12.6.23.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

12.6.24 BRINE SHRIMP, *ARTEMIA SP.*, NAUPLII -- for feeding cultures and test organisms.

12.6.24.1 Newly hatched *Artemia sp.* nauplii are used for food for the stock cultures and test organisms. Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are preferred because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size. (One source that has been found to be acceptable is Aquarium Products, 180L Penrod Ct., Glen Burnie, Maryland 21061). For commercial sources of brine shrimp, *Artemia*, cysts, see Table 2 of Section 5, Facilities, Equipment, and Supplies); and Section 4, Quality Assurance.

12.6.24.2 Each new batch of *Artemia* cysts should be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (Leger, et al., 1985, Leger, et al., 1986) against known suitable reference cysts by performing a side-by-side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be

a previously tested and acceptable batch of cysts, or may be obtained from the Quality Assurance Research Division, EMSL, Cincinnati, OH 45268, 513-569-7325. A sample of newly-hatched *Artemia* nauplii from each new batch of cysts should be chemically analyzed. The *Artemia* cysts should not be used if the concentration of total organochlorine pesticides 0.15 ug/g wet weight or that the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight (For analytical methods see USEPA, 1982).

12.6.24.3 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or an aqueous unionized salt (NaCl) solution prepared with 35 g salt or artificial sea salts per liter, to a 2-L separatory funnel, or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24 h at 27°C. Hatching time varies with incubation temperature and the geographic strain of *Artemia* used (see USEPA, 1985a; USEPA, 1993a; ASTM, 1993).
3. After 24 h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic, and will concentrate at the bottom of the funnel if it is covered for 5-10 minutes with a dark cloth or paper towel. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 min without aeration.
4. Drain the nauplii into a funnel fitted with a ≤150 µm NITEX® or stainless steel screen, and rinse with seawater or equivalent before use.

12.6.24.4 Testing *Artemia* nauplii as food for toxicity test organisms.

12.6.24.4.1 The primary criteria for acceptability of each new supply of brine shrimp cysts is adequate survival, and growth of the mysids. The mysids used to evaluate the acceptability of the brine shrimp nauplii must be the same geographical origin and stage of development (3 to 4 days old) as those used routinely in the toxicity tests. Two 7-day chronic tests are performed side-by-side, each consisting of five replicate test vessels containing five juveniles (25 organisms per test, total of 50

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (x‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with deionized water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

organisms). The juveniles in one set of test chambers is fed reference (acceptable) nauplii and the other set is fed nauplii from the "new" source of *Artemia* cysts.

12.6.24.4.2 The feeding rate and frequency, test vessels, volume of control water, duration of the tests, and age of the *Artemia* nauplii at the start of the test, should be the same as used for the routine toxicity tests.

12.6.24.4.3 Results of the brine shrimp, *Artemia*, nauplii nutrition assay, where there are only two treatments, can be evaluated statistically by use of a t test. The "new" food is acceptable if there are no statistically significant differences in the survival or growth of the mysids fed the two sources of nauplii.

12.6.25 TEST ORGANISMS

12.6.25.1 The test organisms for this method are juveniles of the mysid crustacean, *Holmesimysis costata* (Holmes 1900; previously referred to as *Acanthomysis sculpta*). *H. costata* occurs in the surface canopy of the giant kelp *Macrocystis pyrifera* where it feeds on zooplankters, kelp, epiphytes, and detritus. There are few references to the ecology of this mysid species (Holmquist, 1979; Clutter, 1967, 1969; Green, 1970; Turpen et al., 1994). *H. costata* is numerically abundant in kelp forest habitats and is considered to be an important food source for kelp forest fish (Clark 1971, Mauchline 1980). Mysids are called opossum shrimp because females brood their young in an abdominal pouch, the marsupium. *H. costata* eggs develop for about 20 days in the marsupium before the young are released as juveniles; broods are released at night during molting. Females release their first brood at 55 to 70 days post-release (at 12°C), and may have multiple broods throughout their approximately 120-day life.

12.6.25.2 *H. costata* has been used in previous toxicity studies with a variety of toxicants (Tatem and Portzer, 1985; Davidson et al., 1986; Machuzac and Mikel, 1987; Reish and Lemay, 1988; Asato, 1988; Martin et al., 1989; Singer et al., 1990; 1991; Hunt et al., In Press). Mysids are useful as toxicity test organisms because of their widespread availability, ecological importance, sensitivity to toxicants, and amenability to laboratory culture

(Nimmo et al., 1977; Mauchline, 1980; Gentile et al., 1982; Lussier et al., 1985).

12.6.25.3 Species Identification

12.6.25.3.1 Laboratories unfamiliar with the test organism should collect preliminary samples to verify species identification. Refer to Holmquist (1979) or send samples of mysids and any similar co-occurring organisms to a qualified taxonomist. Request certification of species identification from any organism suppliers. Records of verification should be maintained along with a few preserved specimens.

12.6.25.3.2 There have been recent revisions to the taxonomy of *H. costata*. Previous authors have referred to this species as *Acanthomysis sculpta*. However, Holmquist's (1979) review considers previous references to *Acanthomysis sculpta* in California to be synonymous with *Holmesimysis costata*; we consider Holmquist's designation to be definitive.

12.6.25.4 Obtaining Broodstock

12.6.25.4.1 *H. costata* can be collected by sweeping a small-mesh (0.5 - 1 mm) hand net through the water just under the surface canopy blades of giant kelp *Macrocystis pyrifera*. Although this method collects mysids of all sizes, attention should be paid to the number of gravid females collected because these are used to produce the juvenile mysids used in toxicity testing. Mysids should be collected from waters remote from sources of pollution to minimize the possibility of physiological or genetic adaptation to toxicants.

12.6.25.4.2 Mysids can be transported for a short time (< 3 hours) in tightly covered 20 liter plastic buckets. The buckets should be filled to the top with seawater from the collection site, and should be gently aerated or oxygenated to maintain dissolved oxygen above 60% saturation. Transport temperatures should remain within 3°C of the temperature at the collection site.

12.6.25.4.3 For longer transport times of up to 36 hours, mysids can be shipped in sealed plastic bags filled with seawater. The following transport procedure has been used successfully: 1)

fill the plastic bag with one liter of dilution water seawater, 2) saturate the seawater with oxygen by bubbling pure oxygen for at least 10 minutes, 3) place 25-30 adult mysids, or up to 100 juvenile mysids in each bag, 4) for adults add about 20 *Artemia* nauplii per mysid, for 100 juveniles add a pinch (10 to 20 mg) of ground Tetramin® flake food and 200 newly-hatched *Artemia* nauplii, 5) seal the bag securely, eliminating any airspace, then 6) place it within a second sealed bag in an ice chest. Do not overfeed mysids in transport, as this may deplete dissolved oxygen, causing stress or mortality in transported mysids. A well insulated ice chest should be cooled to approximately 15°C by adding one 1-liter blue ice block for every five 1-liter bags of mysids (a temperature range of 12 to 16°C is tolerable). Wrap the ice in newspaper and a plastic bag to insulate it from the mysid bags. Pack the bags tightly to avoid shifting within the cooler.

12.6.25.5 Broodstock Culture and Handling

12.6.25.5.1 After collection, the mysids should be transported directly to the laboratory and placed in seawater tanks or aquaria equipped with flowing seawater or adequate aeration and filtration. Initial flow rates should be adjusted so that any temperature change occurs gradually (0.5°C per hour). The water temperature should be held at 15 ± 1°C. **Note:** Mysids collected north of Pt. Conception, California, should be held and tested at 13 ± 1°C.

12.6.25.5.2 Mysids can be cultured in tanks ranging from 4 to 1000 liters. Tanks should be equipped with gentle aeration and blades of *Macrocystis* to provide habitat. Static culture tanks can be used if there is constant aeration, temperature control, and frequent water changes (one half the water volume changed at least twice a week). Maintain culture density below 20 animals per liter by culling out adult males or juveniles.

12.6.25.5.3 Adult mysids should be fed 100 *Artemia* nauplii per mysid per day. Juveniles should be fed 5 to 10 newly released *Artemia* nauplii per juvenile per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. Static chambers should be carefully monitored and rations adjusted to

prevent overfeeding and fouling of culture water. Refer to section 12.6.19 for details of *Artemia* culture and quality control.

12.6.25.6 Culture Materials

12.6.25.6.1 Refer to Section 5, Facilities and Equipment, for a discussion of suitable materials to be used in laboratory culture of mysids. Be sure all new materials are properly leached in seawater before use. After use, all culture materials should be washed in soap and water, then rinsed with seawater before re-use.

12.6.25.7 Test Organisms

12.6.25.7.1 Approximately 150 gravid female mysids should be isolated to provide approximately 400 juveniles for each set of toxicity tests (5 juveniles/chamber x 30 reference toxicant chambers and approximately 35 effluent chambers, plus additional mysids so that only healthy active juveniles are used in the test). Gravid females can be identified by their large, extended marsupia filled with (visible) eyed juveniles. Marsupia appear distended and gray when females are ready to release young, due to presence of the juveniles.

12.6.25.7.2 Gravid females are easily isolated from other mysids using the following technique: (1) use a small dip net to capture about 100 mysids from the culture tank, (2)transfer the mysids to a screen-bottomed plastic tube (150 μm-mesh, 25-cm diam.) partly immersed in a water bath or bucket, (3)lift the screen-tube out of the water to immobilize mysids on the damp screen, (4)gently draw the gravid females off the screen with a suction bulb and fire-polished glass tube (5-mm bore), (5) collect the gravid females in a separate screen tube. Re-immerse the screen continuously during the isolation process; mysids should not be exposed to air for more than a few seconds at a time.

12.6.25.7.3 Four or five days before a toxicity test begins, transfer gravid females into a removable, 2-mm-mesh screened cradle suspended within an aerated 80-liter aquarium. Before transfer, make sure there are no juveniles in with the adult females. Extraneous juveniles are excluded to avoid

inadvertently mixing them with the soon-to-be released juveniles used in testing. Provide the gravid females with newly hatched *Artemia* nauplii (approximately 200 per mysid) to help stimulate juvenile release. *Artemia* can be provided continuously throughout the night from an aerated reservoir holding approximately 75,000 *Artemia*. Direct the flow from the feeder into the screened compartment with the females, and add a few blades of *Macrocystis* for habitat. The females are placed within the screened compartment so that as the juveniles are released, they can swim through the mesh into the bottom of the aquarium. Outflows on flow-through aquaria should be screened (150-μm-mesh) to retain juveniles and allow some *Artemia* to escape.

12.6.25.7.4 Juveniles are generally released at night, so it is important to turn off all lights at night to promote release. In the morning, the screened compartment containing the females should be removed and placed in a separate aquarium. Juveniles should be slowly siphoned through a wide-diameter hose into a 150-μm-mesh screen-bottom tube (25 cm diam.) immersed in a bucket filled with clean seawater. Once the release aquarium is emptied, it should be washed with hot fresh water to eliminate stray juveniles that might mix with the next cohort.

12.6.25.7.5 After collection, the number of juveniles should be estimated visually or by counting subsamples with a small beaker. If there are not enough juveniles to conduct the necessary tests, they can be mixed with juveniles from one previous or subsequent release so that the test is initiated with three and/or four-day old juveniles. Initial experiments indicate that mysids 2-days-old and younger survive poorly in toxicity tests and that mysids older than four days may vary in their toxicant sensitivity or survival rate (Hunt et al., 1989; Martin et al., 1989).

12.6.25.7.6 Test juveniles should be transferred to additional screen-tubes (or to 4-liter static beakers if flowing seawater is unavailable). The screen-tubes are suspended in a 15-liter bucket so that dilution water seawater (0.5 liter/min) can flow into the tube, through the screen, and overflow from the bucket. Check water flow rates (< one liter/min) to make sure that juveniles or *Artemia* nauplii are not forced down onto the screen. The height of the bucket determines the level of water in the screen tube. About 200 to 300 juveniles can be held in each screen-tube (200 juveniles per static 4-liter beaker). Juveniles

should be fed 40 newly hatched *Artemia* nauplii per mysid per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. A blade of *Macrocystis* (well rinsed in seawater) should be added to each chamber. Chambers should be gently aerated and temperature controlled at 15 ± 1°C (or 13 ± 1°C if collected north of Pt. Conception). Half of the seawater in static chambers should be changed at least once between isolation and test initiation.

12.6.25.7.7 The day juveniles are isolated is designated day 0 (the morning after their nighttime release). The toxicity test should begin on day three or four. For example, if juveniles are isolated on Friday, the toxicity test should begin on the following Monday or Tuesday.

12.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

12.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

12.8 CALIBRATION AND STANDARIZATION

12.8.1 See Section 4, Quality Assurance.

12.9 QUALITY CONTROL

12.9.1 See Section 4, Quality Assurance.

12.10 TEST PROCEDURES

12.10.1 TEST DESIGN

12.10.1.1 The test consists of at least five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain five replicates of a brine control.

12.10.1.2 Effluent concentrations are expressed as percent effluent.

12.10.2 TEST SOLUTIONS

12.10.2.1 Receiving waters

12.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using five replicates chambers per test, each containing 200 mL would require approximately 1 L or more of sample per test per renewal.

12.10.2.2 Effluents

12.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100% HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

12.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

12.10.2.2.3 The volume of effluent required for a 75% renewal of five replicates per concentration for five concentrations of effluent and two controls, each containing 200 mL of test solution, is approximately 370 mL.

12.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

12.10.2.3 Dilution Water

12.10.2.3.1 Dilution water should be uncontaminated 1-μm-filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

12.10.2.4 Reference Toxicant Test

12.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

12.10.2.4.2 The preferred reference toxicant for mysids is zinc sulfate ($ZnSO_4 \cdot 7H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a 10,000 μg/L zinc stock solution by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

12.10.2.4.3 Reference toxicant solutions should be five replicates each of 0 (control), 10, 18, 32, and 56, and 100 μg/L total zinc. Prepare one liter of each concentration by adding 0, 1.0, 1.8, 3.2, 5.6, and 10.0 mL of stock solution, respectively, to one-liter volumetric flasks and fill with dilution water. Start with control solutions and progress to the highest concentration to minimize contamination.

12.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use juvenile originating from or released from the same pool of gravid females. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

12.10.3 START OF THE TEST

12.10.3.1 Prior to Beginning the Test

12.10.3.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

12.10.3.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (13 or 15 ± 1°C) and maintained at that temperature during the addition of dilution water.

12.10.3.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (13 or 15 ± 1°C).

12.10.3.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the mysids have been examined at the end of the test.

12.10.3.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

12.10.3.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly and filled with test solutions, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

12.10.3.2 Randomized Assignment of Mysids to Test Chambers

12.10.3.2.1 The juvenile mysids must be randomized before placing them into the test chambers. Pool all of the test juveniles into a 1-liter beaker. Using a 10-mL wide-bore pipet or fire-polished glass tube (approximately 2-3 mm inside diameter), place one or two juveniles into as many plastic cups as there are test chambers (including reference toxicant chambers). These cups should contain enough clean dilution seawater to maintain water quality and temperature during the transfer process (approximately 50 mL per cup). When each of the cups contains one or two juveniles, repeat the process, adding mysids until each cup contains 5 animals.

12.10.3.2.2 Carefully pour or pipet off excess water in the cups, leaving less than 5 mL with the test mysids. This 5 mL volume can be estimated visually after initial measurements. Carefully pour or pipet the juveniles into the test chambers immediately after reducing the water volume. Gently rocking the water back and forth before pouring may help prevent juveniles from clinging to the walls of the randomization cups. Juveniles can become trapped in drops; have a squirt bottle ready to gently rinse down any trapped mysids. If more than 5 mLs of water are added to the test solution with the juveniles, report the amount on the data sheet. Be sure that all water used in culture, transfer, and test solutions is within 1°C of the test temperature. Because of the small volumes involved in the transfer process, temperature control is best accomplished in a constant-temperature room.

12.10.3.2.3 Verify that all five animals are in the test chambers by counting the number in each chamber after transfer. This initial count is important because mysids unaccounted for at the end of the test are assumed to be dead.

12.10.4 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

12.10.4.1 The light quality and intensity should be at ambient laboratory conditions are generally adequate. Light intensity should be 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle. A 30 minute phase-in/out period is recommended.

12.10.4.2 The water temperature in the test chambers should be maintained at 13 or 15 ± 1°C. It is critical that the test water temperature be maintained at 13 ± 1°C (for mysids collected north of Pt. Conception, California) or 15 ± 1°C (for mysids collected south of Pt. Conception, California). If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

12.10.4.3 The test salinity should be in the range of 34 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

12.10.4.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

12.10.5 DISSOLVED OXYGEN (DO) CONCENTRATION

12.10.5.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

12.10.6 FEEDING

12.10.6.1 *Artemia* nauplii are prepared as described above.

12.10.6.2 The feeding rates in the test beakers should be closely controlled to avoid overfeeding and fouling of test

solutions. Add 40 newly hatched *Artemia* nauplii per mysid per day. *Artemia* nauplii should be well rinsed with clean seawater and concentrated so that no more than one mL of seawater is added during feeding. (Use a 100-μm-mesh screen tube for rinsing and concentrating the nauplii; see Section 12.6.24.3). Test performance may be enhanced by feeding half the ration twice daily. If mysids die during the course of the experiment, the ration should be reduced proportionally. The mysids should not be fed on day 7.

12.10.7 DAILY CLEANING OF TEST CHAMBERS

12.10.7.1 Before the renewal of test solutions, uneaten and dead *Artemia*, dead mysids and other debris are removed from the bottom of the test chambers with a pipette. As much of the uneaten *Artemia* as possible should be removed from each chamber to ensure that the mysids eat primarily newly hatched nauplii. By placing the test chambers on a light box, inadvertent removal of live mysids can be greatly reduced because they can be more easily seen. If a mysid is lost during siphoning, note the test chamber from it came, and reduce the initial count from five to four for that chamber when calculating survival at the end of the test.

12.10.8 OBSERVATIONS DURING THE TEST

12.10.8.1 Routine Chemical and Physical Observations

12.10.8.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

12.10.8.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

12.10.8.1.3 Record all the measurements on the data sheet.

12.10.8.2 Routine Biological Observations

12.10.8.2.1 The number of live mysids are counted and recorded each day. Dead animals and excess food should be removed with a pipette before test solutions are renewed. This is necessary to avoid cannibalism and to prevent fouling of test solutions.

12.10.8.2.2 Protect the mysids from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of the dead mysids, carefully. Make sure the mysids remain immersed during the performance of the above operations.

12.10.9 TEST SOLUTION RENEWAL

12.10.9.1 The test duration is 7 days. Because effluent toxicity may change over short time periods in test chambers, the test solutions must be renewed after 48 h and 96 h. Prepare renewal test solutions in the same way as initial test solutions. Remove three quarters of the original test solution from each chamber, taking care to avoid losing or damaging mysids. This can be done by siphoning with a small-bore (2 to 3 mm) fire-polished glass tube or pipet. Attach the glass tube to clear plastic tubing fitted with a pinch clamp so that the siphon flow can be stopped quickly if necessary to release entrained mysids. It is convenient to siphon old solutions into a small (500 mL) chamber in order to check to make sure that no mysids have been inadvertently removed during solution renewals. If a mysid is siphoned, return it to the test chamber and note it on the data sheet. Follow the chamber randomization sheet to siphon first from the controls, then work sequentially to the highest test concentration to avoid cross-contamination.

12.10.9.2 To minimize disturbance to the juvenile mysids, refill the chambers to the 200-mL mark by carefully siphoning new test solution into the test chambers using small diameter plastic tubing attached to a bent clean glass rod that directs incoming solution upward or to the side to slow the current and minimize turbulence.

12.10.9.3 The effluent or receiving water used in the test is stored in an incubator or refrigerator at 4°C. Plastic chambers such as 8-20 L cubitainers have proven suitable for effluent collection and storage. For on-site toxicity studies no more than 24 h should elapse between collection of the effluent and

use in a toxicity test (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.10.9.4 Approximately 1 h before test initiation, a sufficient quantity of effluent or receiving water sample is warmed to 13 ± 1°C or 15 ± 1°C to prepare the test solutions. A sufficient quantity of effluent should be warmed to make the test solutions.

12.10.10 TERMINATION OF THE TEST

12.10.10.1 Ending the Test

12.10.10.1.1 Record the time the test is terminated.

12.10.10.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control.

12.10.10.1.3 On the last day of the test, examine each test chamber, and remove and record any dead mysids. Sum the cumulative total of all mortalities observed in each test chamber over the 7 days of the test, subtract this from the initial number of mysids (5), and verify the number of survivors. Immobile mysids that do not respond to a stimulus are considered dead. The stimulus should be two or three gentle prods with a disposable pipet. Mysids that exhibit any response clearly visible to the naked eye are considered living. The most commonly observed movement in moribund mysids is a quick contraction of the abdomen. This or any other obvious movement qualifies a mysid as alive.

12.10.10.2 Weighing

12.10.10.2.1 To prepare mysids for weighing at the end of the exposure period, remove any remaining dead mysids, then carefully pour the contents of the test chamber through a small mesh screen (<300μm). Count the mysids before screening, and take care to keep track of them on the screen. Make sure mortality counts have already been recorded. Briefly dip the screen containing the mysids in deionized water to rinse away the salt. Using fine point forceps, carefully transfer the mysids from the screen to a preweighed and labelled micro-weigh boat. Carefully fold the

foil weigh boats over the mysids to avoid loss while drying test organisms.

12.10.10.2.2 To prepare weigh boats prior to testing, write the test chamber number on each with a fine felt-tipped marker, dry the ink and weigh boat in a drying oven, allow the dry weigh boats to cool in a desiccator, weigh the weigh boats to the nearest 1 microgram (μg) on a microbalance, and record the weight and chamber number on the data sheet. Place the weighed weigh boats in a clean ziplock bag until ready to use for weighing mysids. The juvenile mysids are very small, and light (60 μg) relative to the weigh boats (4 mg). Take all precautions to make sure weigh boats remain clean and dry during weighing and subsequent storage, so that mysid weights may be accurately determined by subtraction.

12.10.10.2.3 When all mysids are loaded onto weigh boats, arrange them all in a dish, small tray or other small open chamber, and place them in a clean drying oven. Dry for at least 24 hours at 60°C or for at least 6 hours at 105°C. Remove the weigh boats with mysids from the drying oven and place them in a desiccator to cool for one hour. When cool, carefully weigh each weigh boat on a microbalance (accurate to 1 μg). Record the chamber number, mysid weight, weigh boat weight (recorded previously), and number of mysids per weigh boat (replicate) on the data sheet.

12.10.10.3 Endpoint

12.10.10.3.1 Growth is measured as dry weight of surviving mysids. All surviving mysids from a single replicate test chamber are pooled together and weighed, then this total weight is divided by the number of original mysids to obtain the mean dry weight per individual for each replicate, which is used for statistical analysis.

12.10.10.3.2 The percentage of surviving mysids in each chamber at the end of the test will be used for subsequent statistical analysis.

12.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

12.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

12.12 ACCEPTABILITY OF TEST RESULTS

12.12.1 Test results are acceptable only if all the following requirements are met:

(1) Control survival must be at least 75%.

(2) The average weight of control mysids must be at least 40 µg per mysid.

(3) Between replicate variability in the mortality data must be low enough that the minimum significant difference (%MSD) is less than 40% in the reference toxicant test.

(4) Between replicate variability in the weight data must be low enough that the %MSD is less than 50 µg in the reference toxicant test.

(5) Both the mortality NOEC and LC50 must be less than 100 µg/L zinc in the reference toxicant test.

12.13 DATA ANALYSIS

12.13.1 GENERAL

12.13.1.1 Tabulate and summarize the data. Table 4 presents a sample set of survival and growth data.

12.13.1.2 The endpoints of the mysid 7-day chronic test are based on the adverse effects on survival and growth. The LC50 and the IC25 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for survival and growth are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are

performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50 and IC25. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for survival and growth, but included in the estimation of the LC50 and IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

12.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

12.13.2 EXAMPLE OF ANALYSIS OF MYSID, *HOLMESIMYSIS COSTATA*, SURVIVAL DATA

12.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 1 and 2. The response used in the analysis is the proportion of animals surviving in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the LC50 endpoint. Concentrations at which there is no survival in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the LC, EC, and IC endpoints.

12.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

12.13.2.3 If equal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE MYSID, *HOLMESIMYSIS COSTATA*, GROWTH AND SURVIVAL TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	13 ± 1°C (mysids collected north of Pt. Conception) 15 ± 1°C (mysids collected south of Pt. Conception)
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m^2/s$ (Ambient laboratory illumination)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber:	1000 mL
8. Test solution volume:	200 mL
9. Renewal of test solutions:	75% renewal at 48 and 96 hours
10. Age of test organisms:	3 to 4 days post-hatch juveniles
11. No. organisms per test chamber:	5
12. No. replicate chambers per concentration:	5
13. No. mysids per concentration:	25
14. Source of food:	Newly hatched *Artemia* nauplii (less than 24 h old)
15. Feeding regime:	Feed 40 nauplii per larvae daily (dividing into morning and evening feedings)

16. Cleaning:	Siphon during test solution renewal
17. Aeration:	None unless DO falls below 4.0 mg/L, then gently aerate in all cups
18. Dilution water:	Uncontaminated 1-μm-filtered natural seawater or hypersaline brine prepared from natural seawater
19. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
20. Dilution factor:	Effluents: ≥0.5 series Receiving waters: None, or ≥0.5
21. Test duration:	7 days
22. Endpoints:	Survival and growth
23. Test acceptability criteria:	≥75% survival, average dry weight ≥ 0.40 μg in the controls; survival MSD <40%; growth MSD <50 μg; and both survival and growth NOECs must be less than 100 μg/L with zinc
24. Sampling requirements:	For on-site tests, samples must be used within 24 h of the time they are removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
25. Sample volume required:	2 L per renewal

TABLE 4. DATA FOR *HOLMESIMYSIS COSTATA* 7-DAY SURVIVAL AND GROWTH TEST[1]

Treatment	Replicate Chamber	Total Mysids	No. Alive	Prop. Alive	Mean Weight
Control, Brine	1	5	5	1.00	0.051
	2	5	5	1.00	0.050
	3	5	5	1.00	0.040
	4	5	5	1.00	0.064
	5	5	5	1.00	0.039
Control, Dilution	1	5	5	1.00	0.048
	2	5	5	1.00	0.058
	3	5	5	1.00	0.047
	4	5	5	1.00	0.058
	5	5	5	1.00	0.051
1.80%		1	5	5	1.00
					0.055
	2	5	5	1.00	0.048
	3	5	5	1.00	0.042
	4	5	4	0.80	0.041
	5	5	5	1.00	0.052
3.20%		1	5	5	1.00
					0.057
	2	5	4	0.80	0.050
	3	5	5	1.00	0.046
	4	5	5	1.00	0.043
	5	5	4	0.80	0.045

t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

12.13.2.4 Probit Analysis (Finney, 1971; see Appendix G) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit model, the Spearman-Karber method, the Trimmed Spearman-Karber

method, or the Graphical method may be used to estimate the LC50 (see Appendices H-K).

12.13.2.5 The proportion of survival in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each concentration including the control are listed in Table 5. A plot of the survival data is provided in Figure 3.

12.13.2.6 Test for Normality

12.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 6.

12.13.2.6.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

Where: $\overline{X}_i$ = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations.

12.13.2.6.3 For this set of data, n = 25

$$\overline{X} = \frac{1}{25}(0.001) = 0.00$$

$$D = 0.227$$

12.13.2.6.4 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 7.

TABLE 5. MYSID, *HOLMESIMYSIS COSTATA*, SURVIVAL DATA

	Replicate	Control	Concentration (%) 1.80	3.20	5.60	10.00
	1	1.00	1.00	1.00	0.80	0.20
	2	1.00	1.00	0.80	1.00	0.00
RAW	3	1.00	1.00	1.00	1.00	0.00
	4	1.00	0.80	1.00	0.80	0.00
	5	1.00	1.00	0.80	0.80	0.00
ARC SINE	1	1.345	1.345	1.345	1.107	0.464
SQUARE	2	1.345	1.345	1.107	1.345	0.225
ROOT	3	1.345	1.345	1.345	1.345	0.225
TRANS-	4	1.345	1.107	1.345	1.107	0.225
FORMED	5	1.345	1.345	1.107	1.107	0.225
Mean($\bar{Y}_i$)		1.345	1.297	1.250	1.202	0.273
S_i^2		0.000	0.011	0.017	0.017	0.011
i		1	2	3	4	5

12.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ..., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 25 and k = 12. The a_i values are listed in Table 8.

12.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 8.

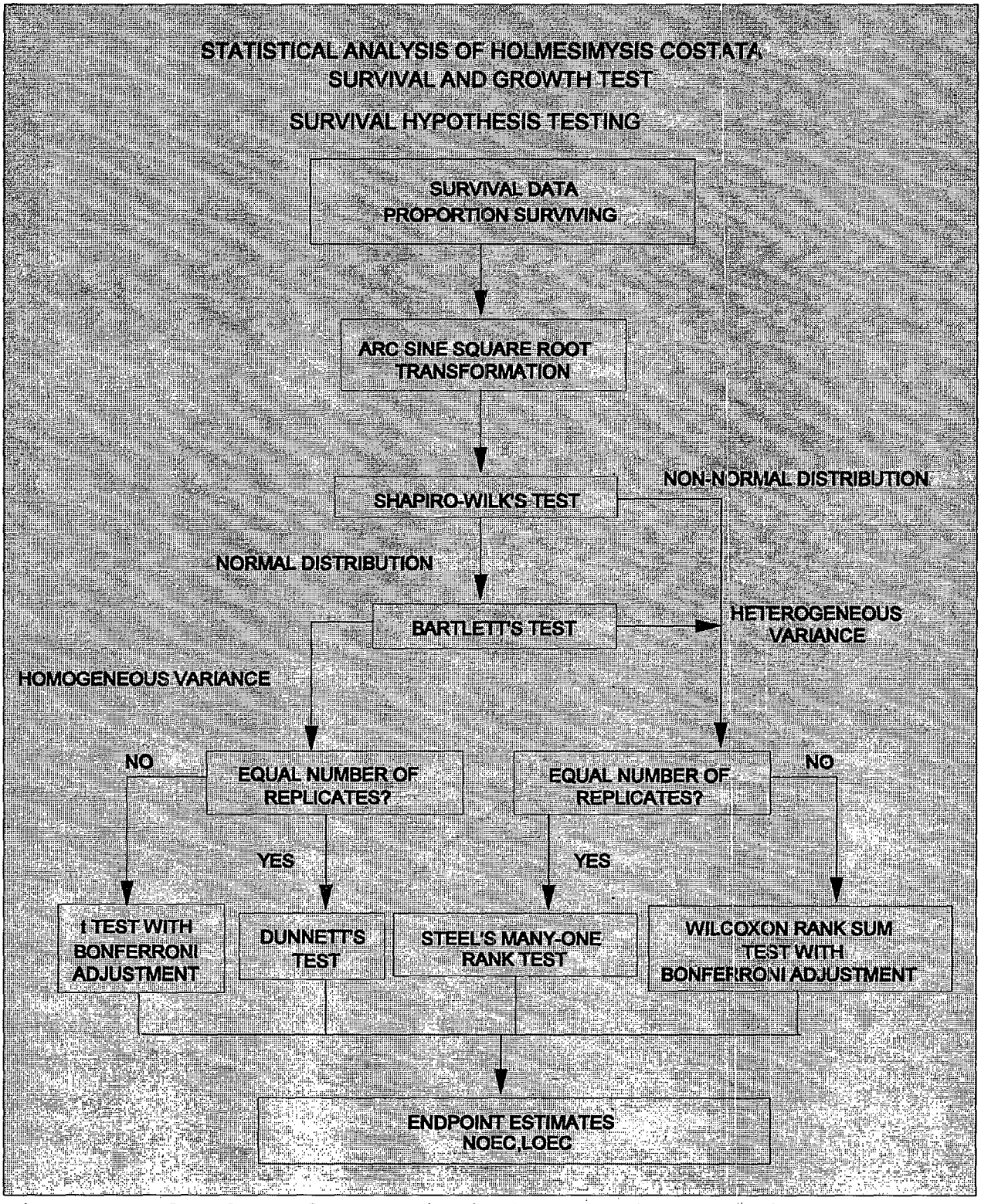

Figure 1. Flowchart for statistical analysis of mysid, *Holmesimysis costata*, survival data by hypothesis testing.

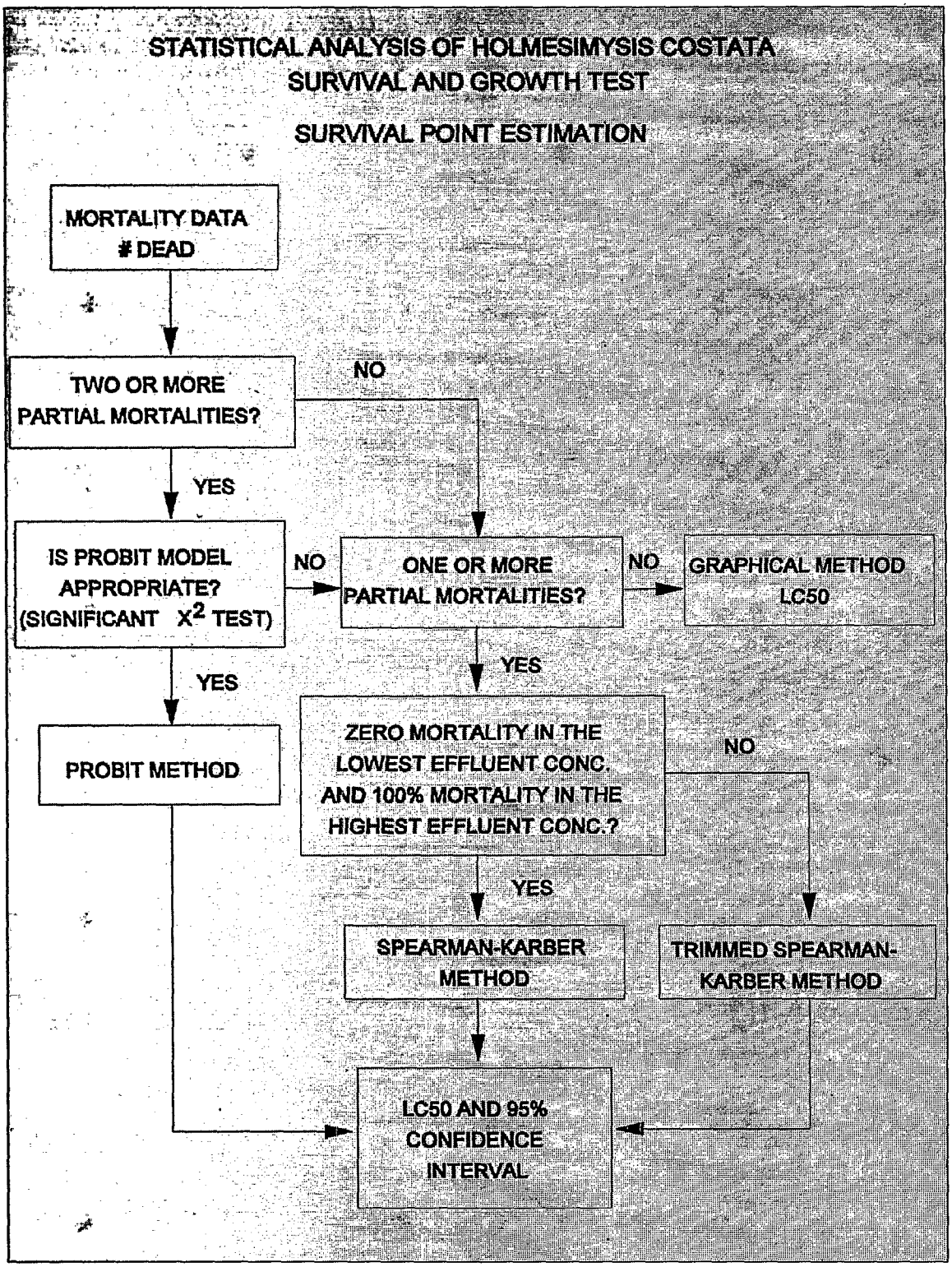

Figure 2.Flowchart for statistical analysis of mysid, *Holmesimysis costata*, survival data by point estimation.

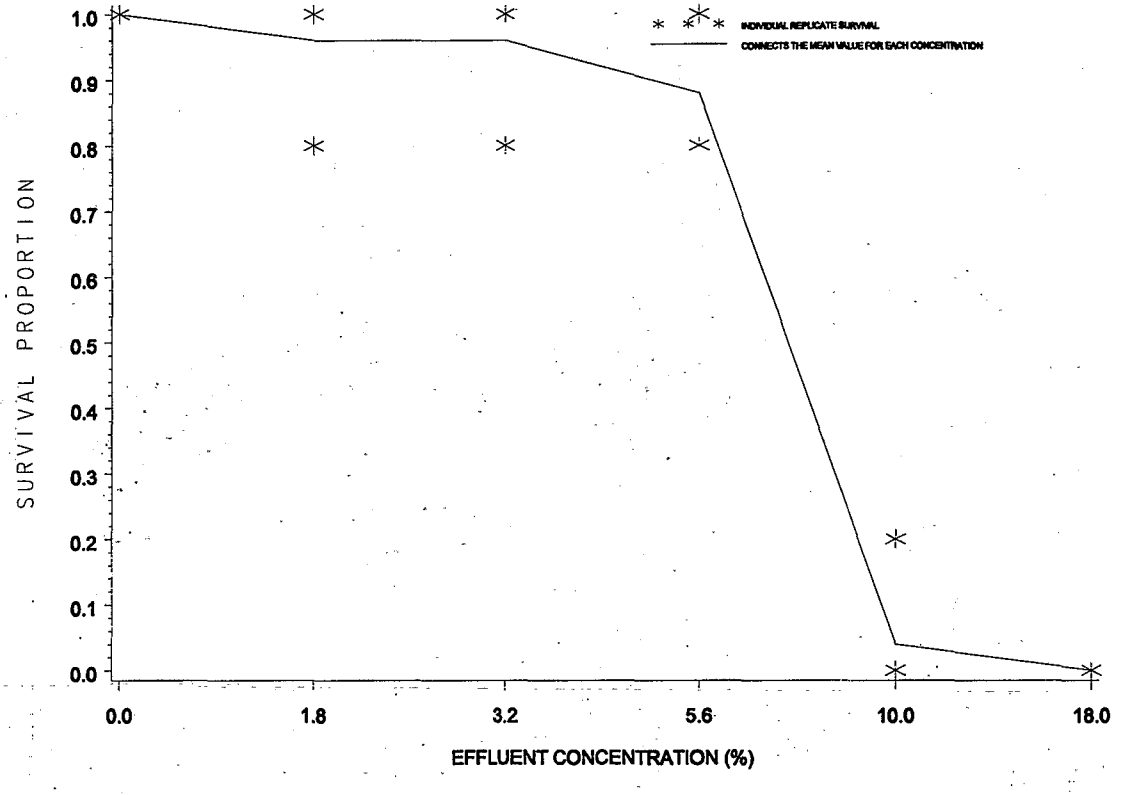

Figure 3. Plot of survival of mysids, *Holmesimysis costata*, at each treatment

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control (Dilution)	Concentration 1.80	3.20	5.60	10.00
1	0.000	0.048	0.095	-0.095	0.191
2	0.000	0.048	-0.143	0.143	-0.048
3	0.000	0.048	0.095	0.143	-0.048
4	0.000	-0.190	0.095	-0.095	-0.048
5	0.000	0.048	-0.143	-0.095	-0.048

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.190	14	0.000
2	-0.143	15	0.000
3	-0.143	16	0.048
4	-0.095	17	0.048
5	-0.095	18	0.048
6	-0.095	19	0.048
7	-0.048	20	0.095
8	-0.048	21	0.095
9	-0.048	22	0.095
10	-0.048	23	0.143
11	0.000	24	0.143
12	0.000	25	0.191
13	0.000		

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4450	0.381	$X^{(25)} - X^{(1)}$
2	0.3069	0.286	$X^{(24)} - X^{(2)}$
3	0.2543	0.286	$X^{(23)} - X^{(3)}$
4	0.2148	0.190	$X^{(22)} - X^{(4)}$
5	0.1822	0.190	$X^{(21)} - X^{(5)}$
6	0.1539	0.190	$X^{(20)} - X^{(6)}$
7	0.1283	0.096	$X^{(19)} - X^{(7)}$
8	0.1046	0.096	$X^{(18)} - X^{(8)}$
9	0.0823	0.096	$X^{(17)} - X^{(9)}$
10	0.0610	0.096	$X^{(16)} - X^{(10)}$
11	0.0403	0.000	$X^{(15)} - X^{(11)}$
12	0.0200	0.000	$X^{(14)} - X^{(12)}$

For this data in this example:

$$W = \frac{1}{0.227} (0.4708)^2 = 0.976$$

12.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 6.6 with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 25 observations is 0.888. Since W = 0.976 is greater than the critical value, conclude that the data are normally distributed.

12.13.2.6.8 Since the variance of the control group is zero, Bartlett's test statistic can not be calculated. Therefore, the survival data variances are considered to be heterogeneous.

12.13.2.6.9 Since the data do not meet the assumption of homogeneity of variance, Steel's Many-one Rank Test will be used to analyze the survival data.

12.13.2.7 Steel's Many-one Rank Test

12.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ... , 10) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

12.13.2.7.2 An example of assigning ranks to the combined data for the control and 1.80% concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are then summed for each concentration level, as shown in Table 11.

12.13.2.7.3 For this example, determine if the survival in any of the concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus compare the rank sums for the survival at each of the various concentration levels with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control) is 17(See Table 5, Appendix E).

12.13.2.8.1 The data used to calculate the LC50 is summarized in Table 12. For this example, although there are two concentrations with partial mortalities, the chi-square test for heterogeneity was significant, indicating that Probit Analysis is inappropriate for this set of data. Inspection of the data reveals that the smoothed, adjusted proportion mortality for the lowest concentration will not be zero, indicating that the Trimmed Spearman-Karber Method is recommended to calculated the LC50 for this dataset.

12.13.2.8.2 For the Trimmed Spearman-Karber analysis, run the USEPA Trimmed Spearman-Karber program, TSK. An example of the program output is provided in Figure 4.

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 1.80% CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion of Total Mortality	Concentration
1	1.107	1.80%
6	1.345	Control
6	1.345	Control
6	1.345	Control
6	1.345	Control
6	1.345	Control
6	1.345	1.80%
6	1.345	1.80%
6	1.345	1.80%
6	1.345	1.80%

TABLE 10. TABLE OF RANKS[1]

		Concentration (%)			
Replicate	Control	1.80	3.20	5.60	10.0
1	1.345(6,6.5,7,8)	1.345(6)	1.345(6.5)	1.107(2)	0.464(5)
2	1.345(6,6.5,7,8)	1.345(6)	1.107(1.5)	1.345(7)	0.225(2.5)
3	1.345(6,6.5,7,8)	1.345(6)	1.345(6.5)	1.345(7)	0.225(2.5)
4	1.345(6,6.5,7,8)	1.107(1)	1.345(6.5)	1.107(2)	0.225(2.5)
5	1.345(6,6.5,7,8)	1.345(6)	1.107(1.5)	1.107(2)	0.225(2.5)

[1]Control ranks are given in the order of the concentration with which they were ranked.

TABLE 11. RANK SUMS

Concentration	Rank Sum
1.80	25.0
3.20	22.5
5.60	20.0
10.00	15.0

TABLE 12. DATA FOR TRIMMED SPEARMAN-KARBER ANALYSIS

	Concentration (%)					
	Control	1.80	3.20	5.60	10.0	18.0
No Dead	0	1	2	3	24	25
No Exposed	25	25	25	25	25	25

12.13.3 EXAMPLE OF ANALYSIS OF MYSID, *HOLMESIMYSIS COSTATA* GROWTH DATA

12.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 5. The response used in the statistical analysis is mean weight per surviving organism per replicate. The IC25 can be calculated for the growth data via a point

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: TEST NUMBER: 1 DURATION: 7 days

TOXICANT : Effluent
SPECIES: Holmesimysis costata

RAW DATA: --- ----	Concentration (%)	Number Exposed	Mortalities
	.00	25	0
	1.80	25	1
	3.20	25	2
	5.60	25	3
	10.00	25	24
	18.00	25	25

SPEARMAN-KARBER TRIM: 4.00%

SPEARMAN-KARBER ESTIMATES:	LC50:	6.95
	95% LOWER CONFIDENCE:	6.22
	95% UPPER CONFIDENCE:	7.76

Figure 4. Output for USEPA Trimmed Spearman-Karber Program, version 1.5.

estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC and LOEC for growth. Concentrations above the NOEC for survival are excluded from the hypothesis test for growth effects.

12.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying

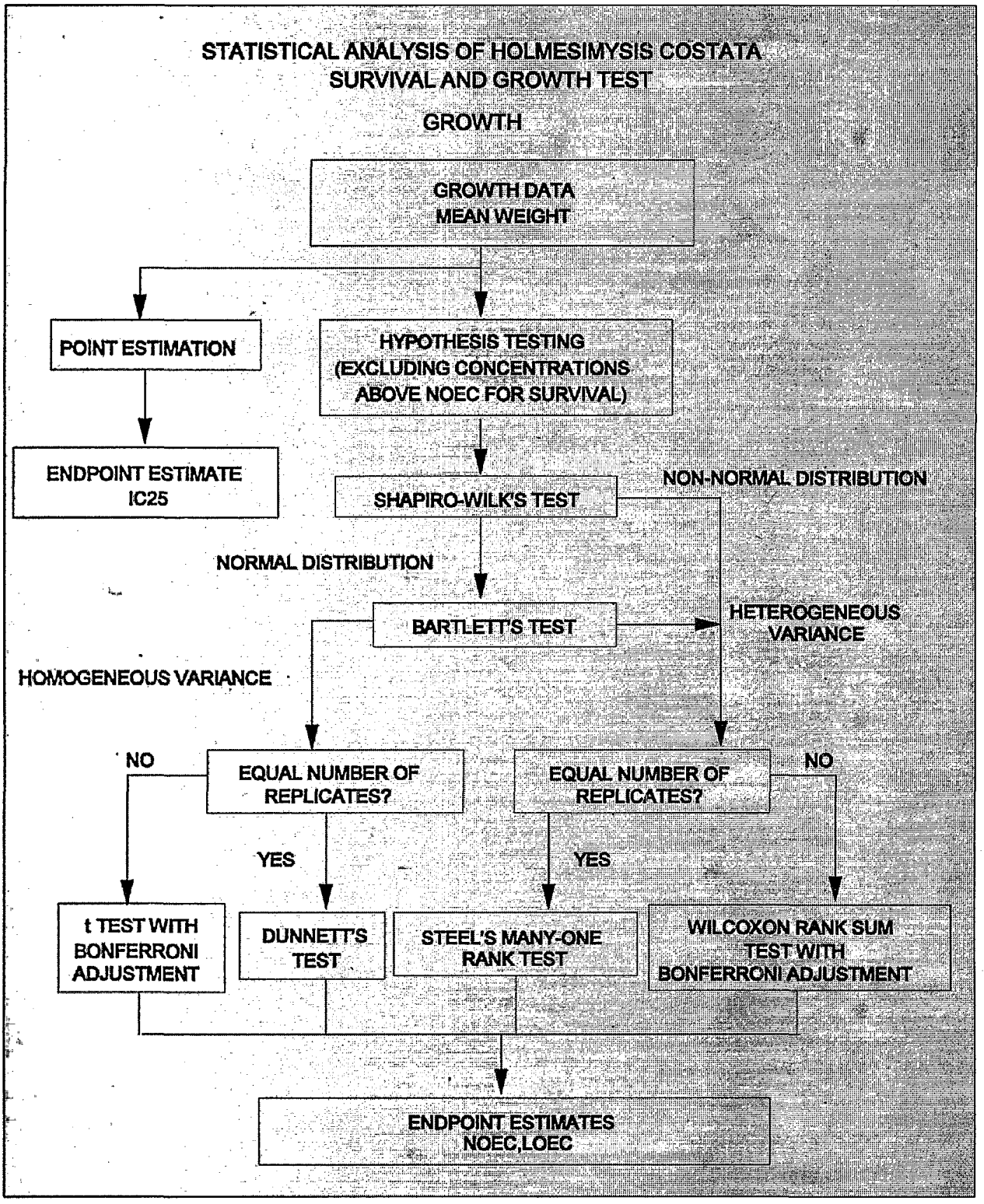

Figure 5. Flowchart for statistical analysis of mysid, *Holmesimysis costata*, growth data.

assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

12.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative. For detailed information on the Bonferroni adjustment, see Appendix D.

12.13.3.4 The data, mean and variance of the observations at each concentration including the control for this example are listed in Table 13. A plot of the data is provided in Figure 6. Since there is significant mortality in the 10.0% concentration, its effect on growth is not considered.

12.13.3.5 Test for Normality

12.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 14.

12.13.3.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

TABLE 13. MYSID, *HOLMESIMYSIS COSTATA*, GROWTH DATA

Replicate	Control	Concentration (%) 1.80	3.20	5.60	10.0
1	0.048	0.055	0.057	0.041	0.033
2	0.058	0.048	0.050	0.040	0.000
3	0.047	0.042	0.046	0.041	0.000
4	0.058	0.041	0.043	0.043	0.000
5	0.051	0.052	0.045	0.040	0.000
Mean($\bar{Y}_i$)	0.052	0.048	0.048	0.041	0.007
S_i^2	0.0000283	0.0000373	0.0000307	0.0000015	0.000218
i	1	2	3	4	5

TABLE 14. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	Concentration (%) 1.80	3.20	5.60
1	-0.004	0.007	0.009	0.000
2	0.006	0.000	0.002	-0.001
3	-0.005	-0.006	-0.002	0.000
4	0.006	-0.007	-0.005	0.002
5	-0.001	0.004	-0.003	-0.001

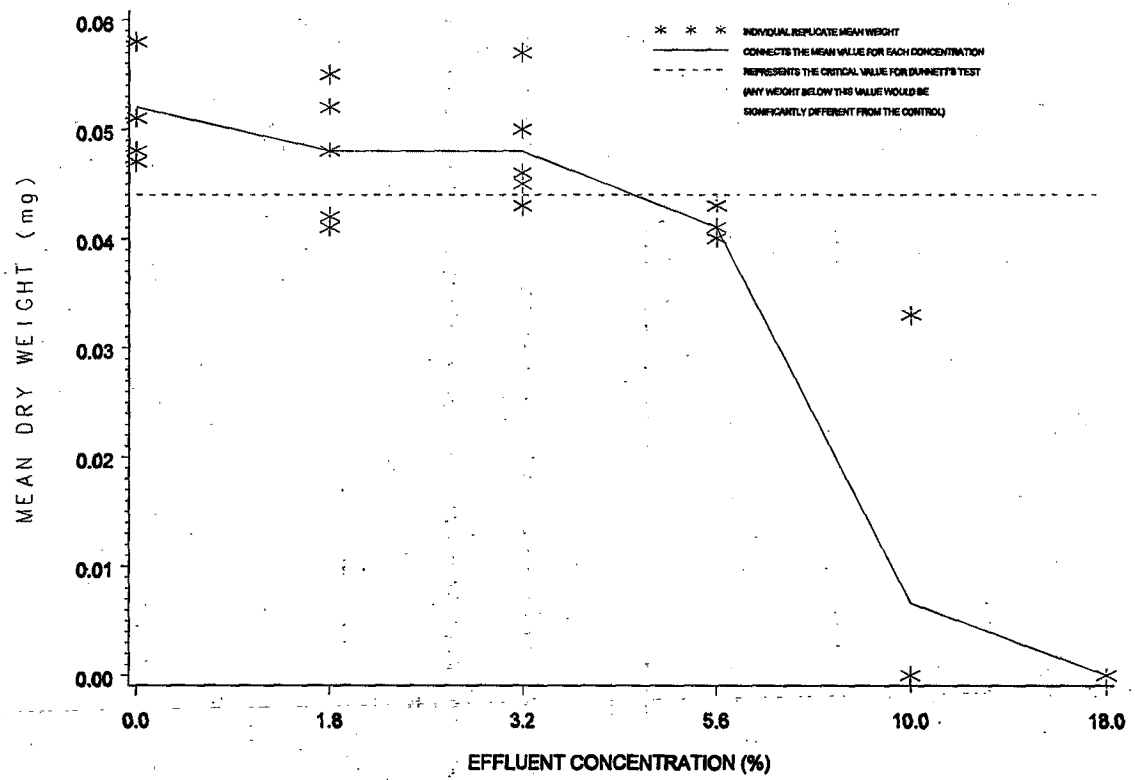

Figure 6. Plot of growth data for mysid, *Holmesimysis costata*, test.

Where: X_i = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations

12.13.3.5.3 For this set of data, n = 20

$$\overline{X} = \frac{1}{20}(0.001) = 0.000$$

$$D = 0.000393$$

12.13.3.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 15.

TABLE 15. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.007	11	0.000
2	-0.006	12	0.000
3	-0.005	13	0.000
4	-0.005	14	0.002
5	-0.004	15	0.002
6	-0.003	16	0.004
7	-0.002	17	0.006
8	-0.001	18	0.006
9	-0.001	19	0.007
10	-0.001	20	0.009

12.13.3.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table 16.

12.13.3.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 16. For this set of data:

$$W = \frac{1}{0.000393}\,(0.0194)^2 = 0.958$$

TABLE 16. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.016	$X^{(20)} - X^{(1)}$
2	0.3211	0.013	$X^{(19)} - X^{(2)}$
3	0.2565	0.011	$X^{(18)} - X^{(3)}$
4	0.2085	0.011	$X^{(17)} - X^{(4)}$
5	0.1686	0.008	$X^{(16)} - X^{(5)}$
6	0.1334	0.005	$X^{(15)} - X^{(6)}$
7	0.1013	0.004	$X^{(14)} - X^{(7)}$
8	0.0711	0.001	$X^{(13)} - X^{(8)}$
9	0.0422	0.001	$X^{(12)} - X^{(9)}$
10	0.0140	0.001	$X^{(11)} - X^{(10)}$

12.13.3.5.7 The decision rule for this test is to compare W as calculated in Subsection 12.13.3.5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of

0.01 and n = 20 observations is 0.868. Since W = 0.958 is greater than the critical value, conclude that the data are normally distributed.

12.13.3.6 Test for Homogeneity of Variance

12.13.3.6.1 The test used to examine whether the variation in mean weight of the mysids is the same across all concentration levels including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i) \ln \overline{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each concentration and the control, $V_i = (n_i - 1)$

p = number of concentration levels including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i = the number of replicates for concentration i.

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

12.13.3.6.2 For the data in this example (See Table 13), all concentrations including the control have the same number of replicates (n_i = 5 for all i). Thus, V_i = 4 for all i.

12.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(16)\ln(0.0000245) - 4\sum_{i=1}^{p}\ln(S_i^2)]/1.104$$

$$= [16(-10.617) - 4(-44.470)]/1.104$$

$$= [-169.872 - (-177.880)]/1.104$$

$$= 7.254$$

12.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with three degrees of freedom, is 9.210. Since B = 7.254 is less than the critical value of 9.210, conclude that the variances are not different.

12.13.3.7 Dunnett's Procedure

12.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 17.

TABLE 17. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations,

$$G = \sum_{i=1}^{P} T_i$$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean weight of the mysids for concentration i in test chamber j)

12.13.3.7.2 For the data in this example:

$$n_1 = n_2 = n_3 = n_4 = 5$$

$$N = 20$$

$$T_1 = Y_{11} + Y_{12} + \ldots + Y_{15} = 0.262$$
$$T_2 = Y_{21} + Y_{22} + \ldots + Y_{25} = 0.238$$
$$T_3 = Y_{31} + Y_{32} + \ldots + Y_{35} = 0.241$$
$$T_4 = Y_{41} + Y_{42} + \ldots + Y_{45} = 0.205$$
$$G = T_1 + T_2 + T_3 + T_4 = 0.946$$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$

$$= \frac{1}{5}(0.225) - \frac{(0.946)^2}{20} = 0.000254$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 0.0455 - \frac{(0.946)^2}{20} = 0.000754$$

$SSW = SST - SSB$

$= 0.000754 - 0.000254 = 0.000500$

$S_B^2 = SSB/(p-1) = 0.000254/(4-1) = 0.0000847$

$S_W^2 = SSW/(N-p) = 0.000500/(20-4) = 0.0000313$

12.13.3.7.3 Summarize these calculations in the ANOVA table (Table 18).

TABLE 18. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	3	0.000254	0.0000847
Within	16	0.000500	0.0000313
Total	19	0.000754	

12.13.3.7.4 To perform the individual comparisons, calculate the *t* statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\overline{Y_1} - \overline{Y_i})}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\overline{Y}_i$ = mean weight for concentration i

$\overline{Y}_1$ = mean weight for the control

S_w = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

12.13.3.7.5 Table 19 includes the calculated *t* values for each concentration and control combination. In this example, comparing the 1.80% concentration with the control the calculation is as follows:

$$t_2 = \frac{(0.052-0.048)}{[0.00559\sqrt{(1/5)+(1/5)}]}$$

$$= 1.131$$

TABLE 19. CALCULATED *t* VALUES

Concentration (ppb)	i	t_i
1.80	2	1.131
3.20	3	1.131
5.60	4	3.111

12.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean weight, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 16 degrees of freedom for error and three concentrations (excluding the control) the approximate critical value is 2.23. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Therefore, the 5.60% concentration has significantly lower mean weight than the control. Hence the NOEC and the LOEC for growth are 3.20% and 5.60%, respectively.

12.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be detected statistically may be calculated.

$$MSD = d\ S_w\sqrt{(1/n_1)+(1/n)}$$

critical value for Dunnett's Procedure

square root of the within mean square

common number of replicates at each
:entration
ssumes equal replication at each concentration)

number of replicates in the control.

his example:

$$MSD = 2.23(0.00559)\sqrt{(1/5)+(1/5)}$$

$$= 2.23\ (0.00559)(0.632)$$

$$= 0.00788$$

efore, for this set of data, the minimum
an be detected as statistically significant is

s represents a 15.2% reduction in mean weight

ation of the ICp

growth data from Table 13 are utilized in this
in the table, the observed means are
-increasing with respect to concentration.
oothed means will be simply the corresponding
he observed means are represented by $\overline{Y}_i$ and the
M_i. Table 20 contains the smoothed means and
plot of the smoothed response curve.

12.13.3.8.5 Using the equation in Section 4.2 from Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1-p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 5.60 + [0.052(1 - 25/100) - 0.041]\ \frac{(10.0 - 5.60)}{(0.0066 - 0.041)}$$

$$= 5.86\%.$$

12.13.3.8.7 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 5.86%. The empirical 95.0% confidence interval for the true mean was 4.9440% to 6.2553%. The computer program output for the IC25 for this data set is shown in Figure 8.

TABLE 20. MYSID, *HOLMESIMYSIS COSTATA*, MEAN GROWTH RESPONSE AFTER SMOOTHING

Toxicant Conc. (%)	i	Response Means Y_i (mg)	Smoothed Means M_i (mg)
Control	1	0.052	0.052
1.80	2	0.048	0.048
3.20	3	0.048	0.048
5.60	4	0.041	0.041
10.00	5	0.0066	0.0066
18.00	6	0.000	0.000

12.14 PRECISION AND ACCURACY

12.12.1 PRECISION

12.12.1.1 Single-Laboratory Precision

12.12.1.1.1 Data on the single laboratory precision of the *Holmesimysis costata* growth and survival test with zinc sulfate

are shown in Table 21. NOECs for mysid survival were either 32 or 56 µg/L Zn. There was also good agreement among LC50s, with a coefficient of variation of 14%. Mysids did not exhibit a growth response at zinc concentrations below those causing significant mortality; NOEC values for growth were always greater than or equal to the highest zinc concentration. IC50 values for growth could not be calculated.

12.12.1.2 Multi-laboratory Precision

12.12.1.2.1 The multi-laboratory data indicate a similar level of test precision (Table 22). The four multi-laboratory tests were conducted over a two year period, and each used split effluent samples tested at two laboratories. Survival NOEC values were the same for both laboratories in three of the four tests, with the NOECs varying by one concentration in the fourth test. The mean coefficient of variation between LC50 values from different laboratories was 21%. The two available comparisons of growth NOEC values indicate similar responses at both laboratories. Growth was the more sensitive indicator of toxicity in three of the four effluent tests.

12.14.2 ACCURACY

12.14.2.1 The accuracy of toxicity tests cannot be determined.

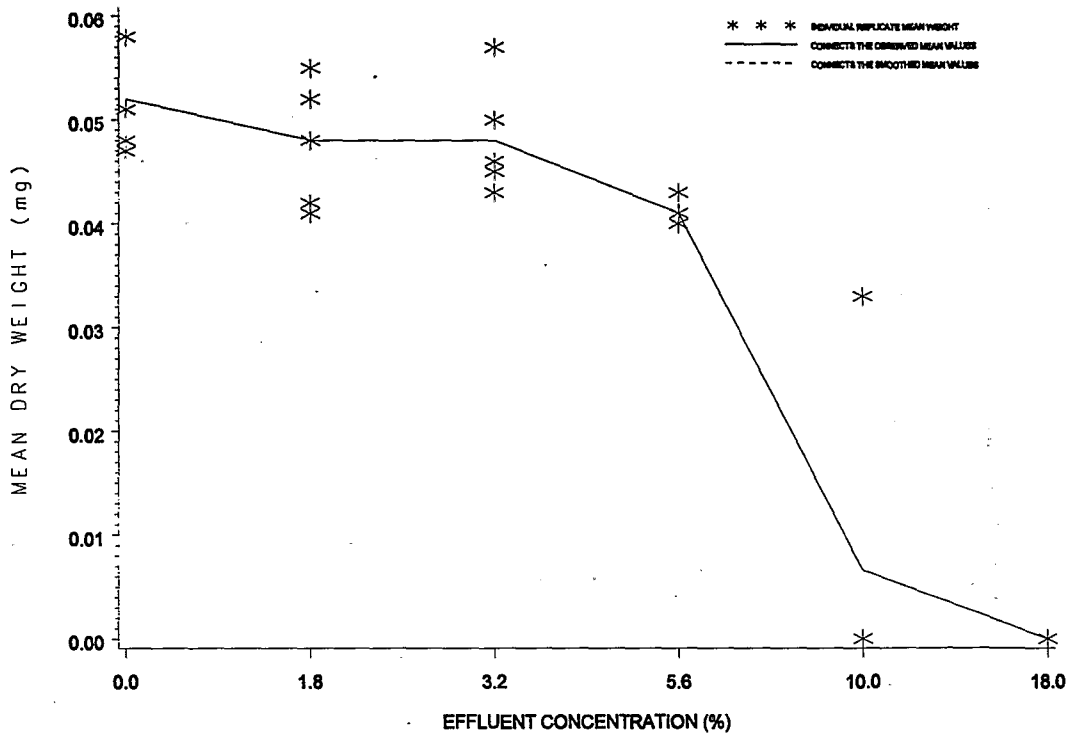

Figure 7. Plot of raw data, observed means, and smoothed means for the mysid, *Holmesimysis costata*, Growth data from tables 13 and 20

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.80	3.20	5.60	10.0	18.0
Response 1	.048	.055	.057	.041	.033	0
Response 2	.058	.048	.050	.040	0	0
Response 3	.047	.042	.046	.041	0	0
Response 4	.058	.041	.043	.043	0	0
Response 5	.051	.052	.045	.040	0	0

*** Inhibition Concentration Percentage Estimate ***.
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: mysid, Holmesimysis costata
Test Duration: 7 days
DATA FILE: mysid.icp
OUTPUT FILE: mysid.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	5	0.000	0.052	0.005	0.052
2	5	1.800	0.048	0.006	0.048
3	5	3.200	0.048	0.006	0.048
4	5	5.600	0.041	0.001	0.041
5	5	10.000	0.007	0.015	0.007
6	5	18.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 5.8174 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 5.8205 Standard Deviation: 0.2673
Original Confidence Limits: Lower: 4.9440 Upper: 6.2553
Expanded Confidence Limits: Lower: 4.5073 Upper: 6.4743
Resampling time in Seconds: 0.22 Random_Seed: 526805435

Figure 8. Output for USEPA Linear Interpolation Program for the IC25.

TABLE 21. SINGLE LABORATORY PRECISION DATA FOR THE MYSID, *HOLMESIMYSIS COSTATA* GROWTH AND SURVIVAL TEST WITH ZINC (ZN µG/L) SULFATE AS THE REFERENCE TOXICANT

Test	Survival		Growth
	NOEC	LC50	NOEC
1	32	47	>32
2	32	59	>32
3	56	62	>56
4	56	65	>56
N	4	4	4
Mean	44	58	>44
SD		7.9	
CV (%)		14	

No growth effect was observed in zinc concentrations below those causing significant mortality (10, 18, 32, 56 and 100 µg/L).

All tests were conducted at MPSL.

TABLE 22. MULTI-LABORATORY PRECISION DATA FOR THE MYSID, *HOLMESIMYSIS COSTATA* GROWTH AND SURVIVAL TEST WITH SPLIT EFFLUENT (%) ON THE SAME DATE.

Test	Effluent Type	Lab	Survival NOEC	Survival LC50	Growth NOEC
1	BKME	OSU	1.0	1.8	0.5[L]
1	BKME	MPSL	1.0	1.3	0.5[L]
			CV=26%		
2	POTW	ATL	3.2	4.1	>3.2[L]
2	POTW	MPSL	3.2	5.1	>3.2[L]
			CV=14%		
3	POTW	SRH	10.0	12.8	na
3	POTW	MPSL	10.0	11.7	3.2[W]
			CV=6%		
4	POTW	SRH	10.0	15.8	5.6[W]
4	POTW	MPSL	5.6	9.1	3.2[W]
			CV=38%		

Mean Interlaboratory CV= 21%

[L] Length was measured as the growth endpoint in tests 1 and 2,
[W] Weight was measured in test 3 and 4.
na Data was not available.

OSU is the Oregon State University Laboratory at the Hatfield Marine Science Center in Newport Oregon.

ATL is Aquatic Testing Laboratory in Ventura, California.

SRH is S.R. Hansen and Associates in Concord, California.

MPSL is the Marine Pollution Studies Laboratory near Monterey, California.

APPENDIX I. MYSID TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a zinc reference toxicant stock solution (10,000 µg/L) by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) to 1 liter of reagent water.

D. Prepare zinc reference toxicant solution of 0 (control) 10, 18, 32, 56 and 100 µg/L by adding 0, 1.0 1.8, 3.2, 5.6 and 10.0 mL of stock solution, respectively, to a 1-L volumetric flask and filling to 1-L with dilution water.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 13 or 15°C and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Four to five days prior to the beginning of the toxicity test, isolate approximately 150 gravid female mysids in a screened (2-mm-mesh) compartment within an aerated 80-liter aquarium (15°C). Add a surplus of *Artemia* nauplii (200 per mysid, static; 500 per mysid, flow-through) to stimulate overnight release of juveniles. Add blades of kelp as habitat.

B. Isolate the newly released juveniles by slowly siphoning into a screen-tube (150-µm-mesh, 25 cm diam.) immersed in a bucket of clean seawater. Transfer juveniles into additional screen-tubes or static 4-liter beakers at a density of approximately 50 juveniles per liter. Juveniles should be fed five to ten newly released *Artemia* nauplii per juvenile per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. Maintain the juveniles for three days at 13 to 15°C, changing the water at least once in static chambers.

C. After three days, begin randomized introduction of juveniles into the test chambers. Place one or two mysids at a time into as many plastic cups as there are test chambers. Repeat the process until each cup has exactly five juvenile mysids.

D. Eliminate excess water from the cups (no more than 5 mL should remain) and pipet the mysids into the test chambers using a wide bore glass tube or pipet (approximately 3 mm ID). Make sure no mysids are left in the randomization cups. Count the number of juveniles in each test chamber to verify that each has five.

E. Remove all dead mysids daily, and add 40 newly hatched *Artemia* nauplii/mysid/day, adjusting feeding to account for mysid mortality.

F. At 48 and 96 hours, renew 75% of the test solution in each chamber.

G. After 7 days, count and record the number of live and dead mysids in each chamber. After counting, use the randomization sheet to assign the correct test concentration to each chamber. Remove all dead mysids.

H. Carefully pour the contents of each test chamber through a small mesh screen (<300μm). Count the mysids and record before screening. Briefly dip the screen containing the mysids in fresh water to rinse away the salt. Carefully transfer the mysids from the screen to a prenumbered, preweighed micro-weigh boat using fine-tipped forceps. Dry for 24 hours at 60°C. Weigh each weigh boat on a microbalance (accurate to 1 μg). Record the chamber number, mysid weight, weigh boat weight (recorded previously), and number of mysids per weigh boat (replicate) on the data sheet.

I. Analyze the data.

J. Include standard reference toxicant point estimate values in the standard quality control charts.

Data Sheet for Juvenile Holmesimysis Toxicity Test

Test Start Date: Start Time: Mysid Source
Test End Date: End Time: Collection/Arrival Date:
Reference Toxicant: Mysid Age at Start:
Sample Source:

Test Cont. #	Toxic Conc.	Number Alive							Total Number Alive	Total Number at Start	Notes and Initials
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
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32											
33											
34											
35											

Computer Data Storage
Disk:
File:

Note: See juvenile growth data on separate sheet.

Data Sheet for Weighing Juvenile Mysids

Test Start Date:
Start Time:
Mysid Source :
Test End Date:
End Time:
Collection/Arrival Date:
Reference Toxicant:

Mysid Age at Start:
Sample Source:
Sample Type:

Test Container Number	Site Code or Concentration	Foil Number	Foil Weight (µg)	Total Weight (µg)	Mysid Wt (Total - Foil) (mg)	Number of Mysids	Weight per Mysid (µg)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
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30							
31							
32							
33							
34							
35							

Computer Data Storage
Disk:
File:
Note: See mysid mortality data on separate sheet.

SECTION 13

PACIFIC OYSTER, *Crassostrea gigas* AND MUSSEL, *Mytilus sp.* EMBRYO-LARVAL DEVELOPMENT TEST METHOD

Adapted from a method developed by
Gary A. Chapman, U.S. EPA, ORD Newport, OR
and Debra L. Denton, U.S. EPA, Region IX

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SECTION 13

PACIFIC OYSTER, *CRASSOSTREA GIGAS*, AND MUSSEL, *MYTILUS SPP.* EMBRYO-LARVAL DEVELOPMENT TEST

13.1 SCOPE AND APPLICATION

13.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the embryos and larvae of several bivalve molluscs, the Pacific oyster (*Crassostrea gigas*) and the mussels (*Mytilus edulis*, *M. californianus*, *M. galloprovincialis*, or *M. trossulus*) in a 48-h static non-renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

13.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

13.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

13.1.4 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

13.1.5 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

13.2 SUMMARY OF METHOD

13.2.1 The method provides step-by-step instructions for performing a 48-h static non-renewal toxicity test using embryos and larvae of the test species to determine the toxicity of

substances in marine and estuarine waters. The test endpoint is normal shell development and should include mortality as a measure of adverse effect.

13.3 **INTERFERENCES**

13.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment, and Supplies).

13.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

13.4 **SAFETY**

13.4.1 See Section 3, Health and Safety

13.5 **APPARATUS AND EQUIPMENT**

13.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult pacific oysters and mussels, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20°C), with appropriate filtration and aeration system.

13.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

13.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature prior to the test.

13.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

13.5.5 Refractometer -- for determining salinity.

13.5.6 Hydrometer(s) -- for calibrating refractometer.

13.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

13.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD 170.1, USEPA, 1979) -- to calibrate laboratory thermometers.

13.5.9 pH and DO meters -- for routine physical and chemical measurements.

13.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

13.5.11 Winkler bottles -- for dissolved oxygen determinations.

13.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

13.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

13.5.14 Glass stirring rods -- for mixing test solutions.

13.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-1000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

13.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 100-1000 mL for making test solutions.

13.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

13.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

13.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

13.5.20 Wash bottles -- for dilution water.

13.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

13.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

13.5.23 Beakers, 50 mL -- for pooling surrogate water samples for chemistry measurements at the end of the test.

13.5.24 Beakers, 250 mL borosilicate glass -- for preparation of test solutions.

13.5.25 Beakers, 1,000 mL borosilicate glass -- for mixing gametes for fertilization of eggs.

13.5.26 Inverted or compound microscope -- for inspecting gametes and making counts of embryos and larvae. The use of an inverted scope is not required, but recommended. Its use reduces the exposure of workers to hazardous fumes (formalin or glutaraldehyde) during the counting of larvae and reduces sample examination time. Alternatively, a Sedgewick-Rafter cell may be used on a regular compound scope.

13.5.27 Counter, two unit, 0-999 -- for recording counts of embryos and larvae.

13.5.28 A perforated plunger -- for maintaining a homogeneous suspension of embryos.

13.5.29 Nytex screens, ca. 75 μm and ca. 37 μm -- for rinsing gametes to separate individual gametes from larger material; for retaining eggs, embryos, or larvae.

13.5.30 60 μm NITEX® filter -- for filtering receiving water.

13.6 **REAGENTS AND SUPPLIES**

13.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

13.6.2 Data sheets (one set per test) -- for data recording (see Figure 1).

13.6.3 Tape, colored -- for labelling test chambers and containers.

13.6.4 Markers, water-proof -- for marking containers, etc.

13.6.5 Parafilm -- to cover graduated cylinders and vessels containing gametes, embryos.

13.6.6 Gloves, disposable -- for personal protection from contamination.

13.6.7 Pipets, serological -- 1-10 mL, graduated.

13.6.8 Pipet tips -- for automatic pipets.

13.6.9 Coverslips -- for microscope slides.

13.6.10 Lens paper -- for cleaning microscope optics.

13.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

13.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

13.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

13.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

13.6.15 Laboratory quality assurance samples and standards -- for the above methods.

13.6.16 Test chambers -- 30 mL, four chambers per concentration. The chambers should be borosilicate glass or nontoxic disposable

plastic labware. The test may be performed in other sized chambers as long as the density of embryos is the same.

13.6.17 Formaldehyde, 37% (Concentrated Formalin) -- for preserving larvae. Note: formaldehyde has been identified as a carcinogen and is irritating to skin and mucous membranes. It should not be used at a concentration higher than necessary to achieve morphological preservation of larvae for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

13.6.19 Reference toxicant solutions (see Section 13.10.2.4 and Section 4, Quality Assurance).

13.6.20 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies and Section 7, Dilution Water).

13.6.21 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

13.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 13.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-μm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

13.6.23 HYPERSALINE BRINES

13.6.23.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

13.6.23.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

13.6.23.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 μm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

13.6.23.4 Freeze Preparation of Brine

13.6.23.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20°C until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

13.6.23.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

13.6.23.4.3 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED BY ADDING DILUTION WATER ONLY OR BRINE ONLY (WITHOUT ADDITION OF DRY SEA SALTS), GIVEN VARIOUS EFFLUENT SALINITIES, DILUTION WATER SALINITIES, AND BRINE SALINITIES, AND MAINTAINING 30% TEST SALINITY.

Effl.	Dilution Water Salinity ‰					Brine Salinity ‰				
‰	31	32	33	34	35	60	70	80	90	100
0	3.23	6.25	9.09	11.76	14.29	50.00	57.14	62.50	66.67	70.00
1	3.33	6.45	9.38	12.12	14.71	50.85	57.97	63.29	67.42	70.71
2	3.45	6.67	9.68	12.50	15.15	51.72	58.82	64.10	68.18	71.43
3	3.57	6.90	10.00	12.90	15.63	52.63	59.70	64.94	68.97	72.16
4	3.70	7.14	10.34	13.33	16.13	53.57	60.61	65.79	69.77	72.92
5	3.85	7.41	10.71	13.79	16.67	54.55	61.54	66.67	70.59	73.68
10	4.76	9.09	13.04	16.67	20.00	60.00	66.67	71.43	75.00	77.78
15	6.25	11.76	16.67	21.05	25.00	66.67	72.73	76.92	80.00	82.35
20	9.09	16.67	23.08	28.57	33.33	75.00	80.00	83.33	85.71	87.50
25	16.67	28.57	37.50	44.44	50.00	85.71	88.89	90.91	92.31	93.33

4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

13.6.23.5 Heat Preparation of Brine

13.6.23.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

13.6.23.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

13.6.23.5.3 Seawater should be filtered to at least 10 μm before being put into the brine generator. The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100% an that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

13.6.23.5.4 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

13.6.23.6 Artificial Sea Salts

13.6.23.6.1 No data from mussel or oyster tests using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

13.6.23.7 Dilution Water Preparation from Brine

13.6.23.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

13.6.23.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 30‰, 100‰ divided by 30‰ = 3.33. The proportion of brine is 1 part in 3.33 (one part brine to 2.33 parts reagent water). To make 1 L of dilution water at 30‰ salinity from a HSB of 100‰, 300 mL of brine and 700 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

13.6.23.8 Test Solution Salinity Adjustment

13.6.23.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always

equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

13.6.23.8.2 Check the pH of all test solutions and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide (see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.6.23.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (30 - SE)/(SB - 30)$$

13.6.23.8.4 This calculation assumes that dilution water salinity is 30 ± 2‰.

13.6.23.9 Preparing Test Solutions

13.6.23.9.1 Ten mL of test solution are needed for each test container. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 1.0 mL of effluent to a 100-mL volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 100-mL mark with dilution water, stopper it, and shake to mix. Pour into a (150-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

13.6.23.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Pour into a (100-250

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (AT X‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Make serial dilutions from the highest test concentration.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION:

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

13.6.23.10 Brine Controls

13.6.23.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 13.6.23.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 30)/(30 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

13.6.24 TEST ORGANISMS, OYSTERS AND MUSSELS

13.6.24.1 The test organisms for this test are the Pacific, oyster, *Crassostrea gigas*, or mussels, *Mytilus spp.* (at least twelve per test). Pacific oysters are native to Japan, but have been cultured commercially on the west coast of the United States for over a century.

13.6.24.2 Species Identification

13.6.24.2.1 The three species of mussels included in this method are presumably native to the west coast. The California mussel (*Mytilus californianus*) is distributed along the exposed rocky coast from Alaska to Baja California and is found from intertidal areas to 150 feet depth. The other two mussels included in this method (*M. trossulus* and *M. galloprovinciallis*) are common in sheltered waters such as bays and estuaries and were previously considered to be west coast populations of *Mytilus edulis*. The two species are both present in central California, with *M.*

galloprovincialis reported from San Francisco Bay to Baja California, and *M. trossulus* reported from Monterey to Alaska.

13.6.24.2.2 Test organisms should be identified to species using morphological features in recognized keys. Separation of the "*M. edulis*" complex, (*M. trossulus*, and *M. galloprovinciallis*) may not be possible without electrophoretic characterization. The geographic source of the *Mytilus spp.* broodstock must be reported.

13.6.24.3 Obtaining Broodstock

13.6.24.3.1 Adult oysters (*Crassostrea gigas*) and mussels (*Mytilus spp.*) can be obtained from commercial suppliers and the mussels can also be collected from the field. Organisms are best shipped in damp towels or seaweed and kept cool (4-12°C). Note: if practical, check the sex ratio of brood stock or request such information from a commercial supplier. A highly skewed sex ratio could result in poor embryo yield.

13.6.24.4 Broodstock Culture and Handling

13.6.24.4.1 The adult bivalves are maintained in glass aquaria or fiberglass troughs or tanks. These are supplied continuously (approximately 5 L/min) with natural seawater, or salt water prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded. Prior to spawning, the animals should be brushed or gently scraped to remove barnacles and other encrusting organisms; this alleviates problems of egg and sperm contamination, especially through potential barnacle spawning.

13.6.24.4.2 Although ambient temperature seawater is usually acceptable for holding, recommended temperatures are 14-15°C for oyster and 8°C for mussels; conditioning bivalves to spawning condition usually requires holding for from 1-8 weeks at a higher temperature (20°C for oysters, 15-18°C for mussels).

13.6.24.4.3 Natural seawater (≥30‰) is used to maintain the adult animals and as a control water in the tests.

13.6.24.4.4 Adult animals used in field studies are transported in insulated boxes or coolers packed with wet kelp or paper

toweling. Upon arrival at the field site, aquaria are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added.

13.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

13.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

13.8 CALIBRATION AND STANDARDIZATION

13.8.1 See Section 4, Quality Assurance.

13.9 QUALITY CONTROL

13.9.1 See Section 4, Quality Assurance.

13.10 TEST PROCEDURES

13.10.1 TEST DESIGN

13.10.1.1 The test consists of at least four replicates of five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain four replicates of a brine control. In addition, at least six extra count controls are prepared in dilution water and the number of embryos in each are counted at the time of test initiation. These counts provide an average initial embryo density that is used in the calculation of test results (see 13.13.1.3). Extra replicates are recommended for water chemistry during the tests (see Section 13.8 and Table 3).

13.10.1.2 Effluent concentrations are expressed as percent effluent.

13.10.2 TEST SOLUTIONS

13.10.2.1 Receiving waters

13.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually

collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 10 mL, and 400 mL for chemical analysis, would require approximately 440 mL of sample per test.

13.10.2.2 Effluents

14.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC). At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100% HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 70% at 30‰ salinity.

13.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

13.10.2.2.3 The volume in each test chamber is 10 mL.

13.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

13.10.2.3 Dilution Water

13.10.2.3.1 Dilution water should be uncontaminated 1-µm-filtered natural seawater or hypersaline brine (prepared from uncontaminated natural seawater) plus reagent water (see Section

7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

13.10.2.4 Reference Toxicant Test

13.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

13.10.2.4.2 The preferred reference toxicant for oysters and mussels is copper chloride ($CuCl_2 \cdot H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a copper reference toxicant stock solution (2,000 mg/L) by adding 5.366 g of copper chloride ($CuCl_2 \cdot 2H_2O$) to 1 liter of reagent water. For each reference toxicant test prepare a copper sub-stock of 3 mg/L by diluting 1.5 mL of stock to one liter with reagent water. Alternatively, certified standard solutions can be ordered from commercial companies.

13.10.2.4.3 Prepare a control (0 µg/L) plus four replicates each of at least five consecutive copper reference toxicant solutions (e.g., from the series 3.0, 4.4, 6.5, 9.5, 13.9, 20.4, and 30.0 µg/L, by adding 0.10, 0.15, 0.22, 0.32, 0.46, 0.68, and 1.00 mL of sub-stock solution, respectively, to 100-mL volumetric flasks and filling to 100-mL with dilution water). Start with control solutions and progress to the highest concentration to minimize contamination.

13.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 30 ± 2‰.

13.10.3 COLLECTION OF GAMETES FOR THE TEST

13.10.3.1 Spawning Induction

13.10.3.1.1 Select at least a dozen bivalves and place them into a container filled with seawater (ca. 20°C for oysters, 15°C for

mussels) and allow time for them to resume pumping (ca. 30 minutes). Mussels will often start pumping following immersion if they have been kept out of water and refrigerated overnight prior to spawning.

TABLE 3. EXAMPLE OF TYPICAL TEST ARRAY SHOWING NUMBER AND TYPES OF TREATMENT CHAMBERS REQUIRED.

TREATMENT	Test Vials	Chemistry Vials
Count Control	6	0
Brine Control	4	1-3
Dilution Water Control	4	1-3
Effluent conc. 1	4	1-3
Effluent conc. 2	4	1-3
Effluent conc. 3	4	1-3
Effluent conc. 4	4	1-3
Effluent conc. 5	4	1-3
TOTAL Chambers = 41-55	34	7-21

13.10.3.1.2 Over a 15-20 minute period, increase the temperature (do not exceed 32°C for oysters, or 20°C for mussels), checking for spawning.

13.10.3.1.3 If no spawning occurs after 30 minutes, replace the water with some at the original temperature and after 15 minutes again increase the temperature as in 13.10.3.2. Although ASTM (1993) cautions against it, the addition of algae into the water can often stimulate spawning of bivalves; if this method is used, the organisms should be moved to clean water once spawning begins. Mussels can also be induced to spawn by injection of 0.5 M KCl into the posterior adductor muscle. Oysters can be induced to spawn by the addition of heat-killed sperm about one hour after initial temperature increase.

13.10.3.2 Pooling Gametes

13.10.3.2.1 When individuals are observed to be shedding gametes, remove each spawner from the tank and place each in a separate container (20°C water for oysters, 15°C for mussels). Alternatively, bivalves can be placed into individual chambers initially (at temperatures per 13.10.5.2) and these placed into a water bath that provides the desired maximum temperature.

13.10.3.2.2 Early in the spawning process, examine a small sample of the gametes from each spawner to confirm sex and to see if the gametes are of adequate quality.

13.10.3.2.3 Place a small amount of sperm from each male onto a microscope slide (well slides work nicely). Examine the sperm for motility; use sperm from those males with the better sperm motility.

13.10.3.2.4 A small sample of the eggs from each female should be examined for the presence of significant quantities of poor eggs (vacuolated, small, or abnormally shaped). If good quality eggs are available from one or more females, questionable batches of eggs should not be used for the test. It is more important to use high quality eggs than it is to use a pooled population of eggs.

13.10.3.2.5 Sperm and egg suspensions that are to be used for preparing the embryo stock should be passed through Nytex screen (ca. 75 μm) to separate out clumps of gametes or extraneous material.

13.10.3.2.6 The pooled eggs are placed into a 1 L beaker and sufficient dilution water added to achieve an egg density of about 5,000-8,000 eggs/mL (objects are just discernible when viewed through the egg suspension) in about 800-900 mL water volume.

13.10.3.3 Fertilization

13.10.3.3.1 Sperm density may vary from one spawning to the next. It is important to use enough sperm to achieve a high percent egg fertilization, but too many sperm can cause polyspermy with resultant abnormal development. To achieve an acceptable level of sperm, several egg suspensions of equal density should be fertilized using a range of sperm volumes,

e.g., 100 mL of egg suspension plus 1, 3, and 10 mL of sperm suspension. This test fertilization should be accomplished within 1 hour of spawning. Use the eggs with the lowest amount of sperm giving normal embryo development after 1.5-2.5 hours after fertilization, as determined by microscopic examination. Usually >90% of the eggs should be fertilized; oysters should have changed from the tear-drop shaped egg to a round single cell zygote; mussels should show a single polar body; or embryos of either species should have advanced to the two-cell stage.

13.10.4 START OF THE TEST

13.10.4.1 Prior to Beginning the Test

13.10.4.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

13.10.4.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (18 or 20 ± 1°C) and maintained at that temperature during the addition of dilution water.

13.10.4.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (18 or 20 ± 1°C).

13.10.4.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and

investigator's name, and safely store it away until after the oysters or mussels have been examined at the end of the test.

13.10.4.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

13.10.4.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

13.10.4.2 Estimation of Embryo Density

13.10.4.2.1 Adjust the embryo suspension to a density of 1,500-3,000/mL. Confirm by counting chamber counts on 1 mL subsamples from a stirred suspension of embryos. Final larval density of 15/mL will provide reasonable precision (150 larvae) and be easier to count than 300 larvae. Add 0.1 mL of the embryo suspension to 10 mL of test solution into each of the randomized test vials. It is extremely important (for a consistent embryo density among test chambers) to maintain a homogeneous distribution of embryos in the stock suspension by regular, slow oscillation of a perforated plunger during embryo distribution.

13.10.4.3 Initial Density Counts

13.10.4.3.1 If tests are conducted on small volumes, using an inverted microscope, the total number of embryos injected into the count controls should be determined as soon as the test has been started. If larger test volumes are used, with counts based upon subsamples, the embros should be resuspended in the water using a perforated plunger. Then subsamples are taken (e.g., 5-10 mL) and the total number of embryos counted in the subsample. Two methods for these counts are to use a counting chamber of the same volume as the subsample, or to screen the embryos using a 37 μm screen and backwash with a smaller volume for small counting chambers. In either procedure, appropriate multiple rinsing is needed to achieve quantitative transfer of embryos.

13.10.4.3.2 Initial counts are required to determine survival in the controls and other treatments. High coefficients of variability in initial counts make survival estimates inexact and may actually decrease the sensitivity of the test.

13.10.4.4 Incubation

13.10.4.4.1 Cover and incubate the chambers in an environmental chamber or by partial immersion in a temperature-controlled water bath for 48 hours.

13.10.4.4.2 At the end of the 48-hour incubation period, examine a count control test chamber (or control test vial if the count controls were transferred to a counting chamber to make the initial counts) under a microscope to check for complete development of control organisms. If development is complete, the test should be ended. If development does not appear to be complete, the test should be continued until complete development occurs (but not beyond 54 hours total test duration).

13.10.5 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

13.10.5.1 The light quality and intensity should be at ambient laboratory conditions. Light intensity should be 10-20 μE/m2/s, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle.

13.10.5.2 The water temperature in the test chambers should be maintained at 18 or 20 ± 1°C. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

13.10.5.3 The test salinity should be in the range of 30 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

13.10.5.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean

polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

13.10.6 DISSOLVED OXYGEN (DO) CONCENTRATION

13.10.6.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

13.10.7 OBSERVATIONS DURING THE TEST

13.10.7.1 Routine Chemical and Physical Observations

13.10.7.1.1 DO is measured at the beginning of the exposure period in each test concentration and in the control.

13.10.7.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in each test concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

13.10.7.1.3 Record all the measurements on the data sheet.

13.10.8 TERMINATION OF THE TEST

13.10.8.1 Ending the Test

13.10.8.1.1 Record the time the test is terminated.

13.10.8.1.2 The pH, dissolved oxygen, and salinity are measured

at the end of the exposure period in one test chamber at each concentration and in the control. If small electrodes are used, these measurements can be performed in a single extra replicate vial set up specifically for this measurement. Measurements should not be made in vials that are to be counted, as larvae may adhere to electrodes, possibly biasing larval counts.

13.10.8.2 Sample Preservation

13.10.8.2.1 To terminate the test, add 0.25 mL of concentrated formalin (37% formaldehyde). It is advisable not to shake the contents at any time following test termination because the larvae may stick to the edge of the chambers. Simply allow the preservative to mix passively and the larvae to settle out. The use of glutaraldehyde instead of formalin is likely to be acceptable, but as no record of its use with this test is known, care should be taken to confirm that glutaraldehyde kills, preserves, and produces no artifacts that would confound the test results.

13.10.8.2.2 Note: Formaldehyde has been identified as a carcinogen and both glutaraldehyde and formaldehyde are irritating to skin and mucus membranes. Neither should be used at higher concentrations than needed to achieve morphological preservation and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

13.10.8.3 Counting

13.10.8.3.1 After addition of preservative, observe all the larvae in each test vial. This can be done by examining the contents of each test vial with an inverted microscope at about 40X-50X magnification or by quantitative transfer of all larvae onto a counting chamber and counting using a compound microscope at about 100X. Using the mechanical stage, carefully count and score all larvae as either normal or abnormal. If substantial numbers of completely developed shells without meat are observed (i.e., > 5 percent of normal larvae), then these shells should be enumerated separately (as dead larvae). "Larvae possessing misshapened or otherwise malformed shells are considered normal, provided development has been completed" (ASTM, 1994). Record the final counts on the data sheet.

13.10.8.3.2 If the number of larvae observed appears to be low in relation to the number inoculated at the beginning of the test, this signifies either mortality and dissolution, or possible adherence to the walls of the vials or incomplete transfer to the counting chamber. Inspect the vials for evidence of the latter two occurrences.

13.10.8.4 Endpoint

13.10.8.4.1 The percentage of embryos that did not survive and develop to live larvae with completely developed shells (i.e., abnormal or dead organisms) is calculated for each treatment replicate (See 13.13.1.3). All larvae are considered live unless they are merely empty shells "without meat" (ASTM, 1994); embryos and larvae that are not yet in the D-hinge stage are counted as abnormal, even if they may have died during the test. Embryos and larvae that die and disintegrate during the test are estimated from initial embryo counts (See N' in 13.13.1.3).

13.10.8.4.2 Unless used as the dilution water, natural seawater controls are only used to check the relative performance of the dilution water controls (e.g., brine controls) required for salinity adjustment. Statistical analysis should use the appropriate dilution water control data.

13.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

13.11.1 A summary of test conditions and test acceptability criteria is listed in Table 4.

TABLE 4. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR, *CRASSOSTREA GIGAS* and *MYTILUS SPP.*, EMBRYO-LARVAL DEVELOPMENT TEST WITH EFFLUENTS AND RECEIVING WATERS

1.	Test type:	Static non-renewal
2.	Salinity:	30 ± 2‰
3.	Temperature:	20 ± 1°C (oysters) 15 or 18 ± 1°C (mussels)*
4.	Light quality:	Ambient laboratory light

5. Light intensity:	10-20 uE/m2/s (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	30 mL
8. Test solution volume:	10 mL
9. No. larvae per chamber:	150-300
10. No. replicate chambers per concentration:	4 (plus 3 chemistry vials)
11. Dilution water:	Uncontaminated 1-μm-filtered natural seawater or hypersaline brine prepared from natural seawater
12. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor:	Effluents: ≥0.5 Receiving waters: None or ≥0.5
14. Test duration:	48 hours (or until complete development up to 54 hours)
15. Endpoint:	Survival and normal shell development
16. Test acceptability criteria:	Control survival must be ≥70% for oyster embryos or ≥50% for mussel embryos in control vials; ≥90% normal shell development in surviving controls; and must achieve a %MSD of <25%

17. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
18. Sample volume required:	1 L per test

*Mussel embryo-larval tests were commonly conducted at 15°C (ASTM, 1994). Experience has shown that many laboratories in northern Californa, Oregon, and Washington often fail to achieve adequate control development at 15°C in 48 hours. It is acceptable to conduct the test at 15°C with the permission of the regulatory authority. Developmental rates may be dependent upon species, local population characteristics, or other factors.

13.12 ACCEPTABILITY OF TEST RESULTS

13.12.1 For tests to be considered acceptable, the following requirements must be met:

(1) The mean survival must be at least 70% for oysters or at least 50% for mussels in the controls.

(2) The percent normal must be at least 90% in the surviving controls.

(3) The minimum significant difference (%MSD) is <25% relative to the control.

13.13 DATA ANALYSIS

13.13.1 GENERAL

13.13.1.1 Tabulate and summarize the data. Calculate the proportion of normally developed larvae for each replicate. A sample set of test data is listed in Table 5.

13.13.1.2 Final calculations are based upon counts of normal larvae and total larvae at test termination, and mean initial embryo count.

13.13.1.3 The percentage of embryos that did not survive or develop to live larvae with completely developed shells (i.e., abnormal or dead organisms) is calculated for each treatment replicate (including controls) using the formula:

$$A = \frac{100\ (N' - B')}{N'}$$

where:

A = percent abnormal and dead organisms
B' = the adjusted number of normal larvae at the end of the test
N' = the initial number of embryos in the test chambers expressed as the mean of the initial counts; and: if N > N', where

N = the actual number of larvae at the end of the test
then: $B' = B\ (N' / N)$

where: B = the actual number of normal larvae at the end of the test but, when $N \leq N'$, then: $B' = B$

The means of "A" are obtained for each treatment concentration, and the latter are corrected for control response using Abbott's formula, as follows:

$$E = \frac{100\ (A - M)}{100 - M}$$

where:

E = the mean percent abnormal/dead corrected for controls
A = the mean percent abnormal/dead
M = the value of A for the controls.

13.13.1.4 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

TABLE 5. DATA FROM BIVALVE DEVELOPMENT TEST

Copper Concentration (µg/L)	Replicate	Initial Density	Number Surviving	Number Normal	Proportion Normal
Control	A	25	22	22	1.00
	B	25	25	24	0.96
	C	25	25	25	1.00
	D	30	30	29	0.97
0.13	A	25	23	22	0.96
	B	30	30	29	0.97
	C	25	25	25	1.00
	D	25	24	23	0.96
0.25	A	25	25	23	0.92
	B	25	19	18	0.95
	C	25	21	19	0.90
	D	25	23	22	0.96
0.50	A	25	11	10	0.91
	B	25	14	13	0.93
	C	25	17	15	0.88
	D	25	15	14	0.93
1.00	A	25	8	7	0.88
	B	25	6	5	0.83
	C	25	8	7	0.88
	D	25	11	9	0.82
2.00	A	25	2	2	1.00

13.13.1.5 The endpoints of toxicity tests using bivalves are based on the reduction in proportion of normally developed larvae. The IC25 is calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for larval development are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

13.13.2 EXAMPLE OF ANALYSIS OF BIVALVE EMBRYO-LARVAL DEVELOPMENT DATA

13.13.2.1 Formal statistical analysis of the embryo-larval development is outlined in Figure 1. The response used in the analysis is the proportion of normally developed surviving larvae in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there is no normal development in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25.

13.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

13.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

13.13.2.4 Example of Analysis of Embryo-Larval Development Data

13.13.2.4.1 Since the response of interest is the proportion of normally developed surviving larvae, each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. Because there are varying numbers of survivors in the replicates, the adjustment for response proportions of zero or one will not be made. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and control are listed in Table 5. The data are plotted in Figure 2.

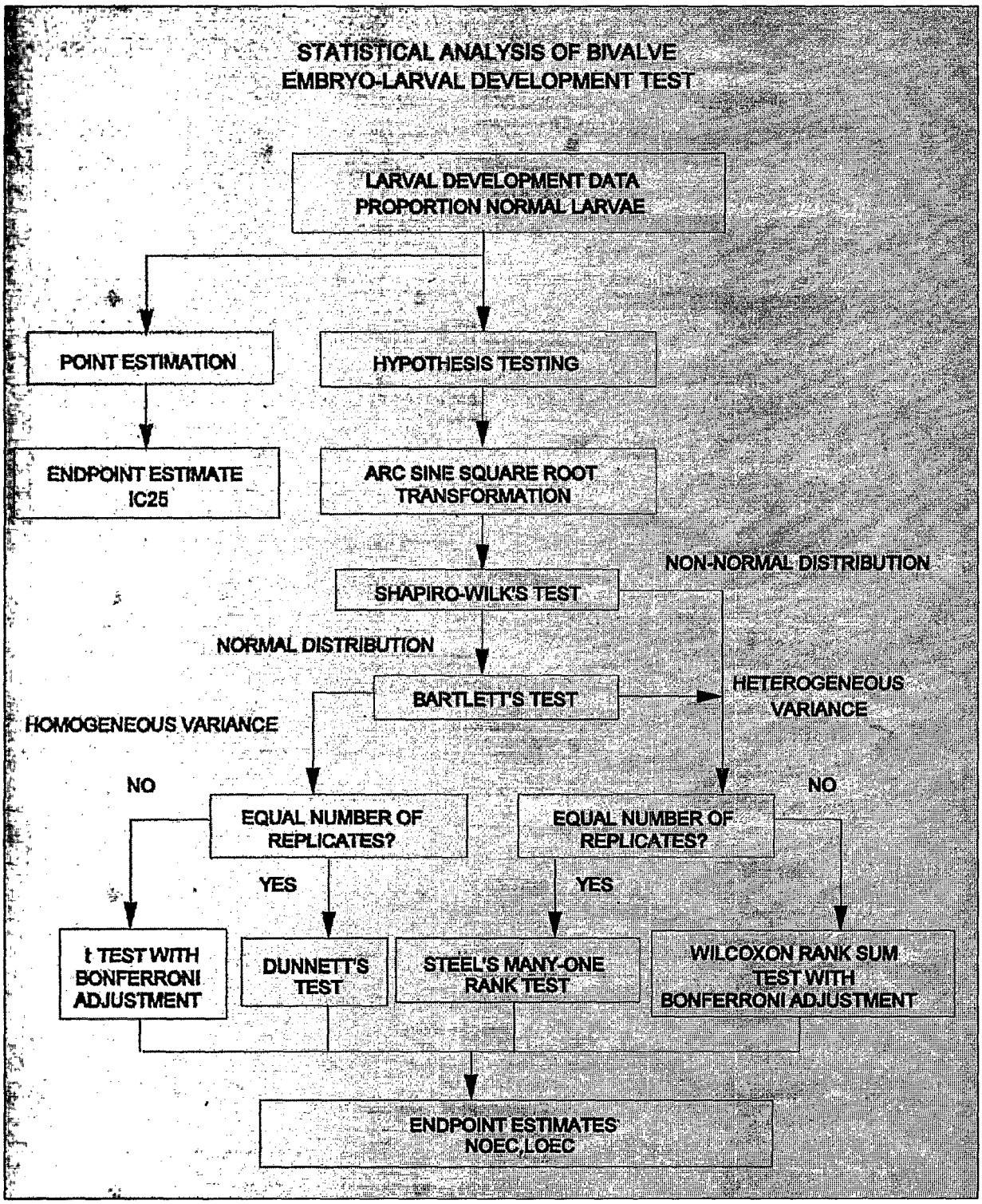

Figure 1. Flowchart for statistical analysis of the pacific oyster, *Crassostrea gigas*, and mussel, *Mytilus spp.*, development data.

13.13.2.5 Test for Normality

13.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

TABLE 6. BIVALVE EMBRYO-LARVAL DEVELOPMENT DATA

		Copper Concentration (µg/L)					
	Replicate	Control	0.13	0.25	0.50	1.00	2.00
	A	1.00	0.96	0.92	0.91	0.88	1.00
RAW	B	0.96	0.97	0.95	0.93	0.83	0.67
	C	1.00	1.00	0.90	0.88	0.88	0.75
	D	0.97	0.96	0.96	0.93	0.82	0.40
ARC SINE	A	1.571	1.369	1.284	1.266	1.217	1.571
SQUARE ROOT	B	1.369	1.397	1.345	1.303	1.146	0.959
TRANSFORMED	C	1.571	1.571	1.249	1.217	1.217	1.047
	D	1.397	1.369	1.369	1.303	1.133	0.685
Mean ($\overline{Y}_i$)		1.477	1.427	1.312	1.272	1.178	1.066
S_i^2		0.01191	0.00945	0.00303	0.00166	0.00203	0.13733
i		1	2	3	4	5	6

13.13.2.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

Where: X_i = the ith centered observation
$\overline{X}$ = the overall mean of the centered observations
n = the total number of centered observations

13.13.2.5.3 For this set of data, n = 24

$$\overline{X} = \frac{1}{24} (-0.002) = 0.000$$

D = 0.4963

13.13.2.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 7

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.381	13	-0.019
2	-0.108	14	-0.006
3	-0.107	15	0.031
4	-0.080	16	0.031
5	-0.063	17	0.033
6	-0.058	18	0.039
7	-0.058	19	0.039
8	-0.055	20	0.057
9	-0.045	21	0.094
10	-0.032	22	0.094
11	-0.030	23	0.144
12	-0.028	24	0.505

13.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 24 and k = 12. The a_i values are listed in Table 8.

13.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 8. For the data in this example:

$$W = \frac{1}{0.4963} (0.6322)^2 = 0.805$$

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.886	$X^{(24)} - X^{(1)}$
2	0.3098	0.252	$X^{(23)} - X^{(2)}$
3	0.2554	0.201	$X^{(22)} - X^{(3)}$
4	0.2154	0.174	$X^{(21)} - X^{(4)}$
5	0.1807	0.120	$X^{(20)} - X^{(5)}$
6	0.1512	0.097	$X^{(19)} - X^{(6)}$
7	0.1245	0.097	$X^{(18)} - X^{(7)}$
8	0.0997	0.088	$X^{(17)} - X^{(8)}$
9	0.0764	0.076	$X^{(16)} - X^{(9)}$
10	0.0539	0.063	$X^{(15)} - X^{(10)}$
11	0.0321	0.024	$X^{(14)} - X^{(11)}$
12	0.0107	0.009	$X^{(13)} - X^{(12)}$

13.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 24 observations is 0.884. Since W = 0.805 is less than the critical value, conclude that the data are not normally distributed.

13.13.2.5.8 Since the data do not meet the assumption of normality, Steel's Many-one Rank Test will be used to analyze the embryo-larval development data.

13.13.2.6 Steel's Many-one Rank Test

13.13.2.6.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ... , 8) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

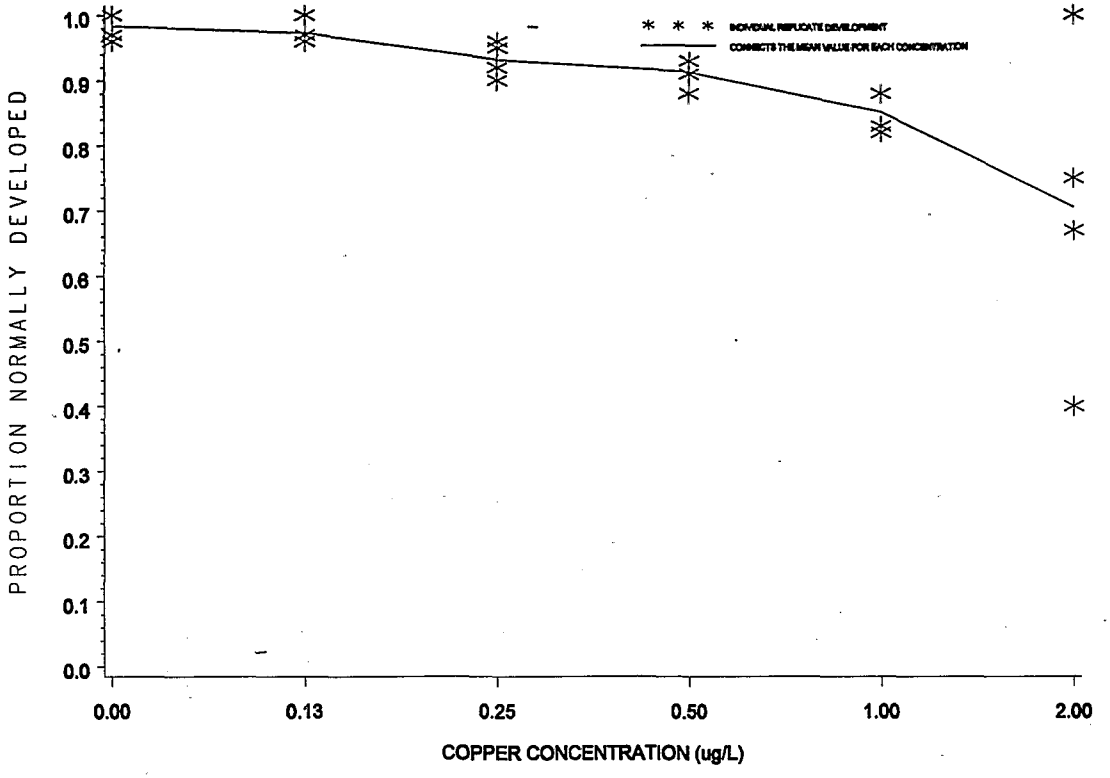

Figure 2. Plot of mean proportion of normaly developed bivalve larvae.

13.13.2.6.2 An example of assigning ranks to the combined data for the control and 0.13 µg/L concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are then summed for each concentration level, as shown in Table 11.

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 0.13 µg/L CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Normal	Concentration
2	1.369	0.13 µg/L
2	1.369	0.13 µg/L
2	1.369	Control
4.5	1.397	0.13 µg/L
4.5	1.397	Control
7	1.571	0.13 µg/L
7	1.571	Control
7	1.571	Control

13.13.2.6.3 For this example, determine if the survival in any of the concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus compare the rank sums for the survival at each of the various concentration levels with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with five concentrations (excluding the control) and four replicates is 10 (See Table 5, Appendix E).

13.13.2.6.4 Since the rank sums for the 0.50 µg/L and 1.00 µg/L concentration levels are equal to the critical value, the proportions of normal development in those concentrations are considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no

TABLE 10. TABLE OF RANKS[1]

Replicate	Control	Copper Concentration (µg/L) 0.13	0.25
1	1.571(7,7.5,7.5,7.5,7)	1.369(2)	1.284(2)
2	1.369(2,4.5,5,5,4)	1.397(4.5)	1.345(3)
3	1.571(7,7.5,7.5,7.5,7)	1.571(7)	1.249(1)
4	1.397(4.5,6,6,6,5)	1.369(2)	1.369(4.5)

Replicate	Copper Concentration (µg/L) (Continued) 0.50	1.00	2.00
1	1.266(2)	1.217(3.5)	1.571(7)
2	1.303(3.5)	1.146(2)	0.959(2)
3	1.217(1)	1.217(3.5)	1.047(3)
4	1.303(3.5)	1.133(1)	0.685(1)

[1]Control ranks are given in the order of the concentration with which they were ranked.

TABLE 11. RANK SUMS

Concentration µg/L Copper)	Rank Sum
0.13	15.5
0.25	10.5
0.50	10.0

other concentration has a significantly lower proportion normal than the control. Because the 0.50 µg/L concentration shows significantly lower normal development than the control while the

higher 2.00 µg/L concentration does not, these test results are considered to have an anomalous dose-response relationship and it is recommended that the test be repeated. If an NOEC and LOEC must be determined for this test, the lowest concentration with significant growth impairment versus the control is considered to the LOEC for growth. Thus, for this test, the NOEC and LOEC would be 0.25 µg/L and 0.50 µg/L, respectively.

13.13.2.7 Calculation of the ICp

13.13.2.7.1 The embryo-larval development data in Table 4 are utilized in this example. As can be seen from Table 4 and Figure 2, the observed means are monotonically non-increasing with respect to concentration (mean response for each higher concentration is not less than or equal to the mean response for the previous concentration and the responses between concentrations do not follow a linear trends). Therefore, it is not necessary to smooth the means prior to calculating the IC. The observed means, represented by $\overline{Y}_i$ become the corresponding smoothed means, M_i. Table 12 contains the response means and smoothed means and Figure 3 gives a plot of the smoothed response curve.

TABLE 12. BIVALVE MEAN LARVAL DEVELOPMENT RESPONSE AFTER SMOOTHING

Copper Conc. (µg/L)	i	Response Means, $\overline{Y}_i$ (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.983	0.983
0.13	2	0.973	0.973
0.25	3	0.932	0.932
0.50	4	0.913	0.913
1.00	5	0.852	0.852
2.00	6	0.705	0.705

13.13.2.7.2 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of normally developed larvae, compared to the controls, would result

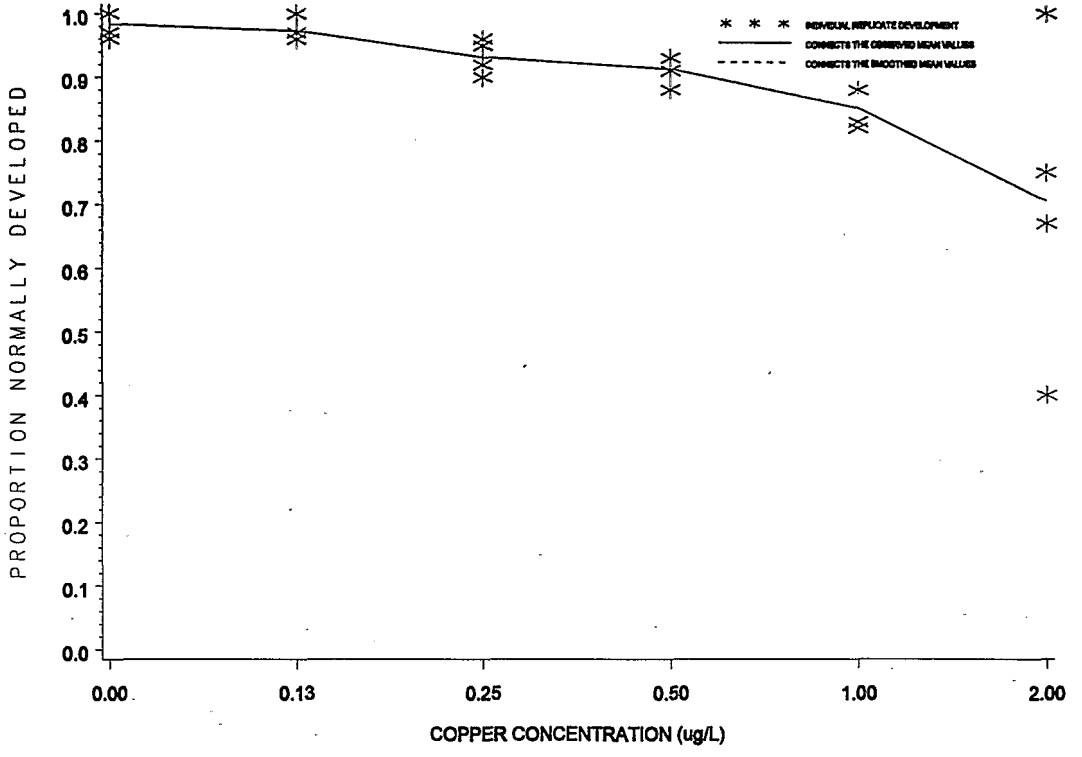

Figure 3. Plot of raw data, observed means, and smoothed means for the bivalve development data from Tables 4 and 12.

in a mean proportion of 0.737, where $M_1(1-p/100) = 0.983(1-25/100)$. Examining the means and their associated concentrations (Table 12), the response, 0.737, is bracketed by C_4 = 1.00 µg/L copper and C_5 = 2.00 µg/L copper.

13.13.2.7.3 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1-p/100) - M_j] \frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 1.00 + [0.983(1 - 25/100) - 0.852] \frac{(2.00 - 1.00)}{(0.705 - 0.852)}$$

$$= 1.78 \text{ µg/L}.$$

13.13.2.7.4 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 1.7839 µg/L. The empirical 95.0% confidence interval for the true mean was not available because the number of resamples which generated an IC25 estimate was not an even multiple of 40. The computer program output for the IC25 for this data set is shown in Figure 4.

13.14 PRECISION AND ACCURACY

13.14.1 PRECISION

13.14.1.1 Single-Laboratory Precision

13.14.1.1.1 Single-laboratory precision data for the *Mytilus spp.* with the reference toxicant cadmium and lyophilized pulp mill effluent with natural seawater are provided in Tables 4-5. The coefficient of variation, based on EC25, is 32.8% to 45.0% for cadmium and 14.2% to 30.6% for lyophilized pulp mill effluent. Single-laboratory precision data for the *Crassostrea gigas* with the reference toxicant cadmium and lyophilized pulp mill effluent with natural seawater are provided in Tables 6-7. The coefficient of variation, based on EC25, is 18.5% to 80.4% for cadmium and 20.8% to 43.3% for lyophilized pulp mill effluent.

13.14.1.2 Multi-laboratory Precision

Conc. ID		1	2	3	4	5	6
Conc. Tested		0	0.13	0.25	0.50	1.00	2.00
Response	1	1.00	0.96	0.92	0.91	0.88	1.00
Response	2	0.96	0.97	0.95	0.93	0.83	0.67
Response	3	1.00	1.00	0.90	0.88	0.88	0.75
Response	4	0.97	0.96	0.96	0.93	0.82	0.40

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date: Test Ending Date:
Test Species: bivalve
Test Duration: 48 hours
DATA FILE: bivalve.icp
OUTPUT FILE: bivalve.i25

Conc. ID	Number Replicates	Concentration ug/L	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	0.983	0.021	0.983
2	4	0.130	0.973	0.019	0.973
3	4	0.250	0.932	0.028	0.932
4	4	0.500	0.913	0.024	0.913
5	4	1.000	0.852	0.032	0.852
6	4	2.000	0.705	0.247	0.705

The Linear Interpolation Estimate: 1.7839 Entered P Value: 25

Number of Resamplings: 80Those resamples not used had estimates above the highest concentration/ %Effluent.

The Bootstrap Estimates Mean: 1.6188 Standard Deviation: 0.1758

No Confidence Limits can be produced since the number of resamples generated is not a multiple of 40.
Resampling time in Seconds: 0.17 Random_Seed: -232404862

Figure 4. ICPIN program output for the IC25.

13.14.1.2.1 Multi-laboratory precision data for *Mytilus spp.* with the reference toxicant, cadmium and lyophilized pulp mill effluent are provided in Tables 12-13. The coefficient of variation for cadmium EC25 is 23.6%, and for effluent EC25 is 14.4% based on five laboratories. Multi-laboratory precision data for *Crassostrea gigas* with the reference toxicant, cadmium and lyophilized pulp mill effluent are provided in Tables 14-15. The coefficient of variation is 21.3% for cadmium EC25 and 14.2% for lyophilized pulp mill effluent EC25, based on results from five laboratories.

13.14.2 ACCURACY

13.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 12. SINGLE AND MULTI-LABORATORY PRECISION OF THE MUSSEL, *MYTILUS SPP.*, DEVELOPMENT TEST PERFORMED WITH CADMIUM CHLORIDE (CD MG/L) AS A REFERENCE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
Oct-92	2.35	1.06	2.42	4.20	4.77
Nov-92	0.86	3.49	3.89	2.21	2.39
Dec-92	1.79	2.51	no data	2.27	3.73
Jan-93	3.69	2.25	6.77	no data	1.57
Feb-93	2.81	2.91	5.85	3.75	3.05
Mar-93	3.71	2.64	2.62	4.89	no data

Mean	2.54	2.48	4.31	3.46	3.10
SD	1.11	0.81	1.94	1.19	1.23
CV (%)	43.9	32.8	45.0	34.3	40.0

# of Labs	Statistic	EC25
5	Mean (N=5) SD CV(%)	3.18 0.75 23.6

These data are from: Pastorok, et al. (1994), West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

TABLE 13. SINGLE AND MULTI-LABORATORY PRECISION OF THE MUSSEL, *MYTILUS SPP.*, DEVELOPMENT TEST PERFORMED WITH LYOPHILIZED PULP MILL EFFLUENT (%) AS THE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
Oct-92	1.78	1.40	2.02	1.83	1.85
Nov-92	1.57	1.94	2.70	1.98	no data
Dec-92	1.74	1.88	3.08	no data	1.87
Jan-93	3.17	2.03	2.46	1.07	no data
Feb-93	1.66	no data	no data	no data	no data
Mar-93	1.85	1.66	1.72	1.82	no data

Mean	1.96	1.78	2.40	1.68	1.86
SD	0.60	0.25	0.54	0.41	0.28
CV (%)	30.6	14.2	22.5	24.5	1.4

# of Labs	Statistic	EC25
5	Mean (n=5) SD CV(%)	1.93 0.28 14.4

These data are from: Pastorok, et al. (1994) West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

TABLE 14. SINGLE AND MULTI-LABORATORY PRECISION OF THE OYSTER, *CRASSOSTREA GIGAS*, DEVELOPMENT TEST PERFORMED WITH CADMIUM CHLORIDE (CD MG/L) AS A REFERENCE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
July-92	1.04	1.54	0.50	0.41	0.56
Aug-92	0.31	1.38	0.30	0.35	no data
Sept-92	0.68	0.20	0.49	no data	no data
Apr-93	no data	0.45	0.51	no data	0.95
May-93	0.46	0.30	1.05	0.52	0.83
June-93	0.26	1.55	0.93	no data	0.83
July-93	0.28	0.82	0.66	1.56	0.90
Mean	0.51	0.89	0.63	0.71	0.81
SD	0.31	0.59	0.27	0.57	0.15
CV (%)	60.6	66.7	42.1	80.4	18.5

# of Labs	Statistic	EC25
5	Mean (n=5) SD CV(%)	0.71 0.15 21.3

These data are from: Pastorok, et al. (1994), West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

TABLE 15. SINGLE AND MULTI-LABORATORY PRECISION OF THE OYSTER, *CRASSOSTREA GIGAS*, DEVELOPMENT TEST PERFORMED WITH LYOPHILIZED PULP MILL EFFLUENT (%) AS THE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
July-92	no data	0.91	1.28	no data	1.43
Aug-92	1.21	1.09	0.98	0.61	no data
Sept-92	0.76	1.66	0.83	no data	no data
Apr-93	0.80	1.10	1.61	1.66	no data
May-93	1.21	0.65	1.90	0.93	0.93
June-93	1.09	1.32	1.72	0.83	0.98
July-93	0.82	0.80	1.56	1.67	1.04

Mean	0.98	1.08	1.41	1.14	1.10
SD.	0.21	0.34	0.40	0.49	0.23
CV (%)	21.6	31.4	28.0	43.3	20.8

# of Labs	Statistic	EC25
5	Mean (n=5) SD CV(%)	1.14 0.16 14.2

These data are from: Pastorok, et al., (1994), West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

APPENDIX I. BIVALVE TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 30 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution.

D. Prepare a series copper reference toxicant concentrations.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 18 or 20°C and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Determine test concentrations and appropriate dilution water based on NPDES permit requirements and guidance from the appropriate regulatory agency.

B. Prepare test solutions by diluting well mixed unfiltered effluent using volumetric pipettes. Use hypersaline brine where necessary to maintain all test solutions at 30 ± 2‰. Include brine controls in tests that use brine.

C. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

D. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

E. Place test chambers in a water bath or environmental chamber set to 18 or 20°C as appropriate for the test species and allow temperature to equilibrate.

F. Measure the test solution temperature daily in a randomly located blank test chamber. Monitor the temperature of the water bath or environmental chamber continuously.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Obtain test organisms and hold or condition as necessary for spawning.

B. On day of test, spawn organisms, examine gametes, pool good eggs, pool good sperm.

C. Fertilize subsets of eggs with a range of sperm concentrations to obtain >90% embryogenesis without polyspermy.

D. Adjust embryo stock suspension density to 1500-3000/mL.

E. Introduce organisms to test chambers (150-300 embryos in 0.1 mL of stock).

F. Count all embryos in each of six extra controls set up for determining mean embryo density and variation. Return these to the test for later examination for developmental rate in controls.

G. Near the end of the 48-hour incubation period examine several of the extra controls to determine if development has reached the prodisoconch stage. If yes, terminate the test at 48 hours; if no, continue the test for up to 54 hours as required for complete development.

H. Terminate the test by addition of formalin.

I. Count larvae and record the number of normal prodisoconch larvae and other larvae in each test vial.

J. Analyze the data.

K. Include standard reference toxicant point estimate values in the standard quality control charts.

Sample data sheet for embryo microscopic examination.

BIVALVE DEVELOPMENT TEST: RESULTS

Bioassay No._____ Date_____

Counter_____

Number	Sample	Abnormal	Normal	%Normal	Notes

SECTION 14

RED ABALONE, *Haliotis rufescens* LARVAL DEVELOPMENT TEST METHOD

Adapted from a method developed by
John W. Hunt and Brian S. Anderson
Institute of Marine Sciences, University of California
Santa Cruz, California

(in association with)
California Department of Fish and Game
Marine Pollution Studies Laboratory
34500 Coast Route 1, Monterey, CA 93940

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SECTION 14

RED ABALONE, *HALIOTUS RUFESCENS* LARVAL DEVELOPMENT TEST METHOD

14.1 SCOPE AND APPLICATION

14.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the larvae of red abalone, *Haliotis rufescens* during a 48-h static non-renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

14.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

14.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

14.1.4 This method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

14.1.5 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

14.2 SUMMARY OF METHOD

14.2.1 This method provides the step-by-step instructions for performing a 48-h static non-renewal test using early development of abalone larvae to determine the toxicity of substances in marine and estuarine waters. The test endpoint is normal shell development.

14.3 **INTERFERENCES**

14.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment, and Supplies).

14.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

14.4 **SAFETY**

14.4.1 See Section 3, Health and Safety.

14.5 **APPARATUS AND EQUIPMENT**

14.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult red abalone, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20°C), with appropriate filtration and aeration system.

14.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

14.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (15°C) prior to the test.

14.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

14.5.5 Refractometer -- for determining salinity.

14.5.6 Hydrometer(s) -- for calibrating refractometer.

14.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

14.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD 170.1, USEPA, 1979) -- to calibrate laboratory thermometers.

14.5.9 pH and DO meters -- for routine physical and chemical measurements.

14.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

14.5.11 Winkler bottles -- for dissolved oxygen determinations.

14.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

14.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

14.5.14 Glass stirring rods -- for mixing test solutions.

14.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-1000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

14.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

14.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

14.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

14.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

14.5.20 Wash bottles -- for dilution water.

14.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

14.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

14.5.23 Beakers, 1,000 mL borosilicate glass -- for mixing gametes for fertilization of eggs.

14.5.24 Beakers, 250 mL borosilicate glass -- for preparation of test solutions.

14.5.25 Counter, two unit, 0-999 -- for recording counts of larvae.

14.5.26 Inverted or compound microscope -- for inspecting gametes and making counts of larvae.

14.5.27 Perforated plunger -- for stirring egg solutions.

14.5.28 Supply of *Macrocystis* or other macroalgae (if holding broodstock for longer than 5 days) -- for feeding abalone.

14.5.29 Stainless steel butter knife, rounded smooth-edged blade (for handling adult abalone). Abalone irons and plastic putty knives have also been used successfully.

14.5.30 Sieve or screened tube, approximately 37 µm-mesh -- for retaining larvae at the end of the test.

14.5.31 60 µm NITEX® filter -- for filtering receiving water.

14.6 **REAGENTS AND SUPPLIES**

14.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

14.6.2 Data sheets (one set per test) -- for data recording (See Appendix I).

14.6.3 Tape, colored -- for labelling test chambers and containers.

14.6.4 Markers, water-proof -- for marking containers, etc.

14.6.5 Parafilm -- to cover graduated cylinders and vessels containing gametes, embryos.

14.6.6 Gloves, disposable -- for personal protection from contamination.

14.6.7 Pipets, serological -- 1-10 mL, graduated.

14.6.8 Pipet tips -- for automatic pipets.

14.6.9 Coverslips -- for microscope slides.

14.6.10 Lens paper -- for cleaning microscope optics.

14.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

14.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

14.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

14.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

14.6.15 Laboratory quality assurance samples and standards -- for the above methods.

14.6.16 Test chambers -- 600 mL, five chambers per concentration. The chambers should be borosilicate glass (for effluents) or nontoxic disposable plastic labware (for reference toxicants). To avoid contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered during the test with safety glass plates or a plastic sheet (6 mm thick).

14.6.17 Formaldehyde, 37% (Concentrated Formalin) -- for preserving larvae. Note: formaldehyde has been identified as a

carcinogen and is irritating to skin and mucous membranes. It should not be used at a concentration higher than necessary to achieve morphological preservation of larvae for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

14.6.18 Tris (hydroxymethyl) aminomethane and hydrogen peroxide (for H_2O_2 spawning method) -- for spawning abalone.

14.6.19 Reference toxicant solutions (see Subsection 14.10.2.4 and see Section 4, Quality Assurance).

14.6.20 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies and Section 7, Dilution Water).

14.6.21 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

14.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 14.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-μm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

14.6.23 HYPERSALINE BRINES

14.6.23.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

14.6.23.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

14.6.23.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 μm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

14.6.23.4 Freeze Preparation of Brine

14.6.23.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20°C until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

14.6.23.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50‰.

14.6.23.4.3 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at

4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

14.6.23.5 Heat Preparation of Brine

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

14.6.23.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat

exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

14.6.23.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

14.6.23.5.3 Seawater should be filtered to at least 10 μm before being put into the brine generator. The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100% and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

14.6.23.5.4 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

14.6.23.6 Artificial Sea Salts

14.6.23.6.1 No data from red abalone tests using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

14.6.23.7 Dilution Water Preparation from Brine

14.6.23.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that

brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

14.6.23.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. The proportion of brine is 1 part plus 1.94 parts reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

14.6.23.8 Test Solution Salinity Adjustment

14.6.23.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

14.6.23.8.2 Check the pH of all test solutions and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide (see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.6.23.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

14.6.23.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

14.6.23.9 Preparing Test Solutions

14.6.23.9.1 Two hundred mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 10 mL of effluent to a 1-liter volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 1-L mark with dilution water, stopper it, and shake to mix. Distribute equal volumes into the replicate test chambers.

14.6.23.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Distribute equal volumes into the replicate test chambers.

14.6.23.10 Brine Controls

14.6.23.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 16.6.23.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

14.6.24 TEST ORGANISMS

14.6.24.1 The test organisms used for this test are red abalone, *Haliotis rufescens*. This large gastropod mollusc is harvested commercially in southern California and supports a popular recreational fishery throughout the state. It consumes a variety of seaweeds and small incidental organisms, and is an important food source for sea otters, lobsters, and octopods (Hines and Pearse 1892). Abalone are "broadcast" spawners that reproduce by equivalent.ejecting large numbers of gametes into the water column, where fertilization takes place externally. Free-swimming larvae hatch as trochophores, then undergo torsion while passing through a veliger stage. Abalone larvae do not feed during their one to three weeks in the plankton, but exist on energy stored in the yolk sack, supplemented perhaps by the uptake of dissolved amino acids. Once larvae come into contact with suitable substrate, they metamorphose and begin to consume benthic algae using a rasp-like tongue (the radula). Red abalone become reproductive after about two years at a length of about 7 cm, and can live for at least 25 years, growing to 30 cm in length. Refer to Hahn (1989) for a review of abalone life history and culture to Martin et al. (1977), Morse et al (1979) and Hunt and Anderson (1989 and 1993) for previous toxicity studies.

14.6.24.2 Species Identification

14.6.24.2.1 Broodstock should be positively identified to species. Epipodal characteristics provide the best means of identification. All California haliotids have a lacey epipodial fringe, except for the red and black abalone, which have smooth, lobed epipodia. The red abalone can be distinguished from the black by shell coloration and by the number of respiratory pores in the shell (reds have 3 to 4, blacks have 5 to 8). For further information on abalone taxonomy consult Owen et al. (1971), and Morris et al. (1980).

14.6.24.3 Obtaining Broodstock

14.6.24.3.1 Mature red abalone broodstock can be collected from rocky substrates from the intertidal to depths exceeding 30 meters. They are found most commonly in crevices in areas where there is an abundance of macroalgae. State collection permits are usually required for collecting abalone. Collection of

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (x‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water.

abalone is regulated by California law. Collectors must obtain a scientific collectors permit from the California Department of Fish and Game and observe any regulations regarding collection, transfer, and maintenance of abalone broodstock.

14.6.24.3.2 While abalone captured in the wild can be induced to spawn, those grown or conditioned in the laboratory have been more dependable. Commercial mariculture facilities in California produce large numbers of abalone, and distribution systems exist to supply live spawners to a number of market areas. In any case, broodstock should be obtained from sources free of contamination by toxic substances to avoid genetic or physiological preadaptation to pollutants.

14.6.24.3.3 Abalone broodstock can be transported for short time periods from the field or supply facility in clean covered plastic buckets filled with seawater. Use compressed air, or battery powered pumps to supply aeration. Compressed oxygen is not recommended because bubbled oxygen may induce unintended spawning (Morse et al., 1977). Maintain water temperatures within 3°C of the temperature at the collecting site. Four abalone in a 15-liter bucket should remain healthy for up to four hours under these conditions.

14.6.24.3.4 Abalone can be transported for up to 30 hours in sealed, oxygen-filled plastic bags containing moist (seawater) polyfoam sponges (Hahn, 1989). Cut the polyfoam into sections (about 20 X 40 cm) and allow them to soak in clean seawater for a few minutes. New sponges should be leached in seawater for at least 24 hours. Rinse the sponges in fresh seawater and wring them out well. Place the polyfoam inside double plastic trash bags, then place the abalone on the moist foam. It is important that there is no standing water in the bags. Put the abalone bags into an ice chest (10 to 15 liter), fill the bags with pure oxygen, squeeze the bags to purge out all the air, then refill with oxygen (approximately three liters of oxygen gas will support eight abalone). Seal the bags (air-tight) with a tie or rubber band. Wrap two small (one-liter) blue ice blocks in sections of newspaper (about 15 pages thick) for insulation, and place the wrapped blue ice in a sealed plastic bag in the chest on top of the abalone bags. Fill any remaining space with packing and seal the box for shipping. Avoid transporting the ice chest in temperatures below freezing or above 30°C.

14.6.24.4 Broodstock Culture and Handling

14.6.24.4.1 At the testing facility, place the abalone in aerated tanks with flowing seawater (1 to 2 liter/min). With high water quality, water flow, and aeration, abalone 8 to 10 cm long can be kept at a density of one per liter of tank space or one per 100 cm^2 of tank surface area, whichever provides the lower density. Density should be cut to a maximum of 0.5 per liter in recirculating systems and to a maximum of 0.25 per liter in static tanks. Tanks should be covered for shade and to prevent escape. Drain and rinse culture tanks twice weekly to prevent build-up of detritus. Remove any dead abalone immediately, and drain and scrub its tank.

14.6.24.4.2 Ideal maintenance temperature is 15 ± 1°C, the toxicity test temperature (see also Leighton, 1974). If broodstock are to be held for longer than 5 days at the testing facility, feed broodstock with blades of the giant kelp, *Macrocystis*. Feed to slight excess; large amounts of uneaten algae will foul culture water. If *Macrocystis* is unavailable, other brown algae (*Nereocystis*, *Egregia*, *Eisenia*) or any fleshy red algae can be substituted (Hahn, 1989).

14.6.24.4.3 Recirculating tanks should be equipped with biological or activated carbon filtration systems and oyster shell beds to maintain water quality. Measure the ammonia content of static or recirculating seawater daily to monitor the effectiveness of the filtration system. Un-ionized ammonia concentrations should not exceed 20 µg/liter and total ammonia concentrations should not exceed 1.0 mg/liter. Supply constant aeration and temperature control. Add only a few blades of algal food at each cleaning to prevent its accumulation and decay.

14.6.24.4.4 When handling abalone, use a rounded, dull-bladed stainless-steel butter knife, abalone iron, or plastic putty knife to release the animal's grip on the substrate. Gently slide the flat dull blade under the foot at the posterior end near the beginning of the shell whorl, and slide it under about two-thirds of the foot. Apply constant pressure to keep the front edge of the blade against the substrate and not up into the foot. Quickly and gently lift the foot off the substrate. A smooth deliberate motion is more effective and less damaging than repeated prying.

14.6.24.4.5 Assess the reproductive condition of the broodstock by examining the gonads, located under the right posterior edge of the shell. An abalone placed upside down on a flat surface will soon relax and begin moving the foot trying to right itself. Take advantage of this movement and use the dull blade to bend the foot away from the gonad area for inspection. The female ovary is jade green, the male testes are cream-colored. When the gonad fully envelopes the dark blue-gray conical digestive gland and is bulky along its entire length, the abalone is ready for spawning (Hahn, 1989). Ripe (recrudescent) spawners have a distinct color difference between the gray digestive gland and the green or cream-colored gonad. Less developed gonads appear gray (in females) or brown (in males).

14.6.24.4.6 Abalone 7 to 10 cm in shell length are recommended in broodstock. They are easier to handle than larger ones, and can be spawned more often (approximately every four months under suitable culture conditions; Ault, 1985). Though spawning fewer eggs than larger abalone, 10 cm abalone will produce over 100,000 eggs at a time (Ault, 1985). Twenty to thirty-five thousand eggs are needed for a single toxicant test, depending on test design. For further information of red abalone culture, see Ebert and Houk (1984) or Hahn (1989).

14.6.24.5 Culture Materials

14.6.24.5.1 See Section 4, Quality Assurance Section for a discussion of suitable materials to be used in laboratory culture of abalone. Be sure all new materials are properly leached in seawater before use. After use, all culture materials should be washed in soap and water, then rinsed with seawater before reuse.

14.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

14.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

14.8 CALIBRATION AND STANDARDIZATION

14.8.1 See Section 4, Quality Assurance.

14.9 QUALITY CONTROL

14.9.1 See Section 4, Quality Assurance.

14.10 TEST PROCEDURES

14.10.1 TEST DESIGN

14.10.1.1 The test consists of at least five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain five replicates of a brine control.

14.10.1.2 Effluent concentrations are expressed as percent effluent.

14.10.2 TEST SOLUTIONS

14.10.2.1 Receiving waters

14.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using five replicate chambers per test, each containing 200 mL would require approximately 1 L of sample per test.

14.10.2.2 Effluents

14.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC,

with one being at least one-half the concentration of the IWC. If 100% HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34% salinity.

14.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

14.10.2.2.3 The volume in each test chamber is 200 mL.

14.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

14.10.2.3 Dilution Water

14.10.2.3.1 Dilution water should be uncontaminated 1-μm-filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

14.10.2.4 Reference Toxicant Test

14.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

14.10.2.4.2 The preferred reference toxicant for red abalone is zinc sulfate ($ZnSO_4 \cdot H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a 10,000 μg/L zinc stock solution by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot H_2O$) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

14.10.2.4.3 Reference toxicant solutions should be five replicates each of 0 (control), 10, 18, 32, and 56, and 100 μg/L total zinc. Prepare one liter of each concentration by adding 0,

1.0, 1.8, 3.2, 5.6, and 10.0 mL of stock solution, respectively, to one-liter volumetric flasks and fill with dilution water. Start with control solutions and progress to the highest concentration to minimize contamination.

14.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

14.10.3 COLLECTION OF GAMETES FOR THE TEST

14.10.3.1 Spawning Induction

14.10.3.1.1 Note: Before beginning the spawning induction process, be sure that test solutions will be mixed, sampled, and temperature equilibrated in time to receive the newly fertilized eggs. Spawning induction generally takes about three hours, but if embryos are ready before test solutions are at the proper temperature, the delay may allow embryos to develop past the one-cell stage before transfer to the toxicant. Transfer can then damage the embryos, leading to unacceptable test results.

14.10.3.1.2 Culture work (spawning, etc.) and toxicant work should be done in separate laboratory rooms, and care should be taken to avoid contaminating organisms prior to testing.

14.10.3.1.3 Ripe abalone can be induced to spawn by stimulating the synthesis of prostoglandin-endoperoxide in the reproductive tissues (Morse et al., 1977). This can be done in two ways: addition of hydrogen peroxide to seawater buffered with Tris (Morse et al., 1977), or irradiation of seawater with ultraviolet light (Kikuchi and Uki, 1974). The first method is preferable for small laboratories because it avoids the cost and maintenance requirements of a UV system. If a UV system is available, this method may be preferable because it is simple, does not use chemicals that could accidentally harm larvae, and is considered to be less likely to force gametes from unripe adults.

14.10.3.1.4 If brood stock are shipped to the laboratory by a supplier, it is important to allow two days or more for laboratory acclimation before spawning induction; this should

increase the probability of achieving a successful spawn of viable gametes. Always bring brood stock up to acclimation temperature slowly to avoid premature spawning.

14.10.3.2 Hydrogen Peroxide Method

14.10.3.2.1 Select four ripe male abalone and four ripe females. Clean their shells of any loose debris. Place the males in one clean polyethylene bucket and the females in another. Cover the buckets with a tight fitting perforated lid, supply the chambers with flowing or recirculating (1 liter/minute) 20-μm-filtered seawater (15°C), and leave the animals without food for 24 to 48 hours to acclimate and eliminate wastes. If flowing seawater is unavailable, keep the spawners in larger (>30 liter) aquaria with aeration at 15 ± 1°C for 24 hours without food to eliminate wastes. Three hours prior to the desired spawning time, drain the buckets, wipe and rinse out mucus and debris, and refill with 6 liters of 1 μm-filtered seawater. If abalone have been kept in larger aquaria, put them in the buckets at this time. Check the abalone from time to time to make sure they remain underwater. Add air stones to the buckets and keep them aerated until spawning begins.

14.10.3.2.1 Dissolve 12.1 g of Tris into 50 mL of reagent water. When the Tris has dissolved completely, mix the hydrogen peroxide (H_2O_2) solution in a separate flask by pouring 10 mL of fresh* refrigerated H_2O_2 (30%) into 40 mL of refrigerated reagent water (1:5 dilution). Pour 25 mL of Tris solution and 25 mL of H_2O_2 solution into each of the spawning buckets (male and female). Stir well to mix; the final concentration in the spawning buckets will be approximately 6 mM Tris (pH = 9.1) and 5 m H_2O_2. Allow the abalone to remain in contact with the chemicals for 2.5 hours at 15 ± 1°C. The chemical reaction is temperature dependent (three hours of contact with H_2O_2 would be necessary at 11°C). Temperatures higher than 15°C are not recommended for spawning. Maintain constant aeration. Since females often begin spawning after the males, it may be useful to induce male spawning 15-30 minutes later, however egg quality should not be compromised if females spawn first (See 14.10.3.3.2 below).

*Note: Hydrogen peroxide loses potency over time. Purchase reagent or certified grade H_2O_2 in small containers (100

mL). Store unopened containers for no more than one year, and discard open containers after one month. Mark the purchase date and opening date on all containers, and keep all containers refrigerated.

14.10.3.2.3 After 2.5 hours, empty the spawning buckets, rinse them well, and refill them to the top with fresh dilution water seawater at the same temperature (15 ± 1°C). Keep the containers clean by siphoning away mucus and debris. Maintain constant aeration until spawning begins, then remove the air stones. The abalone begin spawning about three hours after the introduction of the chemicals (at 15 ± 1°C). Eggs are dark green and are visible individually to the naked eye, sperm appear as white clouds emanating from the respiratory pores.

14.10.3.2.4 If spawning begins before the chemicals have been removed, drain the buckets immediately, discarding any gametes. Rinse the buckets thoroughly and refill with clean, dilution water seawater (15 ± 1°C). Use only the gametes subsequently spawned in clean water for testing.

14.10.3.3 UV Irradiation Method

14.10.3.3.1 Select four ripe male abalone and four ripe females. Clean their shells of any debris. Place the males in one clean polyethylene bucket and the females in another. Cover the buckets with a tight fitting perforated lid, supply the containers with flowing or recirculating (1 liter/minute) 20-μm-filtered seawater (15 ± 1°C), and leave the animals without food for 24 to 48 hours to acclimate and eliminate wastes. If flowing seawater is unavailable, keep the spawners in larger (>30 liter) aquaria with aeration at 15 ± 1°C for 24 hours. Three hours prior to the desired spawning time, drain the buckets, wipe and rinse out mucus and debris, and refill with just enough water to cover the abalone (which should all be placed in the bottom of the bucket). Begin slowly filling the buckets with dilution water seawater (15 ± 1°C) that has passed through the UV sterilization unit. Flow rates to each of the buckets should be 150 mL/min. A low total flow rate (300 mL/minute) in the UV unit is necessary to permit sufficient seawater irradiation. (The sterilization unit should be cleaned and the UV bulb replaced at least once annually). Place the buckets in a water bath at 15 ± 1°C to counter the temperature increase caused by the slow

passage of the water past the UV lamp. Check the containers periodically, and keep them clean by siphoning out any debris. After three hours (± about 1/2 hour), abalone should begin spawning by ejecting clouds of gametes into the water. Eggs are dark green and are visible individually to the naked eye, sperm appear as white clouds emanating from the respiratory pores.

14.10.3.3.2 Note: If past experience or other factors indicate difficulties in achieving synchronous spawning, it may be helpful to induce a second group of females about an hour after the first. This will increase the chances of providing fresh eggs (less than one hour old) for fertilization if males spawn late (see below). Senescence of sperm is seldom a problem because males continue spawning over a longer period of time.

14.10.3.4 Pooling Gametes

14.10.3.4.1 Although it is not necessary, it is preferable to have more than one abalone of each sex spawn. To increase the probability of multiple spawners without risking senescence of the gametes, allow one-half hour after the first individual of the second sex begins to spawn before initiating fertilization. For example, if males spawn first, wait one-half hour after the first female spawns before fertilizing eggs. In most cases this will provide time for more than one of each sex to spawn. More important than multiple spawning, however, is avoiding delay of fertilization. Eggs should be fertilized within one hour of release (Uki and Kikuchi 1974). All sperm should be pooled, and all eggs should be pooled prior to fertilization. This can be accomplished by gentle swirling within the spawning buckets. Note: Take care to avoid contaminating eggs with sperm prior to the intended fertilization time. It is important that development is synchronous among all test embryos.

14.10.3.5 Fertilization

14.10.3.5.1 As the females spawn, allow the eggs to settle to the bottom. If necessary, gently stir to evenly distribute the eggs. Siphon out and discard any eggs that appear clumped together. Eggs are ready to transfer to a third (fertilization) bucket when either: (1) one-half hour has passed since the first individual of the second sex has spawned (2) multiple individuals of each sex have spawned, or 3) there are too many eggs on the

bottom of the bucket to allow evenly distributed eggs to avoid each other. Slowly siphon eggs into a third clean polyethylene bucket containing one or two liters of dilution water seawater (15 ± 1°C). Siphon carefully to avoid damaging the eggs and to avoid collecting any debris from the spawning container. Siphon about 100,000 eggs, enough to make a single even layer on the container bottom. Each egg should be individually distinguishable, and not touching other eggs. If excess eggs are available, siphon them into a second fertilization bucket to be used as a reserve. Keep all containers at 15 ± 1°C. Make sure that water temperatures differ by no more than 1°C when transferring eggs or sperm from one container to another.

14.10.3.5.2 As the males spawn, siphon sperm from directly above the respiratory pore and collect this in a 500 mL flask with filtered seawater. Keep the flask at 15 ± 1°C, and use it as a back-up in case the males stop spawning. If spawning continues renew this reserve every 15 minutes. Usually the males will continue spawning, turning the water in the bucket milky white. As long as the males continue spawning, partially drain and refill the bucket every 15 minutes, replacing old sperm-laden water with fresh seawater (15 ± 1°C). Use the freshest sperm possible for fertilization.

14.10.3.5.3 Make sure eggs are fertilized within one hour of release (Uki and Kikuchi, 1974, see note after Section 14.8.5.2). To fertilize the eggs, collect about 200 mL of sperm-laden water in a small beaker. The sperm concentration in the beaker does not have to be exact, just enough to give a slightly cloudy appearance (approximately 1 to 10 X 10^6 cells/mL in the fertilization bucket). See Hahn (1989) for further information on sperm concentrations and the method for fertilization. Pour the sperm solution into the fertilization bucket containing the clean isolated eggs. Using a hose fitted with a clean glass tube, add dilution water seawater to the fertilization bucket at a low flow rate (<1 liter/min; 15 ± 1°C). Use the water flow to gently roil the eggs to allow them to mix with the sperm and fertilize. When the bucket is about half-full and eggs are evenly mixed, stop the water flow and allow the eggs to settle to the bottom of the bucket (about 15 minutes). Fertilization is then complete.

14.10.3.5.4 Note: Once fertilized eggs have settled to the bottom of the bucket (15 minutes after addition of sperm), the following steps (rinsing, concentrating, and counting the embryos) must proceed without delay to assure that embryos are transferred into the test solutions within about one hour. Embryos must be delivered to the test chambers before the first cell division takes place. (Multicellular embryos are more susceptible to damage in handling, and test endpoint analysis assumes that the first cell division takes place in the toxicant solution).

14.10.3.5.5 After embryos have settled, carefully pour or siphon off the water from above the settled embryos to remove as much of the sperm laden water as possible without losing substantial numbers of embryos. Slowly refill the bucket with dilution water seawater (15 ± 1°C). Allow the embryos to settle, and siphon them into a tall 1000 mL beaker for counting. Siphon at a slow flow rate, and move the siphon along the bottom of the bucket quickly to pick up a large number of embryos in the short amount of time it takes to fill the beaker. Examine a sample of the embryos at 100X magnification. One to one hundred sperm should be visible around the circumference of each embryo, 15 sperm per egg is optimal. If sperm are so dense that the embryos appear fuzzy (>>100 sperm/egg), the abalone may develop abnormally and should not be used.

14.10.4 START OF THE TEST

14.10.4.1 Prior to Beginning the Test

14.10.4.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

14.10.4.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make

the test solutions should be adjusted to the test temperature (15 ± 1°C) and maintained at that temperature during the addition of dilution water.

14.10.4.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (15 ± 1°C).

14.10.4.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the abalone have been examined at the end of the test.

14.10.4.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

14.10.4.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly and filled with test solutions, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

14.10.4.2 Estimation of Embryo Density

14.10.4.2.1 Evenly mix the embryos in the 1000 mL beaker by gentle vertical stirring with a clean perforated plunger. Never allow embryos to settle densely in the bottom of the beaker, and take care not to crush embryos while stirring. Take a sample of the evenly suspended embryos using a 1 mL wide bore graduated pipet. Hold the pipet up to the light and count the individual embryos using a hand counter. Alternatively, empty the contents of the pipet onto a Sedgewick-Rafter slide and count embryos under low magnification on a compound scope. Discard the sampled

embryos after counting. Density of embryos in the beaker should be between 200 and 300 embryos/mL. Dilute if the concentration is too high, let embryos settle and pour off excess water if concentration is too low. Take the mean of five samples from this solution to estimate the number of embryos per milliliter.

14.10.4.3 Delivery of Fertilized Embryos

14.10.4.3.1 Using the estimated embryo density in the 1000 mL beaker, calculate the volume of water that contains 1000 embryos. Remove 1000 embryos (or less for smaller volumes, see Section 14.10.1.3) by drawing the appropriate volume of water from the well-mixed beaker using a 10 mL wide bore pipet. Deliver the embryos into the test chambers directly from the pipet making sure not to touch the pipet to the test solution. Stir the embryo beaker with the plunger before taking aliquots. The temperature of the embryo suspension must be within 1°C of the temperature of the test solution. (As above, all solutions are kept at 15 ± 1°C). Record the volume of water delivered into the test chambers with the embryos. Embryos must be delivered into the test solutions within one hour of fertilization. Immediately after the embryos have been delivered, take a sample from the embryo beaker and examine it under 100X magnification. All embryos should still be in the one-cell stage; record any observations to the contrary on the data sheet.

14.10.4.4 Incubation

14.10.4.4.1 Incubate test organisms for 48 hours in the test chambers at 15 ± 1°C under low lighting (approximately 10 $\mu E/m^2/s$) with 16L:8D photoperiod. Fertilized embryos become trochophore larvae, hatch, and develop into veliger larvae in the test solutions during the exposure period.

14.10.5 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

14.10.5.1 The light quality and intensity should be at ambient laboratory conditions. Light intensity should be 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle.

14.10.5.2 The water temperature in the test chambers should be maintained at 15 ± 1°C. If a water bath is used to maintain the

test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

14.10.5.3 The test salinity should be in the range of 34 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

14.10.5.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

14.10.6 DISSOLVED OXYGEN (DO) CONCENTRATION

14.10.6.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

14.10.7 OBSERVATIONS DURING THE TEST

14.10.7.1 Routine Chemical and Physical Observations

14.10.7.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

14.10.7.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at

least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

14.10.7.1.3 Record all the measurements on the data sheet.

14.10.8 TERMINATION OF THE TEST

14.10.8.1 Ending the Test

14.10.8.1.1 Record the time the test is terminated.

14.10.8.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control.

14.10.8.2 Sample Preservation

14.10.8.2.1 After 48 hours exposure, the abalone larvae are fixed in formalin or glutaraldehyde. The two methods for sample preservation are described. Be sure that samples for physicochemical measurements have been taken before further processing of test solutions.

14.10.8.2.2 At the end of the 48-hour incubation period, remove each test chamber, swirl the solution to suspend all the larvae, and pour the entire contents through a 37 μm-mesh screen. The test solution is discarded and the larvae are retained on the screen. Using streams of filtered seawater from a squeeze bottle, rinse the larvae from the screen through a funnel into 25 mL screw cap vials. Be careful not to hit the larvae directly with the streams of water; rough handling during transfer may cause fragmentation of the larvae, making counting more difficult and less accurate. Add enough buffered formalin to preserve larvae in a 5% solution (some laboratories have successfully preserved larvae with lower formalin concentrations. Under-preserved larvae disintegrate quickly, however, and whole tests may have to be rejected if larvae have not been adequately fixed). Addition of formalin is more accurate if the vials are premarked with lines showing the volume of sample and the volume

of formalin to be added. Alternatively, a 0.05% final glutaraldehyde solution may be substituted. Larvae should be counted within two weeks.

14.10.8.2.3 Note: Formaldehyde has been identified as a carcinogen and both glutaraldehyde and formaldehyde are irritating to skin and mucus membranes. Neither should be used at higher concentrations than needed to achieve morphological preservation and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

14.10.8.3 Counting

14.10.8.3.1 To count the larvae using a standard compound microscope, pipet all the larvae from the bottom of the preservation vial onto a Sedgewick-Rafter counting cell. Examine 100 larvae from each vial under 100X magnification. To best characterize the sample and to avoid bias, select groups of larvae one field of vision at a time, moving to the next field without looking through the lens. Be careful to work across the slide in one direction to avoid recounting the same areas. Count the number of normal and abnormal larvae using hand counters. The percent normal larvae is calculated as the number normal divided by the total number counted. After counting, use a funnel to return the larvae to the vial for future reference.

14.10.8.4 Endpoint

14.10.8.4.1 Examine the shape of the larval shell to distinguish normal from abnormal larvae. Count veliger larvae as normal if they have smoothly curved larval shells that are striated with calcareous deposits and are somewhat opaque. It is common for normal larvae to have a slight curved indentation near the leading edge of the shell. A single indentation is this area is counted as normal.

14.10.8.4.2 Larvae with both multiple indentations and an obvious lack of calcification (i.e. clear appearance in at least part of the shell) are counted as abnormal. The combination of these two features indicates inhibition of a biological process (lack of calcification) and actual damage to the organism (indentations) allowed by the thin shell. Refer to the accompanying photographs (Figure 1) for classification of

marginally deformed larvae. The following types of larvae are also counted as abnormal: (1) larvae that have arrested development (from one cell through trochophore stage), (2) larvae with obvious severe deformations, (3) larvae with broken shells, (4) larval shells separated from the rest of the animal, and (5) larvae found remaining in the egg membrane (however, take care to distinguish these from larvae that may have come in contact with loose egg cases). Record all counts and the test chamber number on the data sheet.

14.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

14.14.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

14.12 **ACCEPTABILITY OF TEST RESULTS**

14.12.1 Test results are acceptable only if all the following requirements are met:

(1) the mean larval normality must be at least 80% in the controls.

(2) the response from 56 µg/L zinc treatment must be significantly different from the control response.

(3) the minimum significant difference (%MSD) is <20% relative to the control for the reference toxicant.

14.13 **DATA ANALYSIS**

14.13.1 GENERAL

14.13.1.1 Tabulate and summarize the data. Calculate the proportion of larvae with normally developed shells for each replicate. A sample set of test data is listed in Table 4.

14.13.1.2 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

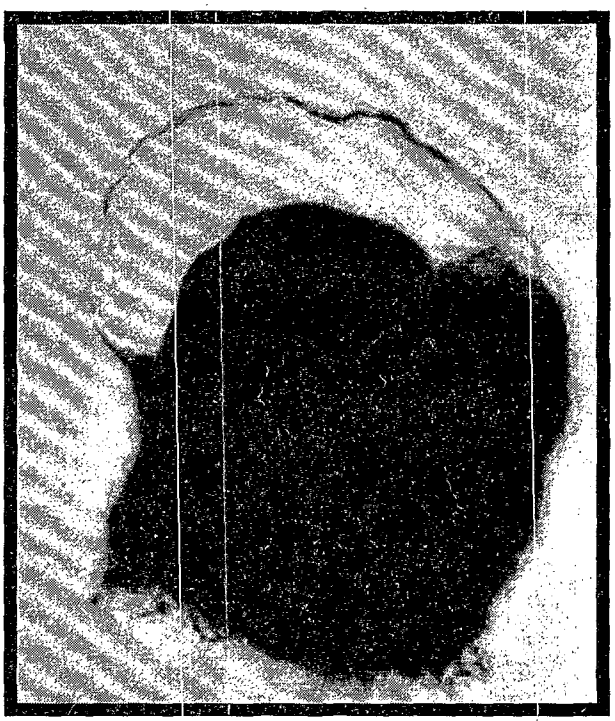
FIGURE 1. 48-HOUR-OLD ABALONE VELIGER LARVAE

Figures 1A -D Provided by John Hunt, Institute of Marine Sciences. Photocopied from:

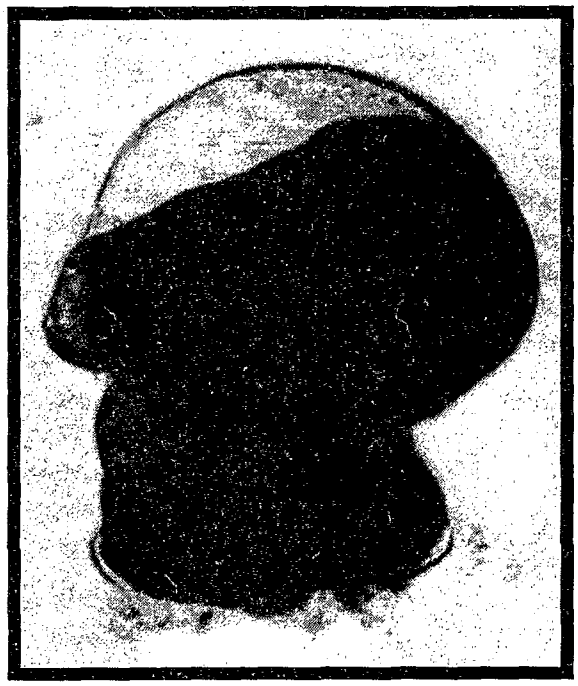
"Marine Bioassay Project Procedures Manual of October, 1990." California State Water Resources Control Board.

The following three pages show 12 photographs of 48-hour-old abalone veliger larvae from effluent toxicity tests. All larvae were taken from intermediate effluent concentrations and were chosen to represent "borderline" cases (i.e. larvae that were slightly affected and are therefore, difficult to categorize as normal or abnormal). In most cases, larvae from lower and higher effluent concentrations are more easily categorized than those shown here; in the lower concentrations they are obviously without shell abnormalities and in the higher concentrations they are severely deformed. These photographs are presented as a visual reference to help standardize test analysis and eliminate bias in the interpretation of marginally deformed larvae. All larvae on the left-hand side of these pages were counted as normal, all larvae on the right-hand side were counted as abnormal.

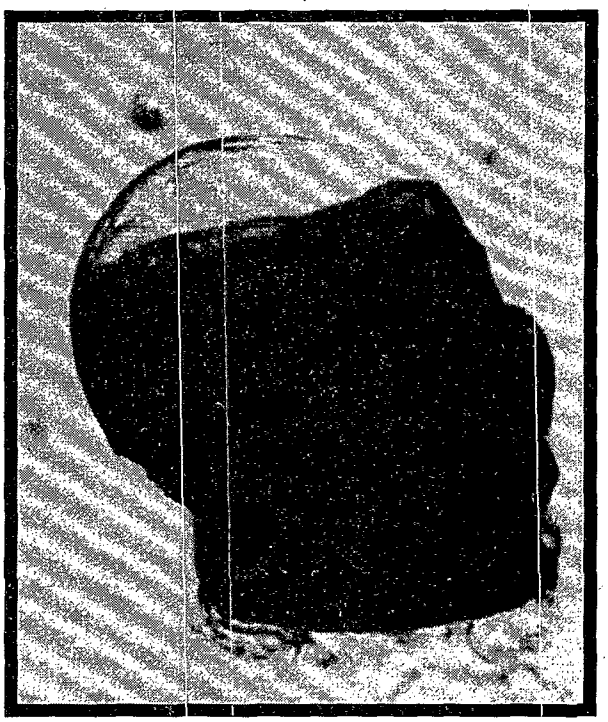
A. Normal larva with well calcified (striated) shell but slight uneven shell outline.



B. Obviously abnormal larva with transparent shell and numerous shell deformities.



C. Normal larva with some shell thinning and mild flattening of shell curvature near the leading edge (left side of photograph).

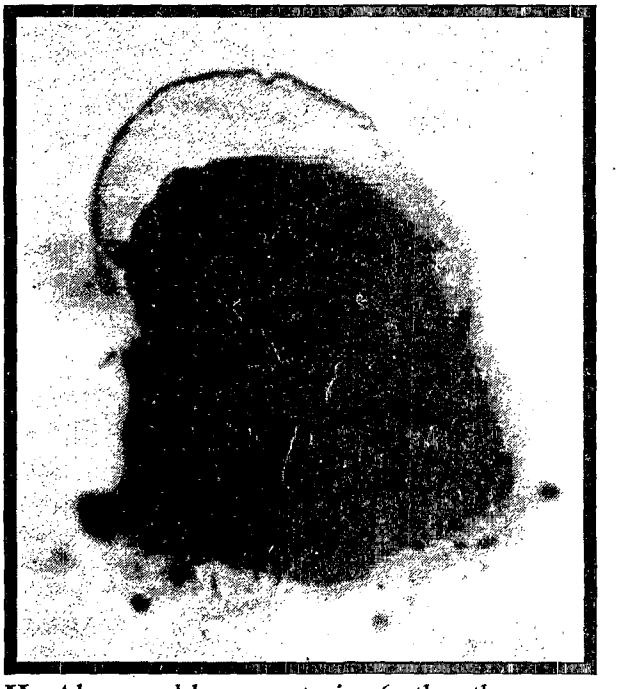


D. Abnormal larva with multiple slight indentations and transparency near the leading edge (left side of photograph)

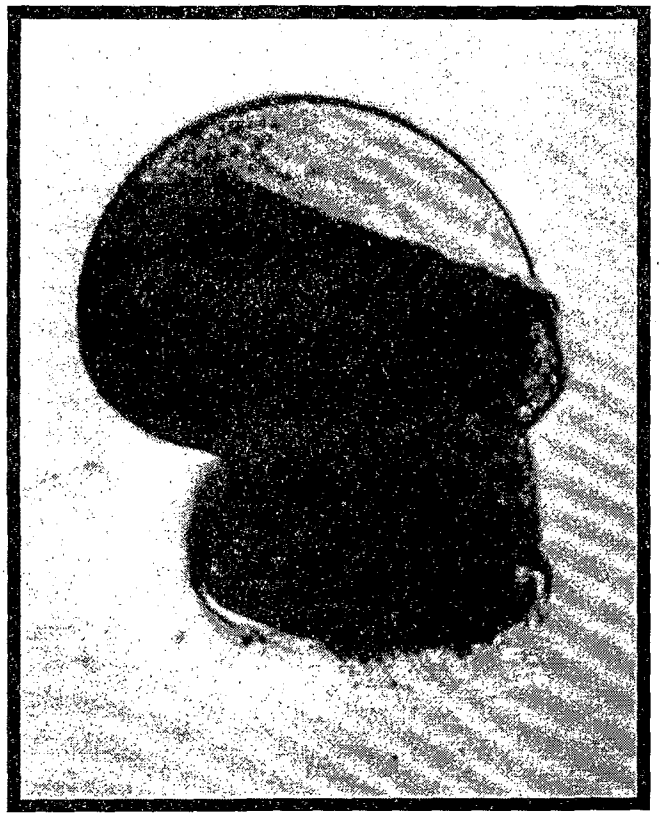
E. Normal larva with well calcified (striated) shell but uneven shell outline.

F. Abnormal larva with transparent shell and large indentation.

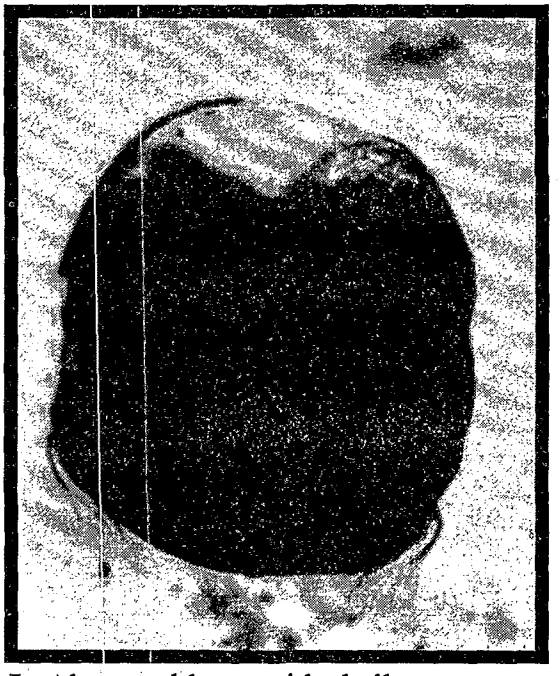
G. Normal larva, anterior (rather than lateral) view. Well striated, smooth rounded shell outline.



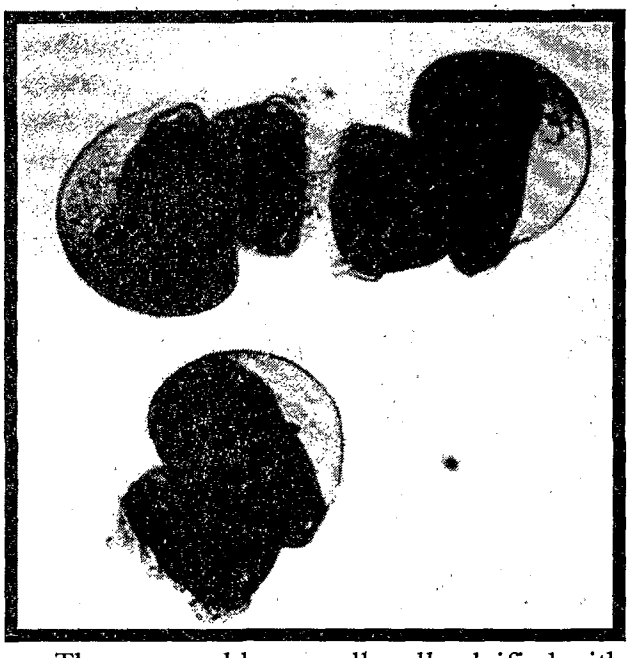
H. Abnormal larva, anterior (rather than lateral) view. Transparent irregular shell with indentations.



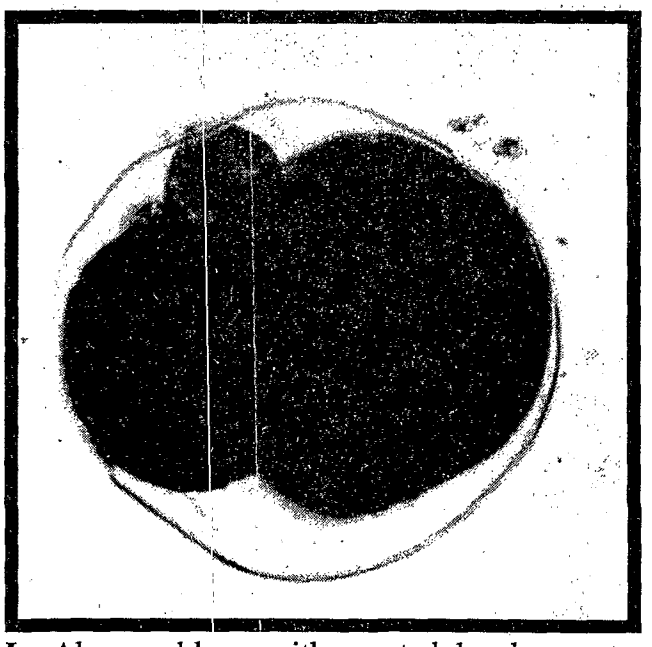
I. Normal larva with well calcified shell and e small indentation at leading edge.



J. Abnormal larva with shell transparencies, indentations, and irregular shape.



Three normal larvae, all well calcified with small indentations at the leading edge.



L. Abnormal larva with arrested development at an early stage. Any larva found within the egg membrane, no matter how ell developed, is counted as abnormal.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR, *HALIOTIS RUFESCENS*, LARVAL DEVELOPMENT TEST WITH EFFLUENTS AND RECEIVING WATERS

1.	Test type:	Static non-renewal
2.	Salinity:	34 ± 2‰
3.	Temperature:	15 ± 1°C
4.	Light quality:	Ambient laboratory light
5.	Light intensity:	10 $\mu E/m^2/s$ (Ambient laboratory levels)
6.	Photoperiod:	16 h light, 8 h darkness
7.	Test chamber size:	600 mL
8.	Test solution volume:	200 mL/replicate
9.	Larvae density per chamber:	5-10 per mL
10.	No. replicate chambers per concentration:	5
11.	Dilution water:	Uncontaminated 1-μm-filtered natural seawater or hypersaline brine plus reagent water
12.	Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13.	Dilution factor:	Effluents: ≥0.5 Receiving waters: None or ≥0.5
14.	Test duration:	48 h
15.	Endpoint:	Normal shell development

16. Test acceptability criteria:	≥80% normal shell development in the controls; must have statistical significant effect at 56 µg/L zinc; must achieve a %MSD of <20%
17. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
18. Sample volume required:	2 L per test

14.13.1.3 The endpoints of toxicity tests using the red abalone are based on the reduction in proportion of normal shell development. The IC25 is calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for larval development are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

14.13.2 EXAMPLE OF ANALYSIS OF RED ABALONE, *HALIOTUS RUFESCENS*, LARVAL DEVELOPMENT DATA

14.13.2.1 Formal statistical analysis of the larval development is outlined in Figure 2. The response used in the analysis is the proportion of larvae with normally developed shells in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there is no normal shell development in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25.

TABLE 4. DATA FROM RED ABALONE, *HALIOTUS RUFESCENS*, DEVELOPMENT TEST

Effluent Concentration (%)	Replicate	No. Larvae Counted	Number Normal	Proportion Normal
Brine	A	100	100	1.00
Control	B	100	98	0.98
	C	100	100	1.00
	D	100	99	0.99
	E	100	99	0.99
Dilution	A	100	99	0.99
Control	B	100	99	0.99
	C	100	99	0.99
	D	100	100	1.00
0.56	A	100	99	0.99
	B	100	99	0.99
	C	100	98	0.98
	D	100	100	1.00
	E	100	100	1.00
1.00	A	100	99	0.99
	B	100	100	1.00
	C	100	99	0.99
	D	100	99	0.99
	E	100	100	1.00
1.80	A	100	99	0.99
	B	100	99	0.99
	C	100	99	0.99
	D	100	98	0.98
	E	100	97	0.97
3.20	A	100	39	0.39
	B	100	57	0.57
	C	100	61	0.61
	D	100	65	0.65
	E	100	80	0.80
5.60	A	100	0	0.00
	B	100	0	0.00
	C	100	0	0.00
	D	100	0	0.00
	E	100	0	0.00
10.00	A	100	0	0.00
	B	100	0	0.00
	C	100	0	0.00
	D	100	0	0.00
	E	100	0	0.00

14.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

14.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

14.13.2.4 Comparison of Brine and Dilution Controls

14.13.2.4.1 This example uses toxicity data from a red abalone, *Haliotus rufescens*, larval development test performed with effluent. The response of interest is the proportion of larvae with normally developed shells, thus each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. Because the example test was run using both brine and dilution controls, the two controls must first be tested for significant differences in the normal shell development proportions. The raw and transformed data, means and variances of the transformed observations for the two controls are listed in Table 5.

14.13.2.4.2 Tests for Normality

14.13.2.4.2.1 In the two sample situation, the distributional assumption is that each sample comes from a normally distributed population. Thus in comparing the brine and dilution controls, the data for each concentration must be separately checked for normality. When the two response groups are tested separately, it is not necessary to center the data.

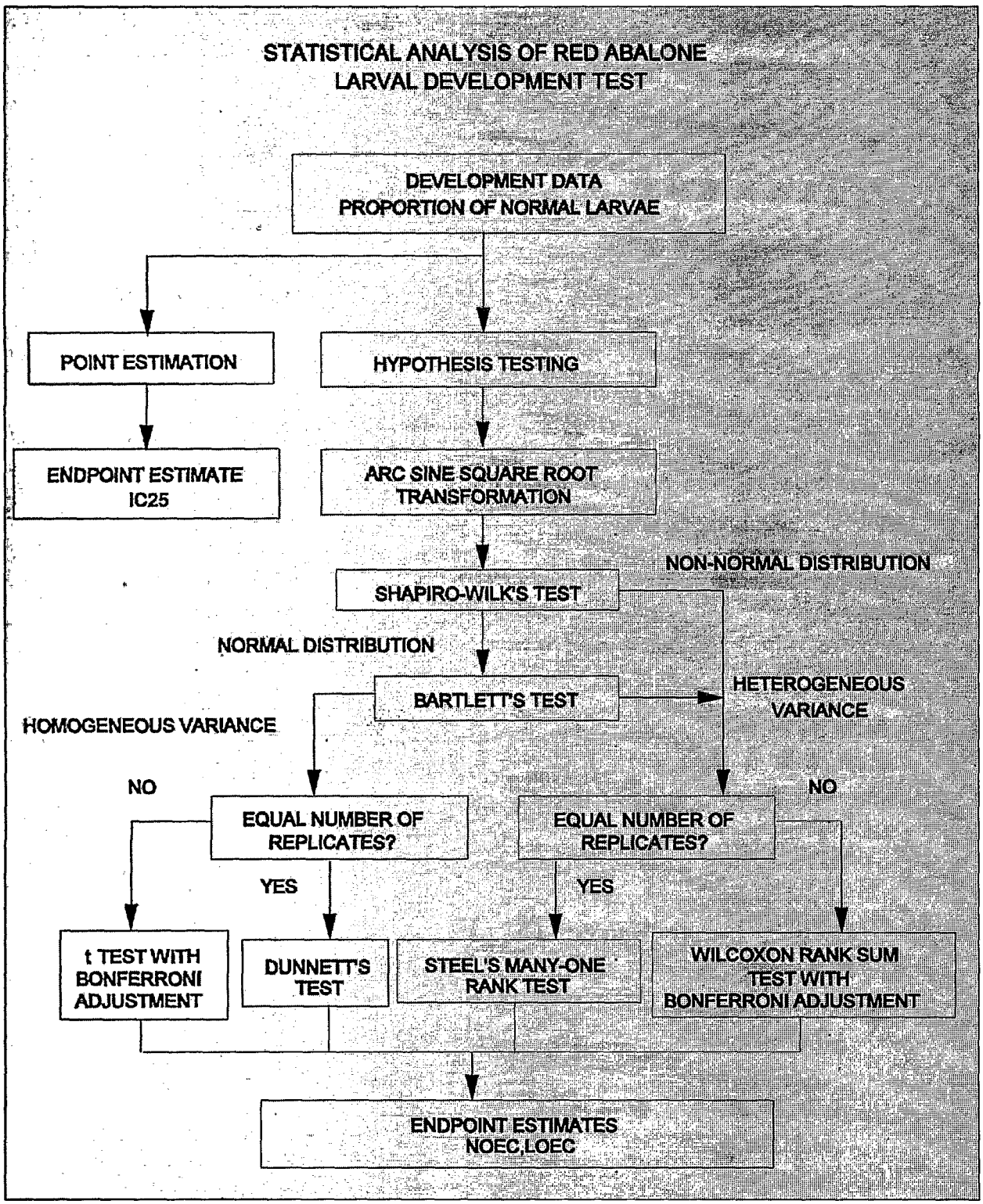


Figure 2. Flowchart for statistical analysis of red abalone, *Haliotus rufescens*, development data.

TABLE 5. RED ABALONE, *HALIOTUS RUFESCENS*, LARVAL DEVELOPMENT DATA FROM BRINE AND DILUTION CONTROLS

	Replicate	Brine Control	Dilution Control
RAW	A	1.00	0.99
	B	0.98	0.99
	C	1.00	0.99
	D	0.99	1.00
	E	0.99	
ARC SINE SQUARE ROOT TRANSFORMED	A	1.521	1.471
	B	1.429	1.471
	C	1.521	1.471
	D	1.471	1.521
	E	1.471	
Mean ($\bar{Y}_i$)		1.483	1.484
S_i^2		0.00152	0.000625
i		1	2

14.13.2.4.2.2 Calculate the denominator, D, of the statistic for each control group:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

14.13.2.4.2.3 For the brine control data,

n = 5

$$\bar{X} = \frac{1}{5}(7.413) = 1.483$$

$$D = 0.00609$$

For the dilution control data,

$$n = 4$$

$$\bar{X} = \frac{1}{4}(5.934) = 1.484$$

$$D = 0.00191$$

14.13.2.4.2.4 Order the observations for each control group from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for the two groups in this example are listed in Table 6.

TABLE 6. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Brine Control		Dilution Control	
i	$X^{(i)}$	i	$X^{(i)}$
1	1.429	1	1.471
2	1.471	2	1.471
3	1.471	3	1.471
4	1.521	4	1.521
5	1.521		

14.13.2.4.2.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the datasets in this example, n = 5 and k = 2 for the brine control group, and n = 4 and k = 2 for the dilution control group. The a_i values are listed in Table 7.

TABLE 7. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
		Brine Control Group	
1	0.6646	0.092	$X^{(5)} - X^{(1)}$
2	0.2413	0.050	$X^{(4)} - X^{(3)}$
		Dilution Control Group	
1	0.6872	0.050	$X^{(4)} - X^{(1)}$
2	0.1667	0.000	$X^{(3)} - X^{(2)}$

14.13.2.4.2.6 Compute the test statistic, W, for each group as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 7. For the data in the brine example:

$$W = \frac{1}{0.00609}\ (0.07321)^2 = 0.880$$

For the data in the dilution example:

$$W = \frac{1}{0.00191}\ (0.03436)^2 = 0.618$$

14.13.2.4.2.7 The decision rule for this test is to compare W as

calculated in Subsection 2.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in the brine control, the critical value at a significance level of 0.01 and n = 5 observations is 0.686. Since W = 0.880 is greater than the critical value, conclude that the brine control data are normally distributed. For the data in the dilution control, the critical value at a significance level of 0.01 and n = 4 observations is 0.687. Since W = 0.618 is less than the critical value, conclude that the dilution control data are not normally distributed.

14.13.2.4.2.8 Since the dilution control data does not meet the normality assumption, the Wilcoxon Rank Sum Test will be used to compare the responses in the two control groups.

14.13.2.4.3 Wilcoxon Rank Sum Test

14.13.2.4.3.1 To perform the Wilcoxon Rank Sum test, combine the data from the two groups and arrange in order from smallest to largest. Assign the rands (1, 2, ..., 9) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation. A table of the ranks is given in Table 8.

TABLE 8. ASSIGNING RANKS TO THE BRINE AND DILUTION CONTROLS FOR THE WILCOXON RANK SUM TEST

Rank	Transformed Proportion Normal	Control Group
1	1.429	Brine
4	1.471	Brine
4	1.471	Brine
4	1.471	Dilution
4	1.471	Dilution
4	1.471	Dilution
8	1.521	Brine
8	1.521	Dilution
8	1.521	Dilution

14.13.2.4.3.2 The ranks are then summed for both of the control groups. For this data, the sum of the ranks in the brine control group is 25 and the sum of the ranks in the dilution control group is 20.

14.13.2.4.3.3 For this situation, we wish to determine if the proportions of normally developed larvae in the two control groups are significantly different. To do this, compare the rank sum of the group with the smaller sample size with some "minimum" or critical rank sum, at or below which the devlopment in the controls would be considered significantly different. At a significance level of 0.05, the minimum rank sum in a test with five replicates in one group and and four replicates in the other is 11 (See Snedecor and Cochran, 1980).

14.13.2.4.3.4 The dilution control sample size is smaller than the sample size of the brine control group so its rank sum is compared to the critical value. Since its rank sum of 20 is greater than the critical value of 11, conclude that the development proportions for the two control groups are not significantly different.

14.13.2.5 Example of Analysis of Larval Development Data

14.13.2.5.1 Since the responses in the two control groups are not significantly different, only the dilution control group will be used in the analysis of the shell development responses for the effluent concentrations. As above, each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and dilution control are listed in Table 9. The data are plotted in Figure 3. Since there is 100% abnormality in all replicates for the 5.6% and 10.0% concentrations, they are not included in the statistical analysis and are considered qualitative abnormality effects.

14.13.2.6 Test for Normality

14.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all

observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 10.

14.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

Where: X_i = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations

14.13.2.6.3 For this set of data, n = 24

$$\overline{X} = \frac{1}{24} (-0.004) = 0.000$$

D = 0.1127

14.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 11.

14.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 24 and k = 12. The a_i values are listed in Table 12.

14.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D} \left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)}) \right]^2$$

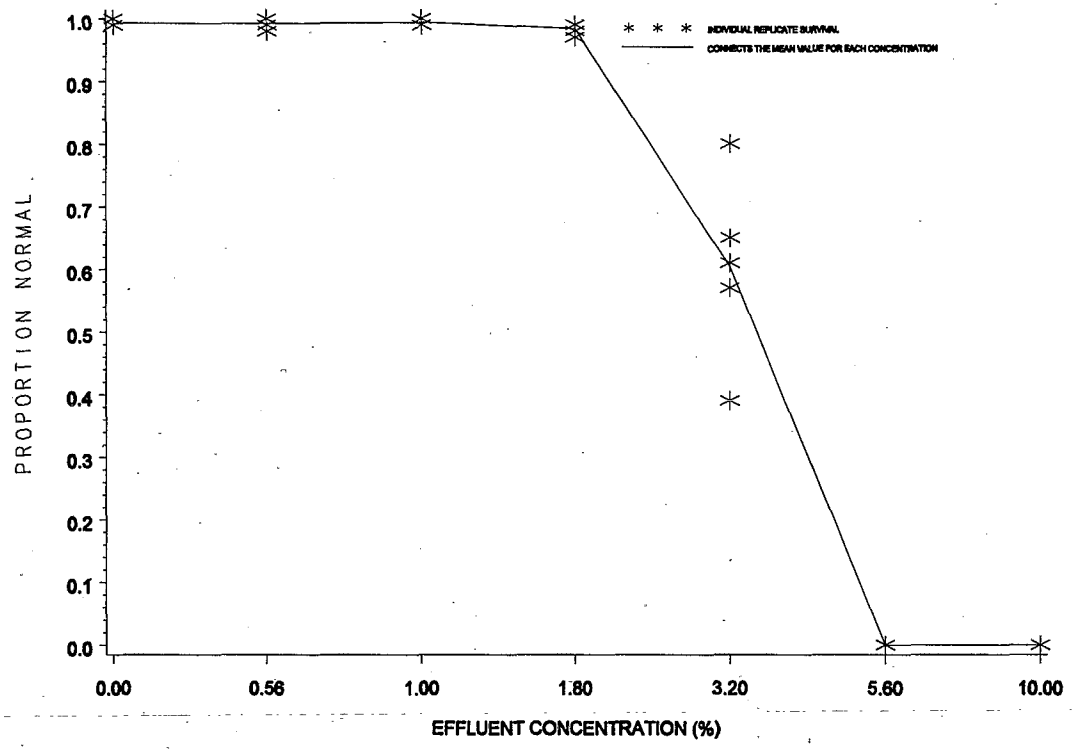


Figure 3. Plot of mean proportion of normally developed red abalone, *Haliotus rufescens* larvae.

TABLE 9. RED ABALONE, *HALIOTUS RUFESCENS*, SHELL DEVELOPMENT DATA

	Replicate	Dilution Control	Effluent Concentration (%) 0.56	1.00	1.80	3.20	5.6	10.0
	A	0.99	0.99	0.99	0.99	0.39	0	0
RAW	B	0.99	0.99	1.00	0.99	0.57	0	0
	C	0.99	0.98	0.99	0.99	0.61	0	0
	D	1.00	1.00	0.99	0.98	0.65	0	0
	E		1.00	1.00	0.97	0.80	0	0
ARC SINE	A	1.471	1.471	1.471	1.471	0.674	-	-
SQUARE ROOT	B	1.471	1.471	1.521	1.471	0.856	-	-
TRANSFORMED	C	1.471	1.429	1.471	1.471	0.896	-	-
	D	1.521	1.521	1.471	1.429	0.938	-	-
	E		1.521	1.521	1.397	1.107	-	-
Mean ($\overline{Y}_i$)		1.484	1.483	1.491	1.448	0.894	-	-
S_i^2		0.000625	0.001523	0.000750	0.001137	0.024288	-	-
i		1	2	3	4	5	6	7

TABLE 10. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	Effluent Concentration (%) 0.56	1.00	1.80	3.20
A	-0.013	-0.012	-0.020	0.023	-0.220
B	-0.013	-0.012	0.030	0.023	-0.038
C	-0.013	-0.054	-0.020	0.023	0.002
D	0.037	0.038	-0.020	-0.019	0.044
E		0.038	0.030	-0.051	0.213

TABLE 11. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.220	13	-0.012
2	-0.054	14	0.002
3	-0.051	15	0.023
4	-0.038	16	0.023
5	-0.020	17	0.023
6	-0.020	18	0.030
7	-0.020	19	0.030
8	-0.019	20	0.037

TABLE 12. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.433	$X^{(24)} - X^{(1)}$
2	0.3098	0.098	$X^{(23)} - X^{(2)}$
3	0.2554	0.089	$X^{(22)} - X^{(3)}$
4	0.2145	0.076	$X^{(21)} - X^{(4)}$
5	0.1807	0.057	$X^{(20)} - X^{(5)}$
6	0.1512	0.050	$X^{(19)} - X^{(6)}$
7	0.1245	0.050	$X^{(18)} - X^{(7)}$
8	0.0997	0.042	$X^{(17)} - X^{(8)}$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 12. For the data in this example:

$$W = \frac{1}{0.1127} (0.2974)^2 = 0.7848$$

14.13.2.5.7 The decision rule for this test is to compare W as calculated in 14.13.2.5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 24 observations is 0.884. Since W = 0.7848 is less than the critical value, conclude that the data are not normally distributed.

14.13.2.5.8 Since the data do not meet the assumption of normality, the Wilcoxon Rank Sum Test with the Bonferroni Adjustment will be used to analyze the shell development data.

14.13.2.6 Wilcoxon Rank Sum Test with the Bonferroni Adjustment

14.13.2.6.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ... , 9) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

14.13.2.6.2 An example of assigning ranks to the combined data for the control and 0.56% concentration is given in Table 13. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 14. The ranks are then summed for each concentration level, as shown in Table 15.

14.13.2.6.3 For this example, determine if the survival in any of the concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus compare the rank sums for the survival at each of the various concentration levels with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control), four control replicates and five concentration replicates is 15 (See Table 5, Appendix F).

TABLE 13. ASSIGNING RANKS TO THE CONTROL AND 0.56% CONCENTRATION LEVEL FOR THE WILCOXON RANK SUM TEST WITH THE BONFERRONI ADJUSTMENT

Rank	Transformed Proportion Normal	Concentration
1	1.429	0.56 %
4	1.471	0.56 %
4	1.471	0.56 %
4	1.471	Control
4	1.471	Control
4	1.471	Control
8	1.521	0.56 %
8	1.521	0.56 %
8	1.521	Control

TABLE 14. TABLE OF RANKS[1]

Replicate	Control	Effluent Concentration (%)			
		0.56	1.00	1.80	3.20
1	1.471(4,3.5,5.5,7)	1.471(4)	1.471(3.5)	1.471(5.5)	0.674(1)
2	1.471(4,3.5,5.5,7)	1.471(4)	1.521(8)	1.471(5.5)	0.856(2)
3	1.471(4,3.5,5.5,7)	1.429(1)	1.471(3.5)	1.471(5.5)	0.896(3)
4	1.521(8,8,9,9)	1.521(8)	1.471(3.5)	1.429(2)	0.938(4)
5		1.521(8)	1.521(8)	1.397(1)	1.107(5)

[1]Control ranks are given in the order of the concentration with which they were ranked.

TABLE 15. RANK SUMS

Concentration (% Effluent)	Rank Sum
0.56	25.0
1.00	26.5
1.80	19.5

14.13.2.6.4 Since the rank sum for the 3.20% concentration level is equal to the critical value, the proportion normal in that concentration is considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no other concentration has a significantly lower proportion normal than the control. Hence, the NOEC and the LOEC are 1.80% and 3.20%, respectively.

14.13.2.7 Calculation of the ICp

14.13.2.7.1 The shell development data in Table 4 are utilized in this example. As can be seen from Table 4 and Figure 4, the observed means are not monotonically non-increasing with respect to concentration (mean response for each higher concentration is not less than or equal to the mean response for the previous concentration and the responses between concentrations do not follow a linear trends). Therefore, the means are smoothed prior to calculating the IC. In the following discussion, the observed means are represented by $\bar{Y}_i$ and the smoothed means by M_i.

14.13.2.7.2 Starting with the control mean, $\bar{Y}_1 = 0.993$ and $\bar{Y}_2 = 0.992$, we see that $\bar{Y}_1 > \bar{Y}_2$. Set $M_1 = \bar{Y}_1$. Comparing $\bar{Y}_2$ to $\bar{Y}_3$, $\bar{Y}_2 < \bar{Y}_3$.

14.13.2.7.3 Calculate the smoothed means:

$$M_2 = M_3 = (\bar{Y}_2 + \bar{Y}_3)/2 = 0.993$$

14.13.2.7.4 Since $\bar{Y}_7 = 0 < \bar{Y}_6 = 0 < \bar{Y}_5 = 0.604 < \bar{Y}_4 = 0.984 < \bar{Y}_3 = 0.993$, set $M_3 = 0.993$, $M_4 = 0.984$, $M_5 = 0.604$, $M_6 = 0$, and set $M_7 = 0$.

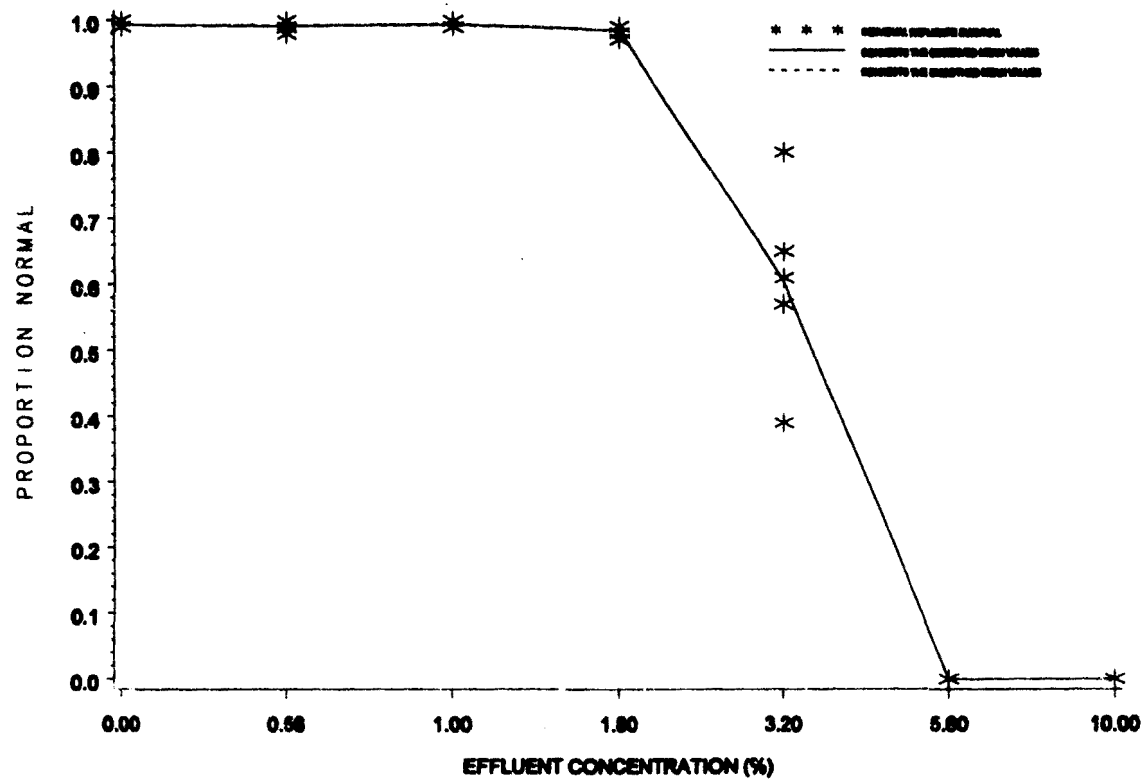


Figure 4. Plot of raw data, observed means, and smoothed means for red abalone, *Haliotus rufescens*, development data from Tables 4 and 16.

TABLE 16. RED ABALONE, *HALIOTUS RUFESCENS*, MEAN SHELL DEVELOPMENT RESPONSE AFTER SMOOTHING

Effluent Conc. (%)	i	Response Means, $\bar{Y}_i$ (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.993	0.993
0.56	2	0.992	0.993
1.00	3	0.994	0.993
1.80	4	0.984	0.984
3.20	5	0.604	0.604
5.60	6	0.000	0.000
10.00	7	0.000	0.000

14.13.2.7.5 Table 16 contains the response means and smoothed means and Figure 4 gives a plot of the smoothed response curve.

14.13.2.7.6 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of fertilized eggs, compared to the controls, would result in a mean proportion of 0.745, where $M_1(1-p/100) = 0.993(1-25/100)$. Examining the means and their associated concentrations (Table 16), the response, 0.745, is bracketed by $C_4 = 1.80\%$ effluent and $C_5 = 3.20\%$ effluent.

14.13.2.7.7 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1-p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 1.8 + [0.993(1 - 25/100) - 0.984]\frac{(3.2 - 1.8)}{(0.604 - 0.984)}$$

$$= 2.68\%.$$

14.13.2.7.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 2.6818%. The empirical 95.0% confidence interval for the true

mean was 2.5000% to 3.1262 %. The computer program output for the IC25 for this data set is shown in Figure 5.

14.14 PRECISION AND ACCURACY

14.14.1 PRECISION

14.14.1.1 Single-Laboratory Precision

14.14.1.1.1 Data on the single laboratory precision of the *Haliotis rufescens* larval development method using zinc sulfate are shown in Table 17. Zinc concentrations were 18, 32, 56, and 100 µg/L. All tests were conducted at the Marine Pollution Studies Laboratory. There was good agreement among test EC50s, with a coefficient of variation of 8%.

14.14.1.2 Multi-laboratory Precision

14.14.1.2.1 The multi-laboratory data indicate a similar level of test precision Table 18. Data are presented for four interlaboratory trials in which either two or three laboratories tested both split effluent samples and reference toxicants. The mean coefficient of variation between EC50 values from different laboratories was 15%.

14.14.2 ACCURACY

14.14.2.1 The accuracy of toxicity tests cannot be determined.

```
Conc. ID          1     2     3     4     5     6     7
-------------------------------------------------------
Conc. Tested      0   .56     1   1.8   3.2   5.6    10
-------------------------------------------------------
Response  1     .99   .99   .99   .99   .39     0     0
Response  2     .99   .99  1.00   .99   .57     0     0
Response  3     .99   .98   .99   .99   .61     0     0
Response  4    1.00  1.00   .99   .98   .65     0     0
Response  5          1.00  1.00   .97   .80     0     0
-------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date:     Test Ending Date:
Test Species: Red Abalone
Test Duration:         48 hours
DATA FILE: abalone.icp
OUTPUT FILE: abalone.i25
-------------------------------------------------------------------
Conc.      Number      Concentration      Response      Std.       Pooled
 ID     Replicates                %         Means       Dev.   Response Means
-------------------------------------------------------------------------
  1          4              0.000          0.993       0.005       0.993
  2          5              0.560          0.992       0.008       0.993
  3          5              1.000          0.994       0.005       0.993
  4          5              1.800          0.984       0.009       0.984
  5          5              3.200          0.604       0.148       0.604
  6          5              5.600          0.000       0.000       0.000
  7          5             10.000          0.000       0.000       0.000
-------------------------------------------------------------------------
The Linear Interpolation Estimate:     2.6818   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:    2.7085 Standard Deviation:     0.1510
Original Confidence Limits:   Lower:      2.5000 Upper:      3.1262
Expanded Confidence Limits:   Lower:      2.4091 Upper:      3.3484
Resampling time in Seconds:     0.27  Random_Seed: -770872716
```

Figure 5. ICPIN program output for the IC25.

TABLE 17. SINGLE LABORATORY PRECISION DATA FOR THE RED ABALONE, *HALIOTIS RUFESCENS* LARVAL DEVELOPMENT TEST WITH ZINC (ZN µG/L) SULFATE AS A REFERENCE TOXICANT

Test Date	NOEC (µg/L)	EC50 (µg/L)
March 1990	32	42[1]
May 1990	32	39[1]
January 1991	18	34[1]
February 1991	18	40[2]
Mean		38.4
SD		3.0
CV (%)		7.8

1 Source: Hunt et al., 1991
2 Source: Anderson et al., 1994

TABLE 18. MULTI-LABORATORY PRECISION OF THE RED ABALONE, *HALIOTIS RUFESCENS* LARVAL DEVELOPMENT TEST PERFORMED WITH ZINC (ZN μG/L) SULFATE AND EFFLUENT (%) AS THE TOXICANTS

Test Date	Toxicant	Lab	NOEC	EC50	CV
March 1990	Effluent	A	>3.2%	nc	
March 1990	Effluent	B	>3.2%	nc	
March 1990	Effluent	C	0.32%	1.83	nc
March 1990	Zinc	A	32	41	
March 1990	Zinc	B	18	28	
March 1990	Zinc	C	18	31	20%
May 1990	Effluent	A	3.2%	4.7	
May 1990	Effluent	D	1.8%	3.5	
May 1990	Effluent	C	3.2%	3.8	16%
May 1990	Zinc	A	32	39	
May 1990	Zinc	D	32	46	
May 1990	Zinc	C	32	37	12%
January 1991	Effluent	A	<0.56%	1.5	
January 1991	Effluent	C	1.25%	1.8	13%
January 1991	Zinc	A	18	34	
January 1991	Zinc	C	32	48	24%
January 1991	Effluent	A	1.0%	2.7	
January 1991	Effluent	C	1.8%	2.8	3.0%

Mean Interlaboratory CV = 15% Interlaboratory CV based on 6 tests for which CV values could be calculated. Source: Hunt et al., 1991.

nc = indicates that the CV could not be calculated because only one laboratory observed a 50% effect and calculated an EC50.

APPENDIX I. RED ABALONE TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a zinc reference toxicant stock solution (10,000 µg/L) by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) to 1 liter of reagent water.

D. Prepare zinc reference toxicant solution of 0 (control) 10, 18, 32, 56 and 100 µg/L by adding 0, 1.0 1.8, 3.2, 5.6 and 10.0 mL of stock solution, respectively, to a 1-L volumetric flask and filling to 1-L with dilution water.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 15°C and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Obtain test organisms and hold or condition as necessary for spawning.

B. Induce four male and four female abalone to spawn using either H_2O_2 and Tris or UV irradiated seawater (300 mL/min flow rate through the UV unit). All solutions should be maintained at 15 ± 1°C.

C. Siphon eggs into a fertilization bucket. Add 200 mL of sperm-laden water to fertilize the eggs. Wash the fertilized eggs at least twice by slowly decanting and refilling the chamber with fresh filtered seawater. Temperatures should vary by no more than 1°C between waters used in mixing and refilling.

D. Suspend the embryos evenly in a 1000 mL beaker and count five samples in a 1 mL pipet to estimate embryo density.

E. Pipet 1000 fertilized embryos into each 200 mL test chamber. Be sure temperatures in the embryo beaker and the solutions are at 15 ± 1°C. Incubate for 48 h. For smaller-sized chambers, use proportionately fewer embryos.

F. At the end of the 48 h period, pour the entire test solution with larvae through a 37 µm-meshed screen. Wash larvae from the screen into 25 mL vials. Add buffered formalin to preserve the larvae in a 5% solution or glutaraldehyde for a 0.05% solution. Cap the flask and invert gently to mix.

G. Pipet a sample from each vial onto a Sedgwick-Rafter counting slide and count 100 larvae. Return the larvae to the vials for future reference.

H. Count the number of normal larvae for each replicate and divide by the total counted.

I. Analyze the data.

J. Include standard reference toxicant point estimate values in the standard quality control charts.

Salinity Adjustment Worksheet for Abalone

Date Sampled: Batch:
Date Adjusted: Region:

$$\frac{VS\ \ (TS-SS)}{(SB-TS)}$$

SS = Salinity of Sample
VS = Volume of Sample
TS = Target Salinity (34±2%)
VB = Volume of Brine
SB = Salinity of Brine

VDW = VBL - VBS

VDW = Volume of Dilution Water (Adjusted to 34±2%)
VBL = Largest Volume of Brine added to adjust salinity
VBS = Volume of Brine added to each Sample

Total Volume = VB added + VDW added
(Total volume should be the same for all samples)

Site Code (ID Org #) or concentration	Initial Salinity	TS	Vol. of Brine	Vol.Dil. Water	Total Volume	Final Salinity	Precision and Accuracy for Refractometer
		34±2					
		34±2					
		34±2					
		34±2					
		34±2					
		34±2					
		34±2					
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		34±2					
		34±2					
		34±2					
		34±2					

Initials: Double Checked:

Data Sheet for Mollusc Larval Development Toxicity Test

Test Start Date: Start Time: Mollusc Species:

Test End Date: End Time: Collection/Arrival Date:

Reference Toxicant:Reference Toxicant: Broodstock Source:

Sample Source:

Sample Type: Effluent Ref Tox Solid Elutriate Pore Water WaterSample Type: Effluent Ref Tox Solid Elutriate Pore Water Water

Test Cont. #	Station Code or Concentration	Sample ID #	After 48 hours		Notes
			Normal Larvae	Abnormal Larvae	
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					
21					
22					
23					
24					
25					
26					
27					
28					
29					
30					
31					
32					
33					
34					
35					

Computer Data StorageComputer Data Storage
Disk:
File:

Notes

SECTION 15

PURPLE URCHIN, *Strongylocentrotus purpuratus* AND SAND DOLLAR, *Dendraster excentricus* LARVAL DEVELOPMENT TEST METHOD

Adapted from a method developed by
Steven Bay and Darrin Greenstein
Southern California Coastal Water Research Project
Westminster, CA 92683

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SECTION 15

SEA URCHIN, *Strongylocentrotus purpuratus* AND SAND DOLLAR, *Dendraster excentricus* LARVAL DEVELOPMENT TEST

15.1 SCOPE AND APPLICATION

15.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the developing embryos of the purple sea urchin, *Strongylocentrotus purpuratus*, and the sand dollar, *Dendraster excentricus*, during a 72-h static non-renewal exposure. The effects include the synergistic, antangonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

15.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

15.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

15.1.4 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

15.1.5 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

15.2 SUMMARY OF METHOD

15.2.1 The method provides the step-by-step instructions for

performing a 72-h static non-renewal test using the early development of the purple sea urchin, *Strongylocentrotus purpuratus*, and the sand dollar, *Dendraster excentricus*, to determine the toxicity of substances in marine and estuarine waters. The test endpoint is normal larval development and may include mortality if modified for total counts at test initiation and termination.

15.3 **INTERFERENCES**

15.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

15.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sample Preparation for Toxicity Tests).

15.4 **SAFETY**

15.4.1 See Section 3, Health and Safety.

15.5 **APPARATUS AND EQUIPMENT**

15.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult sea urchins and sand dollars, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20°C), with appropriate filtration and aeration system.

15.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

15.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (15°C) prior to the test.

15.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

15.5.5 Refractometer -- for determining salinity.

15.5.6 Hydrometer(s) -- for calibrating refractometer.

15.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

15.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD 170.1, USEPA, 1979) -- to calibrate laboratory thermometers.

15.5.9 pH and DO meters -- for routine physical and chemical measurements.

15.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

15.5.11 Winkler bottles -- for dissolved oxygen determinations.

15.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

15.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

15.5.14 Glass stirring rods -- for mixing test solutions.

15.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-1000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

15.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 100-1000 mL for making test solutions.

15.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

15.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

15.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

15.5.20 Wash bottles -- for dilution water.

15.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

15.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

15.5.23 Beakers, 5-10 mL borosilicate glass -- for collecting sperm from sand dollars.

15.5.24 Beakers, 250 mL borosilicate glass -- for preparation of test solutions.

15.5.25 Beakers, 100 mL borosilicate glass -- for spawning; to support sea urchins and to collect sea urchin and sand dollar eggs.

15.5.26 Beakers, 1,000 mL borosilicate glass -- for rinsing and settling sea urchin eggs.

16.5.27 Vortex mixer -- to mix sea urchin semen in tubes prior to sampling.

15.5.28 Compound microscope -- for examining gametes, counting sperm cells (200-400x), eggs and embryos and (100x), and examining larvae. Dissecting scopes are sometimes used to count eggs at a lower magnification. One piece of equipment worthy of a special mention is an inverted microscope. The use of an inverted scope is not required, but recommended. Its use reduces the exposure of workers to hazardous fumes (formalin or glutaraldehyde) during the counting of larvae and reduces sample examination time. Alternatively, a Sedgewick-Rafter cell may be used on a regular compound scope.

15.5.29 Counter, two unit, 0-999 -- for recording sperm, egg, embryo, and larval counts.

15.5.30 Sedgwick-Rafter counting chamber -- for counting egg and embryo stock and examining larval development at the end of the test.

15.5.31 Centrifuge tubes, test tubes, or vials -- for holding semen.

15.5.32 Hemacytometers, Neubauer -- for counting sperm.

15.5.33 Siphon hose (3 mm i.d.) -- for removing wash water from settled eggs.

15.5.34 Perforated plunger -- for maintaining homogeneous distribution of eggs and embryos during sampling and distribution to test chambers.

15.5.35 Enamel or plastic tray -- for optional spawning platform.

15.5.36 Nitex® screening (0.5mm mesh) -- cleaning egg solutions.

15.5.37 60 μm NITEX® filter -- for filtering receiving waters.

15.6 **REAGENTS AND SUPPLIES**

15.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

15.6.2 Data sheets (one set per test) -- for data recording (see Figures 1-4).

15.6.3 Tape, colored -- for labelling test chambers and containers.

15.6.4 Markers, water-proof -- for marking containers, etc.

15.6.5 Parafilm -- to cover graduated cylinders and vessels containing gametes and embryos.

15.6.6 Gloves, disposable -- for personal protection from contamination.

15.6.7 Pipets, serological -- 1-10 mL, graduated.

15.6.8 Pipet tips -- for automatic pipets. Note: pipet tips for handling semen should be cut off to produce an opening about 1 mm in diameter; pipet tips for handling eggs should be cut off to produce an opening about 2 mm in diameter. This is necessary to provide smooth flow of the viscous semen, accurate sampling of eggs, and to prevent injury to eggs passing through a restricted opening. A clean razor blade can be used to trim pipet tips.

15.6.9 Coverslips -- for microscope slides.

15.6.10 Lens paper -- for cleaning microscope optics.

15.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

15.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

15.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

15.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

15.6.15 Laboratory quality assurance samples and standards -- for the above methods.

15.6.16 Test chambers -- 30-mL glass scintillation vials with polypropylene caps, four chambers per concentration.

15.6.17 Formaldehyde, 10%, in seawater -- for preserving larvae. Note: formaldehyde has been identified as a carcinogen and is irritating to skin and mucous membranes. It should not be used at a concentration higher than necessary to achieve morphological preservation of larvae for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

15.6.18 Glutaraldehyde, 1% in seawater -- for preserving larvae.

Figure 1. Sample experiment set-up sheet.

SEA URCHIN EMBRYO DEVELOPMENT TEST

EXPERIMENT DESCRIPTION

Bioassay No.___________ Start Date________ Start time_______

Investigator___________ End Date__________ End Time_________

Sample Numbers	Sample	Concentration	Other Information

Figure 2. Sample worksheet for urchin spawning information.

SEA URCHIN DEVELOPMENT TEST

SPAWNING WORKSHEET

Bioassay no._____ Date _____

Spawning

No.	Injection time	Sex	Accepted? (Comments)
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			

Sperm density

#sperm counted=_____

_____

_____ mean=_____

_____

(mean)_____ x (5 x 10^6)=_____ sperm/mL

Egg dilution

eggs counted=_____

_____ mean=_____

_____

(mean)_____ x 100=_____ eggs/mL in stock

eggs/mL in stock ÷ 1,000= _____ Egg dilution factor

Figure 3. Sample worksheet for sea urchin fertilization information.

SEA URCHIN DEVELOPMENT TEST
FERTILIZATION WORKSHEET

Bioassay No. _____
Date_____

_____mL eggs used _____mL dilution water used

Fertilization and initiation

_____mL in egg dilution x 1,000 eggs/mL
= _____eggs in dilution

_____eggs in dilution x 500 sperm/egg
= _____sperm needed

_____sperm needed ÷ _____sperm/mL in sperm dilution=_____mL sperm dilution needed

Percent fertilized after 10 min _____

Time of inoculation_____

Figure 4. Sample data sheet for embryo microscopic examination.

SEA URCHIN DEVELOPMENT TEST: RESULTS

Bioassay No._____ Date_____

Counter_____

Number	Sample	Abnormal	Normal	%Normal	Notes

15.6.19 Acetic acid, 10%, reagent grade, in filtered (10μ) seawater -- for preparing killed sperm dilutions for sperm counts.

15.6.20 Haemo-Sol or equivalent cleaner -- for cleaning hemacytometer and cover slips.

15.6.21 0.5 M KCl solution -- for inducing spawning.

15.6.22 Syringe, disposable, 3 or 5 mL -- for injecting KCl into sea urchins and sand dollars to induce spawning.

15.6.23 Needles, 25 gauge -- for injecting KCl.

15.6.24 Pasteur pipets and bulbs -- for sampling eggs from spawning beakers.

15.6.25 Hematocrit capillary tubes -- for sampling sperm for examination and for loading hemacytometers.

15.6.26 Microscope well-slides -- for pre-test assessment of sperm activity and egg condition.

15.6.27 Reference toxicant solutions (see Section 15.10.2.4 and Section 4, Quality Assurance).

15.6.28 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

15.6.29 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

15.6.30 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 15.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-μm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

15.6.31 HYPERSALINE BRINES

15.6.31.1 Most industrial and sewage treatment effluents

entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

15.6.31.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

15.6.31.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 μm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

15.6.31.4 Freeze Preparation of Brine

15.6.31.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20°C until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

15.6.31.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

15.6.31.4.3 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

15.6.31.5 Heat Preparation of Brine

15.6.31.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

15.6.31.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

15.6.31.5.3 Seawater should be filtered to at least 10 μm before being put into the brine generator. The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

15.6.31.5.4 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

15.6.31.6 Artificial Sea Salts

15.6.31.6.1 No data from sea urchin or sand dollar larval tests using sea salts or artificial seawater (e.g., GP2) are available

for evaluation at this time, and their use must be considered provisional.

15.6.31.7 Dilution Water Preparation from Brine

15.6.31.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

15.6.31.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. The proportion is 1 part brine plus 1.94 parts reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

15.6.31.8 Test Solution Salinity Adjustment

15.6.31.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

15.6.31.8.2 Check the pH of all test solutions and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute

hydrochloric acid or sodium hydroxide (see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

15.6.31.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

15.6.31.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

15.6.31.9 Preparing Test Solutions

15.6.31.9.1 Ten mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (≤6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 1.0 mL of effluent to a 100-mL volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 100-mL mark with dilution water, stopper it, and shake to mix. Pour into a (150-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

15.6.31.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Pour into a (100-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (AT X‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Make serial dilutions from the highest test concentration.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION:

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

15.6.31.10 Brine Controls

15.6.31.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 15.6.31.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

15.6.32 TEST ORGANISMS, PURPLE URCHINS

15.6.32.1 Sea Urchins, *Strongylocentrotus purpuratus* (approximately 6 of each sex per test).

15.6.32.2 Species Identification

15.6.32.2.1 Although identification of purple sea urchins, *Strogylocentrotus purpuratus*, is usually a simple matter of confirming general body color, size, and spine appearance, those unfamiliar with the species should seek confirmation from local experts.

15.6.32.3 Obtaining Broodstock

15.6.32.3.1 Adult sea urchins (*Strongylocentrotus purpuratus*) can be obtained from commercial suppliers or collected from uncontaminated intertidal areas. State collection permits are usually required for collection of sea urchins and collection is prohibited or restricted in some areas. The animals are best transported "dry," surrounded either by moist seaweed or paper towels dampened with seawater. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock which can prematurely induce spawning.

15.6.32.4 Broodstock Culture and Handling

15.6.32.4.1 The adult sea urchins are maintained in glass aquaria or fiberglass tanks. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or salt water prepared from commericial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded.

15.6.32.4.2 Although ambient temperature seawater is usually acceptable, maintaining sea urchins in spawning condition usually requires holding at a relatively constant temperature. The culture unit should be capable of maintaining a constant temperature between 10 and 14°C with a water temperature control device.

15.6.32.4.3 Food for sea urchins -- kelp, recommended, but not necessarily limited to, *Laminaria sp.*, *Hedophyllum sp.*, *Nereocystis sp.*, *Macrocystis sp.*, *Egregia sp.*, *Alaria sp.* or romaine lettuce. The kelp should be gathered from known uncontaminated zones or obtained from commerical supply houses whose kelp comes from known uncontaminated areas, or romaine lettuce. Fresh food is introduced into the tanks at least several times a week. Sun dried (12-24 hours) or oven dried (60°C overnight) kelp, stores well at room temperature or frozen, rehydrates well and is adequate to maintain sea urchins for long periods. Decaying food and fecal pellets are removed as necessary to prevent fouling.

15.6.32.4.4 Natural seawater (>30‰) is used to maintain the adult animals and (≥32‰) as a control water in the tests.

15.6.32.4.5 Adult male and female (if sexes known) animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added. Healthy animals will attach to the kelp or aquarium within hours.

15.6.32.4.6 To successfully maintain about 25 adult animals for seven days at a field site, 40-L glass aquaria using aerated,

recirculating, clean saline water (32‰) and a gravel bed filtration system, are housed within a water bath, such as an INSTANT OCEAN® Aquarium. The sexes should be held separately if possible.

15.6.33 TEST ORGANISMS, SAND DOLLARS

15.6.33.1 Sand Dollars, *Dendraster excentricus*, (approximately 6 of each sex per test).

15.6.33.2 Species Identification

15.6.33.2.1 Although identification of sand dollars, *Dendraster excentricus*, is usually a simple matter of confirming general body appearance, those unfamiliar with the species should seek confirmation from local experts.

15.6.33.3 Obtaining Broodstock

15.6.33.3.1 Adult sand dollars (*Dendraster excentricus*) can be obtained from commercial suppliers or collected from subtidal zones (most areas) or from intertidal zones of some sheltered waters (e.g., Puget Sound). State collection permits may be required for collection of sand dollars and collection prohibited or restricted in some areas. The animals are best transported "dry," surrounded either by moist seaweed or paper towels dampened with seawater. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock which can prematurely induce spawning.

15.6.33.4 Broodstock Culture and Handling

15.6.33.4.1 The adult sand dollars are maintained in glass aquaria or fiberglass tanks. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or saltwater prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded. For longer periods than a few days, several centimeters or more of a sand substrate may be desirable.

15.6.33.4.2 Although ambient temperature seawater is usually acceptable, maintaining sand dollars in spawning condition usually requires holding at a relatively constant temperature.

The culture unit should be capable of maintaining a constant temperature between 8 and 12°C with a water temperature control device.

15.6.33.4.3 Sand dollars will feed on suspended or benthic materials such as phytoplankton, benthic diatoms, etc. No reports of laboratory populations being maintained in spawning condition over several years are known. It is probably most convenient to obtain sand dollars, use them, and then discard them after they cease to produce good quality gametes.

15.6.33.4.4 Natural seawater (>30‰) is used to maintain the adult animals and (≥32‰) as a control water in the tests.

15.6.33.4.5 Adult male and female (if sexes known) animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, trays or aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added.

15.6.33.4.6 To successfully maintain about 25 adult animals for seven days at a field site, 40-L glass aquaria using aerated, recirculating, clean saline water (>30‰) are housed within a water bath, such as an INSTANT OCEAN® Aquarium. The sexes should be held separately if possible.

15.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION AND STORAGE

15.7.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sampling Preparation for Toxicity Tests.

15.8 CALIBRATION AND STANDARDIZATION

15.8.1 See Section 4, Quality Assurance.

15.9 QUALITY CONTROL

15.9.1 See Section 4, Quality Assurance.

15.10 TEST PROCEDURES

15.10.1 TEST DESIGN

15.10.1.1 The test consists of at least four replicates of five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain four replicates of a brine control.

15.10.1.2 Effluent concentrations are expressed as percent effluent.

15.10.2 TEST SOLUTIONS

15.10.2.1 Receiving waters

15.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 μm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 10 mL, and 400 mL for chemical analysis, would require approximately 440 mL of sample per test.

15.10.2.2 Effluents

15.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100% HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

15.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

15.10.2.2.3 The volume in each test chamber is 10 mL.

15.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

15.10.2.3 Dilution Water

15.10.2.3.1 Dilution water should be uncontaminated 1-μm-filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

15.10.2.4 Reference Toxicant Test

15.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

15.10.2.4.2 The preferred reference toxicant for sea urchins and sand dollars is copper chloride ($CuCl_2 \cdot H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a copper reference toxicant stock solution (2,000 mg/L) by adding 5.366 g of copper chloride ($CuCl_2 \cdot 2H_2O$) to 1 liter of reagent water. For each reference toxicant test prepare a copper sub-stock of 3 mg/L by diluting 1.5 mL of stock to one liter with reagent water. Alternatively, certified standard solutions can be ordered from commercial companies.

15.10.2.4.3 Prepare a control (0 μg/L) plus four replicates each of at least five consecutive copper reference toxicant solutions (e.g., from the series 3.0, 4.4, 6.5, 9.5, 13.9, 20.4, and 30.0 μg/L, by adding 0.10, 0.15, 0.22, 0.32, 0.46, 0.68, and 1.00 mL of sub-stock solution, respectively, to 100-L volumetric flasks

and filling to 100-mL with dilution water). Alternatively, certified standard solutions can be ordered from commercial companies. Start with control solutions and progress to the highest concentration to minimize contamination.

15.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

15.10.3 COLLECTION OF GAMETES FOR THE TEST

15.10.3.1 Spawning Induction

15.10.3.1.1 Pour seawater into 100 mL beakers and place in 15°C bath or room. Allow to come to temperature. Select a sufficient number of sea urchins or sand dollars (based upon recent or past spawning success) so that three of each sex are likely to provide gametes of acceptable quantity and quality for the test. During optimal spawning periods this may only require six animals, three of each sex, when the sexes are known from prior spawning. During other periods, especially if the sex is not known, many more animals may be required.

15.10.3.1.2 Care should be exercised when removing sea urchins from holding tanks so that damage to tube-feet is minimized. Following removal, sea urchins should be placed into a container lined with seawater-moistened paper towels to prevent reattachment.

15.10.3.1.3 Place each sand dollar, oral side up, on a 100 mL beaker filled with 15°C seawater or each sea urchin onto a clean tray covered with several layers of seawater moistened paper towels.

15.10.3.1.4 Handle sexes separately once known; this minimizes the chance of accidental egg fertilization. Throughout the test process, it is best if a different worker, different pipets, etc. are used for males (semen) and females (eggs). Frequent washing of hands is a good practice.

15.10.3.1.5 Fill a 3 or 5 mL syringe with 0.5 M KCl and inject

0.5 mL through the soft periostomal membrane of each sea urchin (See Figure 5) or into the oral opening each sand dollar. If sexes are known, use a separate needle for each sex. If sexes are not known, rinse the needle with hot tap water between each injection. This will avoid the accidental injection of sperm from males into females. Note the time of injection on the data sheet.

15.10.3.1.6 Spawning of sea urchins is sometimes induced by holding the injected sea urchin and gently shaking or swirling it for several seconds. This may provide an additional physical stimulus, or may aid in distributing the injected KCl.

15.10.3.1.7 Place the sea urchins onto the beakers or tray (oral side down). Place the sand dollars onto the beakers (oral side up). Females will release orange (sea urchins) or purple (sand dollars) eggs and males will release cream-colored semen.

15.10.3.1.8 As gametes begin to be shed, note the time on the data sheet and separate the sexes. Place male sand dollars with the oral side up atop a small (5-10 mL) glass beaker filled with 12°C seawater. Leave spawning sea urchin males on tray or beaker (oral side down) for semen collection. Female sand dollars and sea urchins are left to shed eggs into the 100-mL beakers.

15.10.3.1.9 If sufficient quantities of gametes are available, only collect gametes for the first 15 min after each animal starts releasing. This helps to insure good quality gametes. As a general guideline, do not collect gametes from any individual for more than 30 minutes after the first injection.

15.10.3.1.10 If no spawning occurs after 5 or 10 minutes, a second 0.5 mL injection may be tried. If animals do not produce sufficient gametes following injection of 1.0 mL of KCl, they should probably not be reinjected as this seldom results in acquisition of good quality gametes and may result in mortality of adult urchins.

15.10.3.1.11 Sections 15.10.4.2 and 15.10.6.4 describe collection and dilution of the sperm and eggs. While some of the gamete handling needs to be in a specific order, parts of the procedure can be done simultaneously while waiting for gametes to settle.

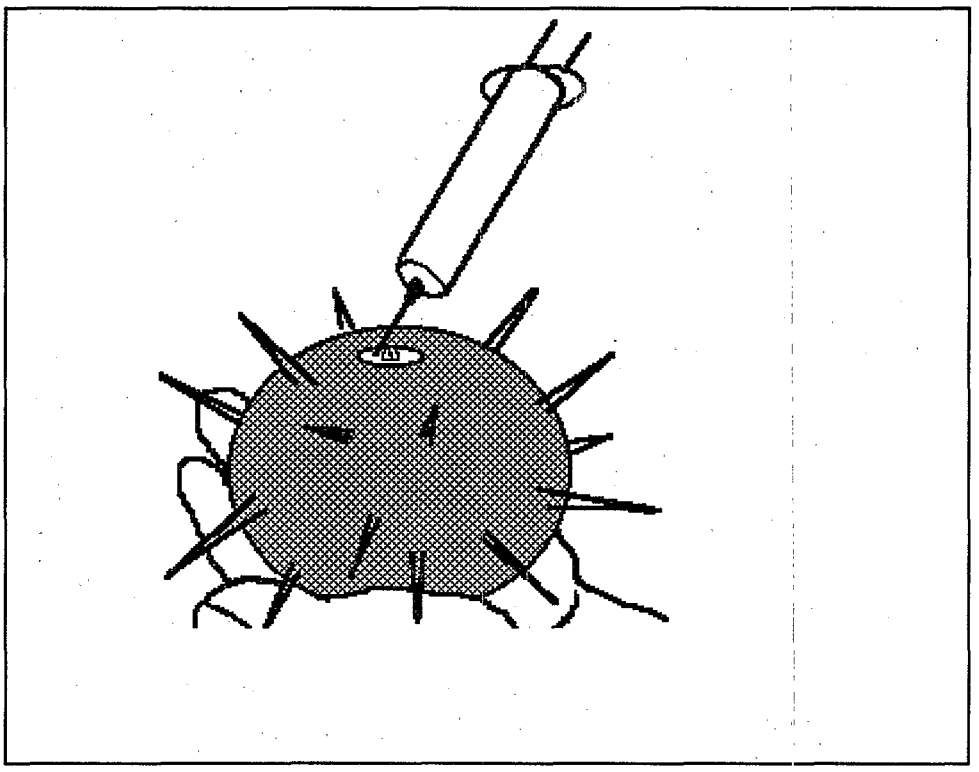


Figure 5. Showing the location and orientation used in the injection of KCl into sea urchins to stimulate spawning.

15.10.3.2 Collection of Sperm

15.10.3.2.1 Sea urchin semen should be collected dry (directly from the surface of the sea urchin), using either a Pasteur pipette or a 0.1 mL autopipette with the end of the tip cut off so that the opening is at least 2 mm. Pipette semen from each male into separate 1-15 mL conical test tubes, stored in an ice water bath.

15.10.3.3 Viability of Sperm

15.10.3.3.1 Early in the spawning process, place a very small amount of sperm from each male sea urchin or sand dollar into

dilution water on a microscope slide (well slides work nicely). Examine the sperm for motility; use sperm from males with high sperm motility.

15.10.3.4 Pooling of Sperm

15.10.3.4.1 Pool equal quantities of semen from each of the sea urchin males that has been deemed good. If possible, 0.025 mL should be pooled from each of those used and a total of at least 0.05 mL of pooled semen should be available. Sperm collected from good male sand dollars should be pooled after first decanting off the overlying water (the final sand dollar sperm density usually is between $2x10^9$ and $2x10^{10}$ sperm/mL).

15.10.3.5 Storage of Sperm

15.10.3.5.1 Cover each test tube or beaker with a cap or parafilm, as air exposure of semen may alter its pH through gas exchange and reduce the viability of the sperm. Keep sperm covered and on ice or refrigerated (<5°C). The sperm should be used within 4 h of collection.

15.10.4 PREPARATION OF SPERM DILUTION FOR USE IN THE TEST

15.10.4.1 Sperm Dilution

15.10.4.1.1 When ready to use sperm, mix by agitating the tube with a vortex mixer. Add about 0.025 mL of semen to a 100 mL beaker containing 50 mL of 15°C dilution water. Stir this solution thoroughly with a Pasteur pipette. A drop of egg solution from each female may be placed on a well slide and a small amount of sperm solution added to test fertilization. If no fertilization membrane forms on eggs from any female, then new gametes should be collected. Keep the sperm dilution covered and at 15°C until ready for use. This dilution should be used to fertilize the eggs within 1.5 hours of being made.

15.10.4.2 Sperm Density Determination

15.10.4.2.1 Take 0.5 mL subsample of the sperm solution and add it to 5 mL of 10% acetic acid in a 50 mL graduated cylinder, to kill the sperm. Bring the volume to 50 mL with dilution water. Mix by inversion and place one drop of the killed sperm solution

onto each side of a hemocytometer. Let sperm settle for about 15 minutes. Count the number of sperm in 80 small squares on each side of the hemocytometer. If the counts for each side are within 80% of one another, then take the mean of those two counts. If the counts are not that close, then refill the hemocytometer, recount and take the mean of the four counts. Use the following equations to determine sperm density and record the results on the spawning worksheet (Figure 2).

$$\#\text{sperm/mL} = \frac{(\text{dilution})(\text{count})(\text{hemacytometer conversion factor})(\text{mm3/mL})}{\text{number of squares counted}}$$

dilution=100
conversion factor=4000
mm^3/mL=1000
number of squares=80

Therefore:

$$\#\text{sperm/mL} = (\text{count})(5 \times 10^6) \qquad \text{(Equation 2A)}$$

15.10.5 PREPARATION OF EGG SUSPENSION FOR USE IN THE TEST

15.10.5.1 Acceptability of Eggs

15.10.5.1.1 Place a small sample of eggs from each female in the counting chamber and examine eggs with the microscope. Look for the presence of significant quantities of immature or abnormal appearing eggs (germinal vesicle present, unusually large or small or irregularly shaped). Do not use the eggs from females having more than 10% abnormal eggs or from females whose eggs did not fertilize during the test in Section 15.10.5.1.

15.10.5.2 Pooling of Eggs

15.10.5.2.1 Allow eggs to settle in the collection beakers. Decant some of the water from the collection beakers taking care not to pour off many eggs. Pour the remaining sea urchin eggs

through the Nitex® screen (to remove fecal material and other debris) into a 1 liter beaker. Repeat with each of the "good" females. Bring the volume up to about 600 mL with dilution water. Allow the eggs to settle to the bottom again. Siphon off about 400 mL of the overlying water and then bring back up to 600 mL with dilution water. Do not allow the temperature to rise above the 15°C test temperature; somewhat cooler temperatures for holding would be acceptable.

15.10.5.2.2 Pooled sand dollar eggs should be treated gently and no additional screening or rinsing step is recommended. Mix well once just before subsampling for egg stock calculations. This is best done in a large graduated cylinder appropriate for the number of eggs available. Cover with parafilm and invert gently several times.

15.10.5.3 Density of Eggs

15.10.5.3.1 Using a plunger, mix the sea urchin egg suspension well. While continuing to mix, remove a 10 mL sample and place in a 1 liter graduated cylinder. Bring the volume up to 1 liter with dilution water. Mix this dilution well and remove a 1 mL sample to a counting cell. Count all the eggs in the 1 mL sample. Repeat the process and take the mean of the two counts. Calculate the number of eggs per mL in the stock solution using Equation 3 and record the results.

# of eggs in count x 100= # eggs/mL in stock (Equation 3)

15.10.5.4 Dilution of Eggs

15.10.5.4.1 When using scintillation vials as the test chamber, the final concentration of eggs in the diluted stock must be 250 eggs/0.25 mL, which is equal to 1,000 eggs/mL. To calculate the dilution factor for the eggs, use Equation 4. (If larger test chambers are used, the total number of eggs used will be greater and the stock solution density may be adjusted, but the final concentration of eggs in the test solutions must remain 25 eggs/mL).

# of eggs/mL in stock ÷ 1,000= Dilution factor (Equation 4)

15.10.5.4.2 The dilution factor must be greater than one. If

not, concentrate the eggs and recount (starting at Section 15.4.5.3 The dilution factor minus 1 equals the number of parts of water that go with one part of eggs in the final dilution. For example: if the dilution factor were 5.3, then 4.3 parts of water would be used with 1 part eggs.

15.10.5.4.3 Make a dilution of the egg stock so that there is more than enough volume to perform the bioassay.

15.10.5.5 Fertilization of Eggs

15.10.5.5.1 The recommended initial sperm to egg ratio for fertilization of the eggs is 500:1. Calculate the volume of sperm dilution (Section 15.10.5.1) to add to the egg dilution, by using the following equations and record the results (Figure 3).

volume of egg dilution x 1,000 eggs/mL= total # of eggs in dilution (Equation 5A)

total # of eggs in dilution x 500 sperm/egg= # of sperm needed (Equation 5B)

# of sperm needed ÷ # sperm/mL in sperm dilution= mL of sperm solution (Equation 5C)

15.10.5.5.2 Add this volume of the sperm dilution to the egg dilution and mix gently with a plunger. Wait 10 min, then check for fertilization. If fertilization is not at least 90%, add a second volume of the sperm dilution. Wait 10 min and recheck. If fertilization is still not 90%, then the test must be restarted with different gametes.

15.10.5.5.3 The test should be initiated within 1 hour of fertilization being achieved.

15.10.6 START OF THE TEST

15.10.6.1 Prior to Beginning the Test

15.10.6.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case

should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

15.10.6.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (15 ± 1°C) and maintained at that temperature during the addition of dilution water.

15.10.6.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (15 ± 1°C).

15.10.6.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the sea urchins or sand dollars have been examined at the end of the test.

15.10.6.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

15.10.6.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly and filled with test solutions, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

15.10.6.1.6 If mortality is to be included as an endpoint, at least 5 extra control chambers should be set up and identified on the randomization sheet as initial count chambers.

15.10.6.2 Delivery of Fertilized Eggs

15.10.6.2.1 Gently mix the solution of fertilized eggs. Deliver 0.25 mL of egg solution to each vial, using an automatic pipette with the tip cut off to provide at least a 0.5 mm opening. Deliver the embryos into the test chambers directly from the pipette, taking care not to touch the pipette to the test solution. The egg solution temperature must be within 1°C of the test solutions. Keep the eggs well mixed during the delivery procedure.

15.10.6.3 Incubation

15.10.6.3.1 The embryos are incubated for 72 hours in the test chambers at 15 ± 1°C at ambient light level.

15.10.6.3.2 The optional extra control chambers for initial counts should be counted as soon as possible after test initiation. If they are sampled and counted in a non-destructive manner they may be returned to the test but used only as a check for larval developmental rate. They must not be used for for routine control counts at the end of the test.

15.10.7 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

15.10.7.1 The light quality and intensity should be at ambient laboratory conditions. Light intensity should be 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle.

15.10.7.2 The water temperature in the test chambers should be maintained at 15 ± 1°C. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

15.10.7.3 The test salinity should be in the range of 34 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.10.7.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the

test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

15.10.8 DISSOLVED OXYGEN (DO) CONCENTRATION

15.10.8.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

15.10.9 OBSERVATIONS DURING THE TEST

15.10.9.1 Routine Chemical and Physical Observations

15.10.9.1.1 The DO should be measured in each test solution at the beginning of the exposure period.

15.10.9.1.2 The temperature, pH, and salinity should be measured in all each test solution at the beginning of the exposure period. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

15.10.9.1.3 Record all the measurements on the data sheet.

15.10.9.2 Routine Biological Observations

15.10.9.2.1 Developing embryos do not need to be monitored during the test under normal circumstances.

15.10.10 TERMINATION OF THE TEST

15.10.10.1 Ending the Test

15.10.10.1.1 Record the time the test is terminated.

15.10.10.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control(s).

15.10.10.2 Sample Preservation

15.10.10.2.1 To terminate the test, add 1.0 mL of 37% (concentrated) buffered formalin to each sample to give a final formalin concentration of 4%. As an alternate fixative, 0.5 mL of 1.0% glutaraldehyde may be used, in each test chamber. Tightly cap and gently mix each chamber and store for later evaluation. (If the test is performed in larger chambers, a 10 mL subsample of well mixed test solution is to be taken from each chamber and preserved).

15.10.10.3 Counting

15.10.10.3.1 It is recommended that the embryos be examined within one week of preservation. Longer storage times may also be used, but run the risk of sample degradation due to improper preservation. Larvae can be counted directly in the scintillation vials using an inverted microscope. If an inverted scope is not available, then samples should be loaded into a Sedgewick-Rafter cell, as follows. The embryos should first be allowed to settle to the bottom of the sample chamber. All but about 1 mL of the overlying liquid should then be removed. All of the remaining liquid containing the embryos should then be transferred to the counting chamber. Whichever scope is used, the embryos should be examined at about 100x power. The first 100 embryos encountered are counted using a multi-unit handcounter to track normal versus abnormal larvae. Record the data by sample number on a data sheet (Figure 4).

15.10.10.3.2 Mortality can be determined only if: (1) all surviving larvae are counted (either in the test vials with an inverted microscope or by <u>total</u> transfer to a counting chamber); or (2) the test solution is stirred with a plunger and

quantitative subsampling is conducted followed by total larval counts on the subsample. The latter procedure requires homogeneous distribution of larvae in the test solution, quantitative transfer of larvae (without adherence to transfer hardware or test chambers), and accurate volume measurements. Mortality is most important to consider with point estimates (e.g., EC25) or when mortality occurs at the NOEC for normal development.

15.10.10.4 Endpoint

15.10.10.4.1 Normal Larvae

15.10.10.4.1.1 Normally developed pluteus larvae have several distinctive characteristics:

(1) The larvae should have a pyramid shape with a pair of skeletal rods that extend at least half the length of the long axis of the larvae (Figure 6D).

(2) The gut should be differentiated into three parts (Figure 6E). If the gut appears lobed and constricts distally in specimens with an obstructed view (e.g., Figure 6D), then normal gut development may be inferred.

(3) Development of post-oral arms has begun.

15.10.10.4.2 Abnormal Larvae

15.10.10.4.2.1 Larvae need only be scored as abnormal or normal to conduct the test, but the categories of abnormalities may be tracked as well. Abnormal larvae should fit into one of the following categories:

(1) Pathological prehatched: Embryos at the single or multi-cell stage with the fertilization membrane still visible.

(2) Pathological hatched: larvae that have no fertilization membrane and demonstrate an extensive

degree of malformation or necrosis. Most of these larvae appear as dark balls of cells or dissociated blobs of cells.

(3) Inhibited: larvae at the blastula or gastrula stage that have no gut differentiation or have no or underdeveloped skeleton. These larvae appear to be developing regularly, but are at a stage earlier than attained by control organisms (e.g., Figure 6A-C).

(4) Gut abnormalities: larvae whose overall appearance is normal, but have guts that are lacking, undifferentiated, abnormally shaped or project outside of the larvae (exogastrulated).

(5) Skeletal abnormalities: larvae whose overall appearance is normal, but have missing spicules, extraneous spicules or rods growing in abnormal directions. Note: Some larvae may exhibit a separation of the rods at the apex. This may be caused by preservation and should not be termed abnormal. Since the test is started with already fertilized eggs, any unfertilized eggs that are encountered should not be counted as either normal or abnormal, but should be ignored.

15.11 SUMMARY OF TEST CONDITIONS

15.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

15.12 ACCEPTABILITY OF TEST RESULTS

15.12.1 Test results are acceptable only if all the following requirements are met:

(1) larval normality must be at least 80% in the controls.

(2) the minimum significant difference (%MSD) is ≤20% relative to the controls.

15.13 DATA ANALYSIS

15.13.1 GENERAL

15.13.1.1 Tabulate and summarize the data. Calculate the

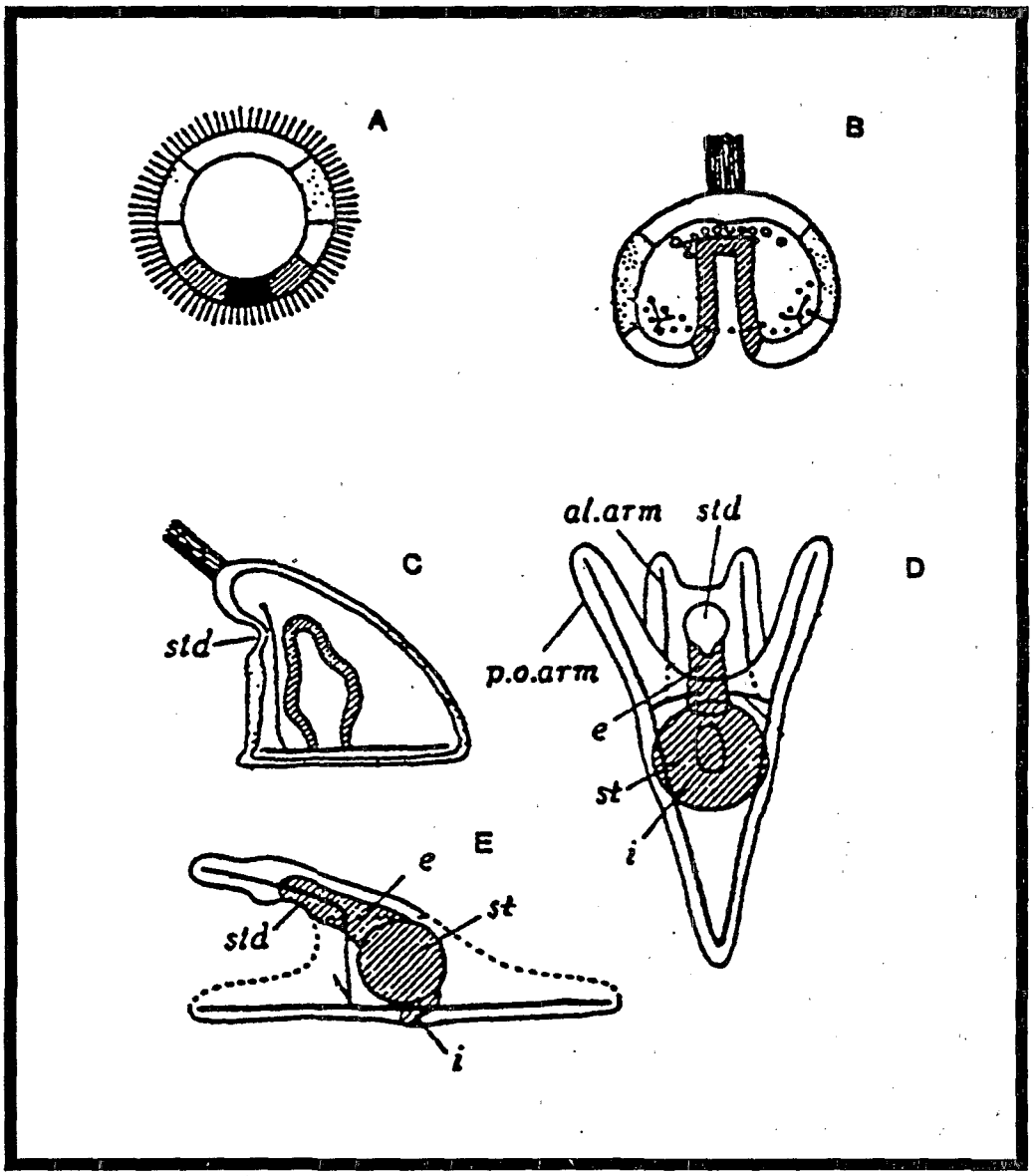


Figure 6. Stages of sea urchin embryo development (modified from Kume and Dan 1957). A. blastula; B. gastrula; C. prism; D. pluteus (frontal view); E. pluteus (lateral view). *al.arm*: anterior lateral arm, *e*: esophagus, *i*: intestine, *st*: stomach, *std*: stomodaeum.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE PURPLE URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, AND SAND DOLLAR, *DENDRASTER EXCENTRICUS* EMBRYO DEVELOPMENT TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	15 ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m_2/s$ (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	30 mL
8. Test solution volume:	10 mL
9. No. replicate chambers per concentration:	4
10. Dilution water:	Uncontaminated 1-μm-filtered natural seawater or hypersaline brine prepared from natural seawater
11. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
12. Dilution factor:	Effluents: ≥0.5 Receiving waters: 100% receiving water and a control
13. Test duration:	72 ± 2 hr
14. Endpoint:	Normal development; mortality can be included
15. Test acceptability criteria:	≥80% normal shell development in the controls; must achieve a %MSD of <25%

16. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
17. Sample volume required:	1 L per test

proportion of normally developed larvae for each replicate. A sample set of test data is listed in Table 4.

15.13.1.2 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

15.13.1.3 The endpoints of toxicity tests using the purple sea urchin are based on the reduction in proportion of normally developed larvae. The IC25 is calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for development are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

15.13.2 EXAMPLE OF ANALYSIS OF PURPLE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, DEVELOPMENT DATA

15.13.2.1 Formal statistical analysis of the larval development data is outlined in Figure 7. The response used in the analysis is the proportion of normally developed larvae in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there are no normally developed larvae in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25.

TABLE 4. DATA FROM PURPLE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, DEVELOPMENT TEST

Copper Concentration (µg/L)	Replicate	No. Larvae Exposed	No. Larvae Normally Developed	Proportion Normal
Control	A	100	87	0.87
	B	100	89	0.89
	C	100	81	0.81
	D	101	89	0.88
	E	74	62	0.84
3.2	A	110	98	0.89
	B	100	82	0.82
	C	100	91	0.91
	D	100	83	0.83
	E	100	89	0.89
5.6	A	102	86	0.84
	B	100	89	0.89
	C	100	85	0.85
	D	107	90	0.84
	E	100	85	0.85
10.0	A	100	70	0.70
	B	100	71	0.71
	C	100	77	0.77
	D	100	74	0.74
	E	100	87	0.87
18.0	A	100	7	0.07
	B	100	12	0.12
	C	100	14	0.14
	D	100	16	0.16
	E	100	10	0.10

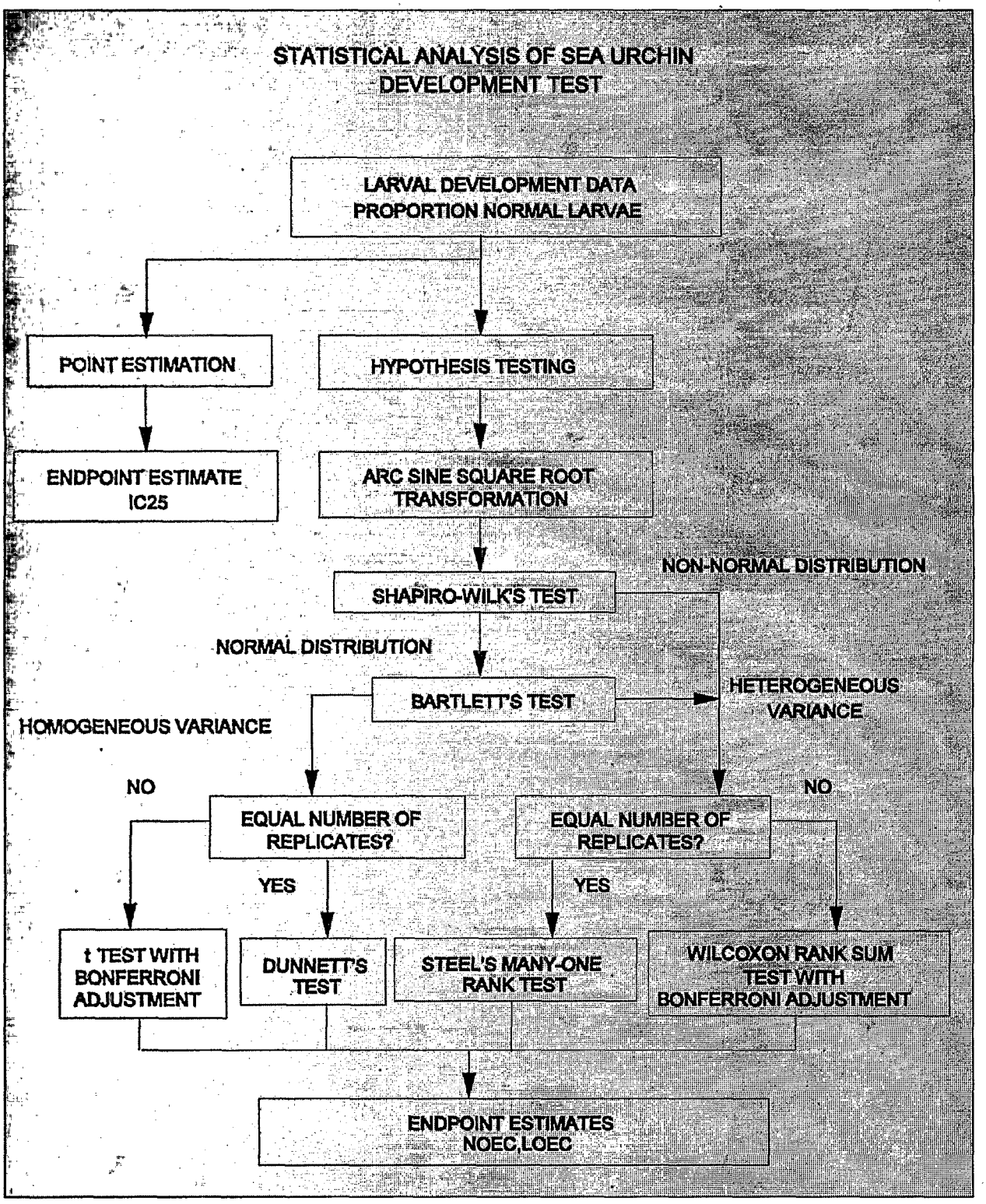


Figure 7. Flowchart for statistical analysis of sea urchin, *Strongylocentrotus purpuratus*, development test.

15.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

15.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

15.13.2.4 Example of Analysis of Development Data

15.13.2.4.1 This example uses toxicity data from a purple sea urchin, *Strongylocentrotus purpuratus*, development test performed with copper. The response of interest is the proportion of normally developed larvae, thus each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each copper concentration and control are listed in Table 5. The data are plotted in Figure 8. Because there is zero normal development in all five replicates of the 32.0 µg/L copper concentration, it was not included in the statistical analysis and is considered a qualitative development effect.

15.13.2.5 Test for Normality

15.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

15.13.2.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations

15.13.2.5.3 For this set of data, n = 25

$$\overline{X} = \frac{1}{25}(-0.001) = 0.000$$

$$D = 0.0680$$

TABLE 5. SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, DEVELOPMENT DATA

		Copper Concentration (µg/L)				
	Replicate	Control	3.2	5.6	10.0	18.0
	A	0.87	0.89	0.84	0.70	0.07
	B	0.89	0.82	0.89	0.71	0.12
RAW	C	0.81	0.91	0.85	0.77	0.14
	D	0.88	0.83	0.84	0.74	0.16
	E	0.84	0.89	0.85	0.87	0.10
	A	1.202	1.234	1.159	0.991	0.268
ARC SINE	B	1.234	1.133	1.234	1.002	0.354
SQUARE ROOT	C	1.120	1.266	1.173	1.071	0.383
TRANSFORMED	D	1.217	1.146	1.159	1.036	0.412
	E	1.159	1.234	1.173	1.202	0.322
Mean ($\overline{Y}_i$)		1.186	1.203	1.180	1.060	0.348
S_i^2		0.00215	0.00351	0.00097	0.00725	0.00311
i		1	2	3	4	5

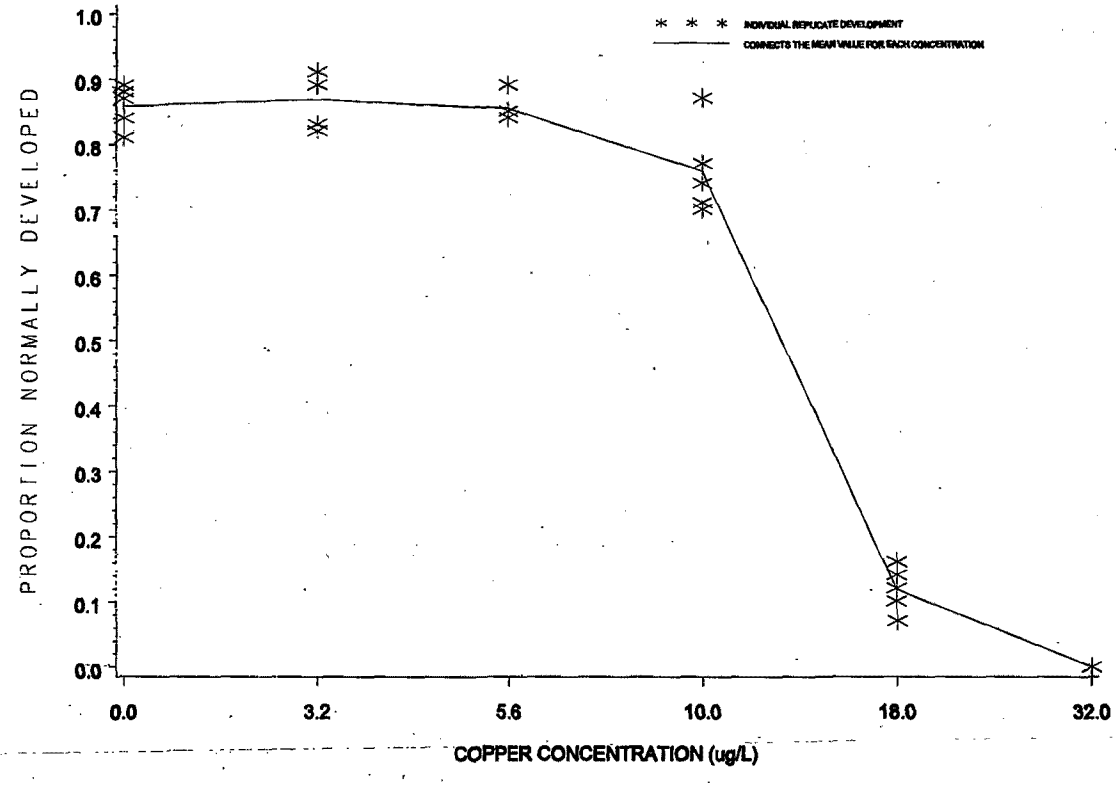


Figure 8. Plot of proportion of normally developed sea urchin, *Strongylocentrotus purpuratus*, larvae,

15.13.2.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 7.

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Copper Concentration (μg/L)				
Replicate	Control	3.2	5.6	10.0	18.0
A	0.016	0.031	-0.021	-0.069	-0.080
B	0.048	-0.070	0.054	-0.058	0.006
C	-0.066	0.063	-0.007	0.011	0.035
D	0.031	-0.057	-0.021	-0.024	0.064
E	-0.027	0.031	-0.007	0.142	-0.026

15.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 25 and k = 12. The a_i values are listed in Table 8.

15.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 8. For the data in this example:

$$W = \frac{1}{0.0680} (0.2545)^2 = 0.953$$

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.080	14	0.006
2	-0.070	15	0.011
3	-0.069	16	0.016
4	-0.066	17	0.031
5	-0.058	18	0.031
6	-0.057	19	0.031
7	-0.027	20	0.035
8	-0.026	21	0.048
9	-0.024	22	0.054
10	-0.021	23	0.063
11	-0.021	24	0.064
12	-0.007	25	0.142
13	-0.007		

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4450	0.222	$X^{(25)} - X^{(1)}$
2	0.3069	0.134	$X^{(24)} - X^{(2)}$
3	0.2543	0.132	$X^{(23)} - X^{(3)}$
4	0.2148	0.120	$X^{(22)} - X^{(4)}$
5	0.1822	0.106	$X^{(21)} - X^{(5)}$
6	0.1539	0.092	$X^{(20)} - X^{(6)}$
7	0.1283	0.058	$X^{(19)} - X^{(7)}$
8	0.1046	0.057	$X^{(18)} - X^{(8)}$
9	0.0823	0.055	$X^{(17)} - X^{(9)}$
10	0.0610	0.037	$X^{(16)} - X^{(10)}$
11	0.0403	0.032	$X^{(15)} - X^{(11)}$
12	0.0200	0.013	$X^{(14)} - X^{(12)}$

15.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For

the data in this example, the critical value at a significance level of 0.01 and n = 25 observations is 0.888. Since W = 0.953 is greater than the critical value, conclude that the data are normally distributed.

15.13.2.6 Test for Homogeneity of Variance

15.13.2.6.1 The test used to examine whether the variation in the proportion of normally developed larvae is the same across all copper concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i) \ln \overline{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each concentration and control,

$V_i = (n_i - 1)$

p = number of concentration levels including the control

n_i = the number of replicates for concentration i.

ln = $\log_e$

i = 1,2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

15.13.2.6.2 For the data in this example (see Table 5), all concentrations including the control have the same number of replicates (n_i = 5 for all i). Thus, V_i = 4 for all i.

15.13.2.6.3 Bartlett's statistic is, therefore:

$$B = [(20)\ln(0.00340) - 4\sum_{i=1}^{p}\ln(S_i^2)] / 1.100$$

$$= [20(-5.6840) - 4(-29.4325)]/1.100$$

$$= 4.050/1.100$$

$$= 3.6818$$

15.13.2.6.4 B is approximately distributed as chi-square with p-1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with 4 degrees of freedom, is 13.28. Since B = 3.6818 is less than the critical value of 13.28, conclude that the variances are not different.

15.13.2.7 Dunnett's Procedure

15.13.2.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 9.

Where: p = number of concentration levels including the control

TABLE 9. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$ Between Sum of Squares

$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the proportion of normal larvae for concentration i in test chamber j)

15.13.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 5$

$N = 25$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 5.932$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 6.013$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 5.898$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 5.302$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} + Y_{55} = 1.739$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 24.884$

$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$
$(137.267)/5 - (24.884)^2/25 = 2.685$

$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$
$27.521 - (24.884)^2/25 = 2.752$

$SSW = SST - SSB = 2.752 - 2.685 = 0.067$

$S_B^2 = SSB/(p-1) = 2.685/(5-1) = 0.6713$

$S_W^2 = SSW/(N-p) = 0.067/(25-5) = 0.0034$

15.13.2.7.3 Summarize these calculations in the ANOVA table (Table 10).

TABLE 10. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	4	2.685	0.6713
Within	20	0.067	0.0034
Total	24	2.752	

15.13.2.7.4 To perform the individual comparisons, calculate the *t* statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\overline{Y}_1 - \overline{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\overline{Y}_i$ = mean proportion normal larvae for concentration i

$\overline{Y}_1$ = mean proportion normal larvae for the control

S_w = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

Since we are looking for a decreased response from the control in the proportion of normally developed larvae, the concentration mean is subtracted from the control mean.

15.13.2.7.5 Table 11 includes the calculated t values for each concentration and control combination. In this example, comparing the 3.2 µg/L copper concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.186 - 1.203)}{0.0583\sqrt{(1/5)+(1/5)}} = -0.461$$

TABLE 11. CALCULATED t VALUES

Copper Concentration (µg/L)	i	t_i
3.2	2	-0.461
5.6	3	0.163
10.0	4	3.417
18.0	5	22.727

15.13.2.7.6 Since the purpose of this test is to detect a significant decrease in the proportion of normally developed larvae, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 20 degrees of freedom for error and four concentrations (excluding the control) the critical value is 2.30. The mean proportion of normally developed larvae for concentration i is considered significantly less than the mean proportion of normally developed larvae for the control if t_i is greater than the critical value. Therefore, the 10.0 µg/L and 18.0 µg/L concentrations have a significantly lower mean proportion of normally developed larvae than the control. Hence the NOEC is 5.6 µg/L copper and the LOEC is 10.0 µg/L copper.

15.13.2.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\ S_w\sqrt{(1/n_1)+(1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

15.13.2.7.8 In this example,

$$MSD = 2.30\ (0.0583)\ \sqrt{(1/5) + (1/5)}$$

$$= 2.30\ (0.0583)\ (0.6325)$$

$$= 0.085$$

15.13.2.7.9 The MSD (0.085) is in transformed units. To determine the MSD in terms of proportion of normally developed larvae, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

$$1.186 - 0.085 = 1.101$$

2. Obtain the untransformed values for the control mean and the difference calculated in step 1 of 13.2.7.9.

$$[\ \text{Sine}\ (1.186)\ ]^2 = 0.859$$

$$[\ \text{Sine}\ (1.101)\ ]^2 = 0.795$$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from step 2 in 14.2.7.9.

$$MSD_u = 0.859 - 0.795 = 0.064$$

15.13.2.7.10 Therefore, for this set of data, the minimum difference in mean proportion of normally developed larvae between the control and any copper concentration that can be detected as statistically significant is 0.064.

15.13.2.7.11 This represents a 7.5% decrease in the proportion of normally developed larvae from the control.

15.13.2.8 Calculation of the ICp

15.13.2.8.1 The development data in Table 4 are utilized in this example. As can be seen from Figure 9, the observed means are not monotonically non-increasing with respect to concentration.

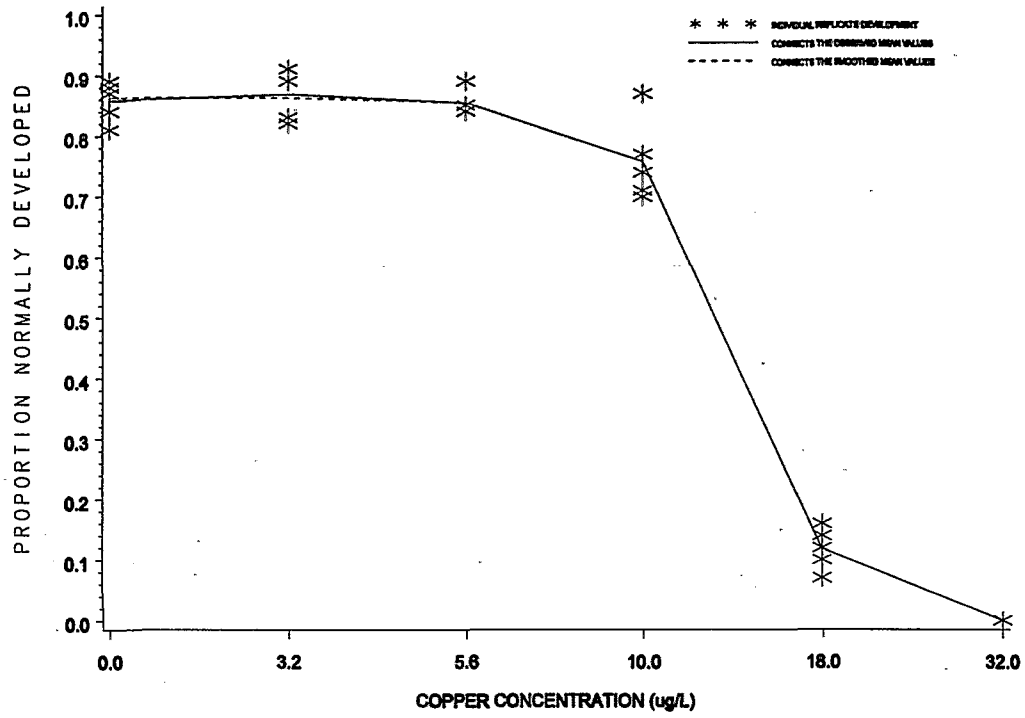


Figure 9. Plot of raw data, observed means, and smoothed means for the sea urchin, *Strogylocentrotus purpuratus*, larval development data from Tables 4 and 12.

Therefore, the means must be smoothed prior to calculating the IC.

15.13.2.8.2 Starting with the observed control mean, $Y_1 = 0.858$, and the observed mean for the lowest copper concentration, $Y_2 = 0.868$, we see that Y_1 is less than Y_2.

15.13.2.8.3 Calculate the smoothed means:

$$M_1 = M_2 = (Y_1 + Y_2)/2 = 0.863$$

15.13.2.8.4 Since $Y_3 = 0.854 > Y_4 = 0.758 > Y_5 = 0.118 > Y_6 = 0.0$, set $M_3 = 0.854$, $M_4 = 0.758$, $M_5 = 0.118$, and $M_6 = 0.0$. Table 12 contains the smoothed means and Figure 8 gives a plot of the smoothed means and the interpolated response curve.

15.13.2.8.5 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of normally developed larvae, compared to the controls, would result in a mean proportion of 0.647, where $M_1(1-p/100) = 0.863(1-25/100)$. Examining the means and their associated concentrations (Table 12), the response, 0.647, is bracketed by $C_4 = 10.0$ µg/L copper and $C_5 = 18.0$ µg/L copper.

TABLE 12. SEA URCHIN, *STRONYLOCENTROTUS PURPURATUS*, MEAN PROPORTION OF NORMALLY DEVELOPED LARVAE

Copper Conc. (µg/L)	i	Response Means, Y_i (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.858	0.863
0.05	2	0.868	0.863
0.10	3	0.854	0.854
0.15	4	0.758	0.758
0.20	5	0.118	0.118
0.40	6	0.000	0.000

15.13.2.8.6 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1-p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	3.2	5.6	10	18	32
Response 1	.87	.89	.84	.70	.07	0
Response 2	.89	.82	.89	.71	.12	0
Response 3	.81	.91	.85	.77	.14	0
Response 4	.88	.83	.84	.74	.16	0
Response 5	.84	.89	.85	.87	.10	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper Chloride
Test Start Date: Test Ending Date:
Test Species: Purple Sea Urchin, Strongylocentrotus purpuratus
Test Duration: 72 hours
DATA FILE: urch_dev.icp
OUTPUT FILE: urch_dev.i25

Conc. ID	Number Replicates	Concentration ug/L	Response Means	Std. Dev.	Pooled Response Means
1	5	0.000	0.858	0.033	0.863
2	5	3.200	0.868	0.040	0.863
3	5	5.600	0.854	0.021	0.854
4	5	10.000	0.758	0.068	0.758
5	5	18.000	0.118	0.035	0.118
6	5	32.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 11.3844 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 11.3702 Standard Deviation: 0.2898
Original Confidence Limits: Lower: 10.7785 Upper: 11.9375
Expanded Confidence Limits: Lower: 10.4756 Upper: 12.2141
Resampling time in Seconds: 0.16 Random_Seed: 83761380

Figure 10. ICPIN program output for the IC25.

$$IC25 = 10.0 + [0.863(1 - 25/100) - 0.758] \frac{(18.0 - 10.0)}{(0.118 - 0.758)}$$

$$= 11.38 \ \mu g/L.$$

15.13.2.8.7 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 11.3844 µg/L. The empirical 95.0% confidence interval for the true mean was 10.7785 µg/L to 11.9375 µg/L. The computer program output for the IC25 for this data set is shown in Figure 10.

15.14 **PRECISION AND ACCURACY**

15.14.1 PRECISION

15.14.1.1 Single Laboratory Precision

15.14.1.1.1 Data on the single-laboratory precision of the development test using copper as a reference toxicant is provided in Table 13. The NOEC varied by only one concentration interval indicating good precision. The coefficient of variation for the EC50 and EC25 were 22% and 21% indicating acceptable precision.

15.14.1.2 Multi-Laboratory Precision

15.14.1.2.1 Data on the multi-laboratory precision of the development test using copper as a reference toxicant is provided in Table 14. The NOEC for laboratory's A and B were identical. The difference in NOEC observed for lab C was probably due the wide range of concentrations used (See Footnote 4). The coefficient of variation for the EC50 was 39%, indicating acceptable interlaboratory precision.

15.14.2 ACCURACY

15.14.2.1 The accuracy of tox1icity tests cannot be determined.

Table 13. SINGLE-LABORATORY PRECISION OF THE PURPLE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, DEVELOPMENT TEST WITH COPPER (CU µG/L) SULFATE AS A REFERENCE TOXICANT[1].

Test Number	NOEC (µg/L)	EC50 (µg/L)	EC25 (µg/L)
1	10.0	19.4	15.1
2	10.0	18.3	15.4
3	5.6	10.8	9.0
4	5.6	14.3	11.0
5	5.6	16.8	12.9
Mean CV(%)		15.9 22.0	12.7 21.0

[1] Tests performed by Marine Pollution Studies Laboratory, Granite Canyon, Monterey California.

TABLE 14. MULTI-LABORATORY PRECISION OF THE PURPLE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, DEVELOPMENT TEST WITH COPPER (CU µG/L) SULFATE AS A REFERENCE TOXICANT.[1]

Lab	NOEC (µg/L)	EC50 (µg/L)
A[2]	10.0	22.5
B[3]	10.0	15.2
C[4]	1.8	10.1
Mean CV(%)		15.9 39.0

[1]Data from labs A and B are from an interlaboratory study using split reference toxicant samples and dilution water. Test performed in August, 1993. Test duration was 72 hr. Concentrations were 3.2, 5.6, 10, 18 and 32 µg/L.

[2]Test performed by Southern California Coastal Water Research Project, Westminster, CA.

[3]Test performed by Marine Pollution Studies Laboratory, Granite Canyon, Monterey California.

[4] Test performed by MEC Analytical Systems, Inc., Tiburon, CA. Test performed in April, 1994. Test duration was 96 hr. Concentrations were 0.1, 0.32, 1.8, 18 and 56 µg/L.

APPENDIX I. SEA URCHIN DEVELOPMENT: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution.

D. Prepare a copper reference toxicant series. Add 10 mL of test solution each vial.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen of each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 15°C and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARTION AND ANALYSIS OF TEST ORGANISMS

A. Obtain test organisms and hold or condition as necessary for spawning.

B. Place six 100 mL beakers of dilution water in 15°C water bath or room. Select 6-8 sea urchins and place on tray covered with seawater moistened paper towels. Induce

spawning by injecting each sea urchin with 0.5 mL of 0.5 M KCl. Place animals back onto tray, oral side down.

C. When spawning begins, note time that each animal begins spawning. Leave males on tray for semen collection. Place spawning females oral side up on 100 mL beakers. Do not collect gametes more than 15 min after spawning begins.

D. Collect semen using either a Pasteur pipette or a 100 µL autopipette. Pipette semen from each male into a separate 5 mL conical test tube, stored in an ice water bath.

E. Check for the motility of sperm from each male.

F. Pool semen by pipetting equal amounts from each "good" male to another centrifuge tube. At least 0.025 mL should be taken from each male and a total of at least 0.05 mL should be available. Cover the tube and store in a refrigerator until ready for use.

G. Finish collecting eggs before diluting semen.

H. Mix pooled semen by agitating on a vortex mixer. Add about 0.025 mL of semen to a 100 mL beaker containing 50 mL of 15°C dilution water. Stir thoroughly with a Pasteur pipette. Test eggs from each female to determine if they can be fertilized.

I. Take 0.5 mL subsample of sperm dilution and add to 5 mL of 10% acetic acid in a 50 mL graduated cylinder. Bring to 50 mL with dilution water. Mix well by inversion and load a drop into each side of hemocytometer. Count the sperm in 80 small squares. Calculate the sperm density using Equation 2A.

J. Examine sample of eggs from each female. Do not use the eggs from any female whose eggs appear abnormal or that did not fertilize in Section G.

K. Decant water from eggs of each usable female and pour through Nitex® screen into a 1 liter beaker. Bring volume up to about 600 mL with dilution water. Allow to resettle, siphon about 400 mL of overlying water and bring back to 600 mL with dilution water.

L. Mix egg solution well and make an accurate 100x dilution using at least 10 mL of the egg solution. Mix the dilution well and count two different 1 mL subsamples in a counting

cell. Use the mean of the two counts in Equation 3 to determine the density of the egg stock.

M. Use Equation 4 to determine the egg dilution factor and make dilution of eggs with dilution water.

N. Use Equations 5 A-C to determine the volume of the sperm dilution that is necessary to fertilize the egg dilution. Add the appropriate volume of sperm and after 10 minutes, check fertilization success.

O. Gently mix the fertilized egg solution with a plunger and deliver 0.25 mL of egg solution to each vial. Make sure that the pipette tip is cut off to provide at least a 0.5 mm opening. Keep egg solution well mixed during addition period.

P. Incubate the embryos for 72 hours at 15 ± 1°C.

Q. Test termination and analysis

R. Perform water quality measurements as at the start.

S. After 72 hours, add 1.0 mL of 37% buffered formalin or 0.5 mL of 1.0% glutaraldehyde to each test chamber. Tightly cap and gently mix each vial.

T. Examine each sample with a microscope and determine the percentage of normally developed embryos.

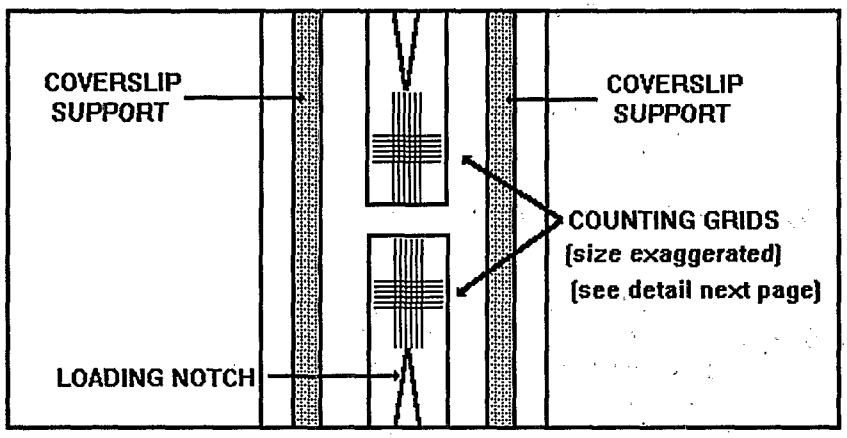
U. Analyze the data.

V. Include standard reference toxicant point estimate values in the standard quality control charts.

APPENDIX II. USING THE NEUBAUER HEMACYTOMETER TO ENUMERATE SEA URCHIN SPERM

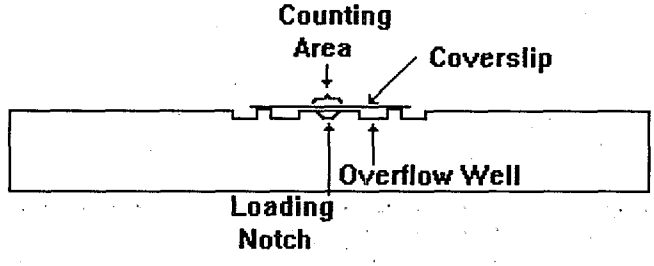
The Neubauer hemacytometer is a specialized microscope slide with two counting grids and a coverslip.

TOP VIEW:

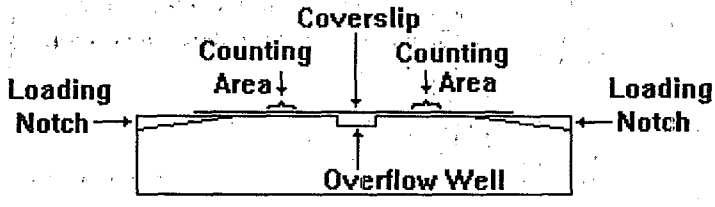


Together, the total area of each grid (1 mm^2) and the vertical distance between the grid and the coverslip (0.1 mm), provide space for a specific microvolume of aqueous sample (0.1 mm^3).

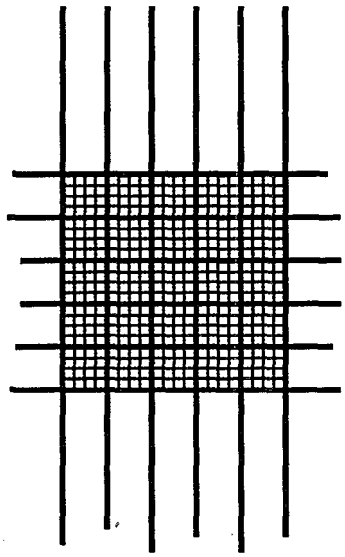
SIDE VIEW:



END VIEW THROUGH MID-CROSS SECTION:



This volume of liquid and the cells suspended therein (e.g., blood cells or sperm cells) represent 1/10,000th of the liquid volume and cell numbers of a full milliliter (cm^3) of the sampled material.



NEUBAUER
HEMACYTOMETER
GRID OF 400 SQUARES

If the full 400-squares of each grid are counted, this represents the number of sperm in 0.1 mm^3. Multiplying this value times 10 yields the sperm per mm^3 (and is the source of the hemacytometer factor of 4,000 squares/mm^3). If this product is multiplied by 1,000 mm^3/cm^3, the answer is the number of sperm in one milliliter of the sample. If the counted sample represents a dilution of a more concentrated original sample, the above answer is multiplied by the dilution factor to yield the cell density in the original sample. If the cells are sufficiently dense, it is not necessary to count the entire 400-square field, and the final calculation takes into account the number of squares actually counted:

$$\text{cells/mL} = \frac{(\text{dilution})\ (4{,}000\ \text{squares/mm}^3)\ (1{,}000\ \text{mm}^3/\text{cm}^3)\ (\text{cell count})}{(\text{number of squares counted})}$$

Thus, with a dilution of 4000 (0.025 mL of semen in 100 mL of dilution water), 80 squares counted, and a count of 100, the calculation becomes:

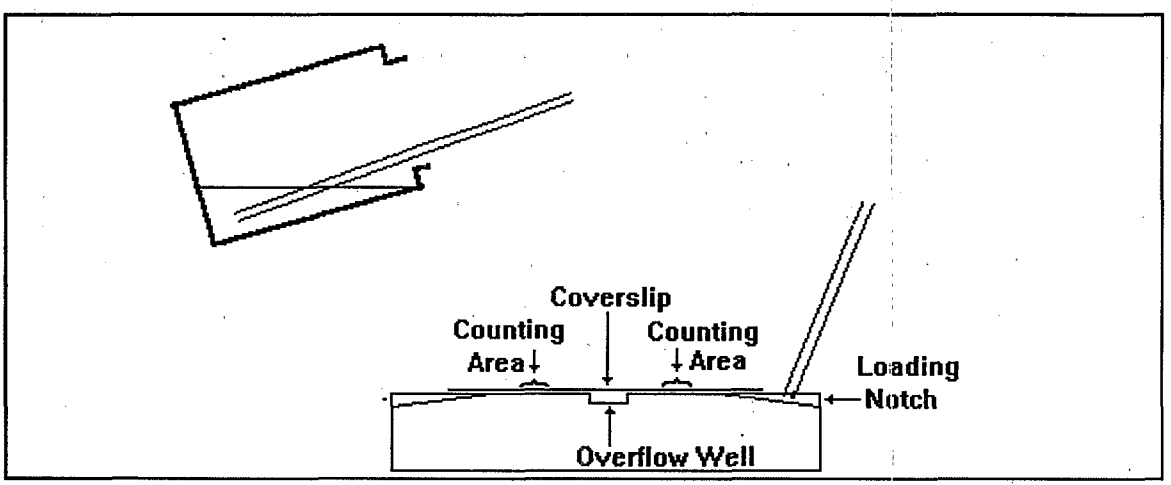
$$\text{cells/mL} = \frac{(4{,}000)\ (4{,}000)\ (1{,}000)\ (100)}{80}$$

$$= 20{,}000{,}000{,}000\ \text{cells/mL}$$

There are several procedures that are necessary for counts to be consistent within and between laboratories. These include mixing the sample, loading and emptying the hematocrit tube, cleaning the hemacytometer and cover slip, and actual counting procedures.

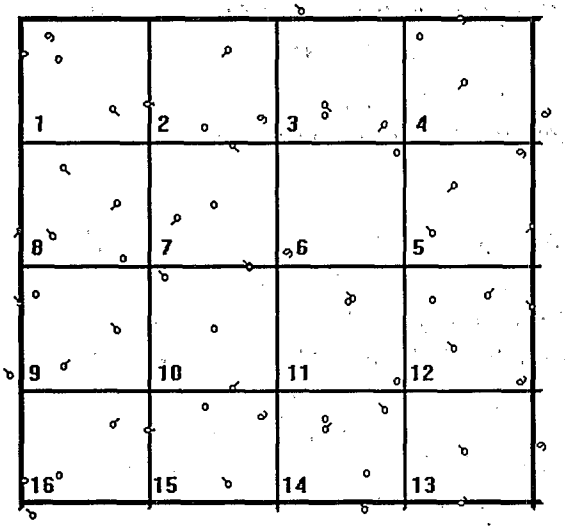
Obviously, if the sample is not homogeneous, subsamples can vary in sperm density. A few extra seconds in mixing can save a lot of wasted minutes in subsequent counting procedures. A full hematocrit tube empties more easily than one with just a little liquid, so withdraw a full sample. This can be expedited by tipping the sample vial.

Because the sperm are killed prior to sampling, they will slowly settle. For this reason, the sample in the hematocrit tube should be loaded onto the hemacytometer as rapidly as possible. Two replicate samples are withdrawn in fresh hematocrit tubes and loaded onto opposite sides of a hemacytometer.

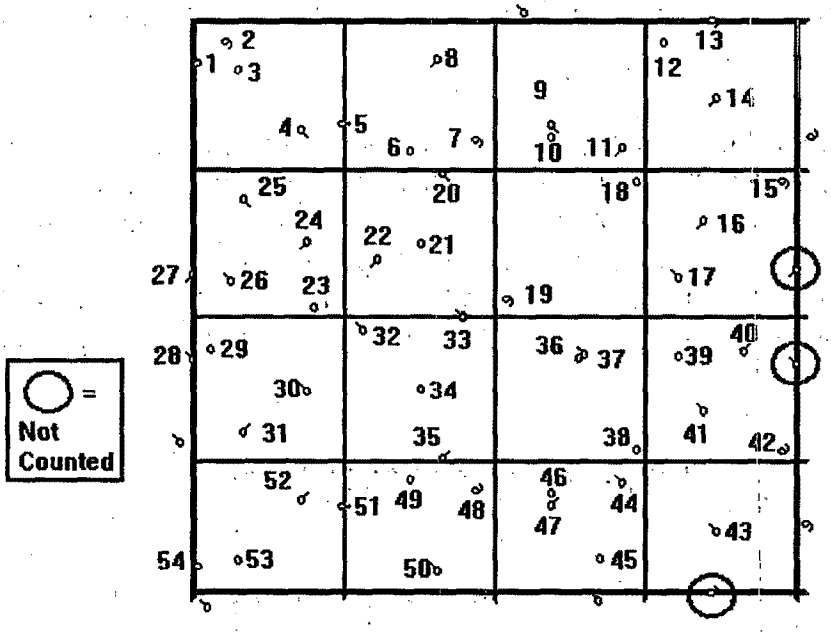


The loaded hemacytometer is left for 15 minutes to allow the sperm to settle onto the counting field. If the coverslip is moved after the samples are loaded, the hemacytometer should be rinsed and refilled with fresh sample. After 15 minutes, the hemacytometer is placed under a microscope and the counting grid located at 100x. Once the grid is properly positioned, the microscope is adjusted to 200x or 400x, and one of the corner squares is positioned for counting (any one of the four corners is appropriate). For consistency, use the same procedure each time (Many prefer to start in the upper left corner of the optical field, and this procedure will be used in the examples given below). Examine the first large square in the selected corner. If no sperm are visible, or if the sperm are so dense or clumped to preclude accurate counting, count a sample with a more appropriate dilution.

In making counts of sperm, it is necessary to adopt a consistent method of scanning the smaller squares and counting sperm that fall upon the lines separating the squares. Count the sperm in the small squares by beginning in the upper left hand corner (square 1) and proceding right to square 4, down to square 5, left to square 8, etc. until all 16 squares are counted.



Because sperm that appear <u>on</u> lines might be counted as being in either square, it is important to avoid double counting or non-counting. For this reason a convention is decided upon and used consistently: paraphrasing the instructions received with one (Hausser Scientific) counting chamber "to avoid counting (sperm) twice, the best practice is to count all touching the top and left, and none touching the lower and right, boundary lines." Whatever convention is chosen, it must be adhered to. The example below shows a sperm count based upon a selected convention of counting sperm that fall on the upper and left lines, but not on the lower or right lines:



In the above illustration, sperm falling on the lower and right lines are not counted. The count begins at the upper left as illustrated in the preceding figure. A typical count sequence is demonstrated by the numbers next to each sperm illustrated. Sperm identified as numbers 1, 5, 13, 20, 27, 28, 33, 51 and 54 touch lines and are counted as being in the square below them or to their right. The circled sperm are not counted as being in this field of 16 small squares (but they would be included in any counts of adjacent squares in which they would be on upper or left hand lines).

Once these counting conventions have been selected, it is advisable to follow another strict protocol outlining the number and sequence of large squares to be counted. Because the sperm may not be randomly distributed across the counting grid, it is recommended to count an array of squares covering the entire grid. The following procedure is recommended:

Count the number of sperm in the first large square.

1. If the number is less than 10, count all 25 squares using the same scanning pattern outlined above (left to right through squares 1 to 5, down to square 6, left through square 10, down to 11, etc.). See pattern no. 3.

2. If the number is between 10 and 19, count 9 large squares using pattern no. 2.

3. If the number is 20 or greater, count 5 large squares using pattern no. 1.

1				2
		3		
4				5

Pattern no. 1

1				2
	4		3	
		5		
	7		6	
8				9

Pattern no. 2

1	2	3	4	5
10	9	8	7	6
11	12	13	14	15
20	19	18	17	16
21	22	23	24	25

Pattern no. 3

The final consideration in achieving good replicate counts is keeping the hemacytometers and coverslips clean. They should be rinsed in distilled water soon after use. The coverslips should be stored in a good biocleanser such as hemasol. For an hour or so prior to use, the hemacytometer slides should also be soaked in the solution. Both slides and coverslips should then be rinsed off with reagent water, blotted dry with a lint-free tissue, and wiped with lens paper.

SECTION 16

PURPLE URCHIN, *Strongylocentrotus purpuratus* AND SAND DOLLAR, *Dendraster excentricus* FERTILIZATION TEST METHOD

Adapted from a method developed by
Gary A. Chapman, U.S. EPA, ORD Newport, OR 97365
and Debra L. Denton, U.S. EPA, Region IX, CA 94105

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SECTION 16

SEA URCHIN, *Strongylocentrotus purpuratus* AND SAND DOLLAR, *Dendraster excentricus* FERTILIZATION TEST

16.1 SCOPE AND APPLICATION

16.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the gametes of sea urchins, (*Strongylocentrotus purpuratus*), or sand dollars (*Dendraster excentricus*) during a static non-renewal 20 minute sperm exposure and a subsequent 20 minute exposure period following the addition of eggs for measuring the fertilizing capacity of the sperm. The effects include the synergistic, antagonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

16.1.2 The purpose of the test is to determine the concentrations of a test substance that reduce egg fertilization by exposed sperm relative to that attained by sperm in control solutions. Concentrations of materials adversely affecting egg fertilization under the conditions of this test are usually acutely and chronically toxic to one or more of several common marine test species and, by extension, are presumably acutely and chronically toxic to other of the many untested marine species.

16.1.3 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

16.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

16.1.5 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s),

consisting of one or more receiving water concentrations and a control.

16.1.6 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

16.2 **SUMMARY OF METHOD**

16.2.1 The method provides the step-by-step instructions for exposing sperm suspensions (appropriate sperm density may first be determined in a trial fertilization test) to effluents or receiving waters for 20 minutes. Eggs are then added to the sperm suspensions and, twenty minutes after the eggs are added, the test is terminated by the addition of a preservative. The percent fertilization is determined by microscopic examination of 100 eggs in an aliquot of eggs from each treatment. The test endpoint is normal egg fertilization.

16.3 **INTERFERENCES**

16.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

16.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sample Preparation for Toxicity Tests).

16.4 **SAFETY**

16.4.1 See Section 3, Health and Safety

16.5 **APPARATUS AND EQUIPMENT**

16.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult sea urchins and sand dollars, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20°C), with appropriate filtration and aeration system.

16.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

16.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (12°C) prior to the test. (Incubators are usually unsatisfactory because test tubes must be removed for addition of sperm and eggs and the small test volumes can rapidly change temperature at normal room temperatures.)

16.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

16.5.5 Refractometer -- for determining salinity.

16.5.6 Hydrometer(s) -- for calibrating refractometer.

16.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

16.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD 170.1, USEPA, 1979) -- to calibrate laboratory thermometers.

16.5.9 pH and DO meters -- for routine physical and chemical measurements.

16.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

16.5.11 Winkler bottles -- for dissolved oxygen determinations.

16.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

16.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

16.5.14 Glass stirring rods -- for mixing test solutions.

16.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-1000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

16.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

16.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

16.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

16.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

16.5.20 Wash bottles -- for dilution water.

16.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

16.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes. Strong solutions of NaOH and formaldehyde should not be held for several month periods in Cubitainers: interaction or leaching into solutions of 0.1 N or 1 N NaOH used for pH adjustment of dilution water has caused poor egg fertilization; formaldehyde similarly stored has induced abberant partial membrane elevation in eggs.

16.5.23 Beakers, 5-10 mL borosilicate glass -- for collecting sperm from sand dollars.

16.5.24 Beakers, 100 mL borosilicate glass -- for spawning; to support sea urchins and to collect sea urchin and sand dollar eggs.

16.5.25 Beakers, 1,000 mL borosilicate glass -- for rinsing and settling sea urchin eggs.

16.5.26 Vortex mixer -- to mix sea urchin semen in tubes prior to sampling.

16.5.27 Compound microscope -- for examining gametes, counting sperm cells (200-400x) and eggs (100x), and examining fertilized eggs. Dissecting scopes are sometimes used to count eggs at a lower magnification.

16.5.28 Counter, two unit, 0-999 -- for recording sperm and egg counts.

16.5.29 Sedgwick-Rafter counting chamber -- for counting egg stock and examining eggs for fertilization at the end of the test.

16.5.30 Hemacytometers, Neubauer -- for counting sperm.

16.5.31 Siphon hose (3 mm i.d.) -- for removing wash water from settled eggs.

16.5.32 Centrifuge tubes, test tubes, or vials -- for holding semen.

16.5.33 Perforated plunger -- for maintaining homogeneous distribution of eggs during sampling and distribution to test tubes.

16.5.34 60 μm NITEX® filter -- for filtering receiving water.

16.6 **REAGENTS AND SUPPLIES**

16.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

16.6.2 Data sheets (one set per test) -- for data recording (see Figures 1 and 2).

16.6.3 Tape, colored -- for labelling test chambers and containers.

16.6.4 Markers, water-proof -- for marking containers, etc.

16.6.5 Parafilm -- to cover graduated cylinders and vessels containing gametes.

16.6.6 Gloves, disposable -- for personal protection from contamination.

16.6.7 Pipets, serological -- 1-10 mL, graduated.

16.6.8 Pipet tips -- for automatic pipets. Note: pipet tips for handling semen should be cut off to produce an opening about 1 mm in diameter; pipet tips for handling eggs should be cut off to produce an opening about 2 mm in diameter. This is necessary to provide smooth flow of the viscous semen, accurate sampling of eggs, and to prevent injury to eggs passing through a restricted opening. A clean razor blade can be used to trim pipet tips.

16.6.9 Coverslips -- for microscope slides.

16.6.10 Lens paper -- for cleaning microscope optics.

16.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

16.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

16.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

16.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

16.6.15 Laboratory quality assurance samples and standards -- for the above methods.

16.6.16 Test chambers -- test tubes, borosilicate glass, 16 x 100 mm or 16 x 125 mm, with caps for conducting the test, four chambers per concentration.

Figure 1. Sample data sheet for spawning record.

Animal No.	Sex	Time Injected	Time Spawn	Comments
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				

Pooled eggs from female nos. _____.

Pooled (_____ mL) of sperm each from male nos. _____.

Figure 2. Sample data sheet for egg and sperm counts.

EGG COUNTS -

Sample	Dilution	Count	Eggs/mL

For 100 mL egg suspension at 2,240 eggs/mL use:

100 mL x 2,240 eggs/mL / (counted eggs/mL) = mL of egg stock

224,000 eggs / _____ eggs/mL = _____ mL

If required stock >100 mL, concentrate egg stock by settling the eggs and decanting off sufficient overlying water to retain:

(_____ eggs/mL / 2,240 eggs/mL) x 100 = _____ % volume

SPERM COUNTS -

Sample	Dilution	Count	Squares	Sperm/mL

$$\text{SPERM/mL} = \frac{(\text{DIL.FACT.})\ (\text{COUNT})\ (4000)\ (1000)}{(\text{NO. SQUARES COUNTED})}$$

16.6.17 Formaldehyde, 10%, in seawater -- for preserving eggs. Note: formaldehyde has been identified as a carcinogen and is irritating to skin and mucous membranes. It should not be used at a concentration higher than necessary to achieve morphological preservation of larvae for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

16.6.18 Glutaraldehyde, 1% in seawater -- for preserving eggs.

16.6.13 pH buffers 4, 7, and 10 (or as per instructions of
16.6.19 Acetic acid, 10%, reagent grade, in filtered (10μ) seawater -- for preparing killed sperm dilutions for sperm counts.

16.6.20 Haemo-Sol or equivalent cleaner -- for cleaning hemacytometer and cover slips.

16.6.21 0.5 M KCl solution -- for inducing spawning.

16.6.22 Syringe, disposable, 3 or 5 mL -- for injecting KCl into sea urchins and sand dollars to induce spawning.

16.6.23 Needles, 25 gauge -- for injecting KCl.

16.6.24 Pasteur pipets and bulbs -- for sampling eggs from spawning beakers.

16.6.25 Hematocrit capillary tubes -- for sampling sperm for examination and for loading hemacytometers.

16.6.26 Microscope well-slides -- for pre-test assessment of sperm activity and egg condition.

16.6.27 Reference toxicant solutions (see 16.10.2.4 and Section 4, Quality Assurance).

16.6.28 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies and Section 7, Dilution Water).

16.6.29 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

16.6.30 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 16.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-μm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

16.6.31 HYPERSALINE BRINES

16.6.31.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

16.6.31.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

16.6.31.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 μm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

16.6.31.4 Freeze Preparation of Brine

16.6.31.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20°C until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

16.6.31.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

16.6.31.4.3 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

16.6.31.5 Heat Preparation of Brine

16.6.31.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is applied, use only oil-free air compressors to prevent contamination.

16.6.31.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the

brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

16.6.31.5.3 Seawater should be filtered to at least 10 μm before being put into the brine generator. The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

16.6.31.5.4 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

16.6.31.6 Artificial Sea Salts

16.6.31.6.1 No data from sea urchin or sand dollar fertilization tests using sea salts are available for evaluation at this time, and their use should be considered provisional. The use of GP2 artificial seawater (Table 2) has been found to provide control fertilization equal to that of natural seawater.

16.6.31.6.2 The GP2 reagent grade chemicals (Table 2) should be mixed with deionized (DI) water or its equivalent in a single batch, never by test concentration or replicate. The reagent water used for hydration should be between 21-26°C. The artificial seawater must be conditioned (aerated) for 24 h before use as the testing medium. If the solution is to be autoclaved, sodium bicarbonate is added after the solution has cooled. A stock solution of sodium bicarbonate is made up by dissolving 33.6 g $NaHCO_3$ in 500 mL of reagent water. Add 2.5 mL of this stock solution for each liter of the GP2 artificial seawater.

16.6.31.7 Dilution Water Preparation from Brine

16.6.31.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent

test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

16.6.31.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. Thus, the proportion is one part brine plus 1.94 parts reagent water). To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

16.6.31.8 Test Solution Salinity Adjustment

16.6.31.8.1 Table 3 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 3.

16.6.31.8.2 Check the pH of all brine mixtures and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide.

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

16.6.31.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

16.6.31.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

16.6.31.9 Preparing Test Solutions

TABLE 2. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR THE PURPLE URCHIN *STRONGYLOCENTROTUS PURPURATUS*, AND SAND DOLLAR *DENDRASTER EXCENTRICUS* TOXICITY TEST[1,2]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	23.90	478.0
Na_2SO_4	4.00	80.0
KCl	0.698	13.96
KBr	0.100	2.00
$Na_2B_4O_7 \cdot 10\ H_2O$	0.039	0.78
$MgCl_2 \cdot 6\ H_2O$	10.80	216.0
$CaCl_2 \cdot 2\ H_2O$	1.50	30.0
$SrCl_2 \cdot 6\ H_2O$	0.025	0.490
$NaHCO_3$	0.193	3.86

[1]Modified GP2 from Spotte et al. (1984)
[2]The constituent salts and concentrations were taken from USEPA (1990b). The salinity is 34.0 g/L.

16.6.31.9.1 Five mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 1.0 mL of effluent to a 100-mL volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 100-mL mark with dilution water, stopper it, and shake to mix. Pour into a (150-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

16.6.31.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For

example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 3) and top off the flask with dilution water. Stopper the flask and shake well. Pour into a (100-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

16.6.31.10 Brine Controls

16.6.31.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 16.6.33.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

16.6.32 TEST ORGANISMS, PURPLE URCHINS

16.6.32.1 Sea Urchins, *Strongylocentrotus purpuratus* (approximately 6 of each sex per test).

16.6.32.2 Adult sea urchins (*Strongylocentrotus purpuratus*) can be obtained from commercial suppliers or collected from uncontaminated intertidal or subtidal areas. State collection permits are usually required for collection of sea urchins and collection is prohibited or restricted in some areas. The animals are best transported "dry," surrounded either by moist seaweed or paper towels dampened with seawater. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock which can prematurely induce spawning.

TABLE 3. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (AT X‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION:

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

16.6.32.3 The adult sea urchins are maintained in glass aquaria or fiberglass tanks. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or salt water prepared from commericial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded.

16.6.32.4 Although ambient temperature seawater is usually acceptable, maintaining sea urchins in spawning condition usually requires holding at a relatively constant temperature. The culture unit should be capable of maintaining a constant temperature between 10 and 14°C with a water temperature control device.

16.6.32.5 Food for sea urchins -- kelp, recommended, but not necessarily limited to, *Laminaria sp.*, *Hedophyllum sp.*, *Nereocystis sp.*, *Macrocystis sp.*, *Egregia sp.*, *Alaria sp.* or romaine lettuce. The kelp should be gathered from known uncontaminated zones or obtained from commerical supply houses whose kelp comes from known uncontaminated areas, or romaine lettuce. Fresh food is introduced into the tanks at least several times a week. Sun dried (12-24 hours) or oven dried (60°C overnight) kelp, stores well at room temperature or frozen, rehydrates well and is adequate to maintain sea urchins for long periods. Decaying food and fecal pellets are removed as necessary to prevent fouling.

16.6.32.6 Natural seawater (>30‰) is used to maintain the adult animals and (≥32‰) as a control water in the tests.

16.6.32.7 Adult male and female (if sexes known) animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added. Healthy animals will attach to the kelp or aquarium within hours.

16.6.32.8 To successfully maintain about 25 adult animals for seven days at a field site, 40-L glass aquaria using aerated, recirculating, clean saline water (32‰) and a gravel bed filtration system, are housed within a water bath, such as an

INSTANT OCEAN® Aquarium. The sexes should be held separately if possible.

16.6.33 TEST ORGANISMS, SAND DOLLARS

16.6.33.1 Sand Dollars, *Dendraster excentricus*, (approximately 6 of each sex per test).

16.6.33.2 Adult sand dollars (*Dendraster excentricus*) can be obtained from commercial suppliers or collected from subtidal zones (most areas) or from intertidal zones of some sheltered waters (e.g., Puget Sound). State collection permits may be required for collection of sand dollars and collection prohibited or restricted in some areas. The animals are best transported "dry," surrounded either by moist seaweed or paper towels dampened with seawater. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock which can prematurely induce spawning.

16.6.33.3 The adult sand dollars are maintained in glass aquaria or fiberglass tanks. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or saltwater prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded. For longer periods than a few days, several centimeters or more of a sand substrate may be desirable.

16.6.33.4 Although ambient temperature seawater is usually acceptable, maintaining sand dollars in spawning condition usually requires holding at a relatively constant temperature. The culture unit should be capable of maintaining a constant temperature between 8 and 12°C with a water temperature control device.

16.6.33.5 Sand dollars will feed on suspended or benthic materials such as phytoplankton, benthic diatoms, etc. No reports of laboratory populations being maintained in spawning condition over several years are known. It is probably most convenient to obtain sand dollars, use them, and then discard them after they cease to produce good quality gametes.

16.6.33.6 Natural seawater (>30‰) is used to maintain the adult animals and (≥32‰) as a control water in the tests.

16.6.33.7 Adult male and female (if sexes known) animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, trays or aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added.

16.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION AND STORAGE

16.7.1 Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sampling Preparation for Toxicity Tests.

16.8 CALIBRATION AND STANDARDIZATION

16.8.1 See Section 4, Quality Assurance.

16.9 QUALITY CONTROL

16.9.1 See Section 4, Quality Assurance.

16.10 TEST PROCEDURES

16.10.1 TEST DESIGN

16.10.1.1 The test consists of at least four effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain four replicates of a brine control. In addition, four extra controls are prepared for egg controls.

16.10.1.2 Effluent concentrations are expressed as percent effluent.

16.10.2 TEST SOLUTIONS

16.10.2.1 Receiving waters

16.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed

through a 60 μm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 5 mL, and 400 mL for chemical analysis, would require approximately 420 mL or more of sample per test.

16.10.2.2 Effluents

16.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100% HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

16.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

16.10.2.2.3 The volume in each test chamber is 5 mL.

16.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

16.10.2.3 Dilution Water

16.10.2.3.1 Dilution water should be uncontaminated 1-μm-filtered natural seawater, or hypersaline brine prepared from uncontaminated natural seawater plus reagent water; or sea salts (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

16.10.2.4 Reference Toxicant Test

16.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

16.10.2.4.2 The preferred reference toxicant for sea uchins and sand dollar is copper chloride ($CuCl_2 \circ H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a copper reference toxicant stock solution (2,000 mg/L) by adding 5.366 g of copper chloride ($CuCl_2 \circ 2H_2O$) to 1 liter of reagent water. For each reference toxicant test prepare a copper sub-stock of 3 mg/L by diluting 1.5 mL of stock to one liter with reagent water. Alternatively, certified standard solutions can be ordered from commercial companies.

16.10.2.4.3 Prepare a control (0 µg/L) plus four replicates each of at least five consecutive copper reference toxicant solutions (e.g., from the series 3.0, 4.4, 6.5, 9.5, 13.9, 20.4, and 30.0 µg/L, by adding 0.10, 0.15, 0.22, 0.32, 0.46, 0.68, and 1.00 mL of sub-stock solution, respectively, to 100-L volumetric flasks and filling to 100-mL with dilution water). Start with control solutions and progress to the highest concentration to minimize contamination.

16.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

16.10.3 COLLECTION OF GAMETES FOR THE TEST

16.10.3.1 Spawning Induction

16.10.3.1.1 Pour seawater into 100 mL beakers and place in 12°C bath or room. Allow to come to temperature. Select a sufficient number of sea urchins or sand dollars (based upon recent or past spawning success) so that three of each sex are likely to provide gametes of acceptable quantity and quality for the test. During optimal spawning periods this may only require six animals, three

of each sex, when the sexes are known from prior spawning. During other periods, especially if the sex is not known, many more animals may be required.

16.10.3.1.2 Care should be exercised when removing sea urchins from holding tanks so that damage to tube-feet is minimized. Following removal, sea urchins should be placed into a container lined with seawater-moistened paper towels to prevent reattachment.

16.10.3.1.3 Place each sand dollar, oral side up, on a 100 mL beaker filled with 12°C seawater or each sea urchin onto a clean tray covered with several layers of seawater moistened paper towels.

16.10.3.1.4 Handle sexes separately once known; this minimizes the chance of accidental egg fertilization. Throughout the test process, it is best if a different worker, different pipets, etc. are used for males (semen) and females (eggs). Frequent washing of hands is a good practice.

16.10.3.1.5 Fill a 3 or 5 mL syringe with 0.5 M KCl and inject 0.5 mL through the soft periostomal membrane of each sea urchin (See Figure 3) or into the oral opening each sand dollar. If sexes are known, use a separate needle for each sex. If sexes are not known, rinse the needle with hot tap water between each injection. This will avoid the accidental injection of sperm from males into females. Note the time of injection (sample data sheet, Figure 1).

16.10.3.1.6 Spawning of sea urchins is sometimes induced by holding the injected sea urchin and gently shaking or swirling it for several seconds. This may provide an additional physical stimulus, or may aid in distributing the injected KCl.

16.10.3.1.7 Place the sea urchins onto the beakers or tray (oral side down). Place the sand dollars onto the beakers (oral side up). Females will release orange (sea urchins) or purple (sand dollars) eggs and males will release cream-colored semen.

16.10.3.1.8 As gametes begin to be shed, note the time on the data sheet and separate the sexes. Place male sand dollars with the oral side up atop a small (5-10 mL) glass beaker filled with 12°C seawater. Leave spawning sea urchin males on tray or beaker

(oral side down) for semen collection. Female sand dollars and sea urchins are left to shed eggs into the 100-mL beakers.

16.10.3.1.9 If sufficient quantities of gametes are available, only collect gametes for the first 15 min after each animal starts releasing. This helps to insure good quality gametes. As a general guideline, do not collect gametes from any individual for more than 30 minutes after the first injection.
16.10.3.1.10 If no spawning occurs after 5 or 10 minutes, a second 0.5 mL injection may be tried. If animals do not produce sufficient gametes following injection of 1.0 mL of KCl, they should probably not be reinjected as this seldom results in acquisition of good quality gametes and may result in mortality of adult urchins.

16.10.3.1.11 Collect the undiluted semen from each male sea urchin, using a 0.1 mL automatic pipet. Store the sperm from each male in a separate, labelled, conical, glass centrifuge tube, covered with a cap or parafilm, on ice. Air exposure of semen may alter its pH through gas exchange and reduce the viability of the sperm. Note: undiluted semen from *Strongylocentrotus purpuratus* typically contains about 4×10^{10} sperm/mL.

16.10.3.1.12 Sections 15.10.4.2 and 15.10.6.4 describe collection and dilution of the sperm and eggs. While some of the gamete handling needs to be in a specific order, parts of the procedure can be done simultaneously while waiting for gametes to settle.

16.10.3.2 Collection of Sperm

16.10.3.2.1 Sea urchin semen should be collected dry (directly from the surface of the sea urchin), using either a Pasteur pipette or a 0.1 mL autopipette with the end of the tip cut off so that the opening is at least 2 mm. Pipette semen from each male into separate 1-15 mL conical test tubes, stored in an ice water bath.

16.10.3.3 Viability of Sperm

16.10.3.3.1 Early in the spawning process, place a very small amount of sperm from each male sea urchin or sand dollar into

dilution water on a microscope slide (well slides work nicely). Examine the sperm for motility; use sperm from males with high sperm motility. It is more important to use high quality sperm than it is to use a pooled population of sperm.

16.10.3.4 Pooling of Sperm

16.10.3.4.1 Pool equal quantities of semen from each of the sea urchin males that has been deemed good. If possible, 0.025 mL should be pooled from each of those used and a total of at least 0.05 mL of pooled semen should be available. Sperm collected from good male sand dollars should be pooled after first decanting off the overlying water (the final sand dollar sperm density usually is between $2x10^9$ and $2x10^{10}$ sperm/mL).

16.10.3.5 Storage of Sperm

16.10.3.5.1 Cover each test tube or beaker with a cap or parafilm, as air exposure of semen may alter its pH through gas exchange and reduce the viability of the sperm. Keep sperm covered and on ice or refrigerated (<5°C). The sperm should be used in a toxicity test within 4 h of collection.

16.10.4 PREPARATION OF EGG SUSPENSION FOR USE IN THE TEST

16.10.4.1 Acceptability of Eggs

16.10.4.1.1 Prior to pooling, a small sample of the eggs from each female should be examined for the presence of significant quantities of poor eggs (vacuolated, small, or irregularly shaped) and mixed with good sperm to determine extent of fertilization. If good quality eggs are available from one or more females, questionable eggs should not be used for the test. It is more important to use high quality eggs than it is to use a pooled population of eggs.

16.10.4.2 Pooling of Eggs

16.10.4.2.1 Allow eggs to settle in the collection beakers. Decant some of the water from the collection beakers taking care not to pour off many eggs. The sea urchin eggs are pooled into a 1 L beaker, and the volume brought to 600 mL with 12°C dilution water. The eggs are suspended by swirling and the eggs allowed

to settle for 15 minutes at 12°C. About 500 mL of the overlying water are siphoned off, the volume brought back to 600 mL with more 12°C dilution water, and the eggs resuspended and allowed to settle for a second 15 minute period. After again siphoning off the overlying 500 mL, the rinsed eggs are gently transferred to either a 100 or a 250 mL graduated cylinder and brought to volume with 12°C dilution water. Eggs are stored at 12°C throughout the pre-test period.

16.10.4.2.2 Pooled sand dollar eggs should be treated gently and no additional rinsing step is recommended. Mix well once just before subsampling for egg stock calculations. This is best done in a large graduated cylinder appropriate for the number of eggs available. Cover with parafilm and invert gently several times.

16.10.4.3 Density of Eggs

16.10.4.3.1 Subsamples of the egg stock are then taken for determining egg density. Place 9 mL of dilution water in each of two 22 mL liquid scintillation vials. Label A and B. Place 1 mL of well-mixed egg stock into vial A. Mix well. (The remaining egg stock is covered with parafilm and stored at 12°C.) Transfer 1 mL of egg suspension from vial A to vial B. Mix contents of vial B and transfer 1 mL of egg suspension B into a Sedgewick-Rafter counting chamber. Count eggs under a compound microscope. If count is <30, count a 1 mL sample from vial A (see sample data sheet, Figure 2).

16.10.4.3.2 Prepare 100 mL of egg stock in dilution water at the final target concentration of 2,240 eggs/mL (224,000 eggs in 100 mL). If the egg stock is >2,240 eggs/mL (A >224 or B >30 eggs/mL), dilute the egg stock by transferring:

224,000 eggs / ____D___ eggs/mL = _____mL

of well-mixed egg stock to a 100 mL graduated cylinder and bring the total volume to 100 mL with dilution water where:

D = (Count A) x 10 or (Count B) x 100.

If the egg stock is <2,240 eggs/mL (A <224 eggs/mL), concentrate the eggs by allowing them to settle and then decant enough water to retain the following percent of the original volume:

(___D___ eggs/mL / 2,240) x 100 = % volume.

16.10.4.3.3 Check the egg stock density. Place 9 mL of dilution water into a 22 mL scintillation vial; add 1 mL of the final egg stock. Mix well and transfer 1 mL into a Sedgewick-Rafter counting chamber. The egg count should be between 200 and 245. Adjust egg stock volume and recheck counts if necessary to obtain counts within this range. Because some eggs (especially sand dollar eggs) may be sensitive to handling, it is advisable to separately prepare egg stocks for the fertilization trial and the definitive test (but use the same pooled batch of eggs).

16.10.5 PREPARATION OF SPERM DILUTION FOR USE IN THE (OPTIONAL) TRIAL FOR ESTIMATING APPROPRIATE SPERM DENSITY FOR TEST

16.10.5.1 A trial fertilization is recommended to reduce the likelihood of a failed test due to inadequate control fertilization or exceeding the maximum acceptable sperm density. However, two other alternative approaches are acceptable:

1) Conduct the test at a low enough sperm density that oversperming does not create test insensitivity. This can be met by using a confirmed sperm stock density of $\leq 5.6 \times 10^6$/mL (this is equivalent to a sperm:egg ratio of ≤500:1 at 200 eggs/mL); or

2) Conduct the test, but include two extra sets of controls, one set receiving only 0.050 mL of the sperm stock and the other receiving 0.2 mL of the sperm stock. The control fertilization in the 0.050 mL sperm stock controls must be at least 5% lower than that in the 0.2 mL sperm stock controls or the test is unacceptable. Confirm that the sperm stock density did not exceed the maximum acceptable density of 3.36×10^7 sperm/mL.

16.10.5.2 Fertilization trial is conducted to determine the sperm density that will provide about 80-100 percent control egg fertilization while avoiding significant "oversperming" that can reduce test sensitivity. Although usually expressed as a sperm:egg ratio (e.g., 1,000:1), because egg density is held constant at 200/mL, the sperm:egg ratio is also a measure of sperm density.

16.10.5.3 It is unacceptable to conduct a definitive toxicity test if the sperm:egg ratio exceeds 3,000:1. This is a cut-off based on gradual loss of test sensitivity at higher sperm densities, even in cases where control fertilization is considerably below 100 percent.

16.10.5.4 It is unnecessary to conduct trials for definitive toxicity tests at sperm:egg ratios below 500:1, because this ratio should never cause significant "oversperming."

16.10.5.5 Sperm density of sea urchin semen or sand dollar sperm suspension is checked by hemocytometer counts and a replicated series of nominal S:E ratios set up (3,000, 1288, 550, 234, and 100:1) based upon appropriate dilution calculations.

16.10.5.6 For sea urchins and sand dollars, prepare a killed sperm preparation for determining the dilution required to obtain a sperm stock (3.36×10^7 sperm/mL) for the maximum sperm density (6×10^5 sperm/200 eggs/mL--3,000:1) needed for the trial. A sperm density of about 1×10^7 is convenient to count. If the approximate sperm density is known, the dilution procedures outlined in Table 4 can be followed without initial sperm counts; the actual trial sperm density must still be determined by subsequent counts. For example (Table 4), if expected sperm density is ca. 5×10^8 dilute 0.2 mL of sperm to 10 mL, if ca. 5×10^9 dilute 0.2 mL of sperm to 100 mL (or 0.025 mL of sperm to 10 mL), if ca. 5×10^{10} dilute 0.040 mL to 200 mL. Table 4 is provided for guidance as a quick reference for dilution volumes if sperm density of pooled semen is can be reasonably estimated, and as a check for mathematical accuracy of formula calculations for sperm dilution.

16.10.5.7 Mix the pooled sea urchin semen (16.10.3.8) by agitating the centrifuge tube for about 5 seconds using a vortex mixer. Very slowly withdraw a subsample of semen using an automatic pipet, wipe off the outside of the pipet tip with tissue, and empty the pipet contents into an Erlenmeyer flask containing the appropriate volume (Table 4) of a sperm killing solution of 1% glacial acetic acid in dilution water (e.g., 10 mL of 10% glacial acetic acid plus 90 mL of dilution water).

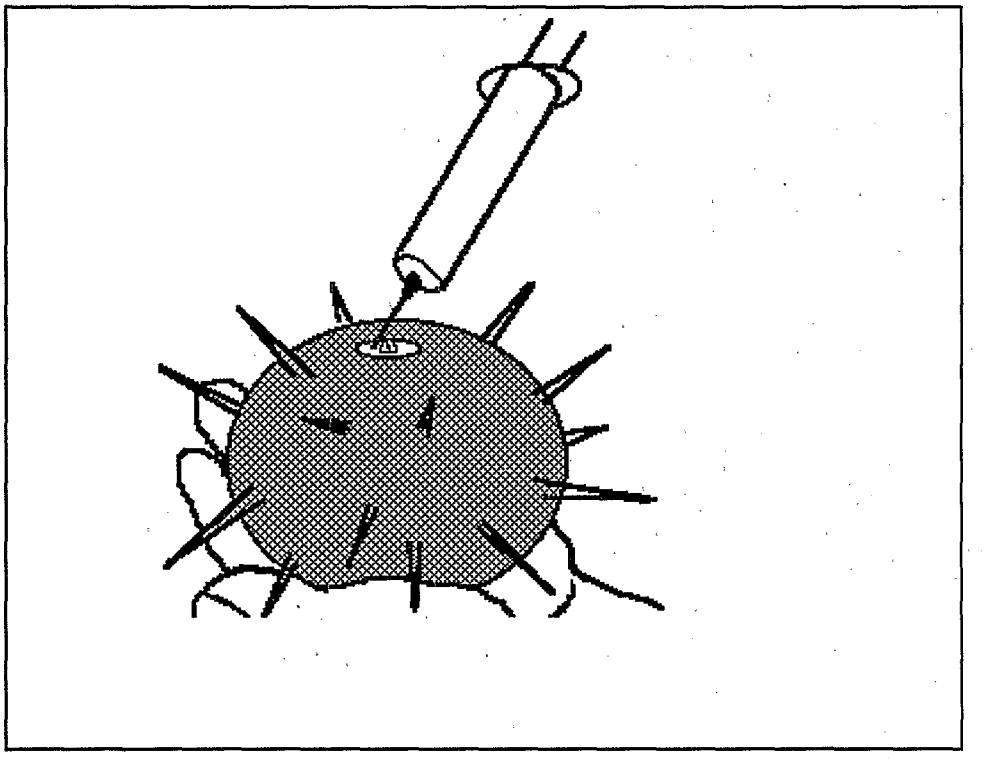


Figure 3. Showing the location and orientation used in the injection of KCl into sea urchins to stimulate spawning.

Repeatedly rinse the residual semen from the pipet tip by filling and emptying until no further cloudy solution is expelled from the pipet (this may require several dozen rinses). Cover the flask with parafilm and mix thoroughly by repeated inversion.

16.10.5.8 Mix the chilled suspension of pooled sand dollar sperm (16.10.5.6) using a stirring rod. Pipet the appropriate volume of sperm suspension (Table 4) into an Erlenmeyer flask containing the appropriate volume (Table 4) of a sperm killing solution of 1% glacial acetic acid in dilution water (e.g., 10 mL of 10% glacial acetic acid plus 90 mL of dilution water).

16.10.5.9 Transfer samples of well-mixed sperm suspension to both sides of two Neubauer hemacytometers. Let the sperm settle 15 min.

16.10.5.10 Count the sperm on one hemacytometer following procedures outlined in Appendix II. If the lower count is at

least 80% of the higher count use the mean count to estimate sperm density in semen and the required dilution volume for the test stock. If the two counts do not agree within 20%, count the two fields on the other hemacytometer. Calculate the sperm density in the semen or sperm suspension using the mean of all four counts unless one count can be eliminated as an obvious outlier.

16.10.5.11 Calculate the volume of dilution water necessary to dilute the sea urchin semen or the sand dollar sperm suspension to the sperm density (sperm/mL) required for the sperm stock for the trial. See Table 5 for recommended dilution procedures; it also provides a quick reference for dilution volumes once sperm density of pooled semen is known, or a check for mathematical accuracy of formula calculations for sperm dilution. Note: table values for sperm densities from $1x10^8$ to $9x10^9$ are for volume (mL) of sperm stock for total volume of 100 mL; table values for sperm densities $\geq 1x10^{10}$ are for dilution water volumes for 0.025 mL of semen. Table 5 is used as follows: given a sperm density in the semen stock (e.g., $4.7x10^9$) find the row containing the integer (characteristic) and the exponent ($4x10^9$) in the left hand column, then read across to the column coresponding to the mantissa (0.7). The value at the intersection of the row and column (0.71 mL) is the volume of semen per 100mL needed for sperm stock to achieve a 3000:1 sperm:egg ratio in the trial.

16.10.5.12 For the approximate sperm:egg ratios dilute the 3000:1 stock as follows:

1288:1	5 mL	3000:1 stock with 6.6 mL dilution water
550:1	2 mL	3000:1 stock with 9.9 mL dilution water
234:1	1 mL	3000:1 stock with 11.8 mL dilution water
100:1	0.5 mL	3000:1 stock with 16.5 mL dilution water

16.10.6 SPERM DENSITY TRIAL

16.10.6.1 The series of trial sperm:egg ratios should include 3,000:1 and several lower ratios. The ratios 100:1, 234:1, 550:1, 1288:1 and 3,000:1 are recommended because they evenly divide the log sperm:egg ratio. Fertilization appears to be a linear function of the log of sperm density (Figure 4). Recommended sperm dilution procedures are given in Table 5.

16.10.6.4 Quantitive evaluation of the sperm density trial should be obtained by counting 100 eggs from each tube until a suitable sperm density can be determined for the definitive test. Examples of sperm density selection are given in Table 6. Percent fertilization may be lower in the test than in the trial because the viability of the stored sperm may decrease during the period of the trial. If the sperm have very good viability (e.g., cases 1 and 2, Table 6), this loss of viability should be small. On the other hand, if viability is inherently poorer (cases 3, 4 and 5, Table 6), the loss of viability could be greater and probably should be taken into account in selecting the sperm density for the test. Case 6 (Table 6) represents a special case in which egg viability may affect the percent fertilization; in this case the asymptote of the fertilization curve is assumed to represent 100% fertilization for purposes of selection of sperm density for the test.

16.10.6.5 Prepare killed sperm preparations of the trial sperm stock suspensions to provide confirmation of the nominal sperm:egg ratios. It saves time if these can be prepared and loaded onto hemacytometers while the trial is being conducted. Alternatively, once the trial has been evaluated, the selected nominal sperm density can be confirmed by direct hemacytometer count.

16.10.6.6 Record all the counts made, select a target sperm:egg ratio for the test, and calculate the dilution of the stored sperm stock needed to provide the necessary sperm density for the definitive test.

16.10.6.7 Table 5 can be used for deriving the volumes needed for preparing the final sperm stock. For a pooled sperm suspension density of 4×10^9 and a target sperm:egg ratio of 500:1, simply read the dilution for the 3000:1 sperm:egg ratio from Figure 5 (0.84 mL / 100 mL) and reduce the sperm volume by 3,000 / 500 = 6. In this case 0.84 / 6 = 0.14 mL; the dilution factor checks out (100 / 0.14 = 714).

TABLE 4. Dilution volume guide for initial count of sperm density to achieve recommended counting density of 1 x 10^7/mL.

Initial Sperm/mL	mL/10mL	mL/100mL	mL/200mL
1 x 10^8	1.000		
2 x 10^8	0.500		
3 x 10^8	0.333		
4 x 10^8	0.250		
5 x 10^8	0.200		
6 x 10^8	0.167		
7 x 10^8	0.143		
8 x 10^8	0.125		
9 x 10^8	0.111		
1 x 10^9	0.100	1.000	
2 x 10^9	0.050	0.500	1.000
3 x 10^9	0.033	0.333	0.667
4 x 10^9	0.025	0.250	0.500
5 x 10^9		0.200	0.400
6 x 10^9		0.167	0.333
7 x 10^9		0.143	0.286
8 x 10^9		0.125	0.250
9 x 10^9		0.111	0.222
1 x 10^{10}		0.100	0.200
2 x 10^{10}		0.050	0.100
3 x 10^{10}		0.033	0.067
4 x 10^{10}		0.025	0.050
5 x 10^{10}			0.040
6 x 10^{10}			0.033
7 x 10^{10}			0.029
8 x 10^{10}			0.025
9 x 10^{10}			0.022

Note: to obtain quantitatively repeatable samples of semen it is important that: (1) the pipet tip have an opening of at least 1 mm; (2) samples be withdrawn slowly to avoid cavitation and entrainment of air in the semen sample; (3) samples not include fragments of broken spines (which usually settle to the test tube bottom upon vortexing); and (4) wiping semen from the pipet tip with tissue be done with care to avoid wicking semen from within the pipet tip.

Bring the indicated volume of sperm stock to 100 mL

Density	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1.00e+08	33.60	30.55	28.00	25.85	24.00	22.40	21.00	19.76	18.67	17.68
2.00e+08	16.80	16.00	15.27	14.61	14.00	13.44	12.92	12.44	12.00	11.59
3.00e+08	11.20	10.84	10.50	10.18	9.88	9.60	9.33	9.08	8.84	8.62
4.00e+08	8.40	8.20	8.00	7.81	7.64	7.47	7.30	7.15	7.00	6.86
5.00e+08	6.72	6.59	6.46	6.34	6.22	6.11	6.00	5.89	5.79	5.69
6.00e+08	5.60	5.51	5.42	5.33	5.25	5.17	5.09	5.01	4.94	4.87
7.00e+08	4.80	4.73	4.67	4.60	4.54	4.48	4.42	4.36	4.31	4.25
8.00e+08	4.20	4.15	4.10	4.05	4.00	3.95	3.91	3.86	3.82	3.78
9.00e+08	3.73	3.69	3.65	3.61	3.57	3.54	3.50	3.46	3.43	3.39
1.00e+09	3.36	3.05	2.80	2.58	2.40	2.24	2.10	1.98	1.87	1.77
2.00e+09	1.68	1.60	1.53	1.46	1.40	1.34	1.29	1.24	1.20	1.16
3.00e+09	1.12	1.08	1.05	1.02	0.99	0.96	0.93	0.91	0.88	0.86
4.00e+09	0.84	0.82	0.80	0.78	0.76	0.75	0.73	0.71	0.70	0.69
5.00e+09	0.67	0.66	0.65	0.63	0.62	0.61	0.60	0.59	0.58	0.57
6.00e+09	0.56	0.55	0.54	0.53	0.53	0.52	0.51	0.50	0.49	0.49
7.00e+09	0.48	0.47	0.47	0.46	0.45	0.45	0.44	0.44	0.43	0.43
8.00e+09	0.42	0.41	0.41	0.40	0.40	0.40	0.39	0.39	0.38	0.38
9.00e+09	0.37	0.37	0.37	0.36	0.36	0.35	0.35	0.35	0.34	0.34

To dilute dense semen: add 0.025 mL of semen into these volumes (mL) of dilution water

Density	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1.00e+10	7.44	8.44	9.44	10.44	11.44	12.44	13.44	14.44	15.44	16.44
2.00e+10	14.88	15.88	16.88	17.88	18.88	19.88	20.88	21.88	22.88	23.88
3.00e+10	22.32	23.32	24.32	25.32	26.32	27.32	28.32	29.32	30.32	31.32
4.00e+10	29.76	30.76	31.76	32.76	33.76	34.76	35.76	36.76	37.76	38.76
5.00e+10	37.20	38.20	39.20	40.20	41.20	42.20	43.20	44.20	45.20	46.20
6.00e+10	44.64	45.64	46.64	47.64	48.64	49.64	50.64	51.64	52.64	53.64
7.00e+10	52.08	53.08	54.08	55.08	56.08	57.08	58.08	59.08	60.08	61.08
8.00e+10	59.52	60.52	61.52	62.52	63.52	64.52	65.52	66.52	67.52	68.52
9.00e+10	66.96	67.96	68.96	69.96	70.96	71.96	72.96	73.96	74.96	75.96

TABLE 5. DILUTION VOLUMES OF SPERM STOCK OF INDICATED DENSITY ($1.0X10^8$ TO $9.9X10^{10}$) TO ACHIEVE THE SPERM STOCK DENSITY ($3.36X10^7$) FOR A 3000:1 SPERM:EGG RATIO.

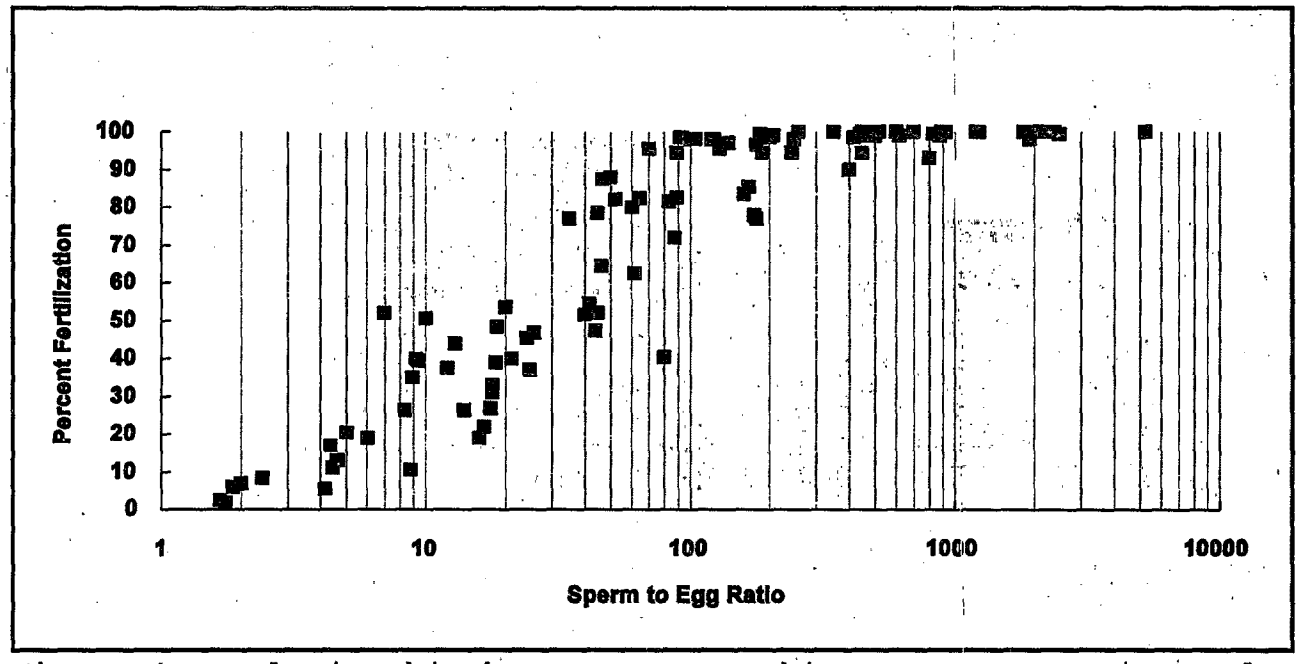


Figure 4. Relationship between sea urchin sperm:egg ration and percent control fertilization from 21 trials conducted by EPA (Feb-May 1991).

16.10.7 OBSERVATIONS DURING THE TEST

16.10.7.1 It is recommended that all observations be made on extra test solution remaining after the test tubes have been filled.

16.10.7.2 DO, pH, and salinity are measured at the beginning of the test. Due to the short duration of the test, no additional measurements of these parameters are required. Temperature is measured several times during the test as outlined in 16.10.7.

16.10.7.3 Record all measurements on the data sheet.

16.10.8 START OF THE DEFINITIVE TEST

16.10.8.1 Prior to Beginning the Test
produced good fertility, or if some produced good fertility.

TABLE 6. EXAMPLES OF RESULTS OF TRIAL FERTILIZATION TESTS WITH SPECIFIED SPERM DENSITIES AND TARGET SPERM DENSITY SELECTION (SPERM:EGG RATIO) FOR THE DEFINITIVE TEST.

sperm: egg	case 1	case 2	case 3	case 4	case 5	case 6
100:1	100*	95*	85	70	40	70
234:1	100	98	95*	80	64	85*
550:1	100	100	98	98*	82	89
1288:1	100	100	100	100	84	90
3000:1	100	100	100	100	88*	90

* recommended selection (interpolation to intermediate sperm:egg ratios may be used if found desirable)

1. If all trials exceed 90% fertilization, select 100:1 (case 1 and case 2).

2. If not all trials exceed 90% fertilization select the lowest sperm:egg ratio that does exceed 90% fertilization (case 3 and case 4).

3. If no trials exceed 90% fertilization, select the highest sperm:egg ratio (case 5) unless fertilization appears to become asymptotic below 100% (case 6).

4. If even the highest sperm:egg ratio fails to achieve 70% fertilization it is probable that an acceptable test cannot be conducted with these gametes.

   11,200 x target S:E ratio = target density; e.g., if target S:E = 500:1, target density = 11,200 x 500 = 5,600,000 sperm/mL. (11,200 = (1,120 eggs/tube)(0.1 mL of sperm stock/tube)).

   (stock sperm/mL)/(target sperm/mL) = dilution; e.g., if stock sperm has $4x10^9$ sperm/mL, then dilution = $4x10^9$ / $5.6x10^6$ = 714

16.10.8.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless

permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

16.10.8.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (12 ± 1°C) and maintained at that temperature during the addition of dilution water.

16.10.8.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (12 ± 1°C).

16.10.8.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the sea urchins and sand dollars have been examined at the end of the test.

16.10.8.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

16.10.8.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

16.10.9.2 Sperm Exposure

16.10.9.2.1 Mix the iced sea urchin semen or sand dollar sperm suspension as described in 16.10.5.7 and 16.10.5.8 (do not kill the sperm). Combine the required volumes of sperm and dilution water and mix this sperm stock well by repeated inversion of the graduate cylinder or beaker. Begin test within 5 minutes. Table 5 (for 3000:1 sperm:egg ratio) can be used to aid in calculating appropriate volumes by reducing the sperm volume or increasing the dilution water volume by the factor:

f = 3000:1 / target sperm:egg ratio

16.10.9.2.2 The test tubes containing 5.0 mL of the various test solutions should have been equilibrated in a 12°C waterbath. Into each test tube, inject 0.100 mL of the sperm stock (except see 16.7.4 and 16.11.4) and note the time of first and last injection. It is important that the injection be performed with care that the entire volume goes directly into the test solution and not onto the side of the test tube. Similarly, the pipet tip should not touch the test solution or the side of the test tube, risking transfer of traces of test solution(s) into the sperm stock. Using repeated single 0.100 mL refill and injection, about 12 tubes per minute is a reasonable injection rate. More rapid rates of injection can be attained with repeating (single fill, multiple injection) pipets. Sperm injection rate (tubes/min) should not exceed that possible for egg injection.

16.10.9.2.3 Unless the test tubes are totally randomized, injection of sperm should be performed by replicate, i.e., the first set of replicates should receive sperm, then the second set, then the third set, etc. The sperm stock solution should be mixed frequently to maintain a homogeneous sperm stock.

16.10.9.2.4 Confirm the sperm density. Pipet 9 mL of sperm stock solution into a vial or test tube containing 1 mL of 10% acetic acid. Fill both sides of a hemacytometer with this dilution after mixing well. Let stand for 15 minutes. Count both sides of the hemacytometer using counting pattern no. 1 outlined in Appendix II and take the average count. For a sperm:egg ratio of 500:1 the stock sperm density will be 5,600,000 sperm/mL. (For counting pattern no. 1, this amounts to a total count of 102 sperm for the five large squares.) Calculate the sperm density in the sperm stock. If either: (1) the stock sperm density is greater than 33,600,000 sperm/mL (S:E

>3,000:1), or (2) the sperm density is more than 2x the target density, the test must be restarted with freshly diluted semen.

16.10.9.2.5 Check the temperature of the test solutions several times during the sperm exposure by including a temperature blank test tube containing 5 mL of dilution water with a thermometer.

16.10.9.3 Adding Eggs to the Test

16.10.9.3.1 Exactly 20 minutes after the sperm addition to the test was begun, begin to add the eggs, with every tube (including egg blanks - 11.7.4) receiving 0.5 mL of egg stock. Follow the same pattern of introduction for the eggs as used with the sperm so that each test tube has a sperm incubation period of 20 minutes. Note the time of start and finish of egg addition. This duration should be within one minute of that used for the sperm.

16.10.9.3.2 In order to maintain the same sperm:egg ratio in each test tube, the eggs must be maintained in a uniform distribution in the water column of the egg stock. Slow, gentle agitation of the egg stock in a beaker using a perforated plunger appears to be the best method of achieving a uniform distribution. Frequent inversion and mixing of egg stock in either a graduated cylinder or a multiple injection pipet may be acceptable.

16.10.9.3.3 The eggs should be injected using a pipet with an opening of at least 2 mm in order to avoid damaging the eggs and to provide sufficient flow to obtain a representative sample.

16.10.9.3.4 Two pair of egg blanks should be included in the test design, one at the beginning of the injection sequence (effluent blank) and one at the end of the injection sequence (egg blank). These tubes receive no sperm. The effluent blank contains the highest concentration of effluent and the egg blank contains dilution water. Examination of the effluent blank will indicate if the effluent induces a false fertilization membrane (a possible event, but probably rare) thus masking toxicity. Examination of the egg blank will indicate if accidentally fertilized eggs were used in the test (this is a minor factor unless a significant portion of the eggs were accidentally fertilized; it can indicate poor laboratory techniques). These

blanks are kept capped until the eggs are added in order to avoid contamination by sperm.

16.10.10 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

16.10.10.1 The echinoderm fertilization test can be conducted in the dark or at ambient laboratory light levels. Due to its short duration, the fertilization test requires no photoperiod.

16.10.10.2 The water temperature in the test chambers should be maintained at 12 ± 1°C. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers. A sensor placed in a temperature blank vial with standard volume of test solution can provide a direct measure of test solution temperature, one which may be more stable than the temperature in the air or water in the medium surrounding the test vials. Do not measure temperatures directly in a test vial, but prepare and handle the temperature blank(s) exactly as the normal control vials. Record the temperature several times between the beginning and the end of the test.

16.10.10.3 The test salinity should be in the range of 34 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

16.10.10.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity.

16.10.11 DISSOLVED OXYGEN (DO) CONCENTRATION

16.10.11.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary

to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

16.10.12 OBSERVATIONS DURING THE TEST

16.10.12.1 Routine Chemical and Physical Observations

16.10.12.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

16.10.12.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

16.10.12.1.3 Record all the measurements on the data sheet.

16.10.13 TERMINATION OF THE TEST

16.10.13.1 Ending the Test

16.10.13.1.1 Record the time the test is terminated.

16.10.13.1.2 Because of the short test duration water quality measurements are not necessary at the end.

16.10.13.2 Sample Preservation

16.10.13.2.1 Exactly 20 minutes after the egg addition, the test should be stopped by the addition of a fixative to kill the sperm and eggs (both unfertilized and fertilized [zygotes]) and to preserve the eggs for examination. Again, the time allotted to fixative addition should be about the same as that for sperm and egg addition and the sequence of addition the same as for the introduction of the gametes.

16.10.13.2.2 The choice of formaldehyde or glutaraldehyde is up to the individual laboratory. There are at least two acceptable procedures: (1) the EPA *Arbacia* method of adding 10% formaldehyde in dilution water at the rate of 2 mL to each test tube; or (2) the addition of 1% glutaraldehyde (vol/vol) in clean seawater at the rate of 0.5 mL to each test tube. Glutaraldehyde should be made up fresh each day. Because concentrated glutaraldehyde is commonly only 25% strength, 1% glutaraldehyde is obtained by diluting the concentrate by 25x (e.g., 4 mL + 96 mL seawater).

16.10.13.2.3 It must be noted that formaldehyde has been identified as a carcinogen and that both glutaraldehyde and formaldehyde are irritating to skin and mucous membranes. Neither should be used at higher concentrations than needed to achieve morphological preservation of eggs for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air. Before using either compound in this method, the user should consult the latest material safety data available.

16.10.13.3 Counting

16.10.13.3.1 Immediately after termination of the test, the tubes are capped (or otherwise covered) and the contents mixed by inversion. They can be stored at room temperature until the eggs are examined for fertilization. Counts should be completed within 48 hours and, if counts extend over two days, should be made by replicate, i.e., count all replicate 1 tubes, then replicate 2, etc.

16.10.13.3.2 At least 100 eggs from each test tube are examined and scored for the presence or absence of an elevated fertilization membrane. Newly fertilized eggs will almost always have a completely elevated membrane around the egg (See Figures 5 and 6). Often a double membrane appears in sea urchin eggs, but following storage, even of only several hours, the inner (hyaline) membrane may disappear. Fertilized eggs may touch the outer membrane, or the membrane(s) may partially collapse. Because these phenomena only occur after preservation, eggs with any elevation of the fertilization membrane are counted as fertilized.

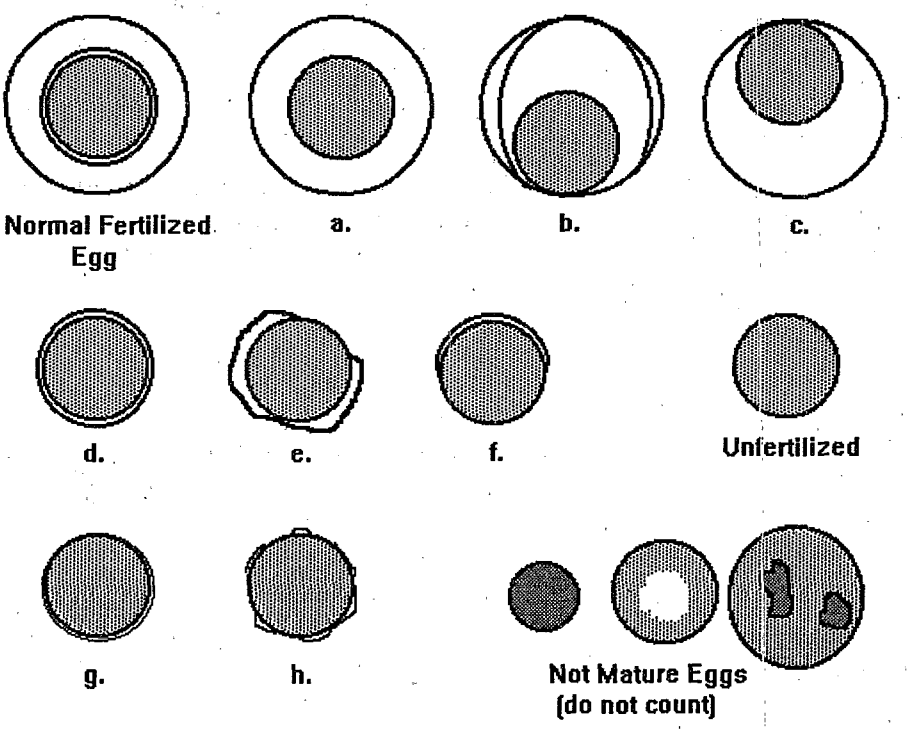


Figure 5. Examples of typical fertilized and unfertilized sea urchin eggs and a number of examples of atypical "fertilized" eggs (a through h). Normal fertilized eggs have an outer fertilization membrane and an inner hyaline membrane. After preservation, the hyaline membrane sometimes disappears (a); in other cases the egg is displaced from the center and contacts the perimeter either inside an enlarged hyaline envelope (b) or with no visible hyaline membrane (c). In some instances there appears to be only a slight elevation of the outer membrane or only the hyaline membrane appears, fully (d), partially (f), or only as a halo (g). In some batches of eggs the membrane(s) appear to be fragile and some collapse (e). In rare cases sperm appear to activate membrane elevation over only segments of the egg leading to a blistered appearance (h). When eggs appearing as those in examples f, g, and h are common in a test, the results should be examined closely to see if their occurrence appears to be dose-related (indicating an effect on fertilization), not dose-related (indicating a problem with egg quality or preservative), or is common in the effluent egg control (indicating an effluent- produced false fertilization). Eggs that are not mature are capable of being fertilized, but should never be counted. These include obviously smaller (often denser) eggs, normal sized eggs with a distinct, clear center, and very large eggs with often irregular color and density.

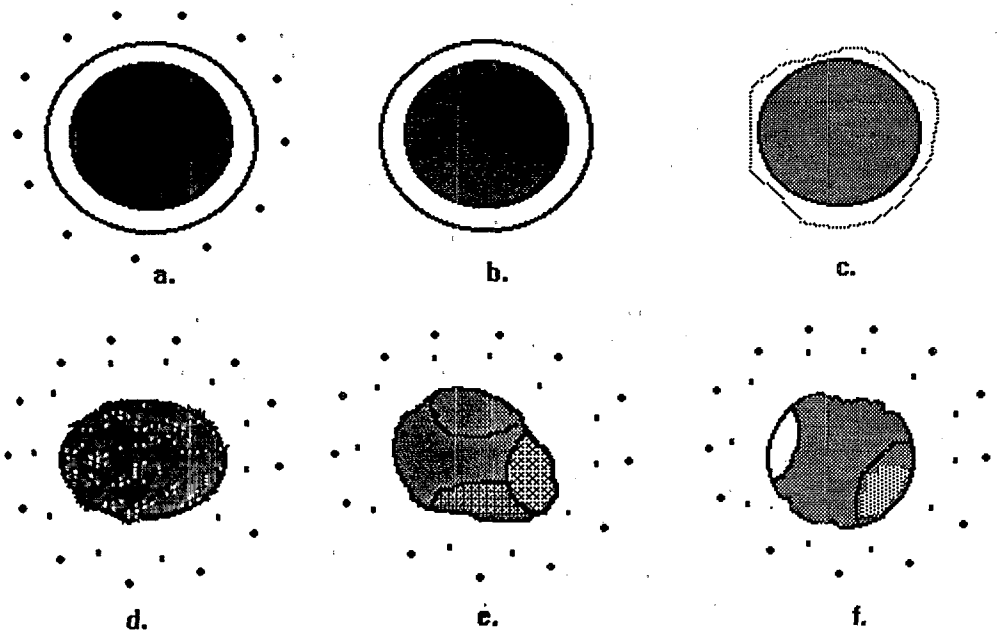


Figure 6. Examples of typical fertilized and unfertilized sand dollar eggs. Nearly all newly released eggs are characterized by a surrounding sphere of small purple chromatophores embedded within the transparent gelatinous coat surrounding the egg. The coat and the chromatophores may be lost or retained in the test and subsequent handling. Typical fertilized eggs are represented by (a) and (b). Some fertilized eggs (c) show only a wispy remnant of the fertilization membrane. Eggs when spawned usually appear as in (d) and (e) or somewhere in between. The more rounded "raisin" appearing egg in (d) is usually superior to the "asteroid" appearing egg in (e) although the latter can provide acceptable test results. However, the more irregularly shaped or vacuolated the eggs appear, the poorer the control fertilization is likely to be. The egg shown in (f), the "pitted olive," never shows a fertilization membrane and should not be counted.

16.10.13.3.3 It is convenient to concentrate the eggs prior to counting. If the eggs are allowed to completely settle (ca 30 minutes after termination and mixing), most of the overlying solution can be removed with a pipet, leaving the eggs concentrated in a much smaller volume. The eggs are then resuspended by filling and emptying a 1 mL pipet about 5 times from the remaining volume and finally transferring 1 mL of the egg suspension into a 1 mL Sedgewick-Rafter counting chamber (other volume counting chambers can be used).

16.10.13.3.4 Failure to completely resuspend the eggs can result in biasing the counts towards higher percent fertilization due to a tendency seen in rare batches of eggs in which unfertile eggs tend to be adhesive. This phenomenon may be further influenced by the choice of preservative, the strength of the preservative, and the period between preservation and counting. However, other sampling procedures may be used once demonstrated not to bias sampling and if no clumping of adhesive eggs is observed in a given test; for example, concentrated eggs may be picked up from the test tube and deposited in a small drop on a microscope slide, or eggs can be scored by examination with the test tubes laying on their sides and viewed at low power or with an inverted microscope.

16.10.13.4 Endpoint

16.10.13.4.1 In a count of at least 100 eggs, record the number of eggs with fertilization membranes and the number of eggs without fertilization membranes.

16.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

16.11.1 A summary of test conditions and test acceptability criteria is listed in Table 7.

TABLE 7. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR, *STRONGYLOCENTROTUS PURPURATUS* AND *DENDRASTER EXCENTRICUS*, FERTILIZATION TEST WITH EFFLUENTS AND RECEIVING WATERS

1.	Test type:	Static non-renewal
2.	Salinity:	34 ± 2‰
3.	Temperature:	12 ± 1°C
4.	Light quality:	Ambient laboratory light during test preparation

5.	Light intensity:	10-20 uE/m^2/s (Ambient laboratory levels)
6.	Test chamber size:	16 x 100 or 16 x 125 mm
7.	Test solution volume:	5 mL
8.	Number of spawners:	Pooled sperm from up to four males and pooled eggs from up to four females are used per test
9.	No. egg and sperm cells per chamber:	About 1,120 eggs and not more than 3,360,000 sperm per test tube
10.	No. replicate chambers per concentration:	4
11.	Dilution water:	Uncontaminated 1-μm-filtered natural seawater or hypersaline brine prepared from natural seawater or artificial sea salts
12.	Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% a control
13.	Dilution factor:	Effluents: ≥0.5 Receiving waters: None or ≥0.5
14.	Test duration:	40 min (20 min plus 20 min)
15.	Endpoint:	Fertilization of eggs
16.	Test acceptability criteria:	≥70% egg fertilization in controls; %MSD of <25%; and appropriate sperm counts
17.	Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, and Sample Preparation for Toxicity Tests)
18.	Sample volume required:	1 L

16.12 ACCEPTABILITY OF TEST RESULTS

16.12.1 Test results are acceptable only if all the following requirements are met:

(1) Egg fertilization at the NOEC must be greater than 80% of that in the controls.

(2) The minimum significant difference (%MSD) is <25% relative to the control.

(3) The sperm count for the final sperm stock must not exceed 33,600,000/mL.

(4) If the sperm count for the final sperm stock is between 5,600,000 and 33,600,000/mL it must not exceed 2x of the target density from the trial, or if no target density was specified for the test (see 11.5.1), the high sperm density controls (0.2 mL sperm stock) must have at least 5% higher fertilization than the low sperm density controls (0.05 mL sperm stock).

(5) Dilution water egg blanks and effluent egg blanks should contain essentially no eggs with fertilization membranes.

16.13 DATA ANALYSIS

16.13.1 GENERAL

16.13.1.1 Tabulate and summarize the data. Calculate the proportion of fertilized eggs for each replicate. A sample set of test data is listed in Table 8.

16.13.1.2 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

16.13.1.3 The endpoints of toxicity tests using the sea urchin and the sand dollar are based on the reduction in proportion of eggs fertilized. The IC25 is calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for fecundity are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints

and for the estimation of the IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

TABLE 8. DATA FROM SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, FERTILIZATION TEST

Effluent Concentration (%)	Replicate	No. of Eggs Counted	No. of Eggs Fertilized	Proportion Fertilized
Control	A	100	97	0.97
	B	100	90	0.90
	C	100	100	1.00
0.05	A	100	100	1.00
	B	100	100	1.00
	C	100	98	0.98
0.10	A	100	100	1.00
	B	100	97	0.97
	C	100	99	0.99
0.15	A	100	98	0.98
	B	100	96	0.96
	C	100	97	0.97
0.20	A	100	94	0.94
	B	100	88	0.88
	C	100	97	0.97
0.40	A	100	43	0.43
	B	100	63	0.63
	C	100	46	0.46
0.60	A	100	2	0.02
	B	100	1	0.01
	C	100	9	0.09
0.80	A	100	0	0.00
	B	100	0	0.00
	C	100	0	0.00

16.13.2 EXAMPLE OF ANALYSIS OF SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, AND SAND DOLLAR, *DENDRASTER EXCENTRICUS*, FERTILIZATION DATA

16.13.2.1 Formal statistical analysis of the fertilization data is outlined in Figure 7.

The response used in the analysis is the proportion of fertilized eggs in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there are no eggs fertilized in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25.

16.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

16.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

16.13.2.4 Example of Analysis of Fecundity Data

16.13.2.4.1 This example uses toxicity data from a sea urchin, *Strongylocentrotus purpuratus*, fertilization test performed with effluent. The response of interest is the proportion of fertilized eggs, thus each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and control are listed in Table 9. The data are plotted in Figure 8. Because there is zero fertilization in all three replicates for the 0.80% effluent concentration, it was not included in the statistical analysis and is considered a qualitative fecundity effect.

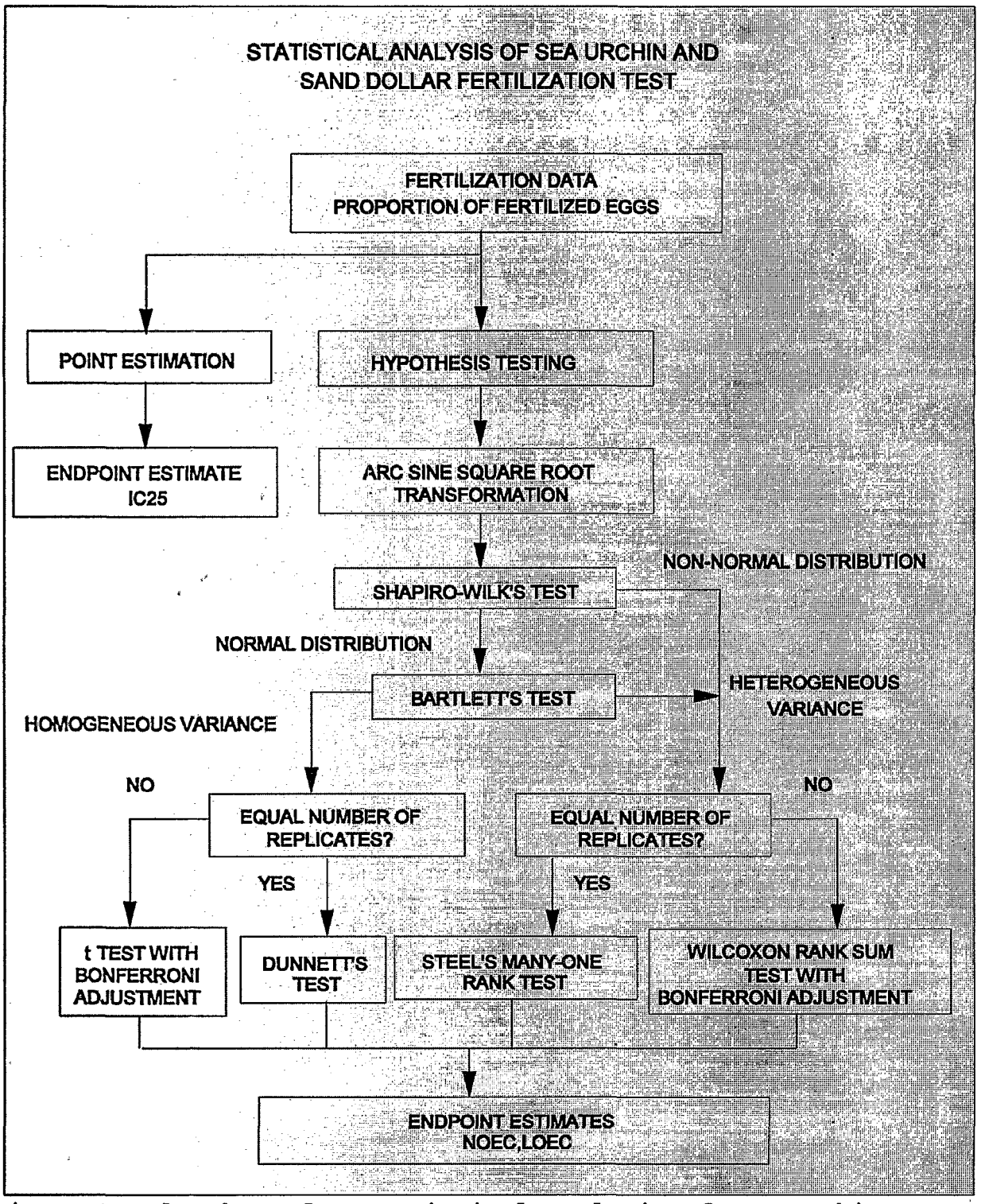


Figure 7. Flowchart for statistical analysis of sea urchin, *Strongylocentrotus purpuratus*, and sand dollar, *Dendraster excentricus*, test.

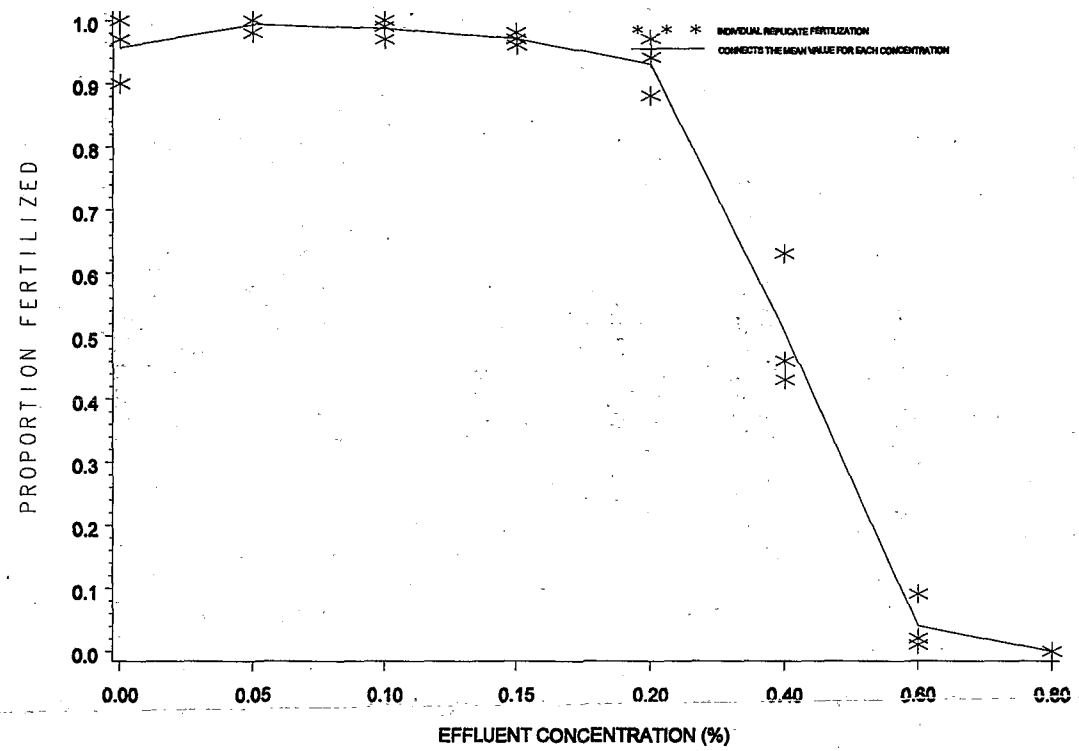


Figure 8. Plot of proportion of fertilized sea urchin, *Strongylocentrotus purpuratus*, eggs

16.13.2.5 Test for Normality

16.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 10.

TABLE 9. SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, FERTILIZATION DATA

		Effluent Concentration (%)						
	Rep.	Control	0.05	0.10	0.15	0.20	0.40	0.60
	A	0.97	1.00	1.00	0.98	0.94	0.43	0.02
RAW	B	0.90	1.00	0.97	0.96	0.88	0.63	0.01
	C	1.00	0.98	0.99	0.97	0.97	0.46	0.09
ARC SINE	A	1.397	1.521	1.521	1.429	1.323	0.715	0.142
SQUARE ROOT	B	1.249	1.521	1.397	1.369	1.217	0.917	0.100
TRANSFORMED	C	1.521	1.429	1.471	1.397	1.397	0.745	0.305
Mean ($\overline{Y}_i$)		1.389	1.490	1.463	1.398	1.312	0.792	0.182
S_i^2		0.01854	0.00282	0.00389	0.00090	0.00819	0.01188	0.01173
i		1	2	3	4	5	6	7

TABLE 10. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Effluent Concentration (%)						
Replicate	Control	0.05	0.10	0.15	0.20	0.40	0.60
A	0.008	0.031	0.058	0.031	0.011	-0.077	-0.040
B	-0.140	0.031	-0.066	-0.029	-0.095	0.125	-0.082
C	0.132	-0.061	0.008	-0.001	0.085	-0.047	0.123

16.13.2.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

Where: X_i = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations

16.13.2.5.3 For this set of data, n = 21

$$\overline{X} = \frac{1}{21} (0.005) = 0.000$$

D = 0.1159

16.13.2.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 11.

16.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 21 and k = 10. The a_i values are listed in Table 12.

16.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D} \left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)}) \right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 12. For the data in this example:

$$W = \frac{1}{0.1159} (0.3345)^2 = 0.9654$$

16.13.2.5.7 The decision rule for this test is to compare W as calculated in 2.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this

TABLE 11. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.140	12	0.008
2	-0.095	13	0.011
3	-0.082	14	0.031
4	-0.077	15	0.031
5	-0.066	16	0.031
6	-0.061	17	0.058
7	-0.047	18	0.085
8	-0.040	19	0.123
9	-0.029	20	0.125
10	-0.001	21	0.132
11	0.008		

TABLE 12. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4643	0.272	$X^{(21)} - X^{(1)}$
2	0.3185	0.220	$X^{(20)} - X^{(2)}$
3	0.2578	0.205	$X^{(19)} - X^{(3)}$
4	0.2119	0.162	$X^{(18)} - X^{(4)}$
5	0.1736	0.124	$X^{(17)} - X^{(5)}$
6	0.1399	0.092	$X^{(16)} - X^{(6)}$
7	0.1092	0.078	$X^{(15)} - X^{(7)}$
8	0.0804	0.071	$X^{(14)} - X^{(8)}$
9	0.0530	0.040	$X^{(13)} - X^{(9)}$
10	0.0263	0.009	$X^{(12)} - X^{(10)}$

example, the critical value at a significance level of 0.01 and n = 21 observations is 0.873. Since W = 0.9654 is greater than the critical value, conclude that the data are normally distributed.

16.13.2.6 Test for Homogeneity of Variance

16.13.2.6.1 The test used to examine whether the variation in the proportion of fertilized eggs is the same across all effluent

concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i) \ln \overline{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each concentration and control,

$V_i = (n_i - 1)$

p = number of concentration levels including the control

n_i = the number of replicates for concentration i.

ln = $\log_e$

i = 1,2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i^2)}{\sum_{i=1}^{p} V_i} \qquad C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{p} 1/V_i - (\sum_{i=1}^{p} V_i)^{-1}]$$

16.13.2.6.2 For the data in this example (see Table 8), all effluent concentrations including the control have the same number of replicates (n_i = 3 for all i). Thus, V_i = 2 for all i.

16.13.2.6.3 Bartlett's statistic is, therefore:

$$B = [(14)\ln(0.008279) - 2\sum_{i=1}^{p} \ln(S_i^2)] / 1.1905$$

$$= [14(-4.7940) - 2(-36.1047)]/1.1905$$

$$= 5.0934/1.1905$$

$$= 4.2784$$

16.13.2.6.4 B is approximately distributed as chi-square with p-1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with 6 degrees of freedom, is 16.81. Since B = 4.2784 is less than the critical value of 16.81, conclude that the variances are not different.

16.13.2.7 Dunnett's Procedure

16.13.2.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 13.

TABLE 13. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{p}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{p} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the proportion of fertilized eggs for concentration i in test chamber j)

16.13.2.7.2 For the data in this example:

$$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = n_7 = 3$$

$$N = 21$$

$$T_1 = Y_{11} + Y_{12} + Y_{13} = 4.167$$
$$T_2 = Y_{21} + Y_{22} + Y_{23} = 4.471$$
$$T_3 = Y_{31} + Y_{32} + Y_{33} = 4.389$$
$$T_4 = Y_{41} + Y_{42} + Y_{43} = 4.194$$
$$T_5 = Y_{51} + Y_{52} + Y_{53} = 3.937$$
$$T_6 = Y_{61} + Y_{62} + Y_{63} = 2.377$$
$$T_7 = Y_{71} + Y_{72} + Y_{73} = 0.547$$

$$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 + T_7 = 24.082$$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$
$$(95.656)/3 - (24.082)^2/21 = 4.269$$

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$
$$32.001 - (24.082)^2/21 = 4.385$$

$$SSW = SST - SSB = 4.385 - 4.269 = 0.116$$

$$S_B^2 = SSB/(p-1) = 4.269/(7-1) = 0.7115$$

$$S_W^2 = SSW/(N-p) = 0.116/(21-7) = 0.0083$$

16.13.2.7.3 Summarize these calculations in the ANOVA table (Table 14).

16.13.2.7.4 To perform the individual comparisons, calculate the *t* statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

TABLE 14. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	6	4.269	0.7115
Within	14	0.116	0.0083
Total	20	4.385	

Where: $\overline{Y}_i$ = mean proportion fertilized eggs for concentration i

$\overline{Y}_1$ = mean proportion fertilized eggs for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

Since we are looking for a decreased response from the control in the proportion of fertilized eggs, the concentration mean is subtracted from the control mean.

16.13.2.7.5 Table 15 includes the calculated *t* values for each concentration and control combination. In this example, comparing the 0.05% concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.389 - 1.490)}{0.0911\sqrt{(1/3)+(1/3)}} = -1.358$$

16.13.2.7.6 Since the purpose of this test is to detect a significant decrease in the proportion of fertilized eggs, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 14 degrees of freedom for error and six concentrations (excluding the control) the critical value is 2.53. The mean proportion of fertilized eggs for concentration i is considered significantly less than the mean proportion of fertilized eggs for the control if t_i is greater than the

critical value. Therefore, the 0.40% and 0.60% concentrations have a significantly lower mean proportion of fertilized eggs than the control. Hence the NOEC is 0.20% effluent and the LOEC is 0.40% effluent.

TABLE 15. CALCULATED t VALUES

Effluent Concentration (%)	i	t_i
0.05	2	-1.358
0.10	3	-0.995
0.15	4	-0.121
0.20	5	1.035
0.40	6	8.026
0.60	7	16.227

16.13.2.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\ S_w\sqrt{(1/n_1)+(1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

16.13.2.7.8 In this example,

$$MSD = 2.53\ (0.0911)\sqrt{(1/3)+(1/3)}$$

$$= 2.53\ (0.0911)(0.8165)$$

$$= 0.188$$

16.13.2.7.9 The MSD (0.188) is in transformed units. To determine the MSD in terms of proportion of fertilized eggs, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

$$1.389 - 0.188 = 1.201$$

2. Obtain the untransformed values for the control mean and the difference calculated in step 1 of 13.2.7.9.

$$[\ \text{Sine}\ (1.389)\ ]^2 = 0.967$$

$$[\ \text{Sine}\ (1.201)\ ]^2 = 0.869$$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from step 2 in 14.2.7.9.

$$MSD_u = 0.967 - 0.869 = 0.098$$

16.13.2.7.10 Therefore, for this set of data, the minimum difference in mean proportion of fertilized eggs between the control and any effluent concentration that can be detected as statistically significant is 0.098.

16.13.2.7.11 This represents a 10.2% decrease in the proportion of fertilized eggs from the control.

16.13.2.8 Calculation of the ICp

16.13.2.8.1 The fertilization data in Table 7 are utilized in this example. As can be seen from Figure 8, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

16.13.3.8.2 Starting with the observed control mean, $Y_1 = 0.957$, and the observed mean for the lowest effluent concentration, $Y_2 = 0.993$, we see that Y_1 is less than Y_2.

16.13.3.8.3 Calculate the smoothed means:

$$M_1 = M_2 = (Y_1 + Y_2)/2 = 0.975$$

16.13.3.8.4 Since $Y_3 = 0.987$ is larger than M_2, average Y_3 with the previous concentrations:

$$M_1 = M_2 = M_3 = (M_1 + M_2 + Y_3)/3 = 0.979.$$

16.13.3.8.5 Since $M_3 > Y_4 = 0.970 > Y_5 = 0.930 > Y_6 = 0.507 > Y_7 = 0.040 > Y_8 = 0.0$, set $M_4 = 0.970$, $M_5 = 0.930$, $M_6 = 0.507$, $M_7 = 0.040$, and $M_8 = 0.0$. Table 16 contains the smoothed means and Figure 10 gives a plot of the smoothed means and the interpolated response curve.

16.13.2.8.6 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of fertilized eggs, compared to the controls, would result in a mean proportion of 0.734, where $M_1(1-p/100) = 0.979(1-25/100)$. Examining the means and their associated concentrations

(Table 16), the response, 0.734, is bracketed by C_5 = 0.20% effluent and C_6 = 0.40% effluent.

16.13.2.8.7 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1-p/100) - M_j] \frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 0.20 + [0.979(1 - 25/100) - 0.930] \frac{(0.40 - 0.20)}{(0.507 - 0.930)}$$

$$= 0.29\%.$$

TABLE 16. SEA URCHIN, *STRONYLOCENTROTUS PURPURATUS*, MEAN PROPORTION OF FERTILIZED EGGS

Effluent Conc. (%)	i	Response Means, Y_i (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.957	0.979
0.05	2	0.993	0.979
0.10	3	0.987	0.979
0.15	4	0.970	0.970
0.20	5	0.930	0.930
0.40	6	0.507	0.507
0.60	7	0.040	0.040
0.80	8	0.000	0.000

16.13.2.8.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 0.2925%. The empirical 95.0% confidence interval for the true mean was 0.2739% to 0.3241%. The computer program output for the IC25 for this data set is shown in Figure 10.

16.14 **PRECISION AND ACCURACY**

16.14.1 PRECISION

16.14.1.1 Single-Laboratory Precision

16.14.1.1.1 Single-laboratory precision data for *Strongylocentrotus purpurtatus* with the reference toxicant copper, tested in natural seawater, are provided in Table 17. The coefficient of variation based on the EC25 is 29%, and on EC50 is 24%, showing acceptable precision. Single-laboratory precision data for *Dendraster excentricus* with the reference toxicant copper, tested in natural seawater, are provided in Tables 18 and 19. The coefficient of variation based on the EC25, is 18% to 29% and EC50, is 21% to 33%, showing acceptable precision.

16.14.1.2 Multi-laboratory Precision

16.14.1.2.1 Multi-laboratory precision data for *Strongylocentrotus purpuratus*, with the reference toxicant copper, tested in natural seawater, are provided in Table 20. The coefficient of variation for the EC25 was 52%, based on data from five laboratories.

16.14.2 ACCURACY

16.14.2.1 The accuracy of toxicity tests cannot be determined.

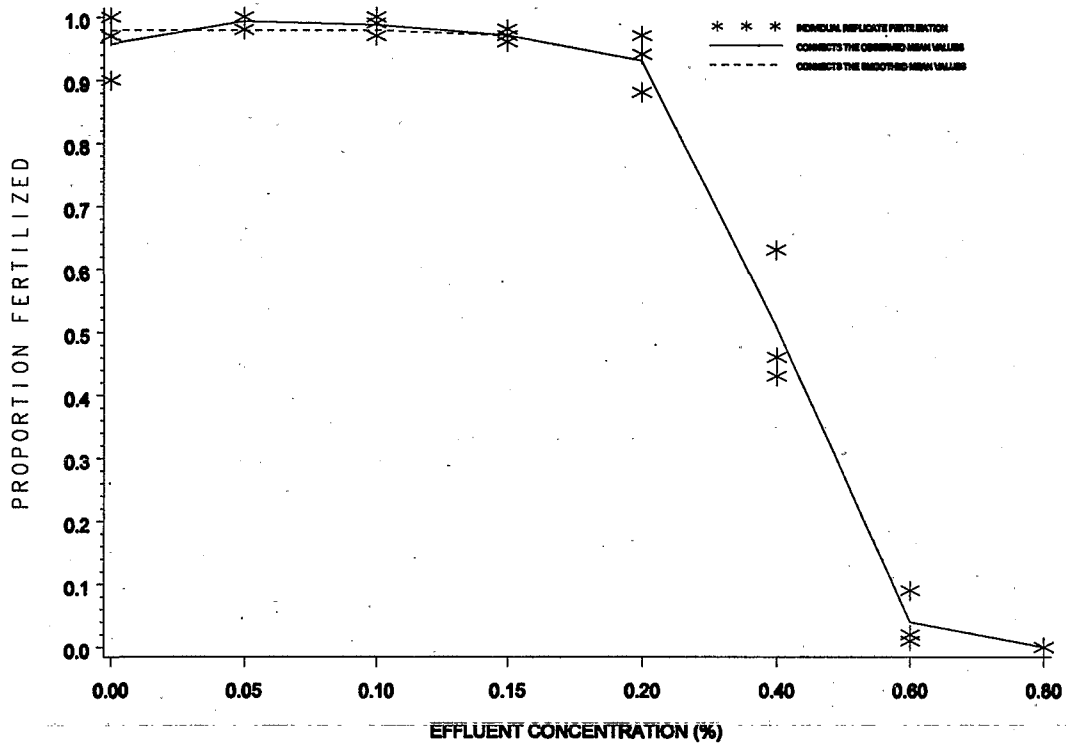


Figure 9. Plot of raw data, observed means, and smoothed means for the sea urchin, *Strongylocentrotus purpuratus*, eggs.

```
Conc. ID          1     2     3     4     5     6     7     8
-------------------------------------------------------------
Conc. Tested      0   .05   .10   .15   .20   .40   .60   .80
-------------------------------------------------------------
Response  1     .97  1.00  1.00   .98   .94   .43   .02     0
Response  2     .90  1.00   .97   .96   .88   .63   .01     0
Response  3    1.00   .98   .99   .97   .97   .46   .09     0
-------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date:     Test Ending Date:
Test Species: Sea Urchin, Strongylocentrotus purpuratus
Test Duration:       40 minutes
DATA FILE: urchin.icp
OUTPUT FILE: urchin.i25
-------------------------------------------------------------------------
Conc.      Number      Concentration      Response       Std.        Pooled
 ID      Replicates                 %       Means        Dev.    Response Means
-------------------------------------------------------------------------
  1           3              0.000          0.957       0.051       0.979
  2           3              0.050          0.993       0.012       0.979
  3           3              0.100          0.987       0.015       0.979
  4           3              0.150          0.970       0.010       0.970
  5           3              0.200          0.930       0.046       0.930
  6           3              0.400          0.507       0.108       0.507
  7           3              0.600          0.040       0.044       0.040
  8           3              0.800          0.000       0.000       0.000
-------------------------------------------------------------------------
The Linear Interpolation Estimate:      0.2925   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:   0.2917 Standard Deviation:     0.0141
Original Confidence Limits:   Lower:      0.2739 Upper:      0.3241
Expanded Confidence Limits:   Lower:      0.2533 Upper:      0.3589
Resampling time in Seconds:      0.22  Random_Seed: -25579058
```

Figure 10. ICPIN program output for the IC25.

TABLE 17. SINGLE LABORATORY PRECISION OF THE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS* FERTILIZATION TEST PERFORMED IN SEAWATER USING GAMETES FROM ADULTS MAINTAINED IN SEAWATER AFTER BEING COLLECTED FROM NATURAL POPULATIONS WITH COPPER (CU µG/L) SULFATE AS THE REFERENCE TOXICANT

Test Number	NOEC (µg/L)	EC25 (µg/L)	EC50 (µg/L)
1	6.9	9.7	14.3
2	23.0	26.2	30.9
3	11.2	19.6	25.8
4	16.0	16.4	31.1
5	15.3	17.8	24.6
6	10.8	18.6	28.3
Mean CV(%)		18.1 29.0	25.8 24.0

Tests performed by Sally Noack, AScI, at EPA's Pacific Ecosystems Branch of ERL-Narragansett, Newport, OR.
Copper concentrations were measured and within 10% of nominal; nominal concentrations were 5, 8, 12, 17, 25, 35, and 50 µg/L.
These tests used only three replicates per concentration.

TABLE 18. SINGLE LABORATORY PRECISION OF THE SAND DOLLAR, *DENDRASTER EXCENTRICUS* FERTILIZATION TEST PERFORMED IN SEAWATER USING GAMETES FROM ADULTS MAINTAINED IN SEAWATER AFTER BEING COLLECTED FROM NATURAL POPULATIONS WITH COPPER (CU µG/L) SULFATE AS THE REFERENCE TOXICANT

Test Date	Test Number	NOEC (µg/L)	EC25 (µg/L)	EC50 (µg/L)
7/11/94	1* 2** 3***	5.0 5.0 -	9.4 14.6 16.0	12.6 17.5 18.6
7/14/94	1* 2** 3***	12.0 <5.0 17.0	16.7 19.6 23.0	20.9 25.8 30.5
7/17/94	1* 2** 3***	8.0 5.0 12.0	15.3 13.5 13.4	17.7 16.4 17.0
7/19/94	1* 2** 3***	12.0 17.0 12.0	12.8 18.6 13.3	15.6 22.1 16.0
Mean	1 2 3 overall		13.5 16.6 16.4	16.7 20.5 20.5
SD	1 2 3 overall		3.2 3.0 4.6	3.5 4.3 6.7
CV(%)	1 2 3 overall		24% 18% 28%	21% 21% 33%

Tests performed at National Council of the Paper Industry for Air and Stream Improvement, Inc. Anacortes, WA.
Copper concentrations were nominal; nominal concentrations were 5, 8, 12, 17, 25, 35, and 50 µg/L.

* Tests conducted with nominal S:E ratio of 147:1
** Tests conducted with nominal S:E ratio of 166:1
*** Tests conducted with nominal S:E ratio of 224:1

TABLE 19. SINGLE LABORATORY PRECISION OF THE SAND DOLLAR, *DENDRASTER EXCENTRICUS* FERTILIZATION TEST PERFORMED IN SEAWATER USING GAMETES FROM ADULTS MAINTAINED IN SEAWATER AFTER BEING COLLECTED FROM NATURAL POPULATIONS WITH COPPER (CU µG/L) SULFATE AS THE REFERENCE TOXICANT.

Test Number	NOEC (µg/L)	EC25 (µg/L)	EC50 (µg/L)
1	17.0	25.8	31.0
2	25.0	34.3	41.8
3	12.0	31.1	43.7
4	8.0	14.2	19.8
5	25.0	27.2	30.5
Mean CV(%)		26.5 29.0	33.4 29.0

Tests performed by Gary Chapman and Debra Denton at EPA's Pacific Ecosystems Branch of ERL-Narragansett, Newport, OR.

Copper concentrations were nominal; nominal concentrations were 5, 8, 12, 17, 25, 35, and 50 µg/L.

TABLE 20. MULTIPLE LABORATORY PRECISION OF THE SEA URCHIN, *STONGYLOCENTROTUS PURPURATUS*, FERTILIZATION TEST PERFORMED WITH COPPER (CU µG/L) SULFATE AS A REFERENCE TOXICANT

Lab	# of Tests	Statistic	EC25 (µg/L)
A	3	Mean SD CV(%)	7.8 3.0 38%
B	2	Mean SD CV(%)	4.0 - -
C	6	Mean SD CV(%)	18.0 5.4 30%
D	2	Mean NA CV(%)	14.9 - -
E	6	Mean SD CV(%)	19.3 10.5 54%

# of Lab Means	Statistic	EC25
5	Mean SD CV(%)	12.8 6.6 52%

Tests performed as part of a methods evaluation effort organized by the US EPA laboratory in Newport, Oregon; tests were conducted in 1991 by volunteer laboratories in California and Washington.

APPENDIX I. PURPLE URCHIN AND SAND DOLLAR TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution (2,000 mg/L) by adding 5.366 g of copper chloride ($CuCl_2 \cdot 2H_2O$) to 1 liter of reagent water. For each reference toxicant test prepare a copper sub-stock of 3 mg/L by diluting 1.5 mL of stock to one liter with reagent water.

D. Prepare a control (0 µg/L) plus at least five consecutive copper reference toxicant solutions (e.g., from the series 3.0, 4.4, 6.5, 9.5, 13.9, 20.4, and 30.0 µg/L, by adding 0.10, 0.15, 0.22, 0.32, 0.46, 0.68, and 1.00 mL of sub-stock solution, respectively, to 100-L volumetric flasks and filling to 100-mL with dilution water).

E. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

F. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

G. Place test chambers in a water bath or environmental chamber set to 12°C and allow temperature to equilibrate.

H. Measure the temperature in several temperature blanks during the course of the test.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Obtain test organisms and hold or condition as necessary for spawning.

B. On day of test, spawn organisms, examine gametes, pool good eggs, pool good sperm.

C. Determine egg and sperm densities and adjust as necessary.

D. Run trial sperm:egg fertilization test (optional).

E. Adjust sperm density for definitive test.

F. Inject sperm into test solutions.

G. 20 minutes later inject eggs into test solutions.

H. 20 minutes after egg addition, stop the test by the addition of preservative.

I. Confirm sperm density in definitive test by hemacytometer counts.

J. Count at least 100 eggs in each test tube.

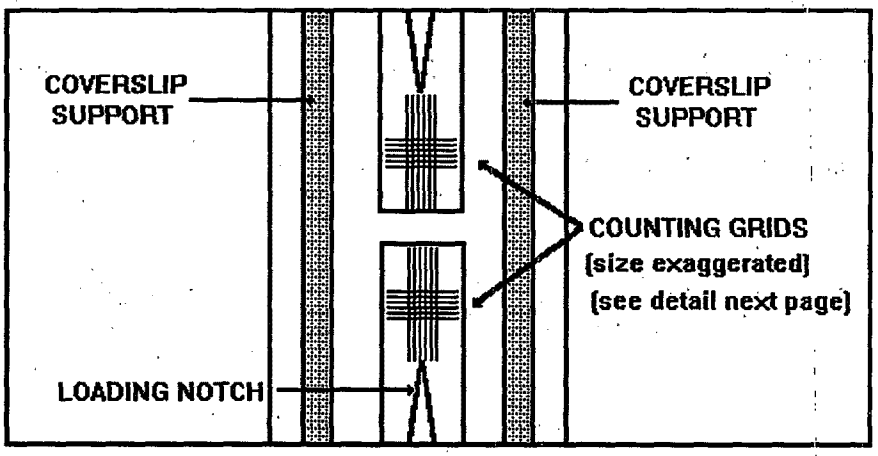
K. Analyze the data.

L. Include standard reference toxicant point estimate values in the standard quality control charts.

APPENDIX II. USING THE NEUBAUER HEMACYTOMETER TO ENUMERATE SEA URCHIN SPERM

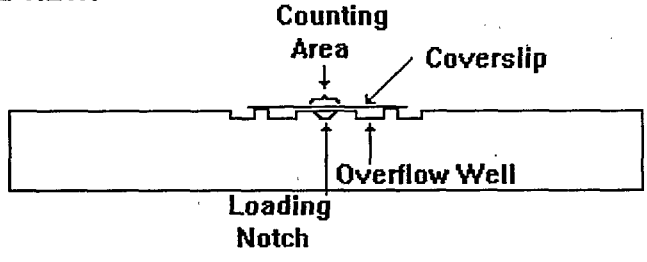
The Neubauer hemacytometer is a specialized microscope slide with two counting grids and a coverslip.

TOP VIEW:

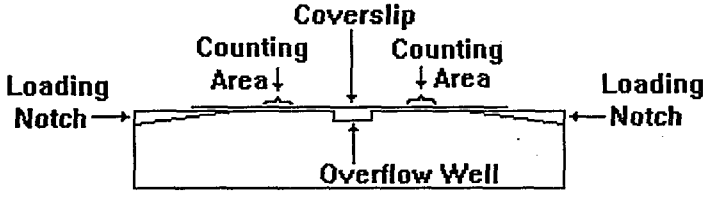


Together, the total area of each grid (1 mm^2) and the vertical distance between the grid and the coverslip (0.1 mm), provide space for a specific microvolume of aqueous sample (0.1 mm^3).

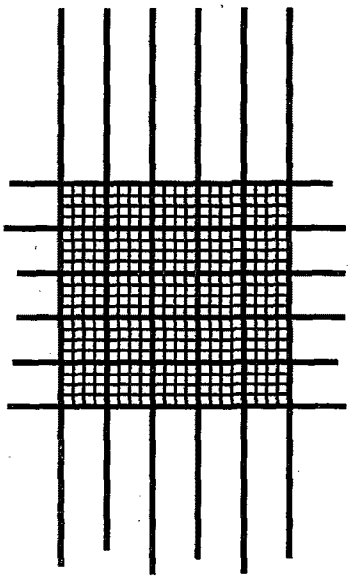
SIDE VIEW:



END VIEW THROUGH MID-CROSS SECTION:



This volume of liquid and the cells suspended therein (e.g., blood cells or sperm cells) represent 1/10,000th of the liquid volume and cell numbers of a full milliliter (cm^3) of the sampled material.



NEUBAUER
HEMACYTOMETER
GRID OF 400 SQUARES

If the full 400-squares of each grid are counted, this represents the number of sperm in 0.1 mm^3. Multiplying this value times 10 yields the sperm per mm^3 (and is the source of the hemacytometer factor of 4,000 squares/mm^3). If this product is multiplied by 1,000 mm^3/cm^3, the answer is the number of sperm in one milliliter of the sample. If the counted sample represents a dilution of a more concentrated original sample, the above answer is multiplied by the dilution factor to yield the cell density in the original sample. If the cells are sufficiently dense, it is not necessary to count the entire 400-square field, and the final calculation takes into account the number of squares actually counted:

$$\text{cells/mL} = \frac{\text{(dilution) (4,000 squares/mm}^3\text{) (1,000 mm}^3\text{/cm}^3\text{) (cell count)}}{\text{(number of squares counted)}}$$

Thus, with a dilution of 4000 (0.025 mL of semen in 100 mL of dilution water), 80 squares counted, and a count of 100, the calculation becomes:

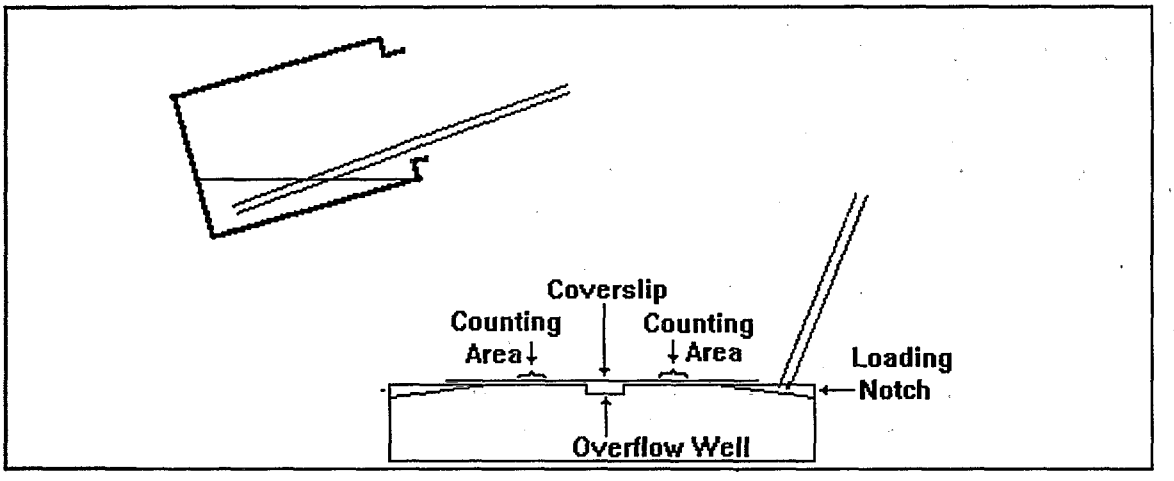
$$\text{cells/mL} = \frac{(4{,}000)\ (4{,}000)\ (1{,}000)\ (100)}{80}$$

$$= 20{,}000{,}000{,}000 \text{ cells/mL}$$

There are several procedures that are necessary for counts to be consistent within and between laboratories. These include mixing the sample, loading and emptying the hematocrit tube, cleaning the hemacytometer and cover slip, and actual counting procedures.

Obviously, if the sample is not homogeneous, subsamples can vary in sperm density. A few extra seconds in mixing can save a lot of wasted minutes in subsequent counting procedures. A full hematocrit tube empties more easily than one with just a little liquid, so withdraw a full sample. This can be expedited by tipping the sample vial.

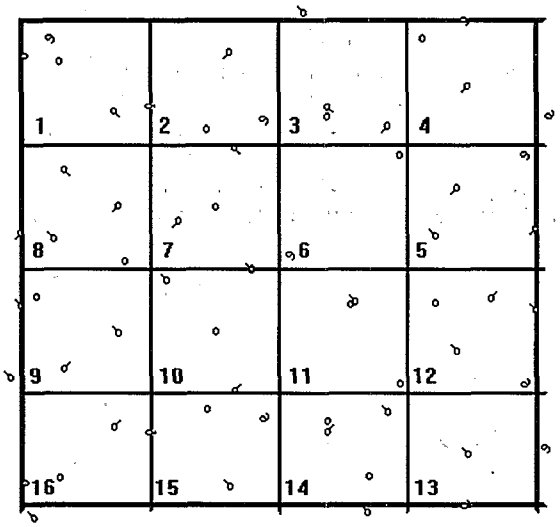
Because the sperm are killed prior to sampling, they will slowly settle. For this reason, the sample in the hematocrit tube should be loaded onto the hemacytometer as rapidly as possible. Two replicate samples are withdrawn in fresh hematocrit tubes and loaded onto opposite sides of a hemacytometer.



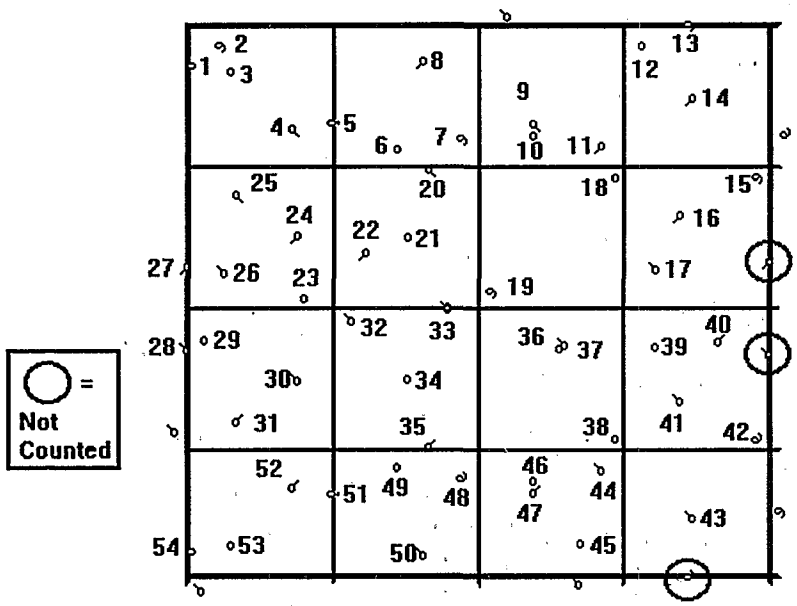
The loaded hemacytometer is left for 15 minutes to allow the sperm to settle onto the counting field. If the coverslip is moved after the samples are loaded, the hemacytometer should be rinsed and refilled with fresh sample. After 15 minutes, the hemacytometer is placed under a microscope and the counting grid located at 100x. Once the grid is properly positioned, the microscope is adjusted to 200x or 400x, and one of the corner squares is positioned for counting (any one of the four corners is appropriate). For consistency, use the same procedure each time (Many prefer to start in the upper left corner of the optical field, and this procedure will be used in the examples given below).

Examine the first large square in the selected corner. If no sperm are visible, or if the sperm are so dense or clumped to preclude accurate counting, count a sample with a more appropriate dilution.

In making counts of sperm, it is necessary to adopt a consistent method of scanning the smaller squares and counting sperm that fall upon the lines separating the squares. Count the sperm in the small squares by beginning in the upper left hand corner (square 1) and proceding right to square 4, down to square 5, left to square 8, etc. until all 16 squares are counted.



Because sperm that appear on lines might be counted as being in either square, it is important to avoid double counting or non-counting. For this reason a convention is decided upon and used consistently: paraphrasing the instructions received with one (Hausser Scientific) counting chamber "to avoid counting (sperm) twice, the best practice is to count all touching the top and left, and none touching the lower and right, boundary lines." Whatever convention is chosen, it must be adhered to. The example below shows a sperm count based upon a selected convention of counting sperm that fall on the upper and left lines, but not on the lower or right lines:



In the above illustration, sperm falling on the lower and right lines are not counted. The count begins at the upper left as illustrated in the preceding figure. A typical count sequence is demonstrated by the numbers next to each sperm illustrated. Sperm identified as numbers 1, 5, 13, 20, 27, 28, 33, 51 and 54 touch lines and are counted as being in the square below them or to their right. The circled sperm are not counted as being in this field of 16 small squares (but they would be included in any counts of adjacent squares in which they would be on upper or left hand lines).

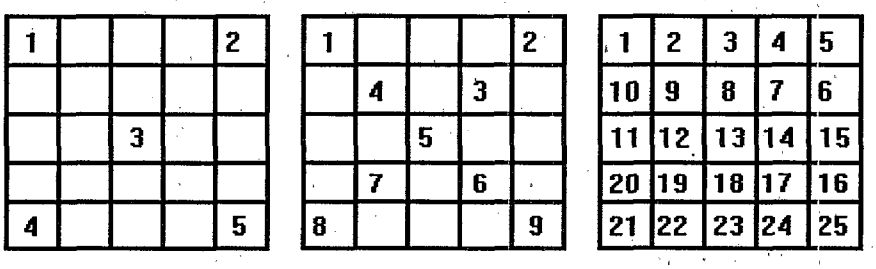
Once these counting conventions have been selected, it is advisable to follow another strict protocol outlining the number and sequence of large squares to be counted. Because the sperm may not be randomly distributed across the counting grid, it is recommended to count an array of squares covering the entire grid. The following procedure is recommended:

Count the number of sperm in the first large square.

1. If the number is less than 10, count all 25 squares using the same scanning pattern outlined above (left to right through squares 1 to 5, down to square 6, left through square 10, down to 11, etc.). See pattern no. 3.

2. If the number is between 10 and 19, count 9 large squares using pattern no. 2.

3. If the number is 20 or greater, count 5 large squares using pattern no. 1.



Pattern no. 1 Pattern no. 2 Pattern no. 3

The final consideration in achieving good replicate counts is keeping the hemacytometers and coverslips clean. They should be rinsed in distilled water soon after use. The coverslips should be stored in a good biocleanser such as hemasol. For an hour or so prior to use, the hemacytometer slides should also be soaked in the solution. Both slides and coverslips should then be rinsed off with reagent water, blotted dry with a lint-free tissue, and wiped with lens paper.

SECTION 17

GIANT KELP, *Macrocystis pyrifera* GERMINATION AND GERM-TUBE GROWTH TEST METHOD

Adapted from a method developed by
Brian S. Anderson and John W. Hunt
Institute of Marine Sciences, University of California
Santa Cruz, California

(in association with)
California Department of Fish and Game
Marine Pollution Studies Laboratory
34500 Coast Route 1, Monterey, CA 93940

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SECTION 17

GIANT KELP, *MACROCYSTIS PYRIFERA* GERMINATION AND GROWTH TEST

17.1 SCOPE AND APPLICATION

17.1.1 This method estimates the chronic toxicity of effluents and receiving water to zoospores and embryonic gametophytes of giant kelp, *Macrocystis pyrifera* during a 48-h static non-renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

17.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

17.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

17.1.4 This method is commonly used in one of two forms: (1) a definitive test, consisting of minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

17.1.5 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

17.2 SUMMARY OF METHOD

17.2.1 This method provides step-by-step instructions for performing a 48-h day static non-renewal toxicity test using giant kelp to determine the toxicity of substances in marine and

estuarine waters. The test endpoints are germination of gameophyte spores and length of embryonic gametophyte germination tubes.

17.3 **INTERFERENCES**

17.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment, and Supplies).

17.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

17.4 **SAFETY**

17.4.1 See Section 3, Health and Safety.

17.5 **APPARATUS AND EQUIPMENT**

17.5.1 Tanks, trays, or aquaria -- for holding and acclimating giant kelp, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20°C), with appropriate filtration and aeration system.

17.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

17.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (15°C) prior to the test.

17.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

17.5.5 Refractometer -- for determining salinity.

17.5.6 Hydrometer(s) -- for calibrating refractometer.

17.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

17.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD 170.1, USEPA, 1979) -- to calibrate laboratory thermometers.

17.5.9 pH and DO meters -- for routine physical and chemical measurements.

17.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

17.5.11 Winkler bottles -- for dissolved oxygen determinations.

17.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

17.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

17.5.14 Glass stirring rods -- for mixing test solutions.

17.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-1000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

17.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

17.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

17.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

17.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

17.5.20 Wash bottles -- for dilution water.

17.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

17.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

17.5.23 Beakers, 250 borosilicate glass -- for mixing test solutions.

17.5.24 Beakers, 1,000 mL borosilicate glass -- for holding sporophyll blades.

17.5.25 Inverted or compound microscope -- for inspecting zoopspores and embryonic gametophytes.

17.5.26 Hemacytometer (bright-line rbc) -- for measuring zoospore density.

17.5.27 Counter, two unit, 0-999 -- for recording counts of zoopspores.

17.5.28 Light meter (irradiance meter w/cosine corrected sensor) -- for measuring light intensity.

17.5.29 Cool white fluorescent lights -- for providing light during incubation of developing gametophytes.

17.5.30 60 μm NITEX® filter -- for filtering receiving water.

17.6 **REAGENTS AND SUPPLIES**

17.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

17.6.2 Data sheets (one set per test) -- for data recording (Figures 1 and 2).

17.6.3 Tape, colored -- for labelling test chambers and containers.

17.6.4 Markers, water-proof -- for marking containers, etc.

17.6.5 Parafilm -- to cover graduated cylinders and vessels.

17.6.6 Gloves, disposable -- for personal protection from contamination.

17.6.7 Pipets, serological -- 1-10 mL, graduated.

17.6.8 Pipet tips -- for automatic pipets.

17.6.9 Coverslips -- for microscope slides.

17.6.10 Lens paper -- for cleaning microscope optics.

17.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

17.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

17.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

17.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

17.6.15 Laboratory quality assurance samples and standards -- for the above methods.

17.6.16 Test chambers -- 600 mL, five chambers per concentration. The chambers should be borosilicate glass (for effluents) or nontoxic disposable plastic labware (for reference toxicants). To avoid contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered during the test with safety glass plates or a plastic sheet (6 mm thick).

17.6.17 Glutaraldehyde -- for specimen preservation - optional; (see Section 17.10.8.2).

17.6.18 Microscope slide (flat) -- for each test chamber to serve as the substratum upon which the zoospores will settle.

17.6.19 Reference toxicant solutions (see Section 17.10.2.4 and see Section 4, Quality Assurance).

17.6.20 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies and Section 7, Dilution Water).

17.6.21 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

17.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 17.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-μm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

17.6.23 HYPERSALINE BRINES

17.6.23.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

17.6.23.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the

microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

17.6.23.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

17.6.23.4 Freeze Preparation of Brine

17.6.23.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from

four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20°C until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

17.6.23.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100%. It is advisable not to exceed about 70% brine salinity unless it is necessary to test effluent concentrations greater than 50%.

17.6.23.4.3 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

17.6.23.5 Heat Preparation of Brine

17.6.23.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

17.6.23.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

17.6.23.5.3 Seawater should be filtered to at least 10 μm before being put into the brine generator. The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

17.6.23.5.4 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

17.6.23.6 Artificial Sea Salts

17.6.23.6.1 No data from giant kelp tests using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

17.6.23.7 Dilution Water Preparation from Brine

17.6.23.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

17.6.23.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the

effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100% and the test is to be conducted at 34‰, 100% divided by 34‰ = 2.94. The proportion of brine is 1 part, plus 1.94 parts reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

17.6.23.8 Test Solution Salinity Adjustment

17.6.23.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

17.6.23.8.2 Check the pH of all test solutions and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide (see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

17.6.23.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

17.6.23.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

17.6.23.9 Preparing Test Solutions

17.6.23.9.1 Two hundred mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution

water. For example, to prepare 1% effluent, add 10 mL of effluent to a 1-liter volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 1-Liter mark with dilution water, stopper it, and shake to mix. Distribute equal volumes into the replicate test chambers.

17.6.23.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Distribute equal volumes into the replicate test chambers.

17.6.23.10 Brine Controls

17.6.23.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 17.6.23.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

17.6.24 TEST ORGANISMS

17.6.24.1 The test organisms for this method are the zoospores of the giant kelp, *Macrocystis pyrifera*. *Macrocystis* is the dominant canopy forming Laminarian alga in southern and central California and forms extensive subtidal forests along the coast. Giant kelp forests support a rich diversity of marine life and provide habitat and food for hundreds of invertebrate and vertebrate species (North, 1971; Foster and Schiel, 1985). It

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (x‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

is an appropriate toxicity test species because of its availability, economic and ecological importance, history of successful laboratory culture (North, 1976; Luning, 1980; Kuwabara, 1981; Deysher and Dean, 1984; Linfield, 1985), and previous use in toxicity testing (Smith and Harrison, 1978; James et al., 1987; Anderson and Hunt, 1988; Hunt et al., 1989; Anderson et al., 1990). Other Laminarian alga species have proven to be useful for laboratory toxicity testing (Chung and Brinkhuis, 1986; Thompson and Burrows, 1984; Hopkin and Kain, 1978; see Thursby et al., 1993 for review).

17.6.24.2 Like all kelps, *Macrocystis* has a life cycle that alternates between a microscopic gametophyte stage and a macroscopic sporophyte stage. It is the sporophyte stage that forms kelp forests. These plants produce reproductive blades (sporophylls) at their base. The sporophylls develop patches (sori) in which biflagellate, haploid zoospores are produced. The zoospores are released into the water column where they swim and eventually settle onto the bottom and germinate. The dioecious spores develop into either male or female gametophytes. The male gametophytes produce flagellated gametes which may fertilize eggs produced by the female gametophytes. Fertilized eggs develop into sporophytes within 12- 15 days, completing the lifecycle.

17.6.24.3 The method described here focuses on germination of the zoospores and the initial growth of the developing gametophytes. It involves the controlled release of zoospores from the sporophyll blades, followed by the introduction of a spore suspension of known density into the test containers. The zoospores swim through the test solution and eventually settle onto glass microscope slides. The settled spores germinate by extruding the cytoplasm of the spore through the germ-tube into the first gametophytic cell. This stage is often referred to as the "dumbell" stage. The two endpoints measured after 48 hours are germination success and growth of the embryonic gametophytes (germ-tube length).

17.6.24.4 Species Identification

17.6.24.4.1 Although there is some debate over the taxonomy of the genus *Macrocystis*, Abbott and Hollenberg (1976) consider only two species in California: *M. pyrifera*, and *M. integrifolia*. The

two are distinguished from each other based on habitat and the morphology of their holdfasts. *Macrocystis pyrifera* occurs subtidally while *M. integrifolia* occurs in the low intertidal and shallow subtidal zones. *Macrocystis pyrifera* has a conical holdfast while *M. integrifolia* has a more flattened, creeping holdfast. Consult Abbott and Hollenberg (1976) for a more detailed taxonomic discussion of the two species.

17.6.24.5 Obtaining Zoospores

17.6.24.5.1 *Macrocystis* zoospores are obtained from the reproductive blades (sporophylls) of the adult plant. The sporophylls are located near the base of the plant just above its conical holdfast. Sporophylls must be collected subtidally and should be collected from at least five different plants in any one location to give a good genetic representation of the population. The sporophylls should be collected from areas free of point and non-point source pollution to minimize the possibility of genetic or physiological adaptation to pollutants. In situations where a thermocline is present at the collection site, the sporophylls should be collected from below the thermocline to ensure adequate spore release. Sporophylls are identified in the field by the presence of darkened patches called sori. The zoospores develop within the sori. In addition, the sporophylls are distinguished from vegetative blades by their thinner width, basal location on the adult plant, and general lack of pneumatocysts (air bladders). Collection of algae is regulated by California law. Collectors must obtain a scientific collector's permit from the California Department of Fish and Game and observe any regulations regarding collection and transport of kelp. For further information regarding sporophyll collection, contact the Marine Pollution Studies Laboratory, 34500 Coast Route 1, Granite Canyon, Monterey CA, 93940, (408) 624-0947.

17.6.24.6 Broodstock Culture and Handling

17.6.24.6.1 After collection, the sporophylls should be kept damp and not exposed to direct sunlight. Avoid immersing the blades in seawater, however, to prevent premature spore release. The sporophylls should be rinsed thoroughly in 0.2 μm filtered seawater to remove diatoms and other epiphytic organisms. The individual blades can be gently rubbed between fingers under

running filtered seawater or brushed with a soft bristled brush. The blades are stored between moist paper towels (lasagna style so that the sporophylls do not overlap each other, and each layer of sporophylls are separated by a layer of paper towels) at approximately 9-12°C until needed. The zoospores must be released within 24 hours of collection to insure their viability. Preliminary data indicate that prolonged storage times may affect test results (Bottomley et al., 1991); however as long as germination rates meet control acceptability criteria this should not affect test results. Sporophylls should be kept shaded to prevent damage to the spores. For holding or transport times longer than approximately six hours, the sporophylls should be placed in an ice chest with blue ice. The blue ice should be wrapped in newspaper (10 layers) for insulation, then plastic to prevent leaking.

17.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

17.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

17.8 CALIBRATION AND STANDARDIZATION

17.8.1 See Section 4, Quality Assurance.

17.9 QUALITY CONTROL

17.9.1 See Section 4, Quality Assurance.

17.10 TEST PROCEDURES

17.10.1 TEST DESIGN

17.10.1.1 The test consists of at least five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain five replicates of a brine control.

17.10.1.2 Effluent concentrations are expressed as percent effluent.

17.10.2 TEST SOLUTIONS

17.10.2.1 Receiving waters

17.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using five replicate chambers per test, each containing 200 mL, analysis would require approximately 1 L of sample per test.

17.10.2.2 Effluents

17.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100% HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

17.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

17.10.2.2.3 The volume in each test chamber is 200 mL.

17.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

17.10.2.3 Dilution Water

17.10.2.3.1 Dilution water should be uncontaminated 1-µm-

filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

17.10.2.4 Reference Toxicant Test

17.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

17.10.2.4.2 The preferred reference toxicant for giant kelp is copper chloride ($CuCl_2 \cdot 2H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a 10,000 µg/L copper stock solution by adding 0.0268 g of copper chloride ($CuCl_2 \cdot 2H_2O$) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

17.10.2.4.3 Reference toxicant solutions should be five replicates each of 0 (control), 5.6, 10, 18, 32, 100, and 180 µg/L total copper. Prepare one liter of each concentration by adding 0, 0.56, 1.0, 1.8, 3.2, 5.6, 10.0, and 18.0 mL of stock solution, respectively, to one-liter volumetric flasks and fill with dilution water. Start with control solutions and progress to the highest concentration to minimize contamination.

17.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use zoospores from the same release. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

17.10.3 RELEASE OF ZOOSPORES FOR THE TEST

17.10.3.1 Zoospores are released by slightly desiccating the sporophyll blades, and then placing them in filtered seawater. To desiccate the sporophylls, blot the blades with paper towels and expose them to air for 1 hour.

17.10.3.2 The number of sporophyll blades needed depends upon their maturity; usually 25-30 blades (~ 100 grams wet weight) are sufficient. After 1 hour the blades should be rinsed again thoroughly using 0.2 μm-filtered seawater, then placed in a one L glass or plastic beaker filled with 0.2 μm filtered seawater at 15-16°C. The release water should never exceed 18°C.

17.10.3.3 After one hour, a sufficient number of zoospores should be present to conduct the test. The presence of zoospores is indicated by a slight cloudiness in the water. To verify whether zoospores are present, periodically sample the solution and observe the sample microscopically (100x).

17.10.3.4 To insure that the zoospores are viable and have not begun to germinate before they are exposed to the toxicant, the zoospore release process should not be longer than two hours. If it takes longer than two hours to get an adequate density of zoospores (~7,500 zoospores/mL of test solution), repeat the release process with a new batch of sporophylls.

17.10.3.5 After the zoospores are released, remove the sporophylls and let the spore mixture settle for 30 minutes. After 30 minutes, decant 250 mLs from the top of the spore solution into a separate clean glass beaker. Sample the spore solution and determine the spore density using a bright-line hemacytometer (100x). Spores may be counted directly, or to obtain a more accurate count, fix a sample of spores by mixing nine milliliters of spore solution with 1-mL of 37% buffered formalin (or acetic acid) in a test tube. Shake the sample well before placing it on the hemacytometer.

17.10.3.6 After counting, the density is multiplied by 1.111 to correct for the dilution caused by adding 1 mL of formalin to the sample. Use at least five replicate counts. After the density is determined, calculate the volume of zoospores necessary to give approximately 7,500 spores/mL of test solution. To prevent over-dilution of the test solution, this volume should not exceed 1% of the test solution volume. If this volume exceeds 1% of the test solution volume, it should be noted in the results.

17.10.3.7 Test solutions must be prepared while the zoospores are releasing from the sporophylls. Test solutions must be mixed, sampled, and temperature equilibrated in time to receive

the swimming zoospores as soon as they are counted. Zoospore release and counting should be done in a room separate from that used for toxicant preparation, and care should be taken to avoid contaminating the zoospores prior to testing.

17.10.4 START OF THE TEST

17.10.4.1 Prior to Beginning the Test

17.10.4.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

17.10.4.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (15 ± 1°C) and maintained at that temperature during the addition of dilution water.

17.10.4.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (15 ± 1°C).

17.10.4.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the gametophyte spores have been examined at the end of the test.

17.10.4.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store

separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

17.10.4.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly and filled with test solutions, they can be arranged in

numerical order for convenience, since this will also ensure random placement of treatments.

17.10.4.2 Estimation of Zoospore Density

17.10.4.2.1 After determining the zoospore density and calculating the volume yielding 7,500 zoospores/mL test solution, add this volume to each test chamber (this is the start time of the test). Observe a sample of zoospores microscopically to verify that they are swimming before adding them to the test chambers.

17.10.4.2.2 Incubate the developing gametophytes for 48 hours in the test chambers at 15°C under 50 $\mu E/m^2/s$. The zoospores germinate and develop to the "dumbell" gametophyte stage during the exposure period.

17.10.5 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

17.10.5.1 The lights used in this method are cool white fluorescent lights adjusted to give 50 $\mu E/m^2/s$ at the top of each test chamber. Each test chamber must receive the same quanta of light (50 ± 10 $\mu E/m^2/s$). Areas of increased light can be eliminated by taping the outside of the light diffuser or wrapping the fluorescent bulbs with aluminum foil.

17.10.5.2 The water temperature in the test chambers should be maintained at 15 ± 1°C. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

15.10.5.3 The test salinity should be in the range of 34 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.10.5.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

17.10.6 DISSOLVED OXYGEN (DO) CONCENTRATION

17.10.6.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

17.10.7 OBSERVATIONS DURING THE TEST

17.10.7.1 Routine Chemical and Physical Observations

17.10.7.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

17.10.7.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

17.10.7.1.3 Record all the measurements on the data sheet.

17.10.8 TERMINATION OF THE TEST

17.10.8.1 Ending the Test

17.10.8.1.1 Record the time the test is terminated.

17.10.8.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control.

17.10.8.2 Sample preservation

17.10.8.2.1 In some cases it may be convenient to preserve the kelp cultures for later analysis. Preliminary work by Anderson and Hunt (Marine Pollution Studies Laboratory unpublished data) indicates that cultures can be preserved in 0.1% glutaraldehyde (final concentration) and that preservation has no significant effect on germination or germ-tube growth. Other researchers have used higher glutaraldehyde concentrations and found adequate preservation with no effect on spore germination or gametophyte growth (K. Goodwin, Calif. Inst. of Tech., unpublished data).

17.10.8.2.2 Because data on the effects of preservation are preliminary, it is recommended that anyone interested in preserving kelp cultures for later analysis first demonstrate that preservation does not affect test results. This can be accomplished by comparing germination and germ-tube growth in preserved vs non-preserved kelp cultures. We also recommend that if it is necessary to preserve kelp cultures for later analysis, a complete test should be preserved so that if any replicates are read preserved, all of the replicates should be read preserved. In the case where concurrent reference toxicant and complex effluent tests are conducted, it may be convenient to fix one test in glutaraldehyde and read the other test immediately.

17.10.8.2.3 When fixing kelp cultures, it is important to minimize disturbance to the gametophytes. Make sure that the culture slides are fixed and stored horizontally. We have used disposable petri dishes for preservation chambers; these allow individual replicate slides to be labelled and preserved separately to avoid mixing replicates. **Note:** Glutaraldehyde is toxic. If you intend to use this material as a preservative, study the material data safety sheets from the supplier and

follow strict safety precautions. Make sure test chambers and solutions contaminated with this material are disposed of properly.

17.10.8.3 Counting

17.10.8.3.1 After 48 hours, the test is terminated. Because it takes a considerable amount of time to read the test, reading can begin after 45 hours and must be completed within six hours. Remove the slide without decanting the test solution. The test slide can be lifted from the bottom of the test chamber with a separate clean microscope slide. Blot the bottom on a paper towel and place an 18-mm square cover slip on the slide. Blot the excess water around the edge of the cover slip to eliminate the flow of water under the cover slip.

17.10.8.4 Endpoints

17.10.8.4.1 The endpoints measured for the 48 hour *Macrocystis* method are percent germination success and germination tube length. Germination is considered successful if a germ-tube is present on the settled zoospore. Germination is considered to be unsuccessful if no germination tube is visible. To differentiate between a germinated and non-germinated zoospore, observe the settled zoospores at 400x magnification and determine whether they are circular (non-germinated) or have a protuberance that extends at least one spore diameter (about 3.0 µm) from the edge of the spore (germinated). Spores with a germination tubes less than one spore diameter are considered non-germinated.

17.10.8.4.2 The first 100 spores encountered while moving across the microscope slide are counted for each replicate of each treatment. **Note:** Sewage effluents may contain certain objects, such as ciliates, which look similar to non-germinated kelp spores. It is important to ensure that only kelp spores are counted for this endpoint. Kelp spores are green-brown in color, spherical, and lack mobility. Also, components of the cytoplasm of kelp spores appear to fluoresce a light green color when the spore is slightly out of focus. If a particular object cannot be identified, it should not be counted.

17.10.8.4.3 The growth endpoint is the measurement of the total length of the germination tube from the edge of the original

spore membrane. Only germinated spores with straight germination tubes and within the same focal plane are measured; if a spore is not completely in focus from tip to tip it should not be measured. The spores to be measured are randomly selected by moving the microscope stage to a new field of view without looking through the ocular lens.

17.10.8.4.4 Measure the germination-tube length of the spore whose spore case center is nearest the micrometer in each field; the spores case can be distinguished from the growing tip because it is usually clear (empty) at 48 hours, and it is more circular than the growing tip. If more than one spore case is touching the micrometer, both (or all) germinated spores are measured. A total of 10 spores for each replicate of each treatment are measured. It is easier to measure germ-tube length with a micrometer having a 10 mm linear scale (0.1 mm subdivisions); measure lengths to the nearest micron (typically to the nearest half micrometer unit; see Section 10200E, Standard Methods 17th edition, for micrometer/microscope calibration procedures). In situations where germination is significantly inhibited it may be difficult to find germinated spores for germ-tube growth measurement using the random search technique.

17.10.8.4.5 To expedite reading, the slide can be scanned to find germinated spores if germination is 30% or less. In this situation the first 10 spores encountered are measured for germ-tube length.

17.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

17.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

17.12 ACCEPTABILITY OF TEST RESULTS

17.12.1 For tests to be considered acceptable, the following requirements must be met:

(1) Mean control germination must be at least 70% in the controls.

(2) Mean germination-tube length in the controls must be at least 10 μm in the controls.

(3) The germination-tube growth NOEC must be below 35 µg/liter in the reference toxicant test.

(4) The minimum significant difference (%MSD) is <20% relative to the control for both germination and germ-tube length in the reference toxicant test.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GIANT KELP, *MACROCYSTIS PYRIFERA*, GERMINATION AND GERM-TUBE LENGTH TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	15 ± 1°C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	50 ± 10 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	600 mL
8. Test solution volume:	200 mL/replicate
9. Spore density per test chamber:	7500/mL of test solution
10. No. replicate chambers per concentration:	5
11. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
12. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor:	Effluents: ≥0.5 Receiving waters: None or ≥0.5
14. Test duration:	48 h

15. Endpoints:	Germination and germ-tube length
16. Test acceptability criteria:	≥70% germination in the controls; ≥10 µm germ-tube length in the controls and the NOEC must be below 35 µg/L in the reference toxicant test; must achieve a %MSD of <20 for both germination and germ-tube length in the reference toxicant.
17. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
18. Sample volume required:	2 L per test

17.13 DATA ANALYSIS

17.13.1 GENERAL

17.13.1.1 Tabulate and summarize the data. Table 4 presents a sample set of germination and growth data.

17.13.1.2 The endpoints of the giant kelp 48-hour chronic test are based on the adverse effects on germination and growth. The IC25 endpoints are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for germination and growth are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the IC25 endpoints. Concentrations at which there is no germination in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for germination and growth, but included in the estimation of the

IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

17.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

17.13.2 EXAMPLE OF ANALYSIS OF GIANT KELP, *MACROCYSTIS PYRIFERA*, GERMINATION DATA

17.13.2.1 Formal statistical analysis of the germination data is outlined in Figure 1. The response used in the analysis is the proportion of germinated spores in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there is no germination in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC endpoints.

17.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

17.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

TABLE 4. DATA FROM GIANT KELP, *MACTOCYSTIS PYRIFERA* GERMINATION AND GROWTH TEST

Copper Conc. (µg/L)	Replicate Chamber	Number Counted	Number Germinated	Proportion Germinated	Mean Length
Control	1	100	89	0.89	19.58
	2	100	88	0.88	18.75
	3	100	85	0.85	19.14
	4	100	89	0.89	16.50
	5	100	91	0.91	17.93
5.6	1	100	82	0.82	18.26
	2	100	55	0.55	16.25
	3	100	84	0.84	16.39
	4	100	96	0.96	18.70
	5	100	85	0.85	15.62
10.0	1	100	90	0.90	13.31
	2	100	90	0.90	18.92
	3	100	70	0.70	15.62
	4	100	83	0.83	14.30
	5	100	87	0.87	15.29
18.0	1	100	88	0.88	18.59
	2	100	52	0.52	12.88
	3	100	83	0.83	16.28
	4	100	54	0.54	15.38
	5	100	49	0.49	19.75
32.0	1	100	71	0.71	12.54
	2	100	82	0.82	10.67
	3	100	86	0.86	15.95
	4	100	81	0.81	12.54
	5	100	82	0.82	11.66
56.0	1	100	84	0.84	11.44
	2	100	68	0.68	11.88
	3	100	62	0.62	11.88
	4	100	80	0.80	11.00
	5	100	83	0.83	11.55
100.0	1	100	66	0.66	7.92
	2	100	72	0.72	7.59
	3	100	63	0.63	8.25
	4	100	72	0.72	9.13
	5	100	71	0.71	8.80
180.0	1	100	37	0.37	6.49
	2	100	69	0.69	7.25
	3	100	0	0.00	--
	4	100	32	0.32	7.63
	5	100	48	0.48	8.13

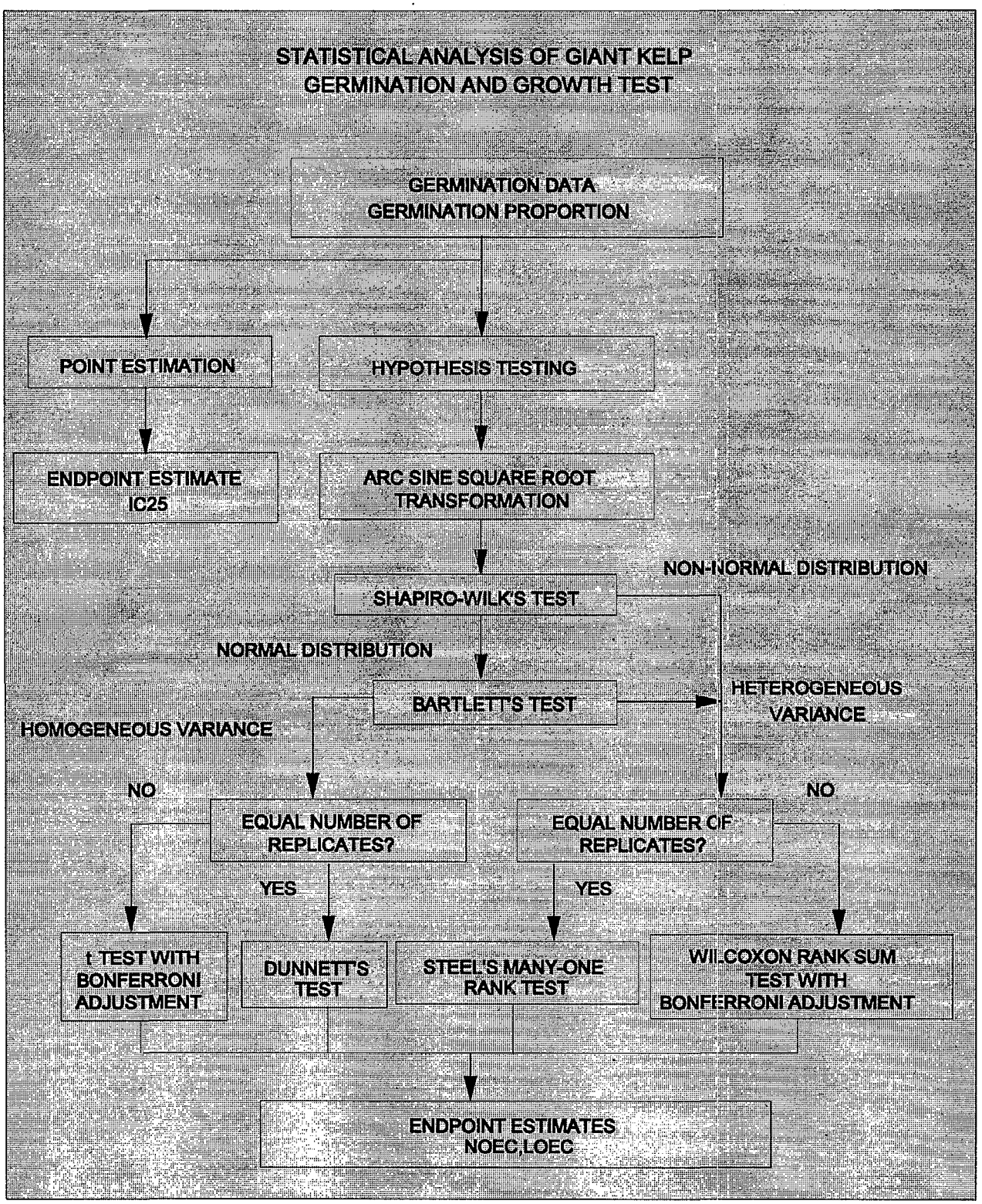

Figure 1. Flowchart for statistical analysis of giant kelp, *Macrocystis pyrifera*, germination data.

17.13.2.4 Example of Analysis of Germination Data

17.12.2.4.1 This example used toxicity data from a giant kelp, Macrocystis pyrifera, germination and growth test performed with copper. The response of interest is the proportion of germinated spores, thus each replicate must be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each concentration including the control are listed in Table 5. A plot of the survival data is provided in Figure 2.

17.13.2.5 Test for Normality

17.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 6.

17.13.2.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

Where: $\overline{X}_i$ = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations.

17.13.2.5.3 For this set of data, n = 40

$$\overline{X} = \frac{1}{40}(-0.002) = 0.000$$

$$D = 0.9281$$

17.13.2.5.4 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

TABLE 5. GIANT KELP, *MACTOCYSTIS PYRIFERA* GERMINATION DATA

COPPER CONCENTRATION ($\mu g/L$)	REPLICATE CHAMBER	RAW DATA	ARC SINE SQUARE ROOT TRANSFORMED	i	MEAN $\overline{Y}$	S_i^2
Control	1	0.89	1.233	1	1.224	0.00114
	2	0.88	1.217			
	3	0.85	1.173			
	4	0.89	1.233			
	5	0.91	1.266			
5.6	1	0.82	1.133	2	1.134	0.03670
	2	0.55	0.835			
	3	0.84	1.159			
	4	0.96	1.369			
	5	0.85	1.173			
10.0	1	0.90	1.249	3	1.167	0.01152
	2	0.90	1.249			
	3	0.70	0.991			
	4	0.83	1.146			
	5	0.87	1.202			
18.0	1	0.88	1.217	4	0.954	0.04423
	2	0.52	0.805			
	3	0.83	1.146			
	4	0.54	0.825			
	5	0.49	0.775			
32.0	1	0.71	1.002	5	1.115	0.00466
	2	0.82	1.133			
	3	0.86	1.187			
	4	0.81	1.120			
	5	0.82	1.133			
56.0	1	0.84	1.159	6	1.058	0.01272
	2	0.68	0.970			
	3	0.62	0.907			
	4	0.80	1.107			
	5	0.83	1.146			
100.0	1	0.66	0.948	7	0.979	0.00191
	2	0.72	1.013			
	3	0.63	0.917			
	4	0.72	1.013			
	5	0.71	1.002			
180.0	1	0.37	0.654	8	0.610	0.11914
	2	0.69	0.980			
	3	0.00	0.050			
	4	0.32	0.601			
	5	0.48	0.765			

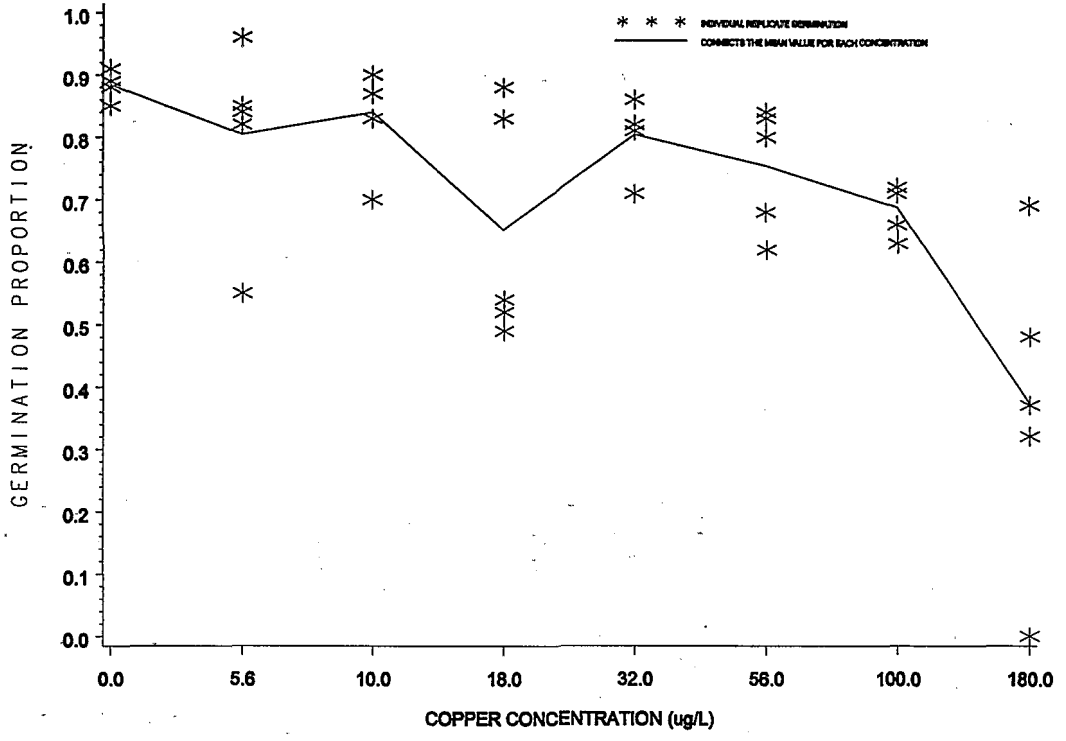

Figure 2. Plot of germination proportions of the giant kelp, *Macrocystis pyrifera*, at each treatment level.

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Copper Concentration (µg/L)						
Rep	Control	5.6	10.0	18.0	32.0	56.0	100.0	180.0
1	0.009	-0.001	0.082	0.263	-0.113	0.101	-0.031	0.044
2	-0.007	-0.299	0.082	-0.149	0.018	-0.088	0.034	0.370
3	-0.051	0.025	-0.176	0.192	0.072	-0.151	-0.062	-0.560
4	0.009	0.235	-0.021	-0.129	0.005	0.049	0.034	-0.009
5	0.042	0.039	0.035	-0.179	0.018	0.088	0.023	0.155

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.560	21	0.018
2	-0.299	22	0.023
3	-0.179	23	0.025
4	-0.176	24	0.034
5	-0.151	25	0.034
6	-0.149	26	0.035
7	-0.129	27	0.039
8	-0.113	28	0.042
9	-0.088	29	0.044
10	-0.062	30	0.049
11	-0.051	31	0.072
12	-0.031	32	0.082
13	-0.021	33	0.082
14	-0.009	34	0.088
15	-0.007	35	0.101
16	-0.001	36	0.155
17	0.005	37	0.192
18	0.009	38	0.235
19	0.009	39	0.263
20	0.018	40	0.370

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 7.

17.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2,...., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 40 and k = 20. The a_i values are listed in Table 8.

17.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 8. For this data in this example:

$$W = \frac{1}{0.9281}(0.9230)^2 = 0.918$$

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.3964	0.930	$X^{(40)} - X^{(1)}$
2	0.2737	0.562	$X^{(39)} - X^{(2)}$
3	0.2368	0.414	$X^{(38)} - X^{(3)}$
4	0.2098	0.368	$X^{(37)} - X^{(4)}$
5	0.1878	0.306	$X^{(36)} - X^{(5)}$
6	0.1691	0.250	$X^{(35)} - X^{(6)}$
7	0.1526	0.217	$X^{(34)} - X^{(7)}$
8	0.1376	0.195	$X^{(33)} - X^{(8)}$
9	0.1237	0.170	$X^{(32)} - X^{(9)}$
10	0.1108	0.134	$X^{(31)} - X^{(10)}$
11	0.0986	0.100	$X^{(30)} - X^{(11)}$
12	0.0870	0.075	$X^{(29)} - X^{(12)}$
13	0.0759	0.063	$X^{(28)} - X^{(13)}$
14	0.0651	0.048	$X^{(27)} - X^{(14)}$
15	0.0546	0.042	$X^{(26)} - X^{(15)}$
16	0.0444	0.035	$X^{(25)} - X^{(16)}$
17	0.0343	0.029	$X^{(24)} - X^{(17)}$
18	0.0244	0.016	$X^{(23)} - X^{(18)}$
19	0.0146	0.014	$X^{(22)} - X^{(19)}$
20	0.0049	0.000	$X^{(21)} - X^{(20)}$

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 5.6 µg/L CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Germinated	Concentration
1	0.835	5.6 µg/L
2	1.133	5.6 µg/L
3	1.159	5.6 µg/L
4.5	1.173	5.6 µg/L
4.5	1.173	Control
6	1.217	Control
7.5	1.233	Control
7.5	1.233	Control
9	1.266	Control
10	1.369	5.6 µg/L

TABLE 10. TABLE OF RANKS[1]

Rep.	Control	Concentration (µg/L) 5.6	10.0
1	1.233(7.5,6.5,8.5,8.5,8.5,8.5,8.5)	1.133(2)	1.249(8.5)
2	1.217(6,5,6.5,7,7,7,7)	0.835(1)	1.249(8.5)
3	1.173(4.5,3,5,5,6,6,6)	1.159(3)	0.991(1)
4	1.233(7.5,6.5,8.5,8.5,8.5,8.5,8.5)	1.369(10)	1.146(2)
5	1.266(9,10,10,10,10,10,10)	1.173(4.5)	1.202(4)

Rep.	Concentration (µg/L) (Continued) 18.0	32.0	56.0	100.0	180.0
1	1.217(6.5)	1.002(1)	1.159(5)	0.948(2)	0.654(3)
2	0.805(2)	1.133(3.5)	0.970(2)	1.013(4.5)	0.980(5)
3	1.146(4)	1.187(6)	0.907(1)	0.917(1)	0.050(1)
4	0.825(3)	1.120(2)	1.107(3)	1.013(4.5)	0.601(2)
5	0.775(1)	1.133(3.5)	1.146(4)	1.002(3)	0.765(4)

[1]Control ranks are given in the order of the concentration with which they were ranked.

17.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 5.6 with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 40 observations is 0.919. Since W = 0.918 is less than the critical value, conclude that the data are not normally distributed.

17.13.2.5.8 Since the data do not meet the assumption of normality, Steel's Many-one Rank Test will be used to analyze the germination data.

17.13.2.6 Steel's Many-one Rank Test

17.13.2.6.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ... , 10) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

17.13.2.6.2 An example of assigning ranks to the combined data for the control and 5.6 µg/L copper concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are then summed for each concentration level, as shown in Table 11.

17.13.2.6.3 For this example, determine if the survival in any of the concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus compare the rank sums for the survival at each of the various concentration levels with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with seven concentrations (excluding the control) and five replicates is 16 (See Table 5, Appendix E).

17.13.2.6.4 Since the rank sum for the 32.0 µg/L concentration is equal to the critical value and the rank sums for the 56.0, 100.0 and 180.0 µg/L concentrations are less than the critical value, the germination proportions in those concentrations are considered significantly less than that in the control. Hence, the NOEC and the LOEC are considered to be 18.0 µg/L and 32.0 µg/L, respectively.

TABLE 11. RANK SUMS

Concentration	Rank Sum
5.6	20.5
10.0	24.0
18.0	16.5
32.0	16.0
56.0	15.0
100.0	15.0
180.0	15.0

17.13.2.7 Calculation of the ICp

17.13.2.7.1 The germination data from Table 4 and Figure 2 are utilized in this example. As can be seen from the figure, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

17.13.2.7.2 Starting with the observed control mean, $Y_1 = 0.884$ is less than the observed mean for the lowest effluent concentration, $Y_2 = 0.804$, so set $M_1 = 0.884$.

17.13.2.7.3 Comparing Y_2 to $Y_3 = 0.840$, we see that Y_2 is less than Y_3.

17.13.2.7.4 Calculate the smoothed means:

$$M_2 = M_3 = (Y_2 + Y_3)/2 = 0.822$$

17.13.2.7.5 Since M_3 is larger than $Y_4 = 0.652$, set $M_4 = 0.652$. Since $Y_5 = 0.804$ is larger than M_4, these means must be smoothed.

17.13.2.7.6 Calculate the smoothed means:

$$M_4 = M_5 = (M_4 + Y_5)/2 = 0.728.$$

17.13.2.7.7 Since $Y_6 = 0.754$ is larger than M_5, average Y_6 with the two previous concentrations:

$$M_4 = M_5 = M_6 = (M_4 + M_5 + Y_6)/3 = 0.737.$$

17.13.2.7.8 Since $M_6 > Y_7 = 0.688 > Y_8 = 0.372$, set $M_7 = 0.688$ and $M_8 = 0.372$. Table 12 contains the smoothed means and Figure 3 gives a plot of the smoothed means and the interpolated response curve.

17.13.2.7.9 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in germination, compared to the controls, would result in a mean germination of 0.663, where $M_1(1-p/100) = 0.884(1-25/100)$. Examining the smoothed means and their associated concentrations (Table 12), the response, 0.663, is bracketed by $C_7 = 100.0$ µg/L and $C_8 = 180.0$ µg/L.

17.13.2.7.10 Using the equation in Section 4.2 from Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1-p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 100.0 + [0.884(1 - 25/100) - 0.688]\frac{(180.0 - 100.0)}{(0.372 - 0.688)}$$

$$= 106.3 \text{ µg/L}.$$

17.13.2.7.11 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 106.3291 µg/L. The empirical 95.0% confidence interval for the true mean was 94.6667 µg/L to 117.0588 µg/L. The computer program output for the IC25 for this data set is shown in Figure 4.

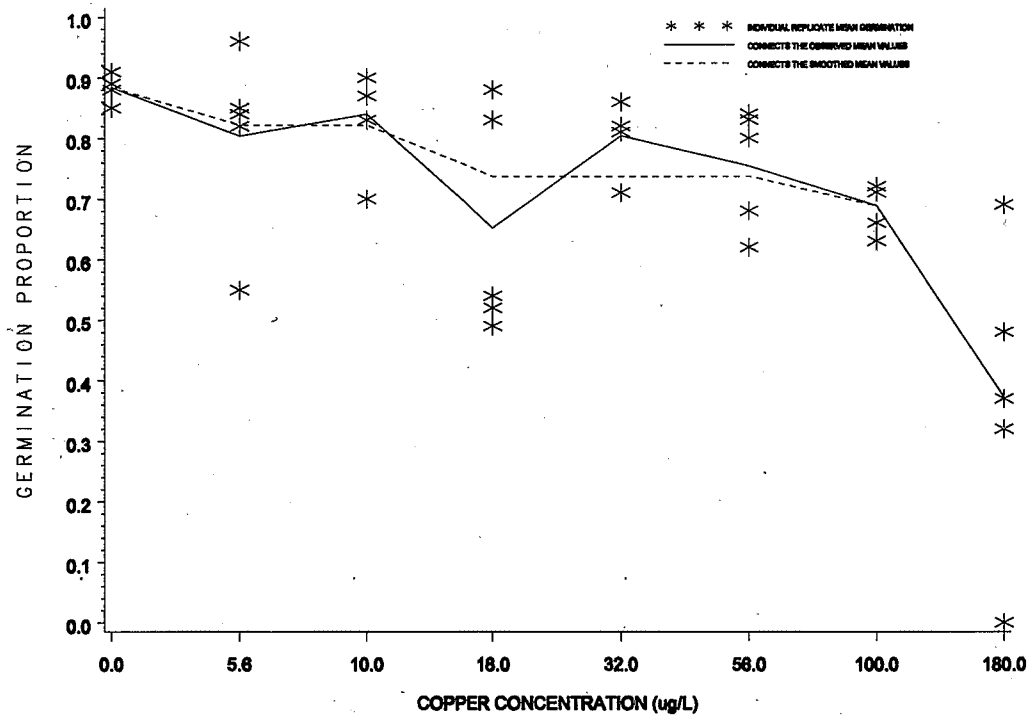

Figure 3. Plot of raw data, observed means, and smoothed means for the giant kelp, *Macrocystis pyrifera*, germination data from Tables 4 and 13.

```
Conc. ID          1     2     3     4     5     6     7     8
-------------------------------------------------------------
Conc. Tested      0   5.6    10    18    32    56   100   180
-------------------------------------------------------------
Response  1     .89   .82   .90   .88   .71   .84   .66   .37
Response  2     .88   .55   .90   .52   .82   .68   .72   .69
Response  3     .85   .84   .70   .83   .86   .62   .63     0
Response  4     .89   .96   .83   .54   .81   .80   .72   .32
Response  5     .91   .85   .87   .49   .82   .83   .71   .48
-------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date:     Test Ending Date:
Test Species: Giant Kelp, Macrocystis pyrifera
Test Duration:         48 hours
DATA FILE: kelpgerm.icp
OUTPUT FILE: kelpgerm.i25
```

Conc. ID	Number Replicates	Concentration ug/L	Response Means	Std. Dev.	Pooled Response Means
1	5	0.000	0.884	0.022	0.884
2	5	5.600	0.804	0.152	0.822
3	5	10.000	0.840	0.083	0.822
4	5	18.000	0.652	0.187	0.737
5	5	32.000	0.804	0.056	0.737
6	5	56.000	0.754	0.098	0.737
7	5	100.000	0.688	0.041	0.688
8	5	180.000	0.372	0.252	0.372

```
The Linear Interpolation Estimate:    106.3291    Entered P Value: 25
-------------------------------------------------------------------
Number of Resamplings:    80
The Bootstrap Estimates Mean: 105.8680 Standard Deviation:      5.6981
Original Confidence Limits:   Lower:     94.6667 Upper:    117.0588
Expanded Confidence Limits:   Lower:     88.8354 Upper:    122.4237
Resampling time in Seconds:      0.28  Random_Seed: 390692880
```

Figure 4. ICPIN program output for the IC25.

17.13.3 EXAMPLE OF ANALYSIS OF GIANT KELP, *MACROCYSTIS PYRIFERA*, GROWTH DATA

17.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 5. The response used in the statistical analysis is mean germ-tube length per replicate. An IC25 can be calculated for the growth data via a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain the NOEC and LOEC for growth.

17.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

17.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative. For detailed information on the Bonferroni adjustment, see Appendix D.

17.13.3.4 The data, mean and variance of the observations at each concentration including the control for this example are listed in Table 13. A plot of the data is provided in Figure 6.

17.13.3.5 Test for Normality

17.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 14.

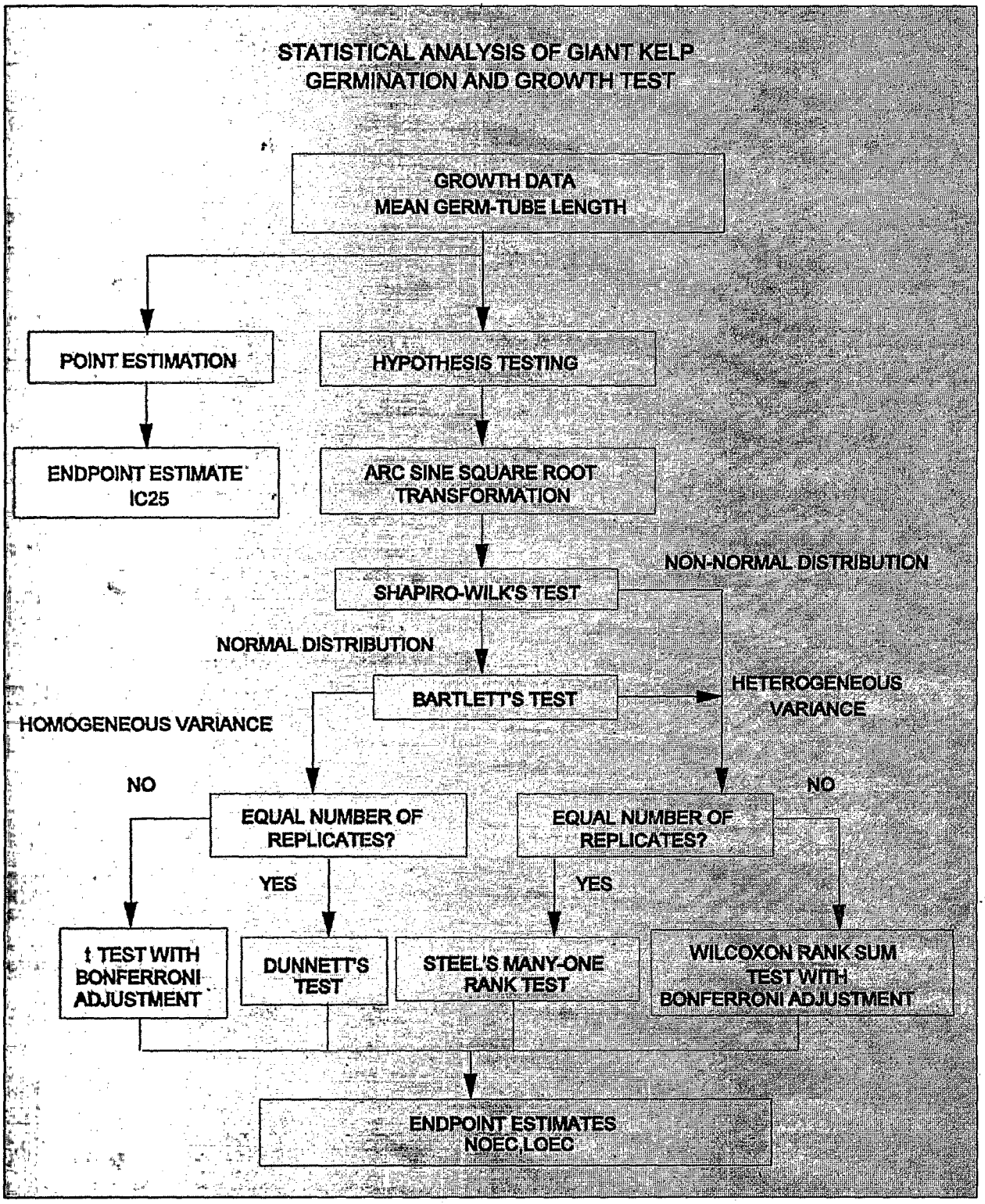

Figure 5. Flowchart for statistical analysis of giant kelp, *Macrocystis pyrifera*, growth data.

TABLE 13. GIANT KELP, *MACROCYSTIS PYRIFERA*, GROWTH DATA

		Copper Concentration (µg/L)						
Rep	Control	5.60	10.0	18.0	32.0	56.0	100.0	180.0
1	19.58	18.26	13.31	18.59	12.54	11.44	7.92	6.49
2	18.75	16.25	18.92	12.88	10.67	11.88	7.59	7.25
3	19.14	16.39	15.62	16.28	15.95	11.88	8.25	--
4	16.50	18.70	14.30	15.38	12.54	11.00	9.13	7.63
5	17.93	15.62	15.29	19.75	11.66	11.55	8.80	8.13
Mean ($\overline{Y}_i$)	18.38	17.04	15.49	16.58	12.67	11.55	8.34	7.38
S_i^2	1.473	1.827	4.498	7.327	3.953	0.133	0.396	0.478
i	1	2	3	4	5	6	7	8

TABLE 14. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Copper Concentration (µg/L)						
Rep	Control	5.6	10.0	18.0	32.0	56.0	100.0	180.0
1	1.20	1.22	-2.18	2.01	-0.13	-0.11	-0.42	-0.89
2	0.37	-0.79	3.43	-3.70	-2.00	0.33	-0.75	-0.13
3	0.76	-0.65	0.13	-0.30	3.28	0.33	-0.09	--
4	-1.88	1.66	-1.19	-1.20	-0.13	-0.55	0.79	0.25
5	-0.45	-1.42	-0.20	3.17	-1.01	0.00	0.46	0.75

17.13.3.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i-1}^{n} (X_i - \overline{X})^2$$

Where: X_i = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations

17.13.3.5.3 For this set of data, n = 39

$$\overline{X} = \frac{1}{39}(-0.03) = 0.000$$

$$D = 79.8591$$

17.13.3.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 15.

TABLE 15. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-3.70	21	-0.11
2	-2.18	22	-0.09
3	-2.00	23	0.00
4	-1.88	24	0.13
5	-1.42	25	0.25
6	-1.20	26	0.33
7	-1.19	27	0.33
8	-1.01	28	0.37
9	-0.89	29	0.46
10	-0.79	30	0.75
11	-0.75	31	0.76
12	-0.65	32	0.79
13	-0.55	33	1.20
14	-0.45	34	1.22
15	-0.42	35	1.66
16	-0.30	36	2.01
17	-0.20	37	3.17
18	-0.13	38	3.28
19	-0.13	39	3.43
20	-0.13		

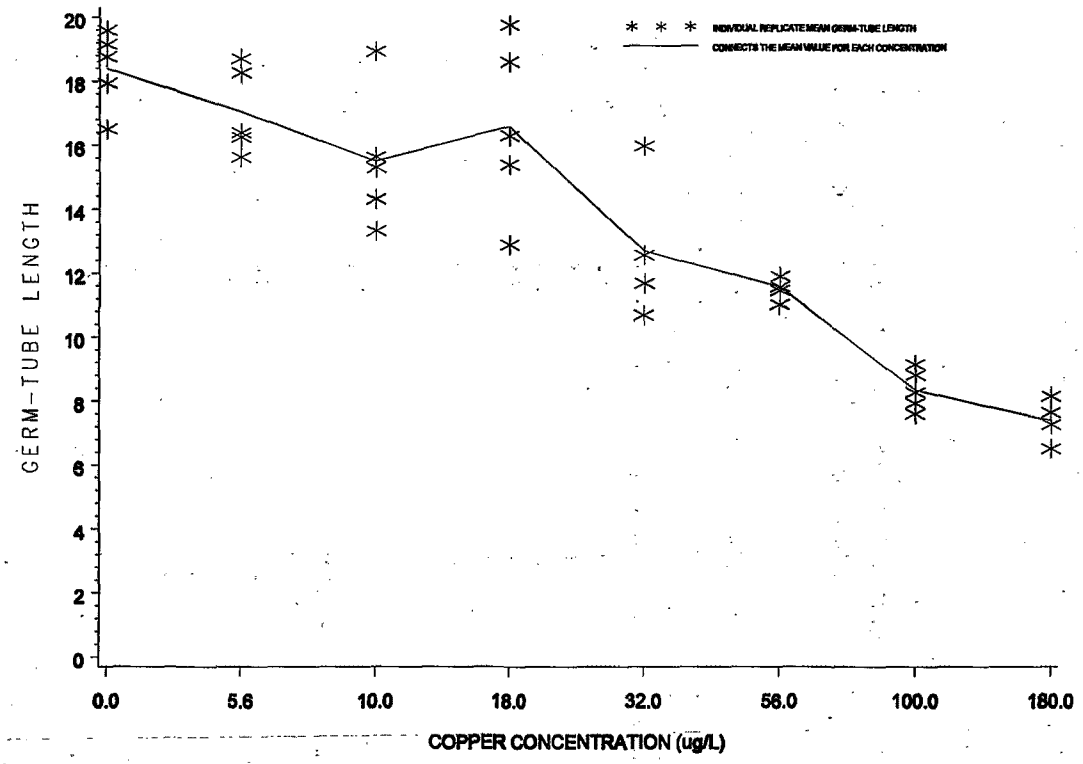

Figure 6. Plot of mean growth data for the giant kelp, *Macrocystis pyrifera*, tests.

17.13.3.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 39 and k = 19. The a_i values are listed in Table 16.

17.13.3.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 16. For this set of data:

$$W = \frac{1}{79.8591}(8.7403)^2 = 0.957$$

TABLE 16. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.3989	7.13	$X^{(39)} - X^{(1)}$
2	0.2755	5.46	$X^{(38)} - X^{(2)}$
3	0.2380	5.17	$X^{(37)} - X^{(3)}$
4	0.2104	3.89	$X^{(36)} - X^{(4)}$
5	0.1880	3.08	$X^{(35)} - X^{(5)}$
6	0.1689	2.42	$X^{(34)} - X^{(6)}$
7	0.1520	2.39	$X^{(33)} - X^{(7)}$
8	0.1366	1.80	$X^{(32)} - X^{(8)}$
9	0.1225	1.65	$X^{(31)} - X^{(9)}$
10	0.1092	1.54	$X^{(30)} - X^{(10)}$
11	0.0967	1.21	$X^{(29)} - X^{(11)}$
12	0.0848	1.02	$X^{(28)} - X^{(12)}$
13	0.0733	0.88	$X^{(27)} - X^{(13)}$
14	0.0622	0.78	$X^{(26)} - X^{(14)}$
15	0.0515	0.67	$X^{(25)} - X^{(15)}$
16	0.0409	0.43	$X^{(24)} - X^{(16)}$
17	0.0305	0.20	$X^{(23)} - X^{(17)}$
18	0.0203	0.04	$X^{(22)} - X^{(18)}$
19	0.0101	0.02	$X^{(21)} - X^{(19)}$

17.13.3.5.7 The decision rule for this test is to compare W as calculated in Subsection 5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 39 observations is 0.917. Since W = 0.957 is greater than the critical value, conclude that the data are normally distributed.

17.13.3.6 Test for Homogeneity of Variance

17.13.3.6.1 The test used to examine whether the variation in mean weight of the mysids is the same across all concentration levels including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i)\ 1n\ \overline{S}^2 - \sum_{i=1}^{P} V_i\ 1n\ S_i^2]}{C}$$

Where: V_i = degrees of freedom for each copper concentration and control, $V_i = (n_i - 1)$

p = number of concentration levels including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i = the number of replicates for concentration i.

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

17.13.3.6.2 For the data in this example (See Table 13), all concentrations including the control have five replicates except the 180 µg/L concentration which has four replicates (n_i = 5 for i = 1 - 7; n_8 = 4). Thus, V_i = 4 for i = 1 - 7 and V_8 = 3.

17.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(31)\ln(2.5761) - \sum_{i=1}^{P} V_i \ln(S_i^2)] / 1.0977$$

$$= [31(0.9463) - [4\ln(1.4729) + \ldots + 3\ln(0.4780)] / 1.0977$$

$$= [29.3353 - 9.4481] / 1.0977$$

$$= 18.12$$

17.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with seven degrees of freedom, is 18.48. Since B = 18.12 is less than the critical value, conclude that the variances are not different.

17.13.3.7 *t* Test with Bonferroni's Adjustment

17.13.3.7.1 To obtain an estimate of the pooled variance for the *t* test with Bonferroni's adjustment, construct an ANOVA table as described in Table 17.

TABLE 17. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N \qquad \text{Between Sum of Squares}$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N \qquad \text{Total Sum of Squares}$$

$$SSW = SST - SSB \qquad \text{Within Sum of Squares}$$

G = the grand total of all sample observations,

$$G = \sum_{i=1}^{P} T_i$$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean length of the germ-tubes for concentration i in test chamber j)

17.13.3.7.2 For the data in this example:

$$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = n_7 = 5; \quad n_8 = 4$$

$$N = 39$$

$$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 91.90$$
$$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 85.22$$
$$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 77.44$$
$$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 82.88$$
$$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} + Y_{55} = 63.36$$
$$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} + Y_{65} = 57.75$$
$$T_7 = Y_{71} + Y_{72} + Y_{73} + Y_{74} + Y_{75} = 41.69$$
$$T_8 = Y_{81} + Y_{82} + Y_{83} + Y_{84} = 29.50$$

$$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 + T_7 + T_8 = 529.74$$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$

$$= 7749.905 - \frac{(529.74)^2}{39} = 554.406$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 7829.764 - \frac{(529.74)^2}{39} = 634.265$$

$$SSW = SST - SSB = 634.265 - 554.406 = 79.859$$

$$S_B^2 = SSB/(p-1) = 554.406/(8-1) = 79.201$$

$$S_W^2 = SSW/(N-p) = 79.859/(39-8) = 2.576$$

17.13.3.7.3 Summarize these calculations in the ANOVA table (Table 18).

TABLE 18. ANOVA TABLE FOR THE *t* TEST WITH BONFERRONI'S ADJUSTMENT EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	7	554.406	79.201
Within	31	79.859	2.576
Total	38	634.265	

17.13.3.7.4 To perform the individual comparisons, calculate the *t* statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\overline{Y}_i$ = mean length for concentration i

$\overline{Y}_1$ = mean length for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

17.13.3.7.5 Table 19 includes the calculated *t* values for each concentration and control combination. In this example, comparing the 5.6 µg/L concentration with the control, the calculation is as follows:

$$t_2 = \frac{(18.38 - 17.04)}{[1.605\sqrt{(1/5)+(1/5)}]}$$

$$= 1.320$$

TABLE 19. CALCULATED t VALUES

Concentration (µg/L)	i	t_i
5.6	2	1.320
10.0	3	2.847
18.0	4	1.773
32.0	5	5.625
56.0	6	6.728
100.0	7	9.891
180.0	8	10.836

17.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean length, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix D. For an overall alpha level of 0.05, 31 degrees of freedom for error and seven concentrations (excluding the control) the approximate critical value is 2.597. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Therefore, the 10.0 µg/L, 32 µg/L, 56.0 µg/L,

100.0 µg/L, 180.0 µg/L concentrations have significantly lower mean length than the control. Because the 10.0 µg/L concentration shows signigicantly lower mean length than the control while the higher 18.0 µg/L concentration does not, these test results are considered to have an anomalous dose-response relationship and it is recommended that the test be repeated. If an NOEC and LOEC must be determined for this test, the lowest concentration with significant growth impairment versus the control is considered to the LOEC for growth. Thus, for this test, the NOEC and LOEC would be 5.6 µg/L and 10.0 µg/L, respectively.

17.13.3.8 Calculation of the ICp

17.13.3.8.1 The growth data from Table 13 and Figure 3 are utilized in this example. As can be seen in the figure, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC

17.13.3.8.2 Starting with the observed control mean, $Y_1 = 18.38$ is greater than the observed mean for the lowest copper concentration, $Y_2 = 17.044$, so set $M_1 = 18.38$. Likewise, Y_2 is greater than the observed mean for the next copper concentration, $Y_3 = 15.488$, so set $M_2 = 17.044$.

17.13.3.8.3 Comparing Y_3 to $Y_4 = 16.576$, we see that Y_3 is less than Y_4.

17.13.3.8.4 Calculate the smoothed means:

$$M_3 = M_4 = (Y_3 + Y_4)/2 = 16.032$$

17.13.3.8.5 Since $M_4 > Y_5 = 12.672 > Y_6 = 11.550 > Y_7 = 8.338 > Y_8 = 7.375$, set $M_5 = 12.672$, $M_6 = 11.550$, $M_7 = 8.338$ and $M_8 = 7.375$. Table 20 contains the smoothed means and Figure 7 gives a plot of the smoothed response curve.

TABLE 20. GIANT KELP, *MACROCYSTIS PYRIFERA*, MEAN GERM-TUBE LENGTHS AFTER SMOOTHING

Copper Conc. (μg/L)	i	Response Means $\bar{Y}_i$ (mm)	Smoothed Means M_i (mm)
Control	1	18.380	18.380
5.6	2	17.044	17.044
10.0	3	15.488	16.032
18.0	4	16.576	16.032
32.0	5	12.672	12.672
56.0	6	11.550	11.550
100.0	7	8.338	8.338
180.0	8	7.375	7.375

17.13.3.8.7 Using the equation in Section 4.2 from Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1-p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 18.0 + [18.380(1 - 25/100) - 16.032]\frac{(32.0 - 18.0)}{(12.672 - 16.032)}$$

$$= 27.36\ \mu g/L.$$

17.13.3.8.6 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in length, compared to the controls, would result in a mean length of 13.785 mm, where $M_1(1-p/100) = 18.380(1-25/100)$. Examining the smoothed means and their associated concentrations (Table 20), the response, 13.785 mm, is bracketed by $C_4 = 18.0\ \mu g/L$ and $C_5 = 32.0\ \mu g/L$.

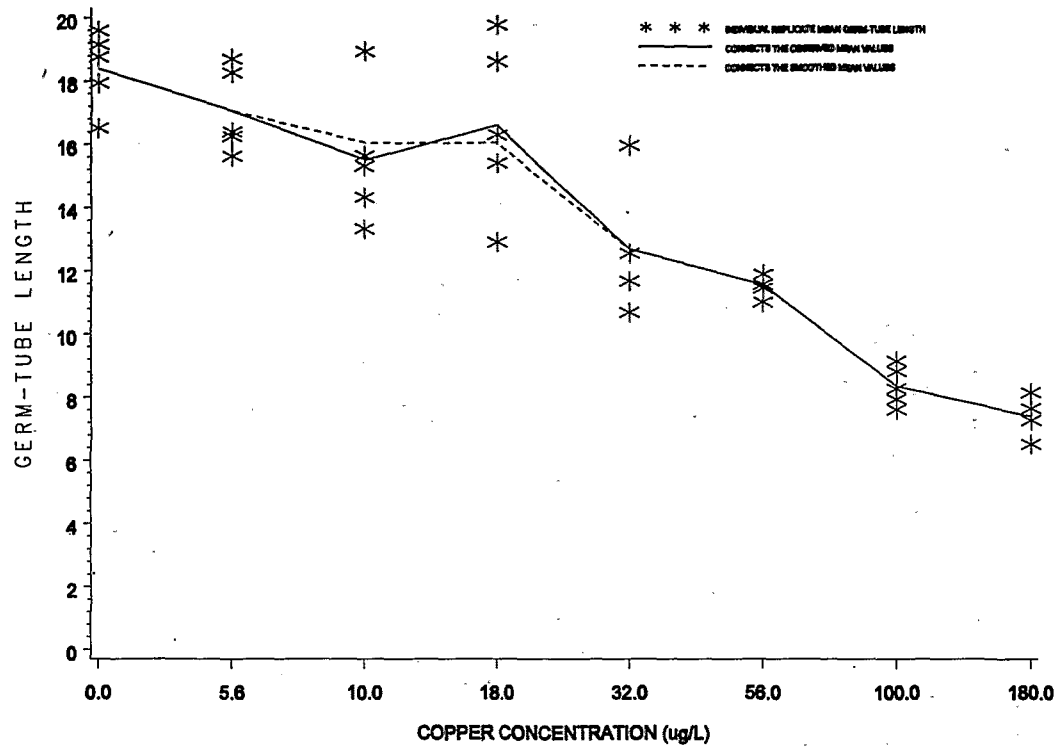

Figure 7. Plot of raw data, observed means, and smoothed means for the giant Kelp, *Macrocystis pyrifera*, growth data from Tables 13 and 20.

17.13.3.8.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 27.3625 µg/L. The empirical 95.0% confidence interval for the true mean was 20.8734 µg/L to 42.3270 µg/L. The computer program output for the IC25 for this data set is shown in Figure 8.

17.14 **PRECISION AND ACCURACY**

17.14.1 PRECISION

17.14.1.1 Single-Laboratory Precision

17.14.1.1.1 Single-laboratory precision data for the giant kelp 48-hour test method with the reference toxicants copper chloride and sodium azide with natural seawater are provided in Tables 21-22. The coefficient of variation (CV) of the germination EC50s using copper was 38.8%; the CV of the germ-tube length IC40s using copper was 32.9% (Table 21). The coefficient of variation (CV) of the germination EC50s using azide was 36.7%; the CV of the germ-tube length IC25s using azide was 30.8%, the CV of the germ-tube length IC50s using azide was 28.4% (Table 22).

17.14.1.2 Multi-laboratory Precision

17.14.1.2.1 Multi-laboratory precision data for the kelp 48-hour test method with the reference toxicant copper chloride are provided in Table 23. The coefficient of variation of the IC50s for the germ-tube length endpoint ranged between 8.4% and 55.5% using copper chloride. The coefficient of variation of the IC50s for the germination endpoint ranged between >1.1% and 67.6% using copper chloride.

17.14.2 ACCURACY

17.14.2.1 The accuracy of toxicity tests cannot be determined.

```
Conc. ID        1    2    3    4    5    6    7    8
-------------------------------------------------------
Conc. Tested    0  5.6   10   18   32   56  100  180
-------------------------------------------------------
Response  1 19.5818.2613.3118.5912.5411.44 7.92 6.49
Response  2 18.7516.2518.9212.8810.6711.88 7.59 7.25
Response  3 19.1416.3915.6216.2815.9511.88 8.25
Response  4 16.5018.7014.3015.3812.5411.00 9.13 7.63
Response  5 17.9315.6215.2919.7511.6611.55 8.80
-------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date:    Test Ending Date:
Test Species: Giant kelp, Macrocystis pyrifera
Test Duration:        48 hours
DATA FILE: kelpgrow.icp
OUTPUT FILE: kelpgrow.i25
```

Conc. ID	Number Replicates	Concentration ug/L	Response Means	Std. Dev.	Pooled Response Means
1	5	0.000	18.380	1.214	18.380
2	5	5.600	17.044	1.352	17.044
3	5	10.000	15.488	2.121	16.032
4	5	18.000	16.576	2.707	16.032
5	5	32.000	12.672	1.988	12.672
6	5	56.000	11.550	0.365	11.550
7	5	100.000	8.338	0.629	8.338
8	4	180.000	7.375	0.691	7.375

```
The Linear Interpolation Estimate:    27.3625   Entered P Value: 25
-------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:  27.5292 Standard Deviation:     4.7812
Original Confidence Limits:   Lower:    20.8734 Upper:    42.3270
Expanded Confidence Limits:   Lower:    17.6289 Upper:    49.8093
Resampling time in Seconds:     0.28  Random_Seed: -35158431
```

Figure 8. ICPIN program output for the IC25.

TABLE 21. SINGLE LABORATORY PRECISION OF THE GIANT KELP, *MACROCYSTIS PYRIFERA* GERMINATION AND GERM-TUBE LENGTH TEST WITH COPPER (CU µG/L) CHLORIDE AS THE REFERENCE TOXICANT

Test Number	Germ-Tube Length		Germination	
	NOEC	IC40	NOEC	EC50
1	<5.6	122.7	10.0	67.5
2	10.0	43.1	18.0	73.5
3	18.0	70.7	18.0	124.3
4	5.6	88.0	56.0	101.6
5	32.0	124.7	56.0	122.9
Mean		89.8		90.7
CV		38.8%		32.9%

Data from Anderson et al., 1994

TABLE 22. SINGLE LABORATORY PRECISION OF THE GIANT KELP, *MACROCYSTIS PYRIFERA* GERMINATION AND GERM-TUBE LENGTH TEST WITH SODIUM AZIDE (MG/L) AS THE REFERENCE TOXICANT

Test Date	Germ-Tube Length			Germination	
	NOEC	IC25	IC50	NOEC	EC50
2/11/92	18.0	39.5	133.7	18.0	52.3
2/18/92	18.0	34.1	96.5	32.0	72.6
6/29/92	32.0	57.5	142.2	32.0	132.1
7/07/92	10.0	33.1	92.5	10.0	79.2
7/15/92	18.0	42.8	138.9	18.0	117.8
7/16/92	5.6	25.0	68.4	10.0	48.3
7/22/92	10.0	30.2	80.6	18.0	62.4
10/09/92	5.6	25.1	80.0	5.6	60.3
7/02/92	10.0	24.8	80.1	18.0	84.0
Mean		34.7	101.4		78.8
CV		30.8%	28.4%		36.7%

Data from Hunt et al., 1991

TABLE 23. MULTI-LABORATORY PRECISION OF THE GIANT KELP, *MACROCYSTIS PYRIFERA* GERMINATION AND GERM-TUBE LENGTH TEST PERFORMED WITH COPPER CHLORIDE (μG/L) AS THE REFERENCE TOXICANT

	Lab	Germ-tube length NOEC	IC40	Germination NOEC	EC50	CV Germ-tube	CV Germination
March 1990	1	5.6	122.7	10.0	46.9	8.4%	>1.1%
	2	32.0	117.8	32.0	46.2		
	3	18.0	104.1	32.0	*		
May 1990	1	10.0	43.1	18.0	112.0	39.9%	59.3%
	2	<5.6	99.1	32.0	164.2		
	3	18.0	68.7	18.0	67.9		
May 1990	1	18.0	70.7	18.0	112.0	45.3%	nc
	2	18.0	91.3	56.0	64.5		
	3	32.0	134.2	32.0	158.0		
December 1990	1	5.6	88.0	56.0	77.7	45.3%	nc
	2	5.6	45.3	18.0	*		
September 1990	1	32.0	124.7	56.0	127.4	55.5%	7.4%
	2	18.0	54.4	56.0	114.8		
September 1989	1	<10.0	89.3**	56.0	115.5	44.5%	67.6%
	2	<10.0	171.8**	56.0	327.7		
November 1989	1	32.0	>180.0	<10.0	>180.0	nc	nc
	2	10.0	>180.0	18.0	>180.0		
May 1988	1	<56.0	232.0***	<56.0	211.0	nc	50.0%
	2	<56.0	*	56.0	100.7		

* No EC50 calculated because response was less than 50%.
** Only concentration means available, therefore no IC40 values were calculated.
nc Not calculated (Insufficient numbers to calculate the coefficient of variation).
*** IC50 value, not IC40

Data from Hunt et al., 1991

APPENDIX I. MACROCYSTIS TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution (10,000 µg/L) by adding 0.0268 of copper chloride ($CuCl_2 \cdot 2H_2O$) to 1 liter of reagent water.

D. Prepare copper reference toxicant solution of 0 (control) 5.6, 10, 18, 32, 100 and 180 µg/L by adding 0, 0.56, 1.0 1.8, 3.2, 10.0 and 18.0 mL of stock solution, respectively, to a 1-L volumetric flask and filling to 1-L with dilution water.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 15°C and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Collect sporophylls and rinse in 0.2 μm filtered seawater. Store at 9-12°C for no more than 24 hours before zoospore release.

B. Blot sporophylls and leave exposed to air for one hour.

C. Place 25-30 sporophylls one liter of 0.2 μm filtered seawater for no more than two hours. The presence of zoospores is indicated by a slight cloudiness in the water.

D. Take a sample of the zoospore solution from the top 5 centimeters of the beaker and determine the spore density using a hemacytometer. Determine the volume of water necessary to give 7,500 spores/mL of test solution. This volume should not exceed one percent of the test solution volume.

E. Verify that the zoospores are swimming, then pipet the volume of water necessary to give 7,500 spores/mL into each of the test chambers. Take zoospores from the top 5 centimeters of the release beaker so that only swimming zoospores are used.

F. At 48 ± 3 hours, count the number of germinated and non-germinated spores of the first 100 spores encountered in each replicate of each concentration. Measure the length of 10 randomly selected germination tubes (or preserve with 0.1% glutaraldehyde for later examination).

G. Analyze the data.

H. Include standard reference toxicant point estimate values in the standard quality control charts.

Data Sheet for Kelp Toxicity Test

Test Start Date: Start Time: Kelp Species :
Test End Date: End Time: Collection/Arrival Date:
Reference Toxicant: Kelp Source:

Sample Source: Microscope Model:

Sample Type: Solid Elutriate Pore Water Water Effluent Ref Tox Micrometer Conversion Factor:

Test Cont. #	Station Code	Number of Spores Germ.	Number of Spores Not Germ.	Length Measurements (in ocular micrometer units)										Notes
				L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
1														
2														
3														
4														
5														
6														
7														
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Computer Data Storage
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File:

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APPENDICES

APPENDIX A

INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

1. STATISTICAL INDEPENDENCE

1.1 Dunnett's Procedure and the *t* test with Bonferroni's adjustment are parametric procedures based on the assumptions that (1) the observations within treatments are independent and normally distributed, and (2) that the variance of the observations is homogeneous across all toxicant concentrations and the control. Of the three possible departures from the assumptions, non-normality, heterogeneity of variance, and lack of independence, those caused by lack of independence are the most difficult to resolve (see Scheffe, 1959). For toxicity data, statistical independence means that given knowledge of the true mean for a given concentration or control, knowledge of the error in any one actual observation would provide no information about the error in any other observation. Lack of independence is difficult to assess and difficult to test for statistically. It may also have serious effects on the true alpha or beta level. Therefore, it is of utmost importance to be aware of the need for statistical independence between observations and to be constantly vigilant in avoiding any patterned experimental procedure that might compromise independence. One of the best ways to help insure independence is to follow proper randomization procedures throughout the test.

2. RANDOMIZATION

2.1 Randomization of the distribution of test organisms among test chambers, and the arrangement of treatments and replicate chambers is an important part of conducting a valid test. The purpose of randomization is to avoid situations where test organisms are placed serially into test chambers, or where all replicates for a test concentration are located adjacent to one another, which could introduce bias into the test results.

2.2 An example of randomization of the distribution of test organisms among test chambers, and an example of randomization of arrangement of treatments and replicate chambers are described using the topsmelt, *Atherinops affinis*, Survival and Growth test. For the purpose of the example, the test design is as follows:

Five effluent concentrations are tested in addition to the control. The effluent concentrations are as follows: 6.25%, 12.5%, 25.0%, 50.0%, and 100.0%. There are five replicate chambers per treatment. Each replicate chamber contains five larvae.

2.3 RANDOMIZATION OF FISH TO REPLICATE CHAMBERS EXAMPLE

2.3.1 Consider first the random assignment of the fish to the replicate chambers. The first step is to label each of the replicate chambers with the control or effluent concentration and the replicate number. The next step is to assign each replicate chamber three double-digit numbers. An example of this assignment is provided in Table A.1. Note that the double digits 00 and 91 through 99 were not used.

2.3.2 The random numbers used to carry out the random assignment of fish to replicate chambers are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double digit number. The first number read identifies the replicate chamber for the first fish taken from the tank. For the example, the first entry in row 2 was chosen as the starting position. The first number in this row is 37. According to Table A.1, this number corresponds to replicate chamber 2 of the 6.25% effluent concentration. Thus, the first fish taken from the tank is to be placed in replicate chamber 2 of the 6.25% effluent concentration.

2.3.3 The next step is to read the double digit number to the right of the first one. The second number identifies the replicate chamber for the second fish taken from the tank. Continuing the example, the second number read in row 2 of Table A.2 is 54. According to Table A.1, this number corresponds to replicate chamber 4 of the 50.0% effluent concentration. Thus, the second fish taken from the tank is to be placed in replicate chamber 4 of the 50.0% effluent concentration.

2.3.4 Continue in this fashion until all the fish have been randomly assigned to a replicate chamber. In order to fill each replicate chamber with ten fish, the assigned numbers will be used more than once. If a number is read from the table that was not assigned to a replicate chamber, then ignore it and continue to the next number. If a replicate chamber becomes filled and a

number is read from the table that corresponds to it, then ignore that value and continue to the next number. The first ten random summarized in Table A.3.2.3.5 Three double-digit numbers were assigned to each replicate chamber (instead of one or two double-digit numbers) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each replicate chamber: the first column of assigned numbers in Table A.1. Whenever the numbers 00 and 31 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

TABLE A.1. RANDOM ASSIGNMENT OF FISH TO REPLICATE CHAMBERS EXAMPLE ASSIGNED NUMBERS FOR EACH REPLICATE CHAMBER

Assigned Numbers	Replicate Chamber
01, 31, 61	Control, replicate chamber 1
02, 32, 62	Control, replicate chamber 2
03, 33, 63	Control, replicate chamber 3
04, 34, 64	Control, replicate chamber 4
05, 35, 65	Control, replicate chamber 5
06, 36, 66	6.25% effluent, replicate chamber 1
07, 37, 67	6.25% effluent, replicate chamber 2
08, 38, 68	6.25% effluent, replicate chamber 3
09, 39, 69	6.25% effluent, replicate chamber 4
10, 40, 70	6.25% effluent, replicate chamber 5
11, 41, 71	12.5% effluent, replicate chamber 1
12, 42, 72	12.5% effluent, replicate chamber 2
13, 43, 73	12.5% effluent, replicate chamber 3
14, 44, 74	12.5% effluent, replicate chamber 4
15, 45, 75	12.5% effluent, replicate chamber 5
16, 46, 76	25.0% effluent, replicate chamber 1
17, 47, 77	25.0% effluent, replicate chamber 2
18, 48, 78	25.0% effluent, replicate chamber 3
19, 49, 79	25.0% effluent, replicate chamber 4
20, 50, 80	25.0% effluent, replicate chamber 5
21, 51, 81	50.0% effluent, replicate chamber 1
22, 52, 82	50.0% effluent, replicate chamber 2
23, 53, 83	50.0% effluent, replicate chamber 3
24, 54, 84	50.0% effluent, replicate chamber 4
25, 55, 85	50.0% effluent, replicate chamber 5
26, 56, 86	100.0% effluent, replicate chamber 1
27, 57, 87	100.0% effluent, replicate chamber 2
28, 58, 88	100.0% effluent, replicate chamber 3
29, 59, 89	100.0% effluent, replicate chamber 4
30, 60, 90	100.0% effluent, replicate chamber 5

TABLE A.2. TABLE OF RANDOM NUMBERS (Dixon and Massey, 1983)

10 09 73 25 33	76 52 01 35 86	34 67 35 43 76	80 95 90 91 17	39 29 27 49 45
37 54 20 48 05	64 89 47 42 96	24 80 52 40 37	20 63 61 04 02	00 82 29 16 65
08 42 26 89 53	19 64 50 93 03	23 20 90 25 60	15 95 33 47 64	35 08 03 36 06
99 01 90 25 29	09 37 67 07 15	38 31 13 11 65	88 67 67 43 97	04 43 62 76 59
12 80 79 99 70	80 15 73 61 47	64 03 23 66 53	98 95 11 68 77	12 27 17 68 33
66 06 57 47 17	34 07 27 68 50	36 69 73 61 70	65 81 33 98 85	11 19 92 91 70
31 06 01 08 05	45 57 18 24 06	35 30 34 26 14	86 79 90 74 39	23 40 30 97 32
85 26 97 76 02	02 05 16 56 92	68 66 57 48 18	73 05 38 52 47	18 62 38 85 79
63 57 33 21 35	05 32 54 70 48	90 55 35 75 48	28 46 82 87 09	83 49 12 56 24
73 79 64 57 53	03 52 96 47 78	35 80 83 42 82	60 93 52 03 44	35 27 38 84 35
98 52 01 77 67	14 90 56 86 07	22 10 94 05 58	60 97 09 34 33	50 50 07 39 98
11 80 50 54 31	39 80 82 77 32	50 72 56 82 48	29 40 52 42 01	52 77 56 78 51
83 45 29 96 34	06 28 89 80 83	13 74 67 00 78	18 47 54 06 10	68 71 17 78 17
88 68 54 02 00	86 50 75 84 01	36 76 66 79 51	90 36 47 64 93	29 60 91 10 62
99 59 46 73 48	87 51 76 49 69	91 82 60 89 28	93 78 56 13 68	23 47 83 41 13
65 48 11 76 74	17 46 85 09 50	58 04 77 69 74	73 03 95 71 86	40 21 81 65 44
80 12 43 56 35	17 72 70 80 15	45 31 82 23 74	21 11 57 82 53	14 38 55 37 63
74 35 09 98 17	77 40 27 72 14	43 23 60 02 10	45 52 16 42 37	96 28 60 26 55
69 91 62 68 03	66 25 22 91 48	36 93 68 72 03	76 62 11 39 90	94 40 05 64 18
09 89 32 05 05	14 22 56 85 14	46 42 75 67 88	96 29 77 88 22	54 38 21 45 98
91 49 91 45 23	68 47 92 76 86	46 16 28 35 54	94 75 08 99 23	37 08 92 00 48
80 33 69 45 98	26 94 03 68 58	70 29 73 41 35	53 14 03 33 40	42 05 08 23 41
44 10 48 19 49	85 15 74 79 54	32 97 92 65 75	57 60 04 08 81	22 22 20 64 13
12 55 07 37 42	11 10 00 20 40	12 86 07 46 97	96 64 48 94 39	28 70 72 58 15
63 60 64 93 29	16 50 53 44 84	40 21 95 25 63	43 65 17 70 82	07 20 73 17 90
61 19 69 04 46	26 45 74 77 74	51 92 43 37 29	65 39 45 95 93	42 58 26 05 27
15 47 44 52 66	95 27 07 99 53	59 36 78 38 48	82 39 61 01 18	33 21 15 94 66
94 55 72 85 73	67 89 75 43 87	54 62 24 44 31	91 19 04 25 92	92 92 74 59 73
42 48 11 62 13	97 34 40 87 21	16 86 84 87 67	03 07 11 20 59	25 70 14 66 70
23 52 37 83 17	73 20 88 98 37	68 93 59 14 16	26 25 22 96 63	05 52 28 25 62
04 49 35 24 94	75 24 63 38 24	45 86 25 10 25	61 96 27 93 35	65 33 71 24 72
00 54 99 76 54	64 05 18 81 59	96 11 96 38 96	54 69 28 23 91	23 28 72 95 29
35 96 31 53 07	26 89 80 93 45	33 35 13 54 62	77 97 45 00 24	90 10 33 93 33
59 80 80 83 91	45 42 72 68 42	83 60 94 97 00	13 02 12 48 92	78 56 52 01 06
46 05 88 52 36	01 39 09 22 86	77 28 14 40 77	93 91 08 36 47	70 61 74 29 41
32 17 90 05 97	87 37 92 52 41	05 56 70 70 07	86 74 31 71 57	85 39 41 18 38
69 23 46 14 06	20 11 74 52 04	15 95 66 00 00	18 74 39 24 23	97 11 89 63 38
19 56 54 14 30	01 75 87 53 79	40 41 92 15 85	66 67 43 68 06	84 96 28 52 07
45 15 51 49 38	19 47 60 72 46	43 66 79 45 43	59 04 79 00 33	20 82 66 95 41
94 86 43 19 94	36 16 81 08 51	34 88 88 15 53	01 54 03 54 56	05 01 45 11 76
98 08 62 48 26	45 24 02 84 04	44 99 90 88 96	39 09 47 34 07	35 44 13 18 80
33 18 51 62 32	41 94 15 09 49	89 43 54 85 81	88 69 54 19 94	37 54 87 30 43
80 95 10 04 06	96 38 27 07 74	20 15 12 33 87	25 01 62 52 98	94 62 46 11 71
79 75 24 91 40	71 96 12 82 96	69 86 10 25 91	74 85 22 05 39	00 38 75 95 79
18 63 33 25 37	98 14 50 65 71	31 01 02 46 74	05 45 56 14 27	77 93 89 19 36
74 02 94 39 02	77 55 73 22 70	97 79 01 71 19	52 52 75 80 21	80 81 45 17 48
54 17 84 56 11	80 99 33 71 43	05 33 51 29 69	56 12 71 92 55	36 04 09 03 24
11 66 44 98 83	52 07 98 48 27	59 38 17 15 39	09 97 33 34 40	88 46 12 33 56
48 32 47 79 28	31 24 96 47 10	02 29 53 68 70	32 30 75 75 46	15 02 00 99 94
69 07 49 41 38	87 63 79 19 76	35 58 40 44 01	10 51 82 16 15	01 84 87 69 38

2.4 RANDOMIZATION OF REPLICATE CHAMBERS TO POSITIONS EXAMPLE

2.4.1 Next consider the random assignment of the 30 replicate chambers to positions within the water bath (or equivalent). Assume that the replicate chambers are to be positioned in a five row by six column rectangular array. The first step is to label the positions in the water bath. Table A.4 provides an example layout. assignments of fish to replicate chambers for the example are

TABLE A.3. EXAMPLE OF RANDOM ASSIGNMENT OF FIRST TEN FISH TO REPLICATE CHAMBERS

Fish	Assignment
First fish taken from tank	6.25% effluent, replicate chamber 2
Second fish taken from tank	50.0% effluent, replicate chamber 4
Third fish taken from tank	25.0% effluent, replicate chamber 5
Fourth fish taken from tank	25.0% effluent, replicate chamber 3
Fifth fish taken from tank	Control, replicate chamber 5
Sixth fish taken from tank	Control, replicate chamber 4
Seventh fish taken from tank	100.0% effluent, replicate chamber 4
Eighth fish taken from tank	25.0% effluent, replicate chamber 2
Ninth fish taken from tank	12.5% effluent, replicate chamber 2
Tenth fish taken from tank	50.0% effluent, replicate chamber 4

TABLE A.4. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE LABELLING THE POSITIONS WITHIN THE WATER BATH

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24
25	26	27	28	29	30

2.4.2 The second step is to assign each of the 30 positions three double-digit numbers. An example of this assignment is provided in Table A.5. Note that the double digits 00 and 91 through 99 were not used.

2.4.3 The random numbers used to carry out the random assignment of replicate chambers to positions are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double-digit number. The first number read identifies the position for the first replicate chamber of the control. For the example, the first entry in row 10 of Table A.2 was chosen as the starting position. The first number in this row was 73. According to Table A.5, this number corresponds to position 13. Thus, the first replicate chamber for the control will be placed in position 13.

2.4.4 The next step is to read the double-digit number to the right of the first one. The second number identifies the position for the second replicate chamber of the control. Continuing the example, the second number read in row 10 of Table A.2 is 79. According to Table A.5, this number corresponds to position 19. Thus, the second replicate chamber for the control will be placed in position 19.

2.4.5 Continue in this fashion until all the replicate chambers have been assigned to a position. The first five numbers read will identify the positions for the control replicate chambers, the second five numbers read will identify the positions for the lowest effluent concentration replicate chambers, and so on. If a number is read from the table that was not assigned to a position, then ignore that value and continue to the next number. If a number is repeated in Table A.2, then ignore the repeats and continue to the next number. The complete randomization of replicate chambers to positions for the example is displayed in Table A.6.

2.4.6 Three double-digit numbers were assigned to each position (instead of one or two) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each position: the first column of assigned numbers in Table A.5. Whenever the numbers 00 and 31 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

3. OUTLIERS

3.1 An outlier is an inconsistent or questionable data point that appears unrepresentative of the general trend exhibited by the majority of the data. Outliers may be detected by tabulation of the data, plotting, and by an analysis of the residuals. An explanation should be sought for any questionable data points. Without an explanation, data points should be discarded only with extreme caution. If there is no explanation, the analysis should be performed both with and without the outlier, and the results of both analyses should be reported.

3.2 Gentleman-Wilk's A statistic gives a test for the condition that the extreme observation may be considered an outlier. For a discussion of this, and other techniques for evaluating outliers, see Draper and John (1981).

TABLE A.5. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE ASSIGNED NUMBERS FOR EACH POSITION

Assigned Numbers	Position
01, 31, 61	1
02, 32, 62	2
03, 33, 63	3
04, 34, 64	4
05, 35, 65	5
06, 36, 66	6
07, 37, 67	7
08, 38, 68	8
09, 39, 69	9
10, 40, 70	10
11, 41, 71	11
12, 42, 72	12
13, 43, 73	13
14, 44, 74	14
15, 45, 75	15
16, 46, 76	16
17, 47, 77	17
18, 48, 78	18
19, 49, 79	19
20, 50, 80	20
21, 51, 81	21
22, 52, 82	22
23, 53, 83	23
24, 54, 84	24
25, 55, 85	25
26, 56, 86	26
27, 57, 87	27
28, 58, 88	28
29, 59, 89	29
30, 60, 90	30

TABLE A.6. EXAMPLE OF RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS:

ASSIGNMENT OF ALL 30 POSITIONS

25.0%	50.0%	6.25%	Control	6.25%	100.0%
25.0%	12.5%	50.0%	25.0%	50.0%	12.5%
Control	12.5%	100.0%	100.0%	6.25%	6.25%
Control	12.5%	100.0%	6.25%	Control	25.0%
100.0%	25.0%	Control	50.0%	50.0%	12.5%

APPENDIX B

VALIDATING NORMALITY AND HOMOGENEITY OF VARIANCE ASSUMPTIONS

1. INTRODUCTION

1.1 Dunnett's Procedure and the *t* test with Bonferroni's adjustment are parametric procedures based on the assumptions that the observations within treatments are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using these tests, to determine if they have been met. Tests for validating the assumptions are provided in the following discussion. If the tests fail (if the data do not meet the assumptions), a nonparametric procedure such as Steel's Many-one Rank Test may be more appropriate. However, the decision on whether to use parametric or nonparametric tests may be a judgement call, and a statistician should be consulted in selecting the analysis.

2. TEST FOR NORMAL DISTRIBUTION OF DATA

2.1 SHAPIRO-WILK'S TEST

2.1.1 One formal test for normality is the Shapiro-Wilk's Test (Conover, 1980). The test statistic is obtained by dividing the square of an appropriate linear combination of the sample order statistics by the usual symmetric estimate of variance. The calculated W must be greater than zero and less than or equal to one. This test is recommended for a sample size of 50 or less. If the sample size is greater than 50, the Kolmogorov "D" statistic (Stephens, 1974) is recommended. An example of the Shapiro-Wilk's test is provided below.

2.2 The example uses growth data from the Mysid Larval Survival and Growth Test. The same data are used later in the discussions of the homogeneity of variance determination in Section 3 of this appendix and Dunnett's Procedure in Appendix C. The data, the mean and variance of the observations at each concentration, including the control, are listed in Table B.1.

TABLE B.1. MYSID, *HOLMESIMYSIS COSTATA*, GROWTH DATA

Replicate	Control	Concentration (%) 1.80	3.20	5.60
1	0.048	0.055	0.057	0.041
2	0.058	0.048	0.050	0.040
3	0.047	0.042	0.046	0.041
4	0.058	0.041	0.043	0.043
5	0.051	0.052	0.045	0.040
Mean ($\bar{Y}_i$)	0.052	0.048	0.048	0.041
S_i^2	0.0000283	0.0000373	0.0000307	0.0000015
i	1	2	3	4

2.3 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table B.2.

TABLE B.2. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	Concentration (%) 1.80	3.20	5.60
1	-0.004	0.007	0.009	0.000
2	0.006	0.000	0.002	-0.001
3	-0.005	-0.006	-0.002	0.000
4	0.006	-0.007	-0.005	0.002
5	-0.001	0.004	-0.003	-0.001

2.4 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations

2.4.1 For this set of data, n = 20

$$\overline{X} = \frac{1}{20}(0.001) = 0.000$$

D = 0.000393

2.5 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table B.3.

TABLE B.3. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.007	11	0.000
2	-0.006	12	0.000
3	-0.005	13	0.000
4	-0.005	14	0.002
5	-0.004	15	0.002
6	-0.003	16	0.004
7	-0.002	17	0.006
8	-0.001	18	0.006
9	-0.001	19	0.007
10	-0.001	20	0.009

2.6 From Table B.4, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table B.5.

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (Conover, 1980)

i \ n	Number of Observations									
	2	3	4	5	6	7	8	9	10	
1	0.7071	0.7071	0.6872	0.6646	0.6431	0.6233	0.6052	0.5888	0.5739	
2	-	0.0000	0.1667	0.2413	0.2806	0.3031	0.3164	0.3244	0.3291	
3	-	-	-	0.0000	0.0875	0.1401	0.1743	0.1976	0.2141	
4	-	-	-	-	-	0.0000	0.0561	0.0947	0.1224	
5	-	-	-	-	-	-	-	0.0000	0.0399	

i \ n	Number of Observations									
	11	12	13	14	15	16	17	18	19	20
1	0.5601	0.5475	0.5359	0.5251	0.5150	0.5056	0.4968	0.4886	0.4808	0.4734
2	0.3315	0.3325	0.3325	0.3318	0.3306	0.3290	0.3273	0.3253	0.3232	0.3211
3	0.2260	0.2347	0.2412	0.2460	0.2495	0.2521	0.2540	0.2553	0.2561	0.2565
4	0.1429	0.1586	0.1707	0.1802	0.1878	0.1939	0.1988	0.2027	0.2059	0.2085
5	0.0695	0.0922	0.1099	0.1240	0.1353	0.1447	0.1524	0.1587	0.1641	0.1686
6	0.0000	0.0303	0.0539	0.0727	0.0880	0.1005	0.1109	0.1197	0.1271	0.1334
7	-	-	0.0000	0.0240	0.0433	0.0593	0.0725	0.0837	0.0932	0.1013
8	-	-	-	-	0.0000	0.0196	0.0359	0.0496	0.0612	0.0711
9	-	-	-	-	-	-	0.0000	0.0163	0.0303	0.0422
10	-	-	-	-	-	-	-	-	0.0000	0.0140

i \ n	Number of Observations									
	21	22	23	24	25	26	27	28	29	30
1	0.4643	0.4590	0.4542	0.4493	0.4450	0.4407	0.4366	0.4328	0.4291	0.4254
2	0.3185	0.3156	0.3126	0.3098	0.3069	0.3043	0.3018	0.2992	0.2968	0.2944
3	0.2578	0.2571	0.2563	0.2554	0.2543	0.2533	0.2522	0.2510	0.2499	0.2487
4	0.2119	0.2131	0.2139	0.2145	0.2148	0.2151	0.2152	0.2151	0.2150	0.2148
5	0.1736	0.1764	0.1787	0.1807	0.1822	0.1836	0.1848	0.1857	0.1864	0.1870
6	0.1399	0.1443	0.1480	0.1512	0.1539	0.1563	0.1584	0.1601	0.1616	0.1630
7	0.1092	0.1150	0.1201	0.1245	0.1283	0.1316	0.1346	0.1372	0.1395	0.1415
8	0.0804	0.0878	0.0941	0.0997	0.1046	0.1089	0.1128	0.1162	0.1192	0.1219
9	0.0530	0.0618	0.0696	0.0764	0.0823	0.0876	0.0923	0.0965	0.1002	0.1036
10	0.0263	0.0368	0.0459	0.0539	0.0610	0.0672	0.0728	0.0778	0.0822	0.0862
11	0.0000	0.0122	0.0228	0.0321	0.0403	0.0476	0.0540	0.0598	0.0650	0.0697
12	-	-	0.0000	0.0107	0.0200	0.0284	0.0358	0.0424	0.0483	0.0537
13	-	-	-	-	0.0000	0.0094	0.0178	0.0253	0.0320	0.0381
14	-	-	-	-	-	-	0.0000	0.0084	0.0159	0.0227
15	-	-	-	-	-	-	-	-	0.0000	0.0076

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (CONTINUED)

i \ n	Number of Observations									
	31	32	33	34	35	36	37	38	39	40
1	0.4220	0.4188	0.4156	0.4127	0.4096	0.4068	0.4040	0.4015	0.3989	0.3964
2	0.2921	0.2898	0.2876	0.2854	0.2834	0.2813	0.2794	0.2774	0.2755	0.2737
3	0.2475	0.2462	0.2451	0.2439	0.2427	0.2415	0.2403	0.2391	0.2380	0.2368
4	0.2145	0.2141	0.2137	0.2132	0.2127	0.2121	0.2116	0.2110	0.2104	0.2098
5	0.1874	0.1878	0.1880	0.1882	0.1883	0.1883	0.1883	0.1881	0.1880	0.1878
6	0.1641	0.1651	0.1660	0.1667	0.1673	0.1678	0.1683	0.1686	0.1689	0.1691
7	0.1433	0.1449	0.1463	0.1475	0.1487	0.1496	0.1505	0.1513	0.1520	0.1526
8	0.1243	0.1265	0.1284	0.1301	0.1317	0.1331	0.1344	0.1356	0.1366	0.1376
9	0.1066	0.1093	0.1118	0.1140	0.1160	0.1179	0.1196	0.1211	0.1225	0.1237
10	0.0899	0.0931	0.0961	0.0988	0.1013	0.1036	0.1056	0.1075	0.1092	0.1108
11	0.0739	0.0777	0.0812	0.0844	0.0873	0.0900	0.0924	0.0947	0.0967	0.0986
12	0.0585	0.0629	0.0669	0.0706	0.0739	0.0770	0.0798	0.0824	0.0848	0.0870
13	0.0435	0.0485	0.0530	0.0572	0.0610	0.0645	0.0677	0.0706	0.0733	0.0759
14	0.0289	0.0344	0.0395	0.0441	0.0484	0.0523	0.0559	0.0592	0.0622	0.0651
15	0.0144	0.0206	0.0262	0.0314	0.0361	0.0404	0.0444	0.0481	0.0515	0.0546
16	0.0000	0.0068	0.0131	0.0187	0.0239	0.0287	0.0331	0.0372	0.0409	0.0444
17	-	-	0.0000	0.0062	0.0119	0.0172	0.0220	0.0264	0.0305	0.0343
18	-	-	-	-	0.0000	0.0057	0.0110	0.0158	0.0203	0.0244
19	-	-	-	-	-	-	0.0000	0.0053	0.0101	0.0146
20	-	-	-	-	-	-	-	-	0.0000	0.0049

i \ n	Number of Observations									
	41	42	43	44	45	46	47	48	49	50
1	0.3940	0.3917	0.3894	0.3872	0.3850	0.3830	0.3808	0.3789	0.3770	0.3751
2	0.2719	0.2701	0.2684	0.2667	0.2651	0.2635	0.2620	0.2604	0.2589	0.2574
3	0.2357	0.2345	0.2334	0.2323	0.2313	0.2302	0.2291	0.2281	0.2271	0.2260
4	0.2091	0.2085	0.2078	0.2072	0.2065	0.2058	0.2052	0.2045	0.2038	0.2032
5	0.1876	0.1874	0.1871	0.1868	0.1865	0.1862	0.1859	0.1855	0.1851	0.1847
6	0.1693	0.1694	0.1695	0.1695	0.1695	0.1695	0.1695	0.1693	0.1692	0.1691
7	0.1531	0.1535	0.1539	0.1542	0.1545	0.1548	0.1550	0.1551	0.1553	0.1554
8	0.1384	0.1392	0.1398	0.1405	0.1410	0.1415	0.1420	0.1423	0.1427	0.1430
9	0.1249	0.1259	0.1269	0.1278	0.1286	0.1293	0.1300	0.1306	0.1312	0.1317
10	0.1123	0.1136	0.1149	0.1160	0.1170	0.1180	0.1189	0.1197	0.1205	0.1212
11	0.1004	0.1020	0.1035	0.1049	0.1062	0.1073	0.1085	0.1095	0.1105	0.1113
12	0.0891	0.0909	0.0927	0.0943	0.0959	0.0972	0.0986	0.0998	0.1010	0.1020
13	0.0782	0.0804	0.0824	0.0842	0.0860	0.0876	0.0892	0.0906	0.0919	0.0932
14	0.0677	0.0701	0.0724	0.0745	0.0765	0.0783	0.0801	0.0817	0.0832	0.0846
15	0.0575	0.0602	0.0628	0.0651	0.0673	0.0694	0.0713	0.0731	0.0748	0.0764
16	0.0476	0.0506	0.0534	0.0560	0.0584	0.0607	0.0628	0.0648	0.0667	0.0685
17	0.0379	0.0411	0.0442	0.0471	0.0497	0.0522	0.0546	0.0568	0.0588	0.0608
18	0.0283	0.0318	0.0352	0.0383	0.0412	0.0439	0.0465	0.0489	0.0511	0.0532
19	0.0188	0.0227	0.0263	0.0296	0.0328	0.0357	0.0385	0.0411	0.0436	0.0459
20	0.0094	0.0136	0.0175	0.0211	0.0245	0.0277	0.0307	0.0335	0.0361	0.0386
21	0.0000	0.0045	0.0087	0.0126	0.0163	0.0197	0.0229	0.0259	0.0288	0.0314
22	-	-	0.0000	0.0042	0.0081	0.0118	0.0153	0.0185	0.0215	0.0244
23	-	-	-	-	0.0000	0.0039	0.0076	0.0111	0.0143	0.0174
24	-	-	-	-	-	-	0.0000	0.0037	0.0071	0.0104
25	-	-	-	-	-	-	-	-	0.0000	0.0035

2.7 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table B.5. For this set of data:

$$W = \frac{1}{0.000393}(0.0194)^2 = 0.958$$

TABLE B.5. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.016	$X^{(20)} - X^{(1)}$
2	0.3211	0.013	$X^{(19)} - X^{(2)}$
3	0.2565	0.011	$X^{(18)} - X^{(3)}$
4	0.2085	0.011	$X^{(17)} - X^{(4)}$
5	0.1686	0.008	$X^{(16)} - X^{(5)}$
6	0.1334	0.005	$X^{(15)} - X^{(6)}$
7	0.1013	0.004	$X^{(14)} - X^{(7)}$
8	0.0711	0.001	$X^{(13)} - X^{(8)}$
9	0.0422	0.001	$X^{(12)} - X^{(9)}$
10	0.0140	0.001	$X^{(11)} - X^{(10)}$

2.8 The decision rule for this test is to compare the computed W to the critical value found in Table B.6. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 20 observations is 0.868. Since W = 0.958 is greater than the critical value, conclude that the data are normally distributed.

2.9 In general, if the data fail the test for normality, a transformation such as to log values may normalize the data. After transforming the data, repeat the Shapiro Wilk's Test for normality.

TABLE B.6. QUANTILES OF THE SHAPIRO WILK'S TEST STATISTIC (Conover, 1980)

n	*0.01*	*0.02*	*0.05*	*0.10*	*0.50*	*0.90*	*0.95*	*0.98*	*0.99*
3	0.753	0.756	0.767	0.789	0.959	0.998	0.999	1.000	1.000
4	0.687	0.707	0.748	0.792	0.935	0.987	0.992	0.996	0.997
5	0.686	0.715	0.762	0.806	0.927	0.979	0.986	0.991	0.993
6	0.713	0.743	0.788	0.826	0.927	0.974	0.981	0.986	0.989
7	0.730	0.760	0.803	0.838	0.928	0.972	0.979	0.985	0.988
8	0.749	0.778	0.818	0.851	0.932	0.972	0.978	0.984	0.987
9	0.764	0.791	0.829	0.859	0.935	0.972	0.978	0.984	0.986
10	0.781	0.806	0.842	0.869	0.938	0.972	0.978	0.983	0.986
11	0.792	0.817	0.850	0.876	0.940	0.973	0.979	0.984	0.986
12	0.805	0.828	0.859	0.883	0.943	0.973	0.979	0.984	0.986
13	0.814	0.837	0.866	0.889	0.945	0.974	0.979	0.984	0.986
14	0.825	0.846	0.874	0.895	0.947	0.975	0.980	0.984	0.986
15	0.835	0.855	0.881	0.901	0.950	0.975	0.980	0.984	0.987
16	0.844	0.863	0.887	0.906	0.952	0.976	0.981	0.985	0.987
17	0.851	0.869	0.892	0.910	0.954	0.977	0.981	0.985	0.987
18	0.858	0.874	0.897	0.914	0.956	0.978	0.982	0.986	0.988
19	0.863	0.879	0.901	0.917	0.957	0.978	0.982	0.986	0.988
20	0.868	0.884	0.905	0.920	0.959	0.979	0.983	0.986	0.988
21	0.873	0.888	0.908	0.923	0.960	0.980	0.983	0.987	0.989
22	0.878	0.892	0.911	0.926	0.961	0.980	0.984	0.987	0.989
23	0.881	0.895	0.914	0.928	0.962	0.981	0.984	0.987	0.989
24	0.884	0.898	0.916	0.930	0.963	0.981	0.984	0.987	0.989
25	0.888	0.901	0.918	0.931	0.964	0.981	0.985	0.988	0.989
26	0.891	0.904	0.920	0.933	0.965	0.982	0.985	0.988	0.989
27	0.894	0.906	0.923	0.935	0.965	0.982	0.985	0.988	0.990
28	0.896	0.908	0.924	0.936	0.966	0.982	0.985	0.988	0.990
29	0.898	0.910	0.926	0.937	0.966	0.982	0.985	0.988	0.990
30	0.900	0.912	0.927	0.939	0.967	0.983	0.985	0.988	0.990
31	0.902	0.914	0.929	0.940	0.967	0.983	0.986	0.988	0.990
32	0.904	0.915	0.930	0.941	0.968	0.983	0.986	0.988	0.990
33	0.906	0.917	0.931	0.942	0.968	0.983	0.986	0.989	0.990
34	0.908	0.919	0.933	0.943	0.969	0.983	0.986	0.989	0.990
35	0.910	0.920	0.934	0.944	0.969	0.984	0.986	0.989	0.990
36	0.912	0.922	0.935	0.945	0.970	0.984	0.986	0.989	0.990
37	0.914	0.924	0.936	0.946	0.970	0.984	0.987	0.989	0.990
38	0.916	0.925	0.938	0.947	0.971	0.984	0.987	0.989	0.990
39	0.917	0.927	0.939	0.948	0.971	0.984	0.987	0.989	0.991
40	0.919	0.928	0.940	0.949	0.972	0.985	0.987	0.989	0.991
41	0.920	0.929	0.941	0.950	0.972	0.985	0.987	0.989	0.991
42	0.922	0.930	0.942	0.951	0.972	0.985	0.987	0.989	0.991
43	0.923	0.932	0.943	0.951	0.973	0.985	0.987	0.990	0.991
44	0.924	0.933	0.944	0.952	0.973	0.985	0.987	0.990	0.991
45	0.926	0.934	0.945	0.953	0.973	0.985	0.988	0.990	0.991
46	0.927	0.935	0.945	0.953	0.974	0.985	0.988	0.990	0.991
47	0.928	0.936	0.946	0.954	0.974	0.985	0.988	0.990	0.991
48	0.929	0.937	0.947	0.954	0.974	0.985	0.988	0.990	0.991
49	0.929	0.937	0.947	0.955	0.974	0.985	0.988	0.990	0.991
50	0.930	0.938	0.947	0.955	0.974	0.985	0.988	0.990	0.991

3. TEST FOR HOMOGENEITY OF VARIANCE

3.1 For Dunnett's Procedure and the t test with Bonferroni's adjustment, the variances of the data obtained from each toxicant concentration and the control are assumed to be equal. Bartlett's Test is a formal test of this assumption. In using this test, it is assumed that the data are normally distributed.

3.2 The data used in this example are growth data from a Mysid Survival and Growth Test, and are the same data used in Appendix C. These data are listed in Table B.7, together with the calculated variance for the control and each toxicant concentration.

TABLE B.7. MYSID, *HOLMESIMYSIS COSTATA*, GROWTH DATA

		Concentration (%)		
Replicate	Control	1.80	3.20	5.60
1	0.048	0.055	0.057	0.041
2	0.058	0.048	0.050	0.040
3	0.047	0.042	0.046	0.041
4	0.058	0.041	0.043	0.043
5	0.051	0.052	0.045	0.040
Mean ($\overline{Y}_i$)	0.052	0.048	0.048	0.041
S_i^2	0.0000283	0.0000373	0.0000307	0.0000015
i	1	2	3	4

3.3 The test statistic for Bartlett's Test (Snedecor and Cochran, 1980) is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i) \ln \overline{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, ($V_i = n_i - 1$)

p = number of levels of toxicant concentration including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i =the number of replicates for concentration i.

$$\overline{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i^2)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{p} 1/V_i - (\sum_{i=1}^{p} V_i)^{-1}]$$

3.4 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value is obtained from a table of the chi-square distribution for p - 1 degrees of freedom and a significance level of 0.01. If B is less than the critical value then the variances are assumed to be equal.

3.5 For the data in this example, all concentrations including the control have the same number of replicates (n_i - 5 for all i). Thus, V_i = 4 for all i. For this data, p = 4, $\overline{S}^2$ = 0.0000245, and C = 1.104. Bartlett's statistic is therefore:

$$B = [(16)\ln(0.0000245) - 4\sum_{i=1}^{p} \ln(S_i^2)]/1.104$$

$$= [16(-10.617) - 4(-44.470)]/1.104$$

$$= [-169.872 - (-177.880)]/1.104$$

$$= 7.254$$

3.6 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value for the test is 9.21 for a significance level of 0.01. Since B = 7.254 is less than 9.21, conclude that the variances are not different.

4. TRANSFORMATIONS OF THE DATA

4.1 When the assumptions of normality and/or homogeneity of variance are not met, transformations of the data may remedy the problem, so that the data can be analyzed by parametric procedures, rather than nonparametric technique such as Steel's Many-one Rank Test or Wilcoxon's Rank Sum Test. Examples of transformations include log, square root, arc sine square root, and reciprocals. After the data have been transformed, the Shapiro-Wilk's and Bartlett's tests should be performed on the transformed observations to determine whether the assumptions of normality and/or homogeneity of variance are met.

4.2 ARC SINE SQUARE ROOT TRANSFORMATION (USEPA, 1993).

4.2.1 For data consisting of proportions from a binomial (response/no response; live/dead) response variable, the variance within the ith treatment is proportional to P_i (1 - P_i), where P_i is the expected proportion for the treatment. This clearly violates the homogeneity of variance assumption required by parametric procedures such as Dunnett's Procedure or the *t* test with Bonferroni's adjustment, since the existence of a treatment effect implies different values of P_i for different treatments, i. Also, when the observed proportions are based on small samples, or when P_i is close to zero or one, the normality assumption may be invalid. The arc sine square root (arc sine $\sqrt{p}$) transformation is commonly used for such data to stabilize the variance and satisfy the normality requirement.

4.2.2 Arc sine transformation consists of determining the angle (in radians) represented by a sine value. In the case of arc sine square root transformation of mortality data, the organism response proportion (proportion dead or affected; proportion surviving) is taken as the sine value, the square root of the sine value is determined, and the angle (in radians) for the square root of the sine value is determined. Whenever the response proportion is 0 or 1, a special modification of the arc sine square root transformation must be used (Bartlett, 1937). An explanation of the arc sine square root transformation and the modification is provided below.

4.2.3 Calculate the response proportion (RP) at each effluent concentration, in this case proportion surviving where:

RP = (number of surviving or unaffected organisms)/(number exposed).

Example: If 12 of 20 animals in a given treatment replicate survive:

RP = 12/20

= 0.60

4.2.4 Transform each RP to its arc sine square root, as follows:

4.2.4.1 For RPs greater than zero or less than one:

Angle (radians) = $\sqrt{RP}$

Example: If RP = 0.60:

Angle = arc sine $\sqrt{0.60}$

= arc sine 0.7746

= 0.8861 radians

4.2.4.2 Modification of the arc sine square root when RP = 0.

Angle (in radians) = arc sine $\sqrt{1/4N}$

Where: N = Number of animals/treatment replicate

Example: If 20 animals are used:

Angle = arc sine $\sqrt{1/80}$

= arc sine 0.1118

= 0.1120 radians

4.2.4.3 Modification of the arc sine square root when RP = 0

Angle = 1.5708 radians - (radians for RP = 0)

Example: Using above value:

Angle = 1.5708 - 0.1120

= 1.4588 radians

APPENDIX C

DUNNETT'S PROCEDURE

1. MANUAL CALCULATIONS

1.1 Dunnett's Procedure (Dunnett, 1955; Dunnett, 1964) is used to compare each concentration mean with the control mean to decide if any of the concentrations differ from the control. This test has an overall error rate of alpha, which accounts for the multiple comparisons with the control. It is based on the assumptions that the observations are independent and normally distributed and that the variance of the observations is homogeneous across all concentrations and control. (See Appendix B for a discussion on validating the assumptions). Dunnett's Procedure uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance. Dunnett's Procedure can only be used when the same number of replicate test vessels have been used at each concentration and the control. When this condition is not met, the *t* test with Bonferroni's adjustment is used (see Appendix D).

1.2 The data used in this example are growth data from a Mysid Survival and Growth Test, and are the same data used in Appendix B. These data are listed in Table C.1.

TABLE C.1. MYSID, *HOLMESIMYSIS COSTATA*, GROWTH DATA

		Concentration (%)		
Replicate	Control	1.80	3.20	5.60
1	0.048	0.055	0.057	0.041
2	0.058	0.048	0.050	0.040
3	0.047	0.042	0.046	0.041
4	0.058	0.041	0.043	0.043
5	0.051	0.052	0.045	0.040
Mean ($\overline{Y}_i$)	0.052	0.048	0.048	0.041
Total (T_i)	0.262	0.238	0.241	0.205
i	1	2	3	4

1.3 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, as described in Table C.2:

TABLE C.2. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of effluent concentrations including the control:

N = the total sample size; $N=\sum_i n_i$

n_i = the number of replicates for concentration "i"

$SST=\sum_{ij} Y_{ij}^2-G^2/N$ Total Sum of Squares

$SSB=\sum_i T_i^2/n_i-G^2/N$ Between Sum of Squares

$SSW=SST-SSB$ Within Sum of Squares

G = the grand total of all sample observations; $G=\sum_{i=1}^{p} T_i$

T_i = the total of the replicate measurements for concentration i

N = the total sample size; $N=\sum_i n_i$

n_i = the number of replicates for concentration i

Y_{ij} = the jth observation for concentration i

1.4 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 5$

$N = 20$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 0.262$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 0.238$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 0.241$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 0.205$

$G = T_1 + T_2 + T_3 + T_4 = 0.946$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$

$$= \frac{1}{5}(0.225) - \frac{(0.946)^2}{20} = 0.000254$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 0.0455 - \frac{(0.946)^2}{20} = 0.000754$$

$SSW = SST - SSB = 0.000754 - 0.000254 = 0.000500$

$S_B^2 = SSB/(p-1) = 0.000254/(4-1) = 0.0000847$

$S_W^2 = SSW/(N-p) = 0.000500/(20-4) = 0.0000313$

1.5 Summarize these data in the ANOVA table, as shown in Table C.3:

TABLE C.3. COMPLETED ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	3	0.000254	0.0000847
Within	16	0.000500	0.0000313
Total	19	0.000754	

1.6 To perform the individual comparisons, calculate the *t* statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\overline{Y}_1 - \overline{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\overline{Y}_1$ = mean for the control

$\overline{Y}_i$ = mean for each concentratioon i

S_w = square root of the within mean square

n_1 = number of replicates in the control

n_i = number of replicates for concentration i.

1.7 Table C.4 includes the calculated *t* values for each concentration and control combination.

TABLE C.4. CALCULATED *t* VALUES

Concentration (ppb)	i	t_i
1.80	2	1.131
3.20	3	1.131
5.60	4	3.111

1.8 Since the purpose of the test is only to detect a decrease in growth from the control, a one-sided test is appropriate. The critical value for the one-sided comparison is read from the table of Dunnett's "*t*" values (Table C.5; this table assumes an equal number of replicates in all treatment concentrations and the control). For this set of data, with an overall alpha level of 0.05, 16 degrees of freedom and three concentrations excluding the control, the critical value is 2.23. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Comparing each of the calculated *t* values in Table C.4 with the critical value, a significant decrease in growth from the control is detected in the 5.60% concentration. Therefore, the NOEC and the LOEC for growth are 3.20% and 5.60%, respectively.

TABLE C.5. DUNNETT'S "T" VALUES (Miller, 1981)

(One-tailed) $d_{\nu,k}$																		
ν \ k	α = .05									α = 0.1								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
5	2.02	2.44	2.68	2.85	2.98	3.08	3.16	3.24	3.30	3.37	3.90	4.21	4.43	4.60	4.73	4.85	4.94	5.03
6	1.94	2.34	2.56	2.71	2.83	2.92	3.00	3.07	3.12	3.14	3.61	3.88	4.07	4.21	4.33	4.43	4.51	4.59
7	1.89	2.27	2.48	2.62	2.73	2.82	2.89	2.95	3.01	3.00	3.42	3.56	3.83	3.96	4.07	4.15	4.23	4.30
8	1.86	2.22	2.42	2.55	2.66	2.74	2.81	2.87	2.92	2.90	3.29	3.51	3.67	3.79	3.88	3.96	4.03	4.09
9	1.83	2.18	2.37	2.50	2.60	2.68	2.75	2.81	2.86	2.82	3.19	3.40	3.55	3.66	3.75	3.82	3.89	3.94
10	1.81	2.15	2.34	2.47	2.56	2.64	2.70	2.76	2.81	2.76	3.11	3.31	3.45	3.56	3.64	3.71	3.78	3.83
11	1.80	2.13	2.31	2.44	2.53	2.60	2.67	2.72	2.77	2.72	3.06	3.25	3.38	3.48	3.56	3.63	3.69	3.74
12	1.78	2.11	2.29	2.41	2.50	2.58	2.64	2.69	2.74	2.68	3.01	3.19	3.32	3.42	3.50	3.56	3.62	3.67
13	1.77	2.09	2.27	2.39	2.48	2.55	2.61	2.66	2.71	2.65	2.97	3.15	3.27	3.37	3.44	3.91	3.56	3.61
14	1.76	2.08	2.25	2.37	2.46	2.53	2.59	2.64	2.69	2.62	2.94	3.11	3.23	3.32	3.40	3.46	3.51	3.56
15	1.75	2.07	2.24	2.36	2.44	2.51	2.57	2.62	2.67	2.60	2.91	3.08	3.20	3.29	3.36	3.42	3.47	3.52
16	1.75	2.06	2.23	2.34	2.43	2.50	2.56	2.61	2.65	2.58	2.88	3.05	3.17	3.26	3.33	3.39	3.44	3.48
17	1.74	2.05	2.22	2.33	2.42	2.49	2.54	2.59	2.64	2.57	2.86	3.03	3.14	3.23	3.30	3.36	3.41	3.45
18	1.73	2.04	2.21	2.32	2.41	2.48	2.53	2.58	2.62	2.55	2.84	3.01	3.12	3.21	3.27	3.33	3.38	3.42
19	1.73	2.03	2.20	2.31	2.40	2.47	2.52	2.57	2.61	2.54	2.83	2.99	3.10	3.18	3.25	3.31	3.36	3.40
20	1.72	2.03	2.19	2.30	2.39	2.46	2.51	2.56	2.60	2.53	2.81	2.97	3.08	3.17	3.23	3.29	3.34	3.38
24	1.71	2.01	2.17	2.28	2.36	2.43	2.48	2.53	2.57	2.49	2.77	2.92	3.03	3.11	3.17	3.22	3.27	3.31
30	1.70	1.99	2.15	2.25	2.33	2.40	2.45	2.50	2.54	2.46	2.72	2.87	2.97	3.05	3.11	3.16	3.21	3.24
40	1.68	1.97	2.13	2.23	2.31	2.37	2.42	2.47	2.51	2.42	2.68	2.82	2.92	2.99	3.05	3.10	3.14	3.18
60	1.67	1.95	2.10	2.21	2.28	2.35	2.39	2.44	2.48	2.39	2.64	2.78	2.87	2.94	3.00	3.04	3.08	3.12
120	1.66	1.93	2.08	2.18	2.26	2.32	2.37	2.41	2.45	2.36	2.60	2.73	2.82	2.90	2.94	2.99	3.03	3.06

1.9 To quantify the sensitivity of the test, the minimum significant difference (MSD) may be calculated. The formula is as follows:

$$MSD = d\ S_w\sqrt{(1/n_1)+(1/n)}$$

Where: d = critical value for the Dunnett's Procedure

S_w = the square root of the within mean square

n = the number of replicates at each concentration, assuming an equal number of replicates at all treatment concentrations

n_1 = number of replicates in the control

For example:

$$MSD = 2.23(0.00559)\sqrt{(1/5)+(1/5)}$$

$$= 2.23\ (0.00559)(0.632)$$

$$= 0.00788$$

1.10 Therefore, for this set of data, the minimum difference between the control mean and a concentration mean that can be detected as statistically significant is 0.00788 mg. This represents a 15.2% reduction in mean weight from the control.

1.11 If the data have not been transformed, the MSD (and the percent decrease from the control mean that it represents) can be reported as is.

1.11.1 In the case where the data have been transformed, the MSD would be in transformed units. In this case carry out the following conversion to determine the MSD in untransformed units.

1.11.2 Subtract the MSD from the transformed control mean. Call this difference D. Next, obtain untransformed values for the control mean and the difference, D. Finally, compute the untransformed MSD as follows:

$$MSD_u = control_u - D_u$$

Where: MSD_u = the minimum significant difference for untransformed data

$Control_u$ = the untransformed control mean

D_u = the untransformed difference

1.11.3 Calculate the percent reduction from the control that MSD_u represents as:

$$\text{Percent Reduction} = \frac{MSD_u}{Control_u} \times 100$$

1.11.3.1 An example of a conversion of the MSD to untransformed units, when the arc sine square root transformation was used on the data, follows.

Step 1. Subtract the MSD from the transformed control mean. As an example, assume the data in Table C.1 were transformed by the arc sine square root transformation. Thus:

$$0.052 - 0.00788 = 0.04412$$

Step 2. Obtain untransformed values for the control mean (0.052) and the difference (0.04412) obtained in Step 1, above.

$$[\text{Sine } (0.052)]^2 = 0.00270$$

$$[\text{Sine } (0.04412)]^2 = 0.00195$$

Step 3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values obtained in Step 2.

$$MSD_u = 0.00270 - 0.00195 = 0.00075$$

In this case, the MSD would represent a 1.4% decrease in survival from the control [(0.00075/0.052)(100)].

2. COMPUTER CALCULATIONS

2.1 This computer program incorporates two analyses: an analysis of variance (ANOVA), and a multiple comparison of treatment means with the control mean (Dunnett's Procedure). The ANOVA is used to obtain the error value. Dunnett's Procedure indicates which toxicant concentration means (if any) are statistically different from the control mean at the 5% level of significance. The program also provides the minimum difference between the control and treatment means that could be detected as statistically significant, and tests the validity of the homogeneity of variance assumption by Bartlett's Test. The multiple comparison is performed based on procedures described by Dunnett (1955).

2.2 The source code for the Dunnett's program is structured into a series of subroutines, controlled by a driver routine. Each subroutine has a specific function in the Dunnett's Procedure, such as data input, transforming the data, testing for equality of variances, computing p values, and calculating the one-way analysis of variance.

2.3 The program compares up to seven toxicant concentrations against the control, and can accommodate up to 50 replicates per concentration.

2.4 If the number of replicates at each toxicant concentration and control are not equal, a *t* test with the Bonferroni adjustment is performed instead of Dunnett's Procedure (see Appendix D).

2.5 The program was written in IBM-PC FORTRAN by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled version of the program can be obtained from EMSL-Cincinnati by sending a diskette with a written request.

2.6 DATA INPUT AND OUTPUT

2.6.1 The mysid growth data from Table C.1 are used to illustrate the data input and output for this program.

2.6.2 Data Input

2.6.2.1 When the program is entered, the user is asked to select the type of data to be analyzed:

1. Response proportions, like survival or fertilization proportions data.
2. Counts and measurements, like offspring counts, cystocarp and algal cell counts, weights, chlorophyll measurements or turbidity measurements.

2.6.2.2 After the type of analysis for the data is chosen, the user has the following options:

1. Create a data file
2. Edit a data file
3. Perform analysis on existing data set
4. Stop

2.6.2.3 When Option 1 (Create a data file) is selected for response proportions, the program prompts the user for the following information:

1. Number of concentrations, including control
2. For each concentration and replicate:
   - number of organisms exposed per replicate
   - number of organisms responding per replicate (organisms surviving, eggs fertilized, etc.)

2.6.2.4 After the data have been entered, the user may save the file on a disk, and the program returns to the main menu (see below).

2.6.2.5 Sample data input is shown in Figure C.1.

2.6.3. Program Output

2.6.3.1 When Option 3 (perform analysis on existing data set) is selected from the menu, the user is asked to select the transformation desired, and indicate whether they expect the means of the test groups to be less or greater than the mean for the control group (see Figure C.2).

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Version 1.5

What type of data do you wish to analyze?

1) response proportions
(like survival data or fertility proportion data)
Note: The program calculates a proportion after prompting for number of exposed organisms and number of responding organisms.

2) counts and measurements
(like offspring counts, cystocarps and algal cell counts, weights, chlorophyll measurements, or turbidity measurements)

Enter "1", "2", (or "q" to quit program): 2

Title ? Appendix C, Dunnett's Procedure Example - Mysid Data

Output to printer or disk file ? P

1) Create a data file
2) Edit a data file
3) Analyze an existing data set
4) Stop

Your choice ? 1

Number of concentrations, including control ? 4

Number of observations for conc. 1 (the control) ? 5

Enter the data for conc. 1 (the control) one observation at a time.

NO. 1? 0.048

NO. 2? 0.058

NO. 3? 0.047

NO. 4? 0.058

NO. 5? 0.051

```
Enter the data for conc.  2 one observation at a time.

NO.  1? 0.055

NO.  2? 0.048

NO.  3? 0.042

NO.  4? 0.041

NO.  5? 0.052

Number of observations for conc.  3 ? 5

Enter the data for conc.  3 one observation at a time.

NO.  1? 0.057

NO.  2? 0.050

NO.  3? 0.046

NO.  4? 0.043

NO.  5? 0.045

Number of observations for conc.  4 ? 5

Enter the data for conc.  4 one observation at a time.

NO.  1? 0.041

NO.  2? 0.040

NO.  3? 0.041

NO.  4? 0.043

NO.  5? 0.040

Do you wish to save the data on disk ? Y

Disk file for output ? c:\mysid.dat
```

```
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 1) Create a data file
 2) Edit a data file
 3) Analyze an existing data set
 4) Stop

Your choice ? 3


File name ? c:\mysid.dat



 Available Transformations
     1)  no transform
     2)  square root
     3)  log10

Your choice ? 1



 Dunnett's test as implemented in this program is
 a one-sided test. You must specify the direction
 the test is to be run; that is, do you expect the
 means for the test concentrations to be less than
 or greater than the mean for the control
 concentration.

Direction for Dunnetts test : L=less than, G=greater than ? L
```

Figure C.2. Example of Choosing Option 3 from the Main Menu of the Dunnett Program.

2.6.3.2 Summary statistics (Figure C.3) for the raw and transformed data, if applicable, the ANOVA table, results of Bartlett's Test, the results of the multiple comparison procedure, and the minimum detectable difference are included in the program output.

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Appendix C, Dunnett's Procedure Example - Mysid Data

Summary Statistics and ANOVA

Transformation = None

Conc.	n	Mean	s.d.	cv%
1 = control	5	.0524	.0053	10.2
2	5	.0476	.0061	12.8
3	5	.0482	.0055	11.5
4*	5	.0410	.0012	3.0

*) the mean for this conc. is significantly less than
the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.006974
This difference corresponds to -13.31 percent of control

Between concentrations
sum of squares = .000333 with 3 degrees of freedom.

Error mean square = .000024 with 16 degrees of freedom.

Bartlett's test p-value for equality of variances = .060

Do you wish to restart the program ?

Figure C.3. Example of Program Output for the Dunnett's Program Using the Data in Table C.1.

APPENDIX D

t TEST WITH BONFERRONI'S ADJUSTMENT

1. The *t* test with Bonferroni's adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus, Dunnett's Procedure is a more powerful test.

2. The *t* test with Bonferroni's adjustment is based on the same assumptions of normality of distribution and homogeneity of variance as Dunnett's Procedure (See Appendix B for testing these assumptions), and, like Dunnett's Procedure, uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance.

3. An example of the use of the *t* test with Bonferroni's adjustment is provided below. The data used in the example are a set of red abalone growth data. Because there are only four replicates in the highest concentration, Dunnett's Procedure cannot be used. The length data are presented in Table D.1.

TABLE D.1. GIANT KELP, *MACROCYSTIS PYRIFERA*, GROWTH DATA

		Copper Concentration (µg/L)						
Rep	Control	5.60	10.0	18.0	32.0	56.0	100.0	180.0
1	19.58	18.26	13.31	18.59	12.54	11.44	7.92	6.49
2	18.75	16.25	18.92	12.88	10.67	11.88	7.59	7.25
3	19.14	16.39	15.62	16.28	15.95	11.88	8.25	--
4	16.50	18.70	14.30	15.38	12.54	11.00	9.13	7.63
5	17.93	15.62	15.29	19.75	11.66	11.55	8.80	8.13
$\bar{Y}_i$	18.38	17.04	15.49	16.58	12.67	11.55	8.34	7.38
S_i^2	1.473	1.827	4.498	7.327	3.953	0.133	0.396	0.478
i	1	2	3	4	5	6	7	8

3.1 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, as described in Table D.2:

TABLE D.2. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)

Where: p = number of effluent concentrations including the control

N = the total sample size; $N=\sum_i n_i$

n_i = the number of replicates for concentration i

$$SST=\sum_{ij} Y_{ij}^2-G^2/N \qquad \text{Total Sum of Squares}$$

$$SSB=\sum_i T_i^2/n_i-G^2/N \qquad \text{Between Sum of Squares}$$

$$SSW=SST-SSB \qquad \text{Within Sum of Squares}$$

Where: G = The grand total of all sample observations; $G=\sum_{i=1}^{P} T_i$

T_i = The total of the replicate measurements for concentration i

Y_{ij} = The jth observation for concentration i

3.2 For the data in this example:

$$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = n_7 = 5; \quad n_8 = 4$$

$$N = 39$$

$$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 91.90$$
$$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 85.22$$
$$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 77.44$$
$$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 82.88$$
$$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} + Y_{55} = 63.36$$
$$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} + Y_{65} = 57.75$$
$$T_7 = Y_{71} + Y_{72} + Y_{73} + Y_{74} + Y_{75} = 41.69$$
$$T_8 = Y_{81} + Y_{82} + Y_{83} + Y_{84} = 29.50$$

$$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 + T_7 + T_8 = 529.74$$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= 7749.905 - \frac{(529.74)^2}{39} = 554.406$$

$$SST = \sum_{i=1}^{p}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 7829.764 - \frac{(529.74)^2}{39} = 634.265$$

$$SSW = SST - SSB = 634.265 - 554.406 = 79.859$$

$$S_B^2 = SSB/(p-1) = 554.406/(8-1) = 79.201$$

$$S_W^2 = SSW/(N-p) = 79.859/(39-8) = 2.576$$

3.3 Summarize these calculations in the ANOVA table (Table D.3):

TABLE D.3. COMPLETED ANOVA TABLE FOR THE *t* TEST WITH BONFERRONI'S ADJUSTMENT EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	7	554.406	79.201
Within	31	79.859	2.576
Total	38	634.265	

3.4 To perform the individual comparisons, calculate the *t* statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\overline{Y}_1 - \overline{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\overline{Y}_i$ = mean for concentration i

$\overline{Y}_1$ = mean for the control

S_w = square root of the within mean square

n_1 = number of replicates in the control.

n_i = number of replicates for concentration i.

3.5 Table D.4 includes the calculated *t* values for each concentration and control combination.

TABLE D.4. CALCULATED t VALUES

Concentration (µg/L)	i	t_i
5.6	2	1.320
10.0	3	2.847
18.0	4	1.773
32.0	5	5.625
56.0	6	6.728
100.0	7	9.891
180.0	8	10.217

3.6 Since the purpose of this test is to detect a significant reduction in mean length, a one-sided test is appropriate. The critical value for this one-sided test is found in Table D.5. For an overall alpha level of 0.05, 31 degrees of freedom for error and seven concentrations (excluding the control) the approximate critical value is 2.597. The mean length for concentration "i" is considered significantly less than the mean length for the control if t_i is greater than the critical value. Comparing each of the calculated t values in Table D.4 with the critical value, the 10.0 µg/L, 32 µg/L, 56.0 µg/L, 100.0 µg/L, 180.0 µg/L concentrations have significantly lower mean length than the control. Because the 10.0 µg/L concentration shows signigicantly lower mean length that the control while the higher 18.0 µg/L concentration does not, these test results are considered to have an anomalous dose-response relationship and it is recommended that the test be repeated. If an NOEC and LOEC must be determined for this test, the lowest concentration with significant growth impairment versus the control is considered to the LOEC for growth. Thus, for this test, the NOEC and LOEC would be 5.6 µg/L and 10.0 µg/L, respectively.

TABLE D.5. CRITICAL VALUES FOR "t" FOR THE t TEST WITH BONFERRONI'S ADJUSTMENT P = 0.05
CRITICAL LEVEL, ONE TAILED

d.f.	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
1	6.314	12.707	19.002	25.452	31.821	38.189	44.556	50.924	57.290	63.657
2	2.920	4.303	5.340	6.206	6.965	7.649	8.277	8.861	9.408	9.925
3	2.354	3.183	3.741	4.177	4.541	4.857	5.138	5.392	5.626	5.841
4	2.132	2.777	3.187	3.496	3.747	3.961	4.148	4.315	4.466	4.605
5	2.016	2.571	2.912	3.164	3.365	3.535	3.681	3.811	3.927	4.033
6	1.944	2.447	2.750	2.969	3.143	3.288	3.412	3.522	3.619	3.708
7	1.895	2.365	2.642	2.842	2.998	3.128	3.239	3.336	3.422	3.500
8	1.860	2.307	2.567	2.752	2.897	3.016	3.118	3.206	3.285	3.356
9	1.834	2.263	2.510	2.686	2.822	2.934	3.029	3.111	3.185	3.250
10	1.813	2.229	2.406	2.634	2.764	2.871	2.961	3.039	3.108	3.170
11	1.796	2.201	2.432	2.594	2.719	2.821	2.907	2.981	3.047	3.106
12	1.783	2.179	2.404	2.561	2.681	2.778	2.863	2.935	2.998	3.055
13	1.771	2.161	2.380	2.533	2.651	2.746	2.827	2.897	2.958	3.013
14	1.762	2.145	2.360	2.510	2.625	2.718	2.797	2.864	2.924	2.977
15	1.754	2.132	2.343	2.490	2.603	2.694	2.771	2.837	2.895	2.947
16	1.746	2.120	2.329	2.473	2.584	2.674	2.749	2.814	2.871	2.921
17	1.740	2.110	2.316	2.459	2.567	2.655	2.729	2.793	2.849	2.899
18	1.735	2.101	2.305	2.446	2.553	2.640	2.712	2.775	2.830	2.879
19	1.730	2.094	2.295	2.434	2.540	2.626	2.697	2.759	2.813	2.861
20	1.725	2.086	2.206	2.424	2.528	2.613	2.684	2.745	2.798	2.846
21	1.721	2.080	2.278	2.414	2.518	2.602	2.672	2.732	2.785	2.832

TABLE D.5. CRITICAL VALUES FOR "*t*" FOR THE *t* TEST WITH BONFERRONI'S ADJUSTMENT
P = 0.05 CRITICAL LEVEL, ONE TAILED (CONTINUED)

df	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
29	1.700	2.046	2.235	2.364	2.463	2.541	2.607	2.664	2.713	2.757
30	1.698	2.043	2.231	2.360	2.458	2.536	2.602	2.658	2.707	2.750
31	1.696	2.040	2.228	2.356	2.453	2.531	2.597	2.652	2.701	2.745
32	1.694	2.037	2.224	2.352	2.449	2.527	2.592	2.647	2.696	2.739
33	1.693	2.035	2.221	2.349	2.445	2.523	2.587	2.643	2.691	2.734
34	1.691	2.033	2.219	2.346	2.442	2.519	2.583	2.638	2.686	2.729
35	1.690	2.031	2.216	2.342	2.438	2.515	2.579	2.634	2.682	2.724
36	1.689	2.029	2.213	2.340	2.435	2.512	2.575	2.630	2.678	2.720
37	1.688	2.027	2.211	2.337	2.432	2.508	2.572	2.626	2.674	2.716
38	1.686	2.025	2.209	2.334	2.429	2.505	2.568	2.623	2.670	2.712
39	1.685	2.023	2.207	2.332	2.426	2.502	2.565	2.619	2.667	2.708
40	1.684	2.022	2.205	2.329	2.424	2.499	2.562	2.616	2.663	2.705
50	1.676	2.009	2.189	2.311	2.404	2.478	2.539	2.592	2.638	2.678
60	1.671	2.001	2.179	2.300	2.391	2.463	2.324	2.576	2.621	2.661
70	1.667	1.995	2.171	2.291	2.381	2.453	2.513	2.564	2.609	2.648
80	1.665	1.991	2.166	2.285	2.374	2.446	2.505	2.556	2.600	2.639
90	1.662	1.987	2.162	2.280	2.369	2.440	2.499	2.549	2.593	2.632
100	1.661	1.984	2.158	2.276	2.365	2.435	2.494	2.544	2.588	2.626
110	1.659	1.982	2.156	2.273	2.361	2.432	2.490	2.540	2.583	2.622
120	1.658	1.980	2.153	2.270	2.358	2.429	2.487	2.536	2.580	2.618
Infinite	1.645	1.960	2.129	2.242	2.327	2.394	2.450	2.498	2.540	2.576

d.f. = Degrees of freedom for MSE (Mean Square Error) from ANOVA.

APPENDIX E

STEEL'S MANY-ONE RANK TEST

1. Steel's Many-one Rank Test is a nonparametric test for comparing treatments with a control. This test is an alternative to Dunnett's Procedure, and may be applied to data when the normality assumption has not been met. Steel's Test requires equal variances across the treatments and the control, but it is thought to be fairly insensitive to deviations from this condition (Steel, 1959). The tables for Steel's Test require an equal number of replicates at each concentration. If this is not the case, use Wilcoxon's Rank Sum Test, with Bonferroni's adjustment (See Appendix F).

2. For an analysis using Steel's Test, for each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to the observation. (Extensive ties would invalidate this procedure). The sum of the ranks within each concentration is then calculated. To determine if the response in a concentration is significantly less than the response in the control, the rank sum for each concentration is compared to the significant values of rank sums given later in the section. In this table, k equals the number of treatments excluding the control and n equals the number of replicates for each concentration and the control.

3. An example of the use of this test is provided below. The test employs embryo-larval development data from a bivalve 48-hour chronic test. The data are listed in Table E.1.

4. For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, 3, ..., 8) to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to each tied observation.

5. An example of assigning ranks to the combined data for the control and 0.13 µg/L copper concentration is given in Table E.2.

This ranking procedure is repeated for each control and concentration combination. The complete set of rankings is listed in Table E.3. The ranks are then summed for each toxicant concentration, as shown in Table E.4.

6. For this set of data, determine if the development in any of the effluent concentrations is significantly lower than the development of the control organisms. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for the development at each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the survival would be considered to be significantly lower than the control. At a probability level of 0.05, the critical rank sum in a test with five concentrations and four replicates per concentration, is 10 (see Table F.4).

7. Since the rank sums for the 0.50 µg/L and 1.00 µg/L concentration levels are equal to the critical value, the proportions of normal development in those concentrations are considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no other concentration has a significantly lower proportion normal than the control. Because the 0.50 µg/L concentration shows signigicantly lower normal development than the control while the higher 2.00 µg/L concentration does not, these test results are considered to have an anomalous dose-response relationship and it is recommended that the test be repeated. If an NOEC and LOEC must be determined for this test, the lowest concentration with significant impairment versus the control is considered to the LOEC for growth. Thus, for this test, the NOEC and LOEC would be 0.25 µg/L and 0.50 µg/L, respectively.

TABLE E.1. BIVALVE EMBRYO-LARVAL DEVELOPMENT DATA

			Copper Concentration (µg/L)				
	Replicate	Control	0.13	0.25	0.50	1.00	2.00
RAW	A	1.00	0.96	0.92	0.91	0.88	1.00
	B	0.96	0.97	0.95	0.93	0.83	0.67
	C	1.00	1.00	0.90	0.88	0.88	0.75
	D	0.97	0.96	0.96	0.93	0.82	0.60
ARC SINE	A	1.571	1.369	1.284	1.266	1.217	1.571
SQUARE ROOT	B	1.369	1.397	1.345	1.303	1.146	0.959
TRANSFORMED	C	1.571	1.571	1.249	1.217	1.217	1.047
	D	1.397	1.369	1.369	1.303	1.133	0.886
Mean ($\overline{Y}_i$)		1.477	1.427	1.312	1.272	1.178	1.116
S_i^2		0.01191	0.00945	0.00303	0.00166	0.00203	0.09644
i		1	2	3	4	5	6

TABLE E.2. ASSIGNING RANKS TO THE CONTROL AND 0.13 µg/L CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Normal	Concentration
2	1.369	0.13 µg/L
2	1.369	0.13 µg/L
2	1.369	Control
4.5	1.397	0.13 µg/L
4.5	1.397	Control
7	1.571	0.13 µg/L
7	1.571	Control
7	1.571	Control

TABLE E.3. TABLE OF RANKS[1]

Replicate	Control	Copper Concentration (μg/L) 0.13	0.25
1	1.571(7,7.5,7.5,7.5,7)	1.369(2)	1.284(2)
2	1.369(2,4.5,5,5,4)	1.397(4.5)	1.345(3)
3	1.571(7,7.5,7.5,7.5,7)	1.571(7)	1.249(1)
4	1.397(4.5,6,6,6,5)	1.369(2)	1.369(4.5)

Replicate	Copper Concentration (μg/L) (Continued) 0.50	1.00	2.00
1	1.266(2)	1.217(3.5)	1.571(7)
2	1.303(3.5)	1.146(2)	0.959(2)
3	1.217(1)	1.217(3.5)	1.047(3)
4	1.303(3.5)	1.133(1)	0.886(1)

[1]Control ranks are given in the order of the concentration with which they were ranked.

TABLE E.4. RANK SUMS

Concentration μg/L Copper)	Rank Sum
0.13	15.5
0.25	10.5
0.50	10.0
1.00	10.0
2.00	13.0

TABLE E.5. SIGNIFICANT VALUES OF RANK SUMS: JOINT CONFIDENCE COEFFICIENTS OF 0.95 (UPPER) and 0.99 (LOWER) FOR ONE-SIDED ALTERNATIVES (Steel, 1959)

	k = number of treatments (excluding control)							
n	2	3	4	5	6	7	8	9
4	11	10	10	10	10	--	--	--
	--	--	--	--	--	--	--	--
5	18	17	17	16	16	16	16	15
	15	--	--	--	--	--	--	--
6	27	26	25	25	24	24	24	23
	23	22	21	21	--	--	--	--
7	37	36	35	35	34	34	33	33
	32	31	30	30	29	29	29	29
8	49	48	47	46	46	45	45	44
	43	42	41	40	40	40	39	39
9	63	62	61	60	59	59	58	58
	56	55	54	53	52	52	51	51
10	79	77	76	75	74	74	73	72
	71	69	68	67	66	66	65	65
11	97	95	93	92	91	90	90	89
	87	85	84	83	82	81	81	80
12	116	114	112	111	110	109	108	108
	105	103	102	100	99	99	98	98
13	138	135	133	132	130	129	129	128
	125	123	121	120	119	118	117	117
14	161	158	155	154	153	152	151	150
	147	144	142	141	140	139	138	137
15	186	182	180	178	177	176	175	174
	170	167	165	164	162	161	160	160
16	213	209	206	204	203	201	200	199
	196	192	190	188	187	186	185	184
17	241	237	234	232	231	229	228	227
	223	219	217	215	213	212	211	210
18	272	267	264	262	260	259	257	256
	252	248	245	243	241	240	239	238
19	304	299	296	294	292	290	288	287

APPENDIX F

WILCOXON RANK SUM TEST

1. Wilcoxon's Rank Sum Test is a nonparametric test, to be used as an alternative to Steel's Many-one Rank Test when the number of replicates are not the same at each concentration. A Bonferroni's adjustment of the pairwise error rate for comparison of each concentration versus the control is used to set an upper bound of alpha on the overall error rate, in contrast to Steel's Many-one Rank Test, for which the overall error rate is fixed at alpha. Thus, Steel's Test is a more powerful test.

2. The use of this test may be illustrated with devlopment data from the red abalone test in Table F.1. The control group has four replicates while each of the concentration levels has five replicates. Since there is 100% abnormality in all replicates for the 5.6% and 10.0% concentrations, they are not included in the statistical analysis and are considered qualitative abnormality effects.

3. For each concentration and control combination, combine the data and arrange the values in order of size, from smallest to largest. Assign ranks to the ordered observations (a rank of 1 to the smallest, 2 to the next smallest, etc.). If ties in rank occur, assign the average rank to each tied observation.

4. An example of assigning ranks to the combined data for the control and effluent concentration 0.56% is given in Table F.2. This ranking procedure is repeated for each of the three remaining control versus test concentration combinations. The complete set of ranks is listed in Table F.3. The ranks are then summed for each effluent concentration, as shown in Table F.4.

5. For this set of data, determine if the development in any of the test concentrations is significantly lower than the development in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for fecundity of each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the fecundity would be considered to be significantly lower than the control. At a

probability level of 0.05, the critical rank in a test with four concentrations (excluding the control), four control replicates, and five concentration replicates is 15 (see Table F.5, for K = 4).

TABLE F.1. RED ABALONE, *HALIOTUS RUFESCENS*, SHELL DEVELOPMENT DATA

			Effluent Concentration (%)					
	Replicate	Dilution Control	0.56	1.00	1.80	3.20	5.6	10.0
	A	0.99	0.99	0.99	0.99	0.39	0	0
RAW	B	0.99	0.99	1.00	0.99	0.57	0	0
	C	0.99	0.98	0.99	0.99	0.61	0	0
	D	1.00	1.00	0.99	0.98	0.65	0	0
	E		1.00	1.00	0.97	0.80	0	0
ARC SINE	A	1.471	1.471	1.471	1.471	0.674	-	-
SQUARE ROOT	B	1.471	1.471	1.521	1.471	0.856	-	-
TRANSFORMED	C	1.471	1.429	1.471	1.471	0.896	-	-
	D	1.521	1.521	1.471	1.429	0.938	-	-
	E		1.521	1.521	1.397	1.107	-	-
Mean ($\overline{Y}_i$)		1.484	1.483	1.491	1.448	0.894	-	-
S_i^2		0.000625	0.001523	0.000750	0.001137	0.024288	-	-
i		1	2	3	4	5	6	7

TABLE F.2. ASSIGNING RANKS TO THE CONTROL AND 0.56% CONCENTRATION LEVEL FOR THE WILCOXON RANK SUM TEST WITH THE BONFERRONI ADJUSTMENT

Rank	Transformed Proportion Normal	Concentration
1	1.429	0.56 %
4	1.471	0.56 %
4	1.471	0.56 %
4	1.471	Control
4	1.471	Control
4	1.471	Control
8	1.521	0.56 %
8	1.521	0.56 %
8	1.521	Control

TABLE F.3. TABLE OF RANKS[1]

Replicate	Control	Effluent Concentration (%) 0.56	1.00	1.80	3.20
1	1.471(4,3.5,5.5,7)	1.471(4)	1.471(3.5)	1.471(5.5)	0.674(1)
2	1.471(4,3.5,5.5,7)	1.471(4)	1.521(8)	1.471(5.5)	0.856(2)
3	1.471(4,3.5,5.5,7)	1.429(1)	1.471(3.5)	1.471(5.5)	0.896(3)
4	1.521(8,8,9,9)	1.521(8)	1.471(3.5)	1.429(2)	0.938(4)
5		1.521(8)	1.521(8)	1.397(1)	1.107(5)

[1]Control ranks are given in the order of the concentration with which they were ranked.

6. Comparing the rank sums in Table F.4 to the appropriate critical rank, the rank sum for the 3.20% concentration level is equal to the critical value, so the proportion normal in that concentration is considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no other concentration has a significantly lower proportion normal than the control. Hence, the NOEC and the LOEC are 1.80% and 3.20%, respectively.

TABLE F.4. RANK SUMS

Concentration (% Effluent)	Rank Sum
0.56	25.0
1.00	26.5
1.80	19.5
3.20	15.0

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
1	3	6	10	16	23	30	39	49	59
	4	6	11	17	24	32	41	51	62
	5	7	12	19	26	34	44	54	66
	6	8	13	20	28	36	46	57	69
	7	8	14	21	29	39	49	60	72
	8	9	15	23	31	41	51	63	72
	9	10	16	24	33	43	54	66	79
	10	10	17	26	35	45	56	69	82
2	3	--	--	15	22	29	38	47	58
	4	--	10	16	23	31	40	49	60
	5	6	11	17	24	33	42	52	63
	6	7	12	18	26	34	44	55	66
	7	7	13	20	27	36	46	57	69
	8	8	14	21	29	38	49	60	72
	9	8	14	22	31	40	51	62	75
	10	9	15	23	32	42	53	65	78
3	3	--	--	--	21	29	37	46	57
	4	--	10	16	22	30	39	48	59
	5	--	11	17	24	32	41	51	62
	6	6	11	18	25	33	43	53	65
	7	7	12	19	26	35	45	56	68
	8	7	13	20	28	37	47	58	70
	9	7	13	21	29	39	49	61	73
	10	8	14	22	31	41	51	63	76
4	3	--	--	--	21	28	37	46	56

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
5	3	--	--	--	--	28	36	46	56
	4	--	--	15	22	29	38	48	58
	5	--	10	16	23	31	40	50	61
	6	--	11	17	24	32	42	52	63
	7	6	11	18	25	34	43	54	66
	8	6	12	19	27	35	45	56	68
	9	7	13	20	28	37	47	59	71
	10	7	13	21	29	39	49	61	74
6	3	--	--	--	--	28	36	45	56
	4	--	--	15	21	29	38	47	58
	5	--	10	16	22	30	39	49	60
	6	--	11	16	24	32	41	51	63
	7	6	11	17	25	33	43	54	65
	8	6	12	18	26	35	45	56	68
	9	6	12	19	27	37	47	58	70
	10	7	13	20	29	38	49	60	73
7	3	--	--	--	--	--	36	45	56
	4	--	--	--	21	29	37	47	58
	5	--	--	15	22	30	39	49	60
	6	--	10	16	23	32	41	51	62
	7	--	11	17	25	33	43	53	65
	8	6	11	18	26	35	44	55	67
	9	6	12	19	27	36	46	58	70
	10	7	13	20	28	38	48	60	72

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
9	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	30	39	48	59
	6	--	10	16	23	31	40	50	62
	7	--	10	17	24	33	42	52	64
	8	--	11	18	25	34	44	55	66
	9	6	11	18	26	35	46	57	69
	10	6	12	19	28	37	47	59	71
10	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	29	38	48	59
	6	--	10	16	23	31	40	50	61
	7	--	10	16	24	32	42	52	64
	8	--	11	17	25	34	43	54	66
	9	6	11	18	26	35	45	56	68
	10	6	12	19	27	37	47	58	71

APPENDIX G

SINGLE CONCENTRATION TOXICITY TEST - COMPARISON OF CONTROL WITH 100% EFFLUENT OR RECEIVING WATER OR COMPARISON OF DILUTION AND BRINE CONTROLS

1. To statistically compare a control with one concentration, such as 100% effluent or the instream waste concentration, a *t* test is the recommended analysis. The *t* test is based on the assumptions that the observations are independent and normally distributed and that the variances of the observations are equal between the two groups.

2. Shapiro-Wilk's test may be used to test the normality assumption (See Appendix B for details). For the two sample case, the datasets must be tested for normality separately. If either set of data does not meet the normality assumption, the nonparametric test, Wilcoxon's Rank Sum Test, may be used to analyze the data. An example of this test is given in Appendix F. Since a control and one concentration are being compared, the K = 1 section of Table F.5 contains the needed critical values for one-sided tests. An additional reference, such as Snedecor and Cochran (1980) must be used to determine critical values for two-sided tests, such as comparing brine and dilution controls.

3. The *F* test for equality of variances is used to test the homogeneity of variance assumption. When conducting the *F* test, the alternative hypothesis of interest is that the variances are not equal.

4. To make the two-tailed *F* test at the 0.01 level of significance, put the larger of the two variances in the numerator of *F*.

$$F = \frac{S_1^2}{S_2^2} \quad \text{where } S_1^2 > S_2^2$$

5. Compare *F* with the 0.005 level of a tabled *F* value with n_1 - 1 and n_2 - 1 degrees of freedom, where n_1 and n_2 are the number of replicates for each of the two groups.

6. A set of mysid growth data from a single-concentration effluent test will be used to illustrate the *F* test. The raw data, mean and variance for the two controls are given in Table G.1. The data from each concentration meets the assumption of normality.

TABLE G.1. MYSID, *HOLMESIMYSIS COSTATA*, GROWTH DATA FROM A SINGLE-CONCENTATION EFFLUENT TEST

	Replicate	Control	Effluent
RAW	A	0.048	0.041
	B	0.058	0.033
	C	0.047	0.044
	D	0.055	0.040
	E	0.051	0.043
Mean ($\bar{Y}_i$)		0.052	0.040
S_i^2		0.0000217	0.0000187
i		1	2

7. Since the variability of the control is greater than the variability of the effluent concentration, S^2 for the control is placed in the numerator of the F statistic and S^2 for the effluent concentration control is placed in the denominator.

$$F = \frac{0.0000217}{0.0000187} = 1.160$$

8. There are 5 replicates for the each groups, so the numerator and denominator degrees of freedom, $n_i - 1$, are both 4. For a two-tailed test at the 0.01 level of significance, the critical F value is obtained from a table of the *F* distribution (Snedecor and Cochran, 1980). The critical *F* value for this test is 23.16. Since 2.41 is not greater than 23.16, conclude that the variances of the brine and dilution controls are homogeneous.

9. Equal Variance *t* Test.

9.1 To perform the *t* test, calculate the following test statistic:

$$t = \frac{\overline{Y}_1 - \overline{Y}_2}{S_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where: $\overline{Y}_1$ = mean for the control

$\overline{Y}_2$ = mean for the effluent concentration

$$S_p = \sqrt{\frac{(n_1 - 1) S_1^2 + (n_2 - 1) S_2^2}{n_1 + n_2 - 2}}$$

S_1^2 = estimate of the variance for the control

S_2^2 = estimate of the variance for the effluent concentration

n_1 = number of replicates for the control

n_2 = number of replicates for the effluent concentration

9.2 Since we are concerned here with a decrease in response from the control, a one-tailed test is appropriate. Thus, we will compare the calculated *t* with a critical *t*, where the critical *t* is at the 5% level of significance with $n_1 + n_2 - 2$ degrees of freedom. If the calculated *t* exceeds the critical *t*, the mean responses are declared different.

9.3 When comparing brine and dilution controls, the concern is for any difference between the two control groups, and a two-tailed test is appropriate. In that case, the calculated *t* would be compared with a critical *t*, where the critical *t* is a two-tailed value at the 5% level of significance with $n_1 + n_2 - 2$ degrees of freedom. If the absolute value of the calculated *t* exceeds the critical *t*, the mean responses are declared different.

9.4 Using the data from Table G.1 to illustrate the *t* test, the calculation of *t* is as follows:

$$t = \frac{0.052 - 0.040}{0.00449\sqrt{\frac{1}{5} + \frac{1}{5}}} = 4.226$$

$$S_p = \sqrt{\frac{(5-1)\,0.0000217 + (5-1)\,0.0000187}{5 + 5 - 2}} = 0.00449$$

Where:

9.5 For a one-tailed test at the 0.05 level of significance and 8 degrees of freedom, the appropriate critical *t* value is 1.860. Note: Table D.5 for K = 1 includes the critical *t* values for comparing two groups in a one-tailed test. Since t = 4.226 is greater than 1.860, conclude that the growth in the effluent concentration is significantly less than the control group growth.

9.6 Critical *t* values for two-tailed tests, such as those needed in comparing a brine control and a dilution control, can be found in a table of the *t* distribution, such as the one in Snedecor and Cochran, 1980. Note that the critical *t* for a two-tailed test is the upper-tail value at the $\alpha/2$ level of significance.

10. UNEQUAL VARIANCE *t* TEST.

10.1 If the *F* test for equality of variance fails, the *t* test is still a valid test. However, the denominator of the *t* statistic is adjusted as follows:

$$t = \frac{\overline{Y}_1 - \overline{Y}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where: $\overline{Y}_1$ = mean for the control

$\overline{Y}_2$ = mean for the effluent concentration

S_1^2 = estimate of the variance for the control

S_2^2 = estimate of the variance for the effluent concentration

n_1 = number of replicates for the control

n_2 = number of replicates for the effluent concentration

10.2 Additionally, the degrees of freedom for the test are adjusted using the following formula:

$$df' = \frac{(n_1-1)\,(n_2-1)}{(n_2-1)\,C^2+(1-C)^2\,(n_1-1)}$$

Where:

$$C = \frac{\frac{S_1^2}{n_1}}{\frac{S_1^2}{n_1}+\frac{S_2^2}{n_2}}$$

10.3 The modified degrees of freedom is usually not an integer. Common practice is to round down to the nearest integer.

10.4 The *t* test is then conducted as the equal variance *t* test. The calculated *t* is compared to the critical *t* at the 0.05 significance level with the modified degrees of freedom. If the calculated *t* exceeds the critical *t*, the mean responses are found to be statistically different.

APPENDIX H

PROBIT ANALYSIS

1. This program calculates the EC1 and EC50 (or LC1 and LC50), and the associated 95% confidence intervals.

2. The program is written in IBM PC Basic for the IBM compatible PC by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled, executable version of the program and supporting documentation can be obtained from EMSL-Cincinnati by sending a written request to EMSL at 3411 Church Street, Cincinnati, OH 45244.

2.1 A set of mortality data from a mysid survival and growth test is given in Table H.1. The program's data input routine is illustrated with this data in Figure H.1. The program begins with a request for the following information:

1. Desired output of abbreviated (A) or full (F) output? (Note: only abbreviated output is shown below.)
2. Output designation (P = printer, D = disk file).
3. Title for the output.
4. The number of exposure concentrations.
5. Toxicant concentration data.

TABLE H.1. DATA FOR PROBIT ANALYSIS

	Concentration (%)					
	Control	1.80	3.20	5.60	10.0	18.0
No. Dead	1	0	3	9	24	25
No. Exposed	25	25	25	25	25	25

2.2 The program output for the abbreviated output options, shown in Figure H.2, includes the following:

1. A table of the observed proportion responding and the proportion responding adjusted for the controls.
2. The calculated chi-square statistic for heterogeneity and the tabular value. This test is one indicator of how well the data fit the model. The program will issue a warning when the test indicates that the data do not fit the model.
3. The estimated LC1 and LC50 values and associated 95% confidence intervals.

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

Do you wish abbreviated (A) or full (F) input/output? A
Output to printer (P) or disk file (D)? P
Title ? Example of Probit Analysis for Appendix H

Number responding in the control group = ? 1
Number of animals exposed in the concurrent control group = ? 25
Number of exposure concentrations, exclusive of controls ? 5

Input data starting with the lowest exposure concentration

Concentration = ? 1.80
Number responding = ? 0
Number exposed = ? 25

Concentration = ? 3.20
Number responding = ? 3
Number exposed = ? 25

Concentration = ? 5.60
Number responding = ? 9
Number exposed = ? 25

Concentration = ? 10.0
Number responding = ? 24
Number exposed = ? 25

Concentration = ? 18.0
Number responding = ? 25
Number exposed = ? 25

Number	Conc.	Number Resp.	Number Exposed
1	1.8000	0	25
2	3.2000	3	25
3	5.6000	9	25
4	10.0000	24	25

Example of Probit Analysis for Appendix H

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	25	1	0.0400	0.0000
1.8000	25	0	0.0000	-.0306
3.2000	25	3	0.1200	0.0930
5.6000	25	9	0.3600	0.3404
10.0000	25	24	0.9600	0.9588
18.0000	25	25	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 3.004
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 7.815

Example of Probit Analysis for Appendix H

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	95% Confidence Limits Lower	Upper
LC/EC 1.00	2.642	1.384	3.519
LC/EC 50.00	5.973	4.998	6.920

Figure H.2. USEPA Probit Analysis Program used for Calculating LC/EC Values, Version 1.5.

APPENDIX I

SPEARMAN-KARBER METHOD

1. The Spearman-Karber Method is a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978). The Spearman-Karber Method estimates the mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Spearman-Karber Method is recommended when partial mortalities occur in the test solutions, but the data do not fit the Probit model.

4. To calculate the LC50 using the Spearman-Karber Method, the following must be true: 1) the smoothed adjusted proportion mortality for the lowest effluent concentration (not including the control) must be zero, and 2) the smoothed adjusted proportion mortality for the highest effluent concentration must be one.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed adjusted proportion mortalities must be between zero and one.

6. The Spearman-Karber Method is illustrated below using a set of mortality data from a Mysid Survival and Growth test. These data are listed in Table I.1.

TABLE I.1. EXAMPLE OF SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A MYSID SURVIVAL AND GROWTH TEST (25 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.08
6.25	2	0.08
12.5	0	0.00
25.0	3	0.12
50.0	16	0.64
100.0	25	1.00

7. Let p_0, p_1, ..., p_k denote the observed response proportion mortalities for the control and k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \leq p_1 \leq \ldots \leq p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \leq p_1 \leq \ldots \leq p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i-1}^s = p_i^s = (p_i + p_{i-1})/2$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

7.1 For the data in this example, because the observed mortality proportions for the control and the 6.25% effluent concentration are greater than the observed response proportions for the 12.5% effluent concentration, the responses for these three groups must be averaged:

$$p_0^s = p_1^s = p_2^s = \frac{0.08+0.08+0.00}{3} = \frac{0.16}{3} = 0.053$$

7.2 Since $p_3 = 0.12$ is larger than p_2^s, set $p_3^s = 0.12$. Similarly, $p_4 = 0.64$ is larger than p_3^s, so set $p_4^s = 0.64$. Finally, $p_5 = 1.00$ is larger than p_4^s, so set $p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table I.2.

TABLE I.2. EXAMPLE OF SPEARMAN-KARBER METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A MYSID SURVIVAL AND GROWTH TEST

Effluent Concentration %	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.08	0.053	0.000
6.25	0.08	0.053	0.000
12.5	0.00	0.053	0.000
25.0	0.12	0.120	0.071

8. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

8.1 For the data in this example, the data for each effluent concentration must be adjusted for control mortality using Abbott's formula, as follows:

$$p_0^a = p_1^a = p_2^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.053 - 0.053}{1 - 0.053} = \frac{0.0}{0.947} = 0.0$$

$$p_3^a = \frac{p_3^s - p_0^s}{1 - p_0^s} = \frac{0.120 - 0.053}{1 - 0.053} = \frac{0.067}{0.947} = 0.071$$

$$p_4^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{0.640 - 0.053}{1 - 0.053} = \frac{0.587}{0.947} = 0.620$$

$$p_5^a = \frac{p_5^s - p_0^s}{1 - p_0^s} = \frac{1.000 - 0.053}{1 - 0.053} = \frac{0.947}{0.947} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table I.2. A plot of the smoothed, adjusted data is shown in Figure I.1.

9. Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k-1} \frac{(p_{i+1}^a - p_i^a)(X_i + X_{i+1})}{2}$$

Where: p_i^a = the smoothed adjusted proportion mortality at concentration i

X_i = the $\log_{10}$ of concentration i

k = the number of effluent concentrations tested, not including the control.

9.1 For this example, the $\log_{10}$ of the estimated LC50, m, is calculated as follows:

$$\begin{aligned} m = {} & [(0.000 - 0.000)(0.7959 + 1.0969)]/2 + \\ & [(0.071 - 0.000)(1.0969 + 1.3979)]/2 + \\ & [(0.620 - 0.071)(1.3979 + 1.6990)]/2 + \\ & [(1.000 - 0.620)(1.6990 + 2.0000)]/2 \end{aligned}$$

$$= 1.64147$$

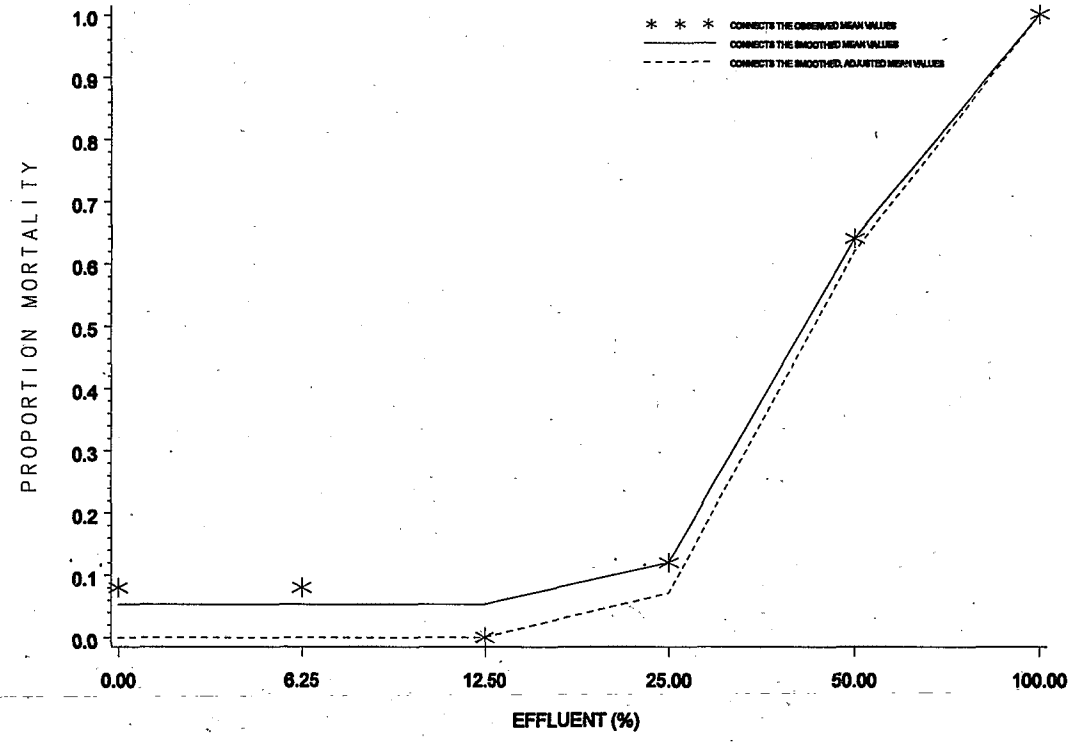

Figure I.1. Plot of observed, smoothed, and adjusted response proportions for mysid, *Holmesimysis costata*, survival data.

10. Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k-1} \frac{p_i^a (1-p_i^a)(X_{i+1}-X_{i-1})^2}{4(n_i-1)}$$

Where: X_i = the $\log_{10}$ of concentration i

n_i = the number of organisms tested at effluent concentration i

p_i^a = the smoothed adjusted observed proportion mortality at effluent concentration i

k = the number of effluent concentrations tested, not including the control.

10.1 For this example, the estimated variance of m, V(m), is calculated as follows:

$$V(m) = (0.000)(1.000)(1.3979 - 0.7959)^2/4(24) + (0.071)(0.929)(1.6990 - 1.0969)^2/4(24) + (0.620)(0.380)(2.0000 - 1.3979)^2/4(24)$$

$$= 0.0011388$$

11. Calculate the 95% confidence interval for m:

$$m \pm 2.0\sqrt{V(m)}$$

11.1 For this example, the 95% confidence interval for m is calculated as follows:

$$1.64147 \pm 2\sqrt{0.0011388} = (1.57398, 1.70896)$$

12. The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking base_{10} antilogs of the above values.

12.1 For this example, the estimated LC50 is calculated as follows:

LC50 = antilog(m) = antilog(1.64147) = 43.8%.

12.2 The limits of the 95% confidence interval for the estimated LC50 are calculated by taking the antilogs of the upper and lower limits of the 95% confidence interval for m as follows:

lower limit: antilog(1.57398) = 37.5%

upper limit: antilog(1.70896) = 51.2%

APPENDIX J

TRIMMED SPEARMAN-KARBER METHOD

1. The Trimmed Spearman-Karber Method is a modification of the Spearman-Karber Method, a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Hamilton, et al, 1977). The Trimmed Spearman-Karber Method estimates the trimmed mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the trimmed mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Trimmed Spearman-Karber Method is recommended only when the requirements for the Probit Analysis and the Spearman-Karber Method are not met.

4. To calculate the LC50 using the Trimmed Spearman-Karber Method, the smoothed, adjusted, observed proportion mortalities must bracket 0.5.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed, adjusted, observed proportion mortalities must be between zero and one.

6. Let $p_0, p_1, \ldots, p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \leq p_1 \leq \ldots \leq p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \leq p_1 \leq \ldots \leq p_k$, with their average. For example, if p_i is less than p_{i-1} then:

$$p^s_{i-1} = p^s_i = (p_i + p_{i-1})/2$$

Where: p^s_i = the smoothed observed proportion mortality for effluent concentration i.

7. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

8. Calculate the amount of trim to use in the estimation of the LC50 as follows:

$$Trim = \max(p_1^a, 1-p_k^a)$$

Where: p_1^a = the smoothed, adjusted proportion mortality for the lowest effluent concentration, exclusive of the control

p_k^a = the smoothed, adjusted proportion mortality for the highest effluent concentration

k = the number of effluent concentrations, exclusive of the control.

The minimum trim should be calculated for each data set rather than using a fixed amount of trim for each data set.

9. Due to the intensive nature of the calculation for the estimated LC50 and the calculation of the associated 95% confidence interval using the Trimmed Spearman-Karber Method, it is recommended that the data be analyzed by computer.

10. A computer program which estimates the LC50 and associated 95% confidence interval using the Trimmed Spearman-Karber Method, can be obtained through EMSL, 3411 Church Street, Cincinnati, OH 45244. The program can be obtained from EMSL-Cincinnati by sending a written request to the above address.

11. The Trimmed Spearman-Karber program automatically performs the following functions:

a. Smoothing.
b. Adjustment for mortality in the control.
c. Calculation of the necessary trim.
d. Calculation of the LC50.
e. Calculation of the associated 95% confidence interval.

12. To illustrate the Trimmed Spearman-Karber method using the Trimmed Spearman-Karber computer program, a set of data from a Topsmelt Larval Survival and Growth test will be used. The data are listed in Table J.1.

TABLE J.1. EXAMPLE OF TRIMMED SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A TOPSMELT LARVAL SURVIVAL AND GROWTH TEST (25 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	0	0.00
6.25	2	0.08
12.5	1	0.04
25.0	5	0.20
50.0	25	1.00
100.0	25	1.00

12.1 The program requests the following input (Figure J.1):
a. Output destination (D = disk file or P = printer).
b. Control data.
c. Data for each toxicant concentration.

12.2 The program output includes the following (Figure J.2):
a. A table of the concentrations tested, number of organisms exposed, and the mortalities.
b. The amount of trim used in the calculation.
c. The estimated LC50 and the associated 95% confidence interval.

```
A:>TSK

TRIMMED SPEARMAN-KARBER METHOD.  VERSION 1.5
ENTER DATE OF TEST:
1
 ENTER TEST NUMBER:
2
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
L
ENTER TEST SPECIES NAME:
Topsmelt
ENTER TOXICANT  NAME:
effluent
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
%
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
25
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
0
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL;  MAX = 10):
5
ENTER THE  5 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
6.25  12.5  25  50  100
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
Y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
25
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
Days
ENTER DURATION OF TEST:
7
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:
2 1 5 25 25
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
Y
```

Figure J.1. Example input for Trimmed Spearman-Karber Method.

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 1 TEST NUMBER: 2 DURATION: 7 Days
TOXICANT: effluent
SPECIES: Topsmelt

RAW DATA: ---	Concentration (%)	Number Exposed	Mortalities
	.00	25	0
	6.25	25	2
	12.50	25	1
	25.00	25	5
	50.00	25	25
	100.00	25	25

SPEARMAN-KARBER TRIM: 6.00%

SPEARMAN-KARBER ESTIMATES: LC50: 30.98
95% LOWER CONFIDENCE: 27.17
95% UPPER CONFIDENCE: 35.32

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

Figure J.2. Example output for Trimmed Spearman-Karber Method.

APPENDIX K

GRAPHICAL METHOD

1. The Graphical Method is used to calculate the LC50. It is a mathematical procedure which estimates the LC50 by linearly interpolating between points of a plot of observed percent mortality versus the base 10 logarithm (log_{10}) of percent effluent concentration. This method does not provide a confidence interval for the LC50 estimate and its use is only recommended when there are no partial mortalities after the data is smoothed and adjusted for control mortality. The only requirement for the Graphical Method is that the observed percent mortalities bracket 50%.

2. For an analysis using the Graphical Method the data must first be smoothed and adjusted for mortality in the control replicates. The procedure for smoothing and adjusting the data is detailed in the following steps.

3. The Graphical Method is illustrated below using a set of mortality data from a Topsmelt Larval Survival and Growth test. These data are listed in Table K.1.

TABLE K.1. EXAMPLE OF GRAPHICAL METHOD: MORTALITY DATA FROM A TOPSMELT LARVAL SURVIVAL AND GROWTH TEST (25 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	1	0.04
6.25	0	0.00
12.5	0	0.00
25.0	0	0.00
50.0	25	1.00
100.0	25	1.00

4. Let p_0, p_1, ..., p_k denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \leq p_1 \leq \ldots \leq p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \leq p_1 \leq \ldots \leq p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i-1}^s = p_i^s = (p_i + p_{i-1})/2$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

4.1 For the data in this example, because the observed mortality proportions for the 6.25%, 12.5%, and 25.0% effluent concentrations are less than the observed response proportion for the control, the values for these four groups must be averaged:

$$p_0^s = p_1^s = p_2^s = p_3^s = \frac{0.04 + 0.00 + 0.00 + 0.00}{4} = \frac{0.04}{4} = 0.01$$

4.2 Since $p_4 = p_5 = 1.00$ are larger then 0.01, set $p_4^s = p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table K.2.

5. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s)/(1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

5.1 Because the smoothed observed proportion mortality for the control group is greater than zero, the responses must be adjusted using Abbott's formula, as follows:

$$p_0^a = p_1^a = p_2^a = p_3^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.01 - 0.01}{1 - 0.0125} = \frac{0.0}{0.99} = 0.0$$

$$p_4^a = p_5^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{1.00 - 0.01}{1 - 0.01} = \frac{0.99}{0.99} = 1.00$$

A table of the smoothed, adjusted response proportions for the effluent concentrations is shown in Table K.2.

TABLE K.2. EXAMPLE OF GRAPHICAL METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A TOPSMELT LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration %	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.04	0.01	0.00
6.25	0.00	0.01	0.00
12.5	0.00	0.01	0.00
25.0	0.00	0.01	0.00
50.0	1.00	1.00	1.00
100.0	1.00	1.00	1.00

5.2 Plot the smoothed, adjusted data on 2-cycle semi-log graph paper with the logarithmic axis (the y axis) used for percent effluent concentration and the linear axis (the x axis) used for observed percent mortality. A plot of the smoothed, adjusted data is shown in Figure K.1.

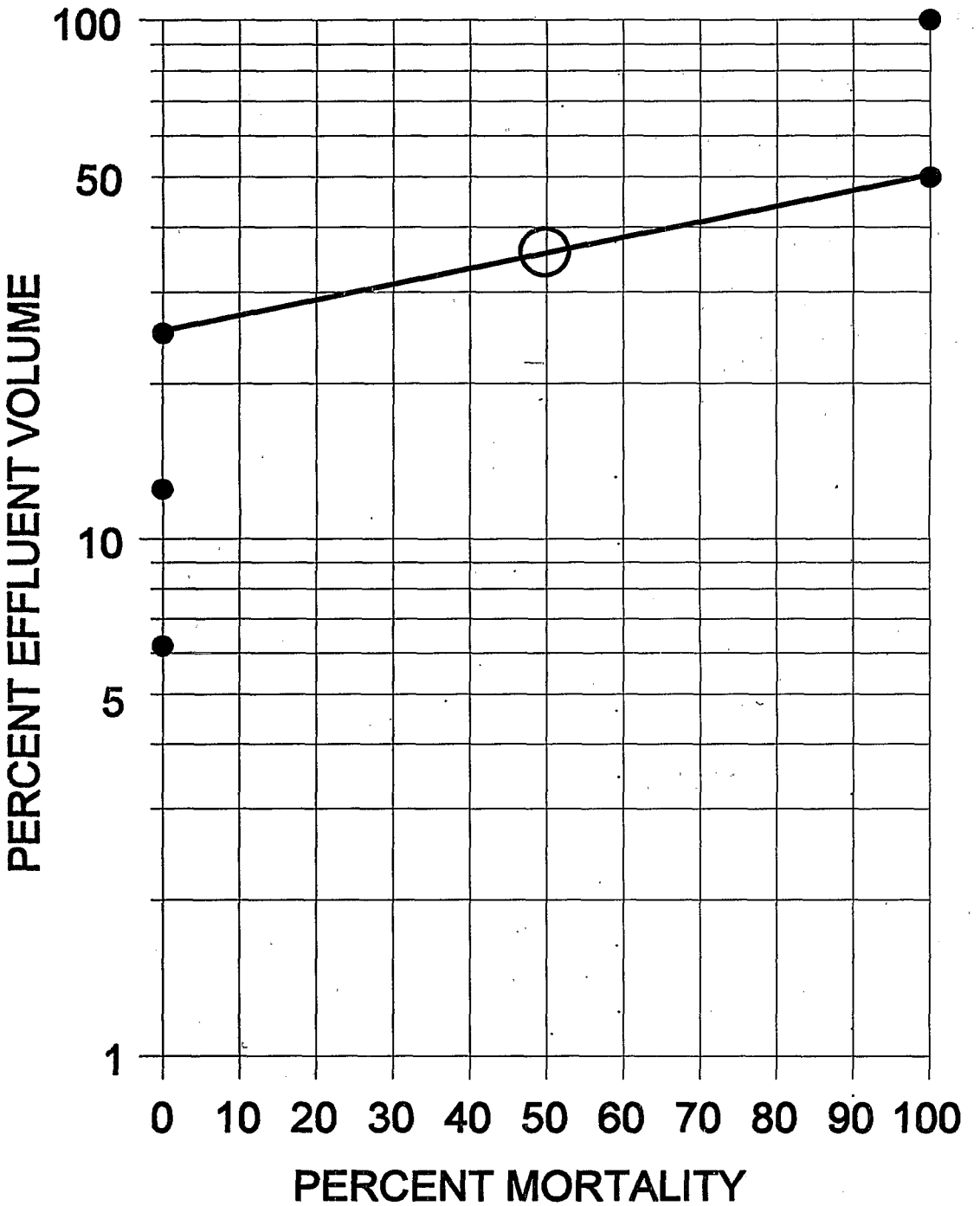

Figure K.1. Plot of the smoothed adjusted response proportions for topsmelt, *Atherinops affinis*, survival data.

6. Locate the two points on the graph which bracket 50% mortality and connect them with a straight line.

7. On the scale for percent effluent concentration, read the value for the point where the plotted line and the 50% mortality line intersect. This value is the estimated LC50 expressed as a percent effluent concentration.

7.1 For this example, the two points on the graph which bracket the 50% mortality line (0% mortality at 25% effluent, and 100% mortality at 50% effluent) are connected with a straight line. The point at which the plotted line intersects the 50% mortality line is the estimated LC50. The estimated LC50 = 35% effluent.

APPENDIX L

LINEAR INTERPOLATION METHOD

1. GENERAL PROCEDURE

1.1 The Linear Interpolation Method is used to calculate a point estimate of the effluent or other toxicant concentration that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms (Inhibition Concentration, or IC). The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests, and the generation of an endpoint from a continuous model that allows a traditional quantitative assessment of the precision of the endpoint, such as confidence limits for the endpoint of a single test, and a mean and coefficient of variation for the endpoints of multiple tests.

1.2 The Linear Interpolation Method assumes that the responses (1) are monotonically non-increasing, where the mean response for each higher concentration is less than or equal to the mean response for the previous concentration, (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. If the data are not monotonically non-increasing, they are adjusted by smoothing (averaging). In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean. Also, no assumption is made about the distribution of the data except that the data within a group being resampled are independent and identically distributed.

2. DATA SUMMARY AND PLOTS

2.1 Calculate the mean responses for the control and each toxicant concentration, construct a summary table, and plot the data.

3. MONOTONICITY

3.1 If the assumption of monotonicity of test results is met, the observed response means ($\overline{Y}_i$) should stay the same or decrease

as the toxicant concentration increases. If the means do not decrease monotonically, the responses are "smoothed" by averaging (pooling) adjacent means.

3.2 Observed means at each concentration are considered in order of increasing concentration, starting with the control mean ($\overline{Y}_1$). If the mean observed response at the lowest toxicant concentration ($\overline{Y}_2$) is equal to or smaller than the control mean ($\overline{Y}_1$), it is used as the response. If it is larger than the control mean, it is averaged with the control, and this average is used for both the control response (M_1) and the lowest toxicant concentration response (M_2). This mean is then compared to the mean observed response for the next higher toxicant concentration ($\overline{Y}_3$). Again, if the mean observed response for the next higher toxicant concentration is smaller than the mean of the control and the lowest toxicant concentration, it is used as the response. If it is higher than the mean of the first two, it is averaged with the first two, and the mean is used as the response for the control and two lowest concentrations of toxicant. This process is continued for data from the remaining toxicant concentrations. A numerical example of smoothing the data is provided below. (Note: Unusual patterns in the deviations from monotonicity may require an additional step of smoothing). Where $\overline{Y}_i$ decrease monotonically, the $\overline{Y}_i$ become M_i without smoothing.

4. LINEAR INTERPOLATION METHOD

4.1 The method assumes a linear response from one concentration to the next. Thus, the ICp is estimated by linear interpolation between two concentrations whose responses bracket the response of interest, the (p) percent reduction from the control.

4.2 To obtain the estimate, determine the concentrations C_J and C_{J+1} which bracket the response M_1 (1 - p/100), where M_1 is the smoothed control mean response and p is the percent reduction in response relative to the control response. These calculations can easily be done by hand or with a computer program as described below. The linear interpolation estimate is calculated as follows:

$$ICp = C_J + [\, M_1\ (1 - p/100) - M_J \,]\ \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

Where:

C_J	=	tested concentration whose observed mean response is greater than $M_1(1 - p/100)$.
C_{J+1}	=	tested concentration whose observed mean response is less than $M_1(1 - p/100)$.
M_1	=	smoothed mean response for the control.
M_J	=	smoothed mean response for concentration J.
M_{J+1}	=	smoothed mean response for concentration J + 1.
p	=	percent reduction in response relative to the control response.
IC*p*	=	estimated concentration at which there is a percent reduction from the smoothed mean control response. The IC*p* is reported for the test, together with the 95% confidence interval calculated by the ICPIN.EXE program described below.

4.3 If the C_J is the highest concentration tested, the IC*p* would be specified as *greater than* C_J. If the response at the lowest concentration tested is used to extrapolate the ICp value, the ICp should be expressed as a *less than the lowest test concentration*.

5. CONFIDENCE INTERVALS

5.1 Due to the use of a linear interpolation technique to calculate an estimate of the ICp, standard statistical methods for calculating confidence intervals are not applicable for the ICp. This limitation is avoided by use a technique known as the bootstrap method as proposed by Efron (1982) for deriving point estimates and confidence intervals.

5.2 In the Linear Interpolation Method, the smoothed response means are used to obtain the ICp estimate reported for the test. The bootstrap method is used to obtain the 95% confidence interval for the true mean. In the bootstrap method, the test data Y_{ji} is randomly resampled with replacement to produce a new set of data Y_{ji}*, that is statistically equivalent to the original data, but a new and slightly different estimate of the IC*p* (IC*p**) is obtained. This process is repeated at least 80 times (Marcus and Holtzman, 1988) resulting in multiple "data" sets, each with an associate ICp* estimate. The distribution of the ICp* estimates derived from the sets of resampled data approximates the sampling distribution of the ICp estimate. The standard error of the ICp is estimated by the standard deviation of the individual ICp* estimates. Empirical confidence intervals are derived from the quantiles of the ICp* empirical distribution. For example, if the test data are resampled a minimum of 80 times, the empirical 2.5% and the 97.5% confidence limits are approximately the second smallest and second largest ICp* estimates (Marcus and Holtzman, 1988).

5.3 The width of the confidence intervals calculated by the bootstrap method is related to the variability of the data. When confidence intervals are wide, the reliability of the IC estimate is in question. However, narrow intervals do not necessarily indicate that the estimate is highly reliable, because of undetected violations of assumptions and the fact that the confidence limits based on the empirical quantiles of a bootstrap distribution of 80 samples may be unstable.

5.4 The bootstrapping method of calculating confidence intervals is computationally intensive. For this reason, all of the calculations associated with determining the confidence intervals for the ICp estimate have been incorporated into a computer program. Computations are most easily done with a computer program such as the revision of the BOOTSTRP program (USEPA, 1988; USEPA, 1989) which is now called "ICPIN" and is described below in subsection 7.

6. MANUAL CALCULATIONS

6.1 DATA SUMMARY AND PLOTS

6.1.1 The data used in this example are the mysid growth data used in the example in Section 14. The data is presented as the mean weight per surviving organism. Table L.1 includes the raw data and the mean growth for each concentration. A plot of the data is provided in Figure L.1.

6.2 MONOTONICITY

6.2.1. As seen in the table, the observed means are monotonically non-increasing with respect to concentration. Therefore, the smoothed means will be simply the corresponding observed mean. The observed means are represented by $\overline{Y}_i$ and the smoothed means by M_i. Table L.2 contains the smoothed means and Figure L.1 gives a plot of the smoothed response curve.

6.3 LINEAR INTERPOLATION

6.3.1 An estimates of the IC25 can be calculated using the Linear Interpolation Method. A 25% reduction in mean weight, compared to the controls, would result in a mean weight of 0.039, where $M_1(1-p/100) = 0.052(1-25/100)$. Examining the smoothed means and their associated concentrations (Table L.2), the response, 0.039 mg, is bracketed by C_4 = 5.60% and and C_5 = 10.0%.

TABLE L.1. MYSID, *HOLMESIMYSIS COSTATA*, GROWTH DATA

		Concentration (%)			
Replicate	Control	1.80	3.20	5.60	10.0
1	0.048	0.055	0.057	0.041	0.033
2	0.058	0.048	0.050	0.040	0.000
3	0.047	0.042	0.046	0.041	0.000
4	0.058	0.041	0.043	0.043	0.000
5	0.051	0.052	0.045	0.040	0.000
Mean($\overline{Y}_i$)	0.052	0.048	0.048	0.041	0.007
i	1	2	3	4	5

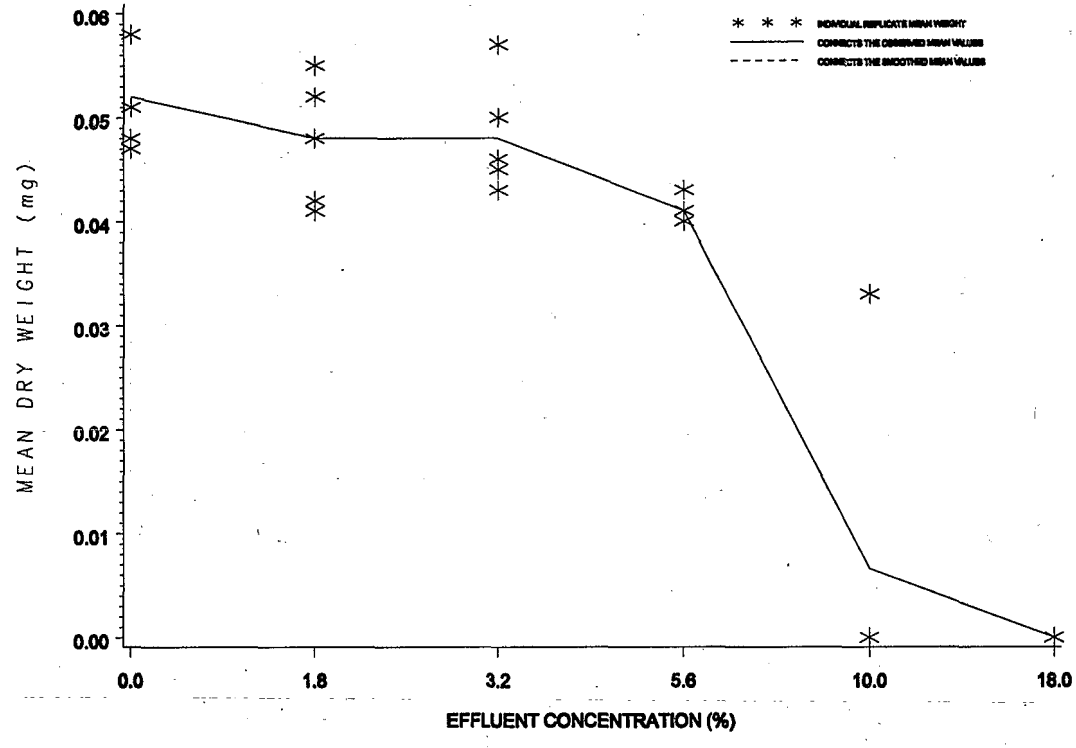

Figure L.1. Plot of raw data, observed means, and smoothed means for the mysid, *Holmesimsys costata*, growth data.

TABLE L.2. MYSID, *HOLMESIMYSIS COSTATA*, MEAN GROWTH RESPONSE AFTER SMOOTHING

Toxicant Conc. (%)	i	Response Means Y_i (mg)	Smoothed Means M_i (mg)
Control	1	0.052	0.052
1.80	2	0.048	0.048
3.20	3	0.048	0.048
5.60	4	0.041	0.041
10.00	5	0.007	0.007
18.00	6	0.000	0.000

6.3.2 Using the equation from section 4.2, the estimate of the IC25 is calculated as follows:

$$ICp = C_J + [\, M_1\ (1 - p/100) - M_J\,]\ \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

$$IC25 = 5.60 + [0.052\ (1 - 25/100) - 0.041]\frac{(10.0 - 5.60)}{(0.007 - 0.041)}$$

$$= 5.86\%$$

6.4 CONFIDENCE INTERVALS

6.4.1 Confidence intervals for the ICp are derived using the bootstrap method. As described above, this method involves randomly resampling the individual observations and recalculating the ICp at least 80 times, and determining the mean ICp, standard deviation, and empirical 95% confidence intervals. For this reason, the confidence intervals are calculated using a computer program called ICPIN. This program is described below and is available to carry out all the calculations of both the interpolation estimate (ICp) and the confidence intervals.

7. COMPUTER CALCULATIONS

7.1 The computer program, ICPIN, prepared for the Linear Interpolation Methods was written in TURBO PASCAL for IBM compatible PCs. The program (version 2.0) has been modified by Computer Science Corporation, Duluth, MN with funding provided by the Environmental Research Laboratory, Duluth, MN (Norberg-King, 1993). The program was originally developed by Battelle Laboratories, Columbus, OH through a government contract supported by the Environmental Research Laboratory, Duluth, MN (USEPA, 1988). A compiled, executable version of the program and supporting documentation can be obtained by sending a written request to EMSL-Cincinnati, 3411 Church Street, Cincinnati, OH 45244.

7.2 The ICPIN.EXE program performs the following functions: 1) it calculates the observed response means ($\bar{Y}_i$) (response means); 2) it calculates the standard deviations; 3) checks the responses for monotonicity; 4) calculates smoothed means (M_i) (pooled response means) if necessary; 5) uses the means, M_i, to calculate the initial ICp of choice by linear interpolation; 6) performs a user-specified number of bootstrap resamples between 80 and 1000 (as multiples of 40); 7) calculates the mean and standard deviation of the bootstrapped ICp estimates; and 8) provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven (Norberg-King, 1993).

7.3 For the ICp calculation, up to twelve treatments can be input (which includes the control). There can be up to 40 replicates per concentration, and the program does not require an equal number of replicates per concentration. The value of p can range from 1% to 99%.

7.4 DATA INPUT

7.4.1 Data is entered directly into the program onscreen. A sample data entry screen in shown in Figure L.2. The program documentation provides guidance on the entering and analysis of data for the Linear Interpolation Method.

ICp Data Entry/Edit Screen Current File:

Conc. ID	1	2	3	4	5	6
Conc. Tested						
Response 1						
Response 2						
Response 3						
Response 4						
Response 5						
Response 6						
Response 7						
Response 8						
Response 9						
Response 10						
Response 11						
Response 12						
Response 13						
Response 14						
Response 15						
Response 16						
Response 17						
Response 18						
Response 19						
Response 20						

F10 for Command Menu Use Arrow Keys to Switch Fields

Figure L.2. ICp data entry/edit screen. Twelve concentration identifications can be used. Data for concentrations are entered in columns 1 through 6. For concentrations 7 through 12 and responses 21-40 the data is entered in additional fields of the same screen.

7.4.2 The user selects the ICp estimate desired (e.g., IC25 or IC50) and the number of resamples to be taken for the bootstrap method of calculating the confidence intervals. The program has the capability of performing any number of resamples from 80 to 1000 as multiples of 40. However, Marcus and Holtzman (1988) recommend a minimum of 80 resamples for the bootstrap method be used and at least 250 resamples are better (Norberg-King, 1993).

7.5 DATA OUTPUT

7.5.1 The program output includes the following (see Figure L.3)

1. A table of the concentration identification, the concentration tested and raw data response for each replicate and concentration.
2. A table of test concentrations, number of replicates, concentration (units), response means (Y_i), standard deviations for each response mean, and the pooled response means (smoothed means; M_i).
3. The linear interpolation estimate of the ICp using the means (M_i). *Use this value for the ICp estimate.*
4. The mean ICp and standard deviation from the bootstrap resampling.
5. The confidence intervals calculated by the bootstrap method for the ICp. Provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven.

7.6 ICPIN program output for the analysis of the mysid growth data in Table L.1 is provided in Figure L.3.

7.6.1 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 5.8174%. The empirical 95% confidence intervals for the true mean was 4.9440% to 6.2553%.

Conc. ID		1	2	3	4	5	6
Conc. Tested		0	1.80	3.20	5.60	10.0	18.0
Response	1	.048	.055	.057	.041	.033	0
Response	2	.058	.048	.050	.040	0	0
Response	3	.047	.042	.046	.041	0	0
Response	4	.058	.041	.043	.043	0	0
Response	5	.051	.052	.045	.040	0	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: mysid, Holmesimysis costata
Test Duration: 7 days
DATA FILE: mysid.icp
OUTPUT FILE: mysid.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	5	0.000	0.052	0.005	0.052
2	5	1.800	0.048	0.006	0.048
3	5	3.200	0.048	0.006	0.048
4	5	5.600	0.041	0.001	0.041
5	5	10.000	0.007	0.015	0.007
6	5	18.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 5.8174 Entered P Value: .25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 5.8205 Standard Deviation: 0.2673
Original Confidence Limits: Lower: 4.9440 Upper: 6.2553
Expanded Confidence Limits: Lower: 4.5073 Upper: 6.4743
Resampling time in Seconds: 0.22 Random_Seed: 526805435

Figure L.3. Example of ICPIN program output for the IC25.

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* U.S. GOVERNMENT PRINTING OFFICE:1995-650-006/22074

Comparability of Suspended-Sediment Concentration and Total Suspended Solids Data

By John R. Gray, G. Douglas Glysson, Lisa M. Turcios, and Gregory E. Schwarz

Water-Resources Investigations Report 00-4191

U.S. Department of the Interior
U.S. Geological Survey

WRIR 00-4191
August 2000

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U. S. GEOLOGICAL SURVEY
Water-Resources Investigations Report 00-4191
Reston, Virginia 2000

U.S. Department of the Interior
Bruce Babbitt, Secretary

U.S. Department of the Interior
Charles G. Groat, Director

For additional information write to:

U.S. Geological Survey
Chief, Office of Surface Water
Mail Stop 415
12201 Sunrise Valley Drive
Reston, VA 20192

Copies of this report can be purchased from:

U.S. Geological Survey
Information Services
Box 25286, Mail Stop 417
Denver Federal Center
Denver, CO 80225-0286

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CONVERSION FACTORS

Multiply SI units	By	To obtain inch-pound units
	Length	
millimeter (mm)	0.03937	inch (in)
	Volume	
liter (L)	33.82	ounce fluid (fl. oz)
liter (L)	2.113	pint (pt)
liter (L)	1.057	quart (qt)
liter (L)	0.2642	gallon (gal)
	Flow	
cubic meter per second (m^3/s)	35.31	cubic foot per second (ft^3/s)
	Mass	
gram (g)	0.03527	ounce, avoirdupois (oz)
gram (g)	0.002205	ounce, avoirdupois (oz)
megagram (Mg)	1.102	ton, short
	Temperature	
degree Celsius (ºC)	F = 1.8 x°C + 32	degree Fahrenheit (ºF)
	Concentration (Mass/Volume)	
milligrams per liter (mg/L)	1.0	parts per million (ppm[1])
milligrams per liter (mg/L)	0.0000334	ounces per quart (oz/qt)

[1]This conversion is true for concentration values <8,000 mg/L. The equivalent value in mg/L for concentrations ≥8,000 ppm can be calculated from table 1, American Society of Testing Material (2000), or by using the following equation:

$$C_{mg/L} = C_{ppm}/(1-C_{ppm}(6.22 \times 10^{-7})$$

where:
$C_{mg/L}$ = sediment concentration, mg/L, and
C_{ppm} = sediment concentration, ppm

Comparability of Suspended-Sediment Concentration and Total Suspended Solids Data

By John R. Gray, G. Douglas Glysson, Lisa M. Turcios, and Gregory E. Schwarz

ABSTRACT

Two laboratory analytical methods — suspended-sediment concentration (SSC) and total suspended solids (TSS) — are predominantly used to quantify concentrations of suspended solid-phase material in surface waters of the United States. The analytical methods differ. SSC data are produced by measuring the dry weight of all the sediment from a known volume of a water-sediment mixture. TSS data are produced by several methods, most of which entail measuring the dry weight of sediment from a known volume of a subsample of the original. An evaluation of 3,235 paired SSC and TSS data, of which 860 SSC values include percentages of sand-size material, shows bias in the relation between SSC and TSS —SSC values tend to increase at a greater rate than their corresponding paired TSS values. As sand-size material in samples exceeds about a quarter of the sediment dry weight, SSC values tend to exceed their corresponding paired TSS values. TSS analyses of three sets of quality-control samples (35 samples) showed unexpectedly small sediment recoveries and relatively large variances in the TSS data. Two quality-control data sets (18 samples) that were analyzed for SSC showed both slightly deficient sediment recoveries, and variances that are characteristic of most other quality-control data compiled as part of the U.S. Geological Survey's National Sediment Laboratory Quality Assurance Program. The method for determining TSS, which was originally designed for analyses of wastewater samples, is shown to be fundamentally unreliable for the analysis of natural-water samples. In contrast, the method for determining SSC produces relatively reliable results for samples of natural water, regardless of the amount or percentage of sand-size material in the samples. SSC and TSS data collected from natural water are not comparable and should not be used interchangeably. The accuracy and comparability of suspended solid-phase concentrations of the Nation's natural waters would be greatly enhanced if all these data were produced by the SSC analytical method.

INTRODUCTION

The importance of fluvial sediment to the quality of aquatic and riparian systems is well established. The U.S. Environmental Protection Agency (1998) identifies sediment as the single most widespread cause of impairment of the Nation's rivers and streams, lakes, reservoirs, ponds, and estuaries.

Reliable, quality-assured sediment and ancillary data are the underpinnings for assessment and remediation of sediment-impaired waters. The U.S. Geological Survey (USGS) has protocols for the collection of sediment data (Edwards and Glysson, 1999) and for laboratory analysis of suspended-sediment samples (Guy, 1969; Matthes and others, 1991; Knott and others, 1992 and 1993; U.S. Geological Survey, 1998 and 1999a). Most of the laboratory analytical methods were adapted or developed by the Federal Interagency Sedimentation Project (1941), approved by the Technical Committee (Glysson and Gray, 1997), and used by most Federal agencies that analyze fluvial-sediment data.

Data collected, processed, and analyzed using consistent protocols are comparable in time and space. Conversely, data obtained using different protocols may not be comparable. The focus of this study is the comparability of suspended-sediment concentration (SSC) and total suspended solids (TSS) data. The terms SSC and TSS are often used interchangeably in the literature to describe the concentration of solid-phase material suspended in a water-sediment mixture, usually expressed in milligrams per liter (mg/L) (Gregory Granato, U.S. Geological Survey, oral commun., 1999; James, 1999). However, given that all other factors are held constant (such as particle density and shape), the analytical procedures for SSC and TSS differ and may produce considerably different results, particularly when sand-size material composes a substantial percentage of the sediment in the sample.

This report compares the SSC and TSS analytical methods and derivative data, and demonstrates which of the data types is the more accurate and reliable. The evaluation is based on historical SSC and TSS data collected and analyzed by the USGS and selected cooperators.

The authors appreciate the assistance of: Stephen S. Anthony, Donna L. Belval, James G. Brown, Ronald D. Evaldi, Herbert S. Garn, John D. Gordon, Stephen D. Preston, Daniel J. Sullivan, Richard J. Wagner and Henry Zajd, Jr. for providing the data used in this report. The formal reviews of Herbert S. Garn, Mary Ellen Ley, and Henry Zajd, Jr., were most appreciated, as were informal reviews by Anne Hoos and Harvey Jobson. Kenneth Pearsall's insights and research significantly enhanced the report. Patricia Greene's and Roger K. Chang's support for developing the tables and figures was invaluable.

Table 1. State in which natural-water samples were collected, collecting organization, collection methods, and devices for obtaining subsamples for suspended-sediment concentration (parameter code 80154) and total suspended solids (parameter code 00530) analyses
[SSC, suspended-sediment concentration; TSS, total suspended solids; USGS, U.S. Geological Survey]

State	Sample Collecting Organization		Sample Collection Method		Subsampling Device	
	SSC (80154)	TSS (00530)	SSC (80154)	TSS (00530)	SSC (80154)	TSS (00530)
Arizona[a]	USGS	USGS	USGS, 1999[i]	USGS, 1999[i]	Churn Splitter	Churn Splitter
Hawaii[b]	USGS	USGS	Automatic Sampler	Automatic Sampler	None	Churn Splitter
Illinois[c]	USGS	USGS	USGS ,1999[i]; Open Bottle	USGS, 1999[i]	Churn Splitter	Churn Splitter
Kentucky[d]	USGS	USGS	USGS	Open Bottle	None	None
Maryland[e]	USGS	USGS	Open Bottle USGS, 1999[i]; Automatic Sampler	USGS, 1999[i]; Automatic Sampler	Churn Splitter	Churn Splitter
Virginia[f]	USGS and Cooperator	USGS and Cooperator	USGS, 1999[i]	USGS, 1999[i]	None	Churn Splitter
Washington[g]	USGS	USGS	USGS, 1999[i]	USGS, 1999[i]	None	Churn Splitter
Wisconsin[h]	USGS	Cooperator	USGS, 1999[i]	Open Bottle	Cone Splitter	Cone Splitter

[a] James G. Brown, U.S. Geological Survey, written commun. (1999).
[b] Stephen S. Anthony, U.S. Geological Survey, written commun. (1999).
[c] Daniel J. Sullivan, U.S. Geological Survey, written commun. (1999).
[d] Ronald D. Evaldi, U.S. Geological Survey, written commun. (1999).
[e] Stephen D. Preston, U.S. Geological Survey, written commun. (1999).
[f] Donna L. Belval, U.S. Geological Survey, written commun. (1999).
[g] Richard J. Wagner, U.S. Geological Survey, written commun. (1999).
[h] Herbert S. Garn, U.S. Geological Survey, written commun. (1999).
[i] See Edwards and Glysson (1999).

FIELD TECHNIQUES AND LABORATORY METHODS

The paired SSC and TSS results used in this evaluation were derived from analyses of natural-water samples collected by the USGS and selected cooperators (table 1). Analyses of all SSC data from natural water were made by USGS sediment laboratories, and analyses of the TSS data were made by USGS and cooperating laboratories. Additionally, 53 quality-control samples were prepared by the USGS and analyzed by a laboratory that provides data to the USGS.

Field Techniques

The large majority of water samples were collected using either the equal-width-increment or the equal-discharge-increment method to obtain a composite sample that is representative of the discharge-weighted SSC (Edwards and Glysson, 1999). Some samples, including those obtained by at least one cooperating agency, were collected by dipping an open bottle to obtain samples for subsequent TSS analysis. Some of the paired SSC and TSS samples were collected in-stream sequentially and submitted to laboratories for analysis as whole samples. The remaining samples were split into subsamples by using a churn splitter or cone splitter (Ward and Haar, 1990; Capel and Larson, 1996; Capel and others, 1995).

Tests performed by the USGS demonstrate that the churn splitter and cone splitter can provide unbiased and acceptably precise (generally within 10 percent of the known value) SSC values as large as about 1,000 mg/L when the mean diameter of sediment particles is less than about 0.25 mm. At SSC values of 10,000 mg/L or more, the bias and precision of SSC values in churn splitter subsamples are considered unacceptable (U.S. Geological Survey, 1997; Wilde and others, 1999).

Cone splitters produce subsamples with SSC values that are adequately representative of the original sample at 10,000 mg/L, but not at 100,000 mg/L. The accuracy of the cone splitter for SSC values between 10,000 mg/L and 100,000 mg/L is unknown and is considered unacceptable at concentrations larger than 100,000 mg/L (U.S. Geological Survey, 1997; Wilde and others, 1999).

Subsampling will typically increase the variance and (or) create bias in the concentration and size distribution of solid-phase material in a subsample. Significant differences in the amount of solid-phase material in some paired samples may have occurred as a result of non-representative splitting of the original samples, or by collecting consecutive in-stream samples under conditions of rapidly varying SSC. Similarly, because the data were obtained by field personnel in eight States as part of unrelated studies, significant differences

may have resulted because of differences in data-collection techniques. However, the probability of significant bias resulting from consistently selecting samples with larger concentrations of sediment for analyses by one of the methods would be small based on the large number of paired data used in the analysis. There is no evidence indicating that methods used for collecting, processing, or selecting subsamples for subsequent analysis introduced bias in the relations between SSC and TSS identified in this evaluation.

Laboratory Methods

Two standard methods are widely cited in the United States for determining the total amount of suspended material in a water sample. These are:

1. Method D 3977-97, "Standard Test Method for Determining Sediment Concentration in Water Samples" of the American Society for Testing and Materials (American Society for Testing and Materials, 2000), and
2. Method 2540 D, "Total Suspended Solids Dried at 103°–105° C" (American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1995).

The differences in these analytical methods, and some variations used to produce TSS data are described below.

Suspended-Sediment Concentration Analytical Method. ASTM Standard Test Method D 3977-97 lists three methods that result in a determination of SSC values in water and wastewater samples:

1. Test Method A – Evaporation: The evaporation method may only be used on sediment that settles within the allotted storage time, which can range from a few days to several months. If the dissolved-solids concentration exceeds about 10 percent of the SSC value, an appropriate correction factor must be applied to the SSC value. The precision and bias of Method A are shown as follows:

[mg/L, milligrams per liter]

Concentration Added, (mg/L)	Concentration Recovered, (mg/L)	Standard Deviation of Test Method (mg/L)	Standard Deviation of Single Operator (mg/L)	Bias, percent
10	9.4	2.5	2.3	-6
1,000	976	36.8	15.9	-2.4
100,000	100,294	532	360	0.3

2. Test Method B- Filtration: The filtration method is used only on samples with concentrations of sand-size material (diameters greater than 0.062 mm) less than about 10,000 mg/L and concentrations of clay-size material of about 200 mg/L. No dissolved-solids correction is needed. The precision and bias of Method B are shown as follows:

[mg/L, milligrams per liter]

Concentration Added, (mg/L)	Concentration Recovered, (mg/L)	Standard Deviation of Test Method (mg/L)	Standard Deviation of Single Operator (mg/L)	Bias, percent
10	8	2.6	2	-20
100	91	5.3	5.1	-9
1,000	961	20.4	14.1	-3.9

3. Test Method C - Wet-sieving filtration: The wet-sieve-filtration method also yields a SSC value, but the method is not as direct as Methods A and B. Method C is used if the percentage of material larger than sand-size particles is desired. The method yields a concentration for the total sample, a concentration of the sand-size particles, and a concentration for the silt- and clay-size particles. A dissolved-solids correction may be needed, depending on the type of analysis done on the fine fraction of the samples and the dissolved-solids concentration of the sample. The precision and bias of Method C are shown as follows:

[mm, millimeters; mg/L, milligrams per liter]

Mixture Number	Sieve Diameter (mm)	Concentration Added (mg/L)	Concentration Recovered (mg/L)	Standard Deviation of Test Method (mg/L)	Standard Deviation of Single Operator (mg/L)	Bias, percent
1	>0.062	1	3.4	2.8	2.4	240
1	<0.062	10	8.7	4.3	2.9	-13
2	>0.062	9	5	5.9	1.9	-44
2	<0.062	91	79	15.2	11	-13
3	>0.062	91	107	12.3	5.9	18
3	<0.062	909	832	87.2	61	-8

These three methods are virtually the same as those used by USGS sediment laboratories and described by Guy (1969). Only the Whatman grade 934AH, 24-mm-diameter filter is used for purposes of standardization. Each method includes retaining, drying at 103°C ±2°C, and weighing all of the sediment in a known mass of a water-sediment mixture (U.S. Geological Survey, 1999a).

Total Suspended Solids Analytical Method. According to the American Public Health Association, American Water Works Association, and Water Pollution Control Federation (1995), the TSS analytical method uses a predetermined volume from the original water sample obtained while the sample is being mixed with a magnetic stirrer. An aliquot of the sample — usually 0.1 L, but a smaller volume if more than 200 mg of residue may collect on the filter — is withdrawn by pipette. The aliquot is passed through a filter, the diameter of which usually ranges from 22 to 125 mm. The filter may be a Whatman grade 934AH, Gelman type A/E, Millipore type AP40; E-D Scientific Specialties grade 161, or another product that gives demonstrably equivalent results. After filtering, the filter and contents are removed and dried at 103° to 105° C, and weighed. No dissolved-solids correction is required. The percentages of sand-size and finer material cannot be determined using the TSS method.

The American Public Health Association, American Water Works Association, and Water Pollution Control Federation (1995) describe the precision for this method as follows: "The standard deviation was 5.2 mg/L (coefficient of variation 33 percent) at 15 mg/L, 24 mg/L (10 percent) at 242 mg/L, and 13 mg/L (0.76 percent) at 1,707 mg/L in studies by two analysts of four sets of 10 determinations each. Single-laboratory analyses of 50 samples of water and wastewater were made with a standard deviation of differences of

2.8 mg/L." The standard provides no indication of the size of particles used in the testing for the method.

In practice, TSS data are produced by a number of variations to the processing methods described in the American Public Health Association, American Water Works Association, and Water Pollution Control Federation (1995). For example:

- For the collection of TSS samples as part of the Chesapeake Bay Program, field staff pump water from a specified depth into a plastic gallon container. The container is vigorously shaken, and 0.2 – 1.0 L of the water-sediment mixture is poured for field filtering and subsequent analysis. (Mary Ley, Interstate Commission on the Potomac River Basin, the State of Maryland and the Commonwealth of Virginia, written commun., 2000).
- One State government laboratory produces TSS data by vigorously shaking the sample and pouring it into a crucible for subsequent analysis. All of the sample is poured into the crucible unless "there is a lot of suspended material," in which case only part of the sample is poured (Lori Sprague, U.S. Geological Survey, written commun., 1999).
- Another laboratory analyzed quality-control samples by using Method 2540D of the American Public Health Association, American Water Works Association, and Water Pollution Control Federation (1995), with the following variation: The sample is shaken vigorously and a third of the desired subsample volume is decanted to a secondary vessel. This process is repeated twice to obtain a single subsample for subsequent filtration, drying and weighing.

The reduction in TSS data comparability is not limited to lack of consistency in processing and analytical methods. According to James (1999), there is generally no agreed upon definition of TSS in regard to storm-water runoff, in part because the settleable part of TSS is not reported in most storm-water studies.

The problem extends to nomenclature. The terms "SSC" and "TSS", or variations thereof, are sometimes attributed to an incorrect data type. For example, a proposed Total Maximum Daily Load for sediment in Stekoa Creek, Georgia (U.S. Environmental Protection Agency, Region 4, written commun., 2000) is based on regional TSS data, which are compiled from U.S. Geological Survey records; the TSS data referred to are actually SSC data. Buchanan and Schoellhamer (1998) refer to "suspended-solids concentration data" for San Francisco Bay. Those data would more appropriately be referred to as SSC, because the total water-sediment mass and all sediment were measured in the analysis (Alan Mlodnosky, USGS, oral commun., 1999).

Part of the problem may be attributable to the origin of the TSS method and subsequent changes in the types of water for which it is recommended for use. Information available from the American Public Health Association and American Water Works Association (1946) makes it clear that the Suspended Solids Method was intended for use for wastewater effluents (Kenneth Pearsall, U.S. Geological Survey, written commun., 2000). This is more or less consistent with the Total Suspended Matter Method, which was "intended for use with wastewaters, effluents, and polluted waters," as listed in the American Public Health Association, American Water Works Association, and Water Pollution Control Federation (1971). A fundamental change took place in 1976, when the Total Suspended Matter Method was deemed suitable for "residue in potable, surface, and saline waters, as well as domestic and industrial wastewaters in the range up to 20,000 mg/L" by the American Public Health Association, American Water Works Association, and Water Pollution Control Federation (1976). The Suspended Solids and Total Suspended Matter Methods described above are predecessors of the "Total Suspended Solids Dried at 103°-105°C" Method, which first appeared in 1985 by that title in the American Public Health Association, American Water Works Association, and Water Pollution Control Federation (1985).

In summary, the evidence indicates that the TSS method was originally designed for wastewater analyses, presumably on samples collected after a settling step at a wastewater treatment facility (hence the term "suspended" in TSS). The American Public Health Association, American Water Works Association, and Water Pollution Control Federation (1976) expanded the TSS Method's applicability in 1976 to include natural water.

Differences Between the SSC and TSS Analytical Methods. The fundamental difference between the SSC and TSS analytical methods stems from preparation of the sample for subsequent filtering, drying, and weighing. A TSS analysis normally entails withdrawal of an aliquot of the original sample for subsequent analysis, although as previously noted, there is evidence of inconsistencies in methods used in the sample preparation phase of the TSS analyses. The SSC analytical method measures all sediment and the mass of the entire water-sediment mixture. Additionally, the percentage of sand-size and finer material can be determined as part of the SSC method, but not as part of the TSS method.

If a sample contains a substantial percentage of sand-size material, then stirring, shaking, or otherwise agitating the sample before obtaining a subsample will rarely produce an aliquot representative of the SSC and particle-size distribution of the original sample. This is a by-product of the rapid settling properties of sand-size material, compared to those for silt- and clay-size material, given virtually uniform densities and shapes as described by Stokes' Law. Aliquots obtained by pipette might be withdrawn from the lower part of the sample where the sand concentration tends to be enriched immediately after agitation, or from a higher part of the sample where the sand concentration is rapidly depleted.

The physical characteristics of a pipette used to withdraw an aliquot, or subsample, can introduce additional errors in subsequent analytical results. The American Public Health Association, American Water Works Association, and Water Pollution Control Federation (1995) specifies use of "wide-bore pipettes" to withdraw aliquots. The tip opening of those recommended for use is about 3 mm in diameter (Kimble-Contes Inc., accessed May 1, 2000). By definition, the upper limit of sand-size material, which is expressed as the median diameter, is 2 mm (Folk, 1980). A natural sediment particle's long axis is almost always larger than its me-

dian axis and can be substantially larger. Hence, a single coarse-grained sand particle or multiple sand-size particles, particularly when present in large concentrations, may clog a 3-mm tip pipette under suction.

If the aforementioned lack of consistency in the TSS analytical procedure extends to variability in diameters of pipette tips used to withdraw TSS aliquots, the size of particles being excluded from the subsample could vary with the type of pipette used. Hence, use of a pipette may cause concentration bias when subsampling if sand-size material is present in the sample.

Based on Stokes' Law, subsamples obtained by pouring sand-rich water-sediment mixtures should be deficient in sand-size material. Because the fine material concentration will not normally be altered by the removal of an aliquot, the differences between the two methods will tend to be more pronounced as the percentage of sand-size material in the sample increases.

Samples collected sequentially in-stream may have different concentrations and size characteristics of solid-phase material. This may be due to natural variations in the amounts and composition of solid-phase material in transport, and to variance and (or) bias that is introduced by sampling procedures. Likewise, a subsample may contain an amount and size distribution of sediment atypical to that of the original. However, any differences in SSC and size-distribution data from paired samples resulting from in-stream variations or sampling procedures would likely occur randomly among the 3,235 paired analyses used in this evaluation.

DESCRIPTION OF DATA USED IN THE EVALUATION

Results of analyses of natural-water samples and of quality-control samples prepared by the USGS were used for this evaluation. Natural-water samples for determination of SSC (parameter code 80154) were collected and analyzed by the USGS (table 1). Natural-water samples for determination of TSS, (parameter code 00530) were collected by the USGS and cooperating agencies, and analyzed by the USGS and cooperating laboratories. A total of 3,235 pairs of SSC and TSS data for natural water were obtained from the files of USGS District offices.

The paired SSC and TSS data were collected at 65 sampling sites in Arizona, Hawaii, Illinois, Kentucky, Maryland, Virginia, Washington, and Wisconsin. All but the 12 sampling sites in Kentucky were at USGS streamflow-gaging stations. The percentage of sand-size material was available for 860, or about 27 percent, of the SSC samples. The SSC and TSS natural-water data used in this evaluation were augmented by analytical results of 53 quality-control samples prepared by the USGS National Sediment Laboratory Quality Assurance Program (Gordon and others, 2000, U.S. Geological Survey, 1998; 1999a; 1999b; 2000b).

Arizona. A total of 122 SSC and TSS sample pairs were collected at a USGS streamflow-gaging station on Pinal Creek at Inspiration Dam near Globe (station number 09498400) in central Arizona from 1982-98. The samples were collected about monthly or bimonthly using techniques described by Edwards and Glysson (1999). A churn splitter was used to obtain subsamples of the water-sediment mixture. The USGS sediment laboratory in Iowa City, Iowa, analyzed the subsamples for SSC and TSS (James G. Brown, U.S. Geological Survey, written commun., 1999).

Hawaii. According to Hill (1996), 13 SSC and TSS sample pairs were collected at three streamflow-gaging stations in the Kamooalii drainage basin, Oahu, Hawaii, from 1985-89, as a component of a large-scale highway-construction study. The SSC samples were collected by a US PS-69 automatic pumping sampler. The TSS samples were collected by a Manning automatic pumping sampler. A churn splitter was used to obtain subsamples for TSS analyses. The SSC samples were analyzed by the USGS sediment laboratory in Oahu. The TSS samples were analyzed by the USGS National Water Quality Laboratory in Denver, Colorado (Stephen S. Anthony, U.S. Geological Survey, written commun., 1999).

Illinois. A total of 223 SSC and TSS sample pairs were collected at 8 USGS streamflow-gaging stations in the upper Illinois River Basin from 1988-90 (Sullivan and Blanchard, 1994). Samples were collected according to techniques described by Edwards and Glysson (1999). A churn splitter was used to obtain subsamples for SSC and TSS analyses. SSC samples were analyzed at the USGS sediment laboratory in Iowa City, Iowa, using the evaporation method. TSS samples were analyzed by an Illinois State laboratory using the nonfilterable residue, gravimetric method (Daniel Sullivan, U.S. Geological Survey, written commun., 1999).

Kentucky. A total of 95 SSC and TSS sample pairs were collected at 12 sampling locations in the Ohio River Basin in May 1999. SSC and TSS samples were collected at each site for one day over several hours at about 1-hour intervals. Samples were collected using an open-bottle sampler because of the low stream velocities. No splitting devices were used to obtain subsamples. The USGS sediment laboratory in Louisville, Kentucky, analyzed the SSC samples. A contract laboratory performed the TSS analyses (Ronald Evaldi, U.S. Geological Survey, written commun., 1999).

Maryland. A total of 1,561 SSC and TSS sample pairs were collected at 6 streamflow-gaging stations in the Patuxent River Basin, Maryland, as part of the USGS Patuxent Nonpoint Source study during the years 1985-98 (Preston and Summers, 1997). The sampling frequency was monthly, with additional samples collected during periods of storm runoff. The monthly base-flow samples were collected using the equal-width-increment method (Edwards and Glysson, 1999), and the storm-runoff samples were collected using an automatic sampler. A churn splitter was used for both monthly and storm samples of both SSC and TSS. The SSC samples were analyzed at USGS sediment laboratories in Lemoyne, Pennsylvania, and Louisville, Kentucky. The TSS samples were analyzed using a pipette and filtration method by a Maryland State laboratory (Stephen D. Preston, U.S. Geological Survey, written commun., 1999).

Virginia. A total of 188 SSC and TSS sample pairs were collected at 7 streamflow-gaging stations in Virginia during the years 1975-95. Paired SSC and TSS samples were collected every other month by the USGS except during some low-flow periods as part of the River Input Monitoring Program (U.S. Geological Survey, 2000a). Techniques described by Edwards and Glysson (1999) were used to collect all samples. A churn splitter was used to obtain subsamples for TSS analyses. The USGS collected most of the samples, except during some low-flow periods when the Virginia Department of Environmental Quality collected the samples. SSC analyses were performed by USGS sediment laboratories. A Virginia State laboratory performed the TSS analyses (Donna L. Belval, U.S. Geological Survey, written commun., 1999).

Washington. A total of 817 SSC and TSS sample pairs were collected at 25 streamflow-gaging stations in Washington during the years 1973-98, as part of various projects. Techniques described by Edwards and Glysson (1999) were used to collect all SSC and TSS samples. A churn splitter was used to obtain subsamples for TSS analyses. The SSC and TSS samples were analyzed at a USGS sediment laboratory in Tacoma, Washington, through September 1982. Thereafter, samples were analyzed at the USGS Cascades Volcano Observatory Sediment Laboratory (Richard J. Wagner, U.S. Geological Survey, written commun., 1999).

Wisconsin. A total of 216 SSC and TSS sample pairs were collected at 3 streamflow-gaging stations on streams in the Lake Michigan watershed, Wisconsin, as part of an evaluation of the differences in results of water-quality monitoring caused by differences in sample-collection methods (Kammerer and others, 1998). Low-flow samples were collected in August and October 1993, and high-flow samples were collected in April-July 1994. The SSC samples were collected using techniques described by Edwards and Glysson (1999). The TSS samples were collected concurrently with the SSC samples by the Wisconsin Department of Natural Resources using an open bottle. Subsamples for SSC and TSS analyses were obtained using a cone splitter. SSC samples were analyzed by the USGS sediment laboratory in Iowa City, Iowa. TSS samples were analyzed by a Wisconsin State laboratory (Herbert S. Garn, U.S. Geological Survey, written commun., 1999).

Quality-Control Data. The SSC and TSS natural-water data used in this evaluation were augmented by analytical results of quality-control samples from a cooperating laboratory. Known amounts of water and sediment were used to constitute quality-control samples as part of the USGS National Sediment Laboratory Quality Assurance Program. The National Sediment Laboratory Quality Assurance Program is designed as an interlaboratory-comparison evaluation to provide a measure of bias and variance of suspended-sediment data analyzed by laboratories operated or used by the USGS. The quality-control samples received by the participating laboratories were identified as such.

The quality-control samples were submitted in five batches to a cooperating laboratory during 1997-99. Of the quality-control samples, the first 35 were shipped as batch numbers 1997-1, 1997-2, and 1998-1 and were analyzed for TSS. Eighteen quality-control samples were shipped as batch numbers 1998-2 and 1999-1 and analyzed for SSC using the evaporation method (Kenneth Pearsall, U.S. Geological Survey, 1999, oral commun.).

COMPARABILITY OF SUSPENDED-SEDIMENT CONCENTRATION AND TOTAL SUSPENDED SOLIDS DATA

Natural-Water Data

The relation between SSC and TSS data was evaluated by comparing all available paired SSC and TSS natural-water data, and subsets of those data for each State. The number of paired SSC and TSS values for selected SSC concentration ranges with and without particle-size data are shown in figure 1.

Of the 3,235 natural-water SSC samples used in this study,

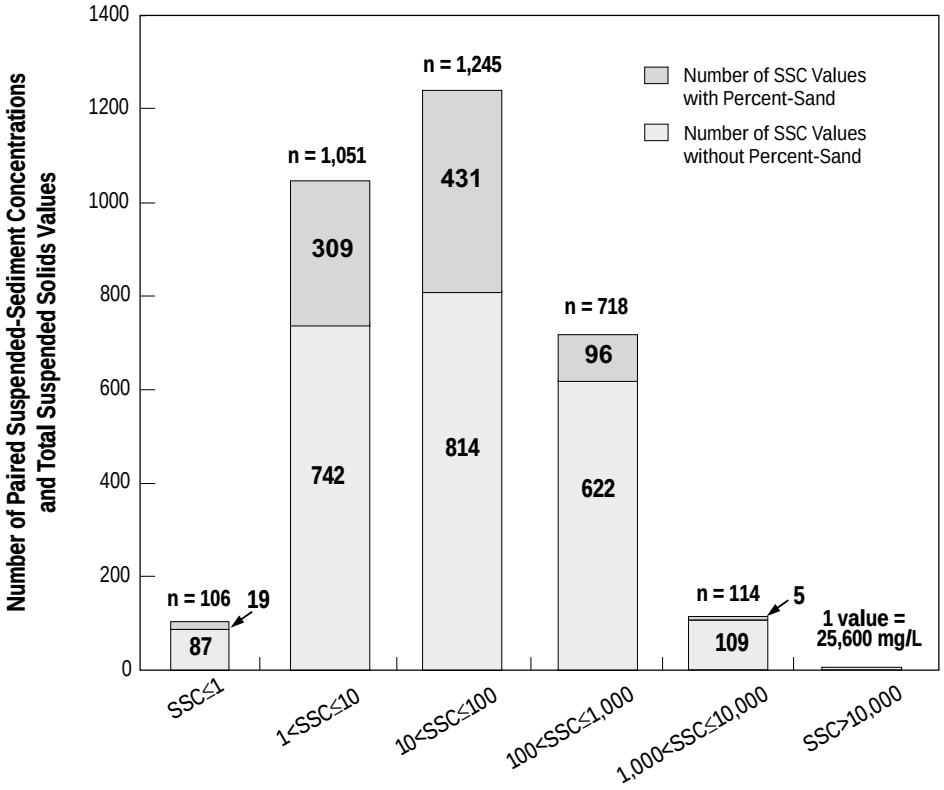

Figure 1. Number of paired suspended-sediment concentration (SSC) values and total suspended solids (TSS) values of the 3,235 data pairs for selected suspended-sediment concentration ranges, in milligrams per liter.

Table 2. Statistical characteristics of paired suspended-sediment concentration (SSC) and total suspended solids (TSS) data for each of eight States, and for the combined data from all States
[mg/L, milligrams per liter; >, greater than]

	SSC Values		SSC Minus TSS				
Source of SSC and TSS Paired Data	Number of values	3rd Quartile mg/L	Number of values >0 mg/L	Percentage of values >0 mg/L for all paired data	Number of values when SSC value is > 3rd Quartile value	Number of values >0 mg/L when SSC value is > 3rd Quartile value	Percentage of values >0 mg/L when SSC value is > 3rd Quartile value
Arizona	122	153.25	93	76%	31	30	97%
Hawaii	13	353.0	13	100%	3	3	100%
Illinois	223	48.5	111	50%	56	34	61%
Kentucky	95	10.2	28	29%	24	9	38%
Maryland	1,561	324.0	1,071	69%	390	328	84%
Virginia	188	16.0	105	56%	44	40	91%
Washington	817	30.0	518	63%	203	179	88%
Wisconsin	216	80.25	184	85%	54	54	100%
All Paired Data[1]	3,235	108.0	2,123	66%	809	672	83%

[1] Based on statistics using all 3,235 paired data; some values vary slightly from those calculated using summary statistics from the eight States.

74 percent had values less than or equal to 100 mg/L; only one value (25,600 mg/L) exceeded 10,000 mg/L (figure 1).

Statistical characteristics of SSC and TSS paired data for each State and for all paired data are given in table 2. Sixty-six percent of all TSS values are smaller than their corresponding paired SSC values. Eighty-three percent of all TSS values are smaller than their paired SSC value when SSC values exceed the 3rd quartile value. For each State except Kentucky (38 percent for 24 paired samples), 61 to 100 percent of the TSS values are smaller than their paired SSC value when SSC values exceed the 3rd quartile value. To summarize, SSC values tend to exceed their corresponding paired TSS values. This tendency becomes stronger at larger values of SSC.

Relations between all 3,235 paired TSS and SSC measurements are shown in figures 2 and 3. According to Glysson and others (2000), there is no simple, straightforward way to adjust TSS data to estimate SSC if paired samples are not available. Relations identified herein are not recommended for use in adjusting TSS data unless supported by additional research.

The data shown in figure 2 are plotted without transformation and include the two ordinary least squares regression lines obtained by regressing TSS on SSC (the lower line) and SSC on TSS (the upper line). Because of measurement errors associated with the collection processing, and analysis of the data, neither line can be interpreted as an unbiased estimate of the true relation

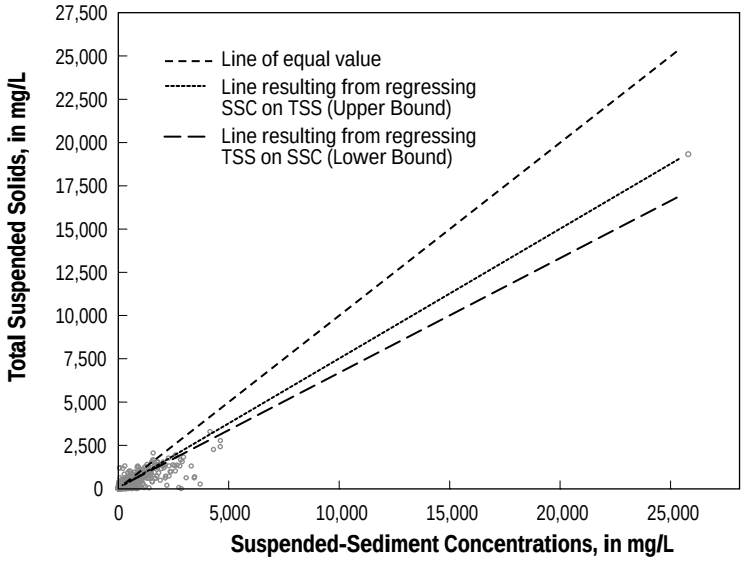

Figure 2. Relation between untransformed values of suspended-sediment concentration and total suspended solids for 3,235 data points.

between the two measurement methods. In fact, the existence of measurement error implies the system of equations describing the two measurements is insufficiently identified, making estimation of an unbiased relation impossible without additional information on the variance of the measurement error for at least one of the measurements (Klepper and Leamer, 1984). However, the two least squares regression lines can be used to bound the true slope and intercept coefficients (Frisch, 1934). In the case of TSS and SSC, the least squares intercepts are very small relative to the range of the data. Consequently, the two regression lines effectively form consistent upper and lower bounds on the true relation between TSS and SSC. These bounds imply that TSS is biased downward relative to SSC by a proportionate amount of 25 to 34 percent. Given the large skew apparent in the data, this finding is tentative and requires confirmation using a statistical or functional transformation yielding homoscedastic residuals.

The relation between SSC and TSS for all 3,235 pairs of transformed data using the base-10 logarithm and the line of equal value are shown in figure 3; the relations for each State and lines of equal value are shown in figure 4. Trends in the scattergrams plotted for all data compared to those with data that were segregated by State show some similarities, including a tendency for the data to plot to the right of the line of equal value, particularly at larger values of SSC.

As described previously, at least two factors associated with the TSS analysis can result in subsamples obtained by pipette or by pouring that are deficient in sand-size material. Rapidly falling sand-size material can be difficult to withdraw representatively, particularly if pipette subsamples are obtained from near the surface and (or) if the subsample is not withdrawn immediately after mixing. Also, coarser sand particles may plug the pipette intake, precluding withdrawal of a representative mixture. Subsamples obtained by pouring are also unlikely to contain representative amounts of sand-size material. In contrast, the amount or percentage of sand-size material in a SSC sample has no effect in bias because all sediment in the original sample is used in the SSC analysis.

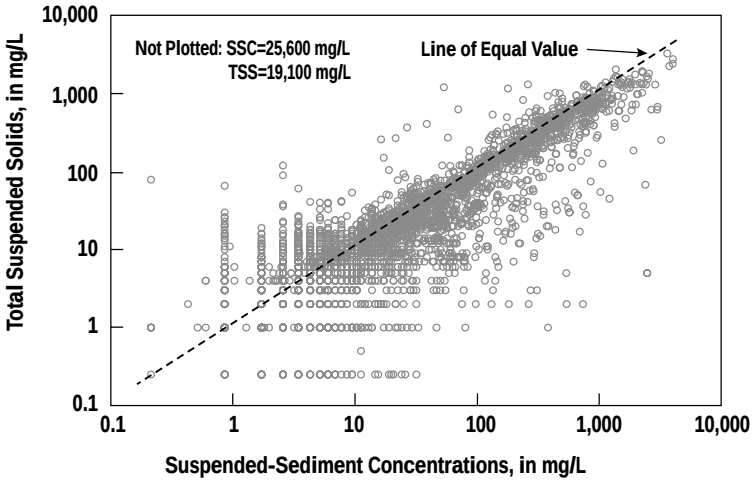

Figure 3. Relation between the base-10 logarithms of suspended-sediment concentration (SSC) and total suspended solids (TSS) for 3,235 data pairs in the scattergrams plotted. All SSC and TSS values less than 0.25 mg/L were set equal to 0.25 mg/L to enable plotting the data on logarithmic coordinates.

The relation between sand-size material and TSS bias was examined using the 860 paired SSC and TSS values for which the amounts of material coarser and finer than 0.062 mm in the SSC sample are known. Percent sand-size material, percent finer material, and the total mass of sand-size material were included in the analysis. All but one of the paired data associated with particle sizes are for streams in Illinois, Virginia, and Washington.

The relation between percent sand-size material associated with the SSC sample, and the SSC minus TSS remainder is shown in figure 5. No bias is apparent when sand-size material composes less than about a quarter of the sample's sediment mass. Above about a third sand-size material, the large majority of the SSC values exceed their paired TSS values. The increase in bias at larger SSC values as percent sand-size values increase is consistent with the observation that splitting original samples that contain a substantial percentage of sand-size material will rarely produce subsamples with a SSC or particle-size distribution similar to those of the original.

Splitting samples that contain small percentages of sand-size material are more likely to produce subsamples with concentrations and particle-size distributions similar to the original. The relation between TSS and the concentration of material finer than 0.062 mm for 860 of the paired SSC and TSS data with associated particle-size distribution data is shown in figure 6. The concentration of fine material was calculated as follows:

$$C_{<0.062mm} = SSC\,[1 - (Percent_{\geq 0.062mm}/100)]$$

where

$C_{<0.062mm}$ is the concentration of material finer than 0.062 mm in diameter,

SSC is suspended-sediment concentration, and

$Percent_{\geq 0.062mm}$ is percent sand-size material associated with the SSC value.

At TSS values that exceed about 5 mg/L of fine material, the SSC and TSS data are more or less evenly distributed around the line of equal value (figure 6). This suggests that the TSS method can provide relatively unbiased results when the large majority of material in a sample is finer than 0.062 mm.

The importance of bias in the relation between SSC and TSS characterized in figure 3 can be magnified when TSS data are used to compute sediment discharges. Sediment discharges increase when the product of water discharge and SSC increases (Porterfield, 1972). Additionally, the mobility of coarse material tends to increase with larger flow velocities. Because of the strong tendency for SSC to exceed TSS at larger values of SSC (see figures 3 and 4), calculating discharges of TSS will usually result in underestimates of

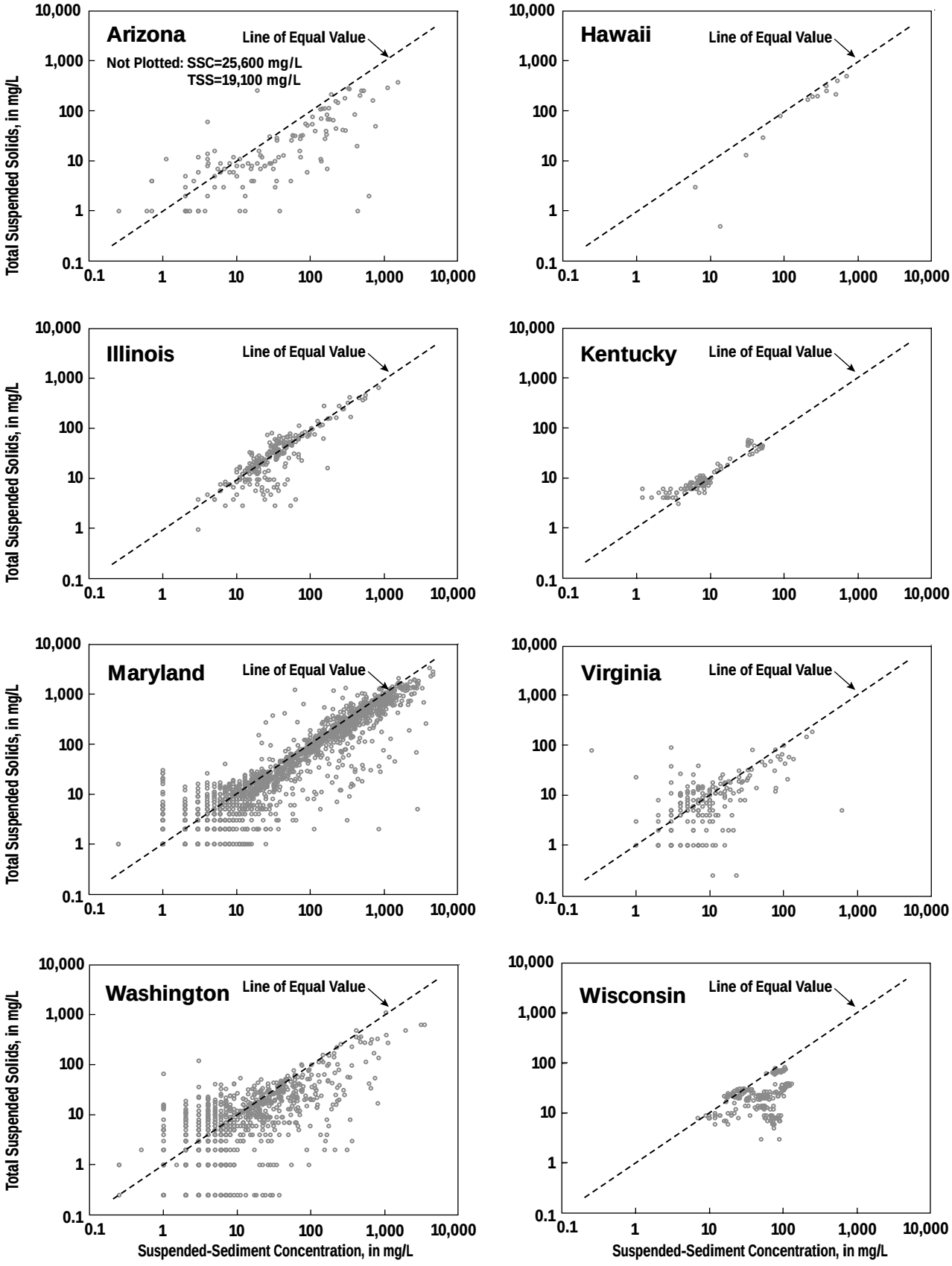

Figure 4. Relation between the base-10 logarithms of suspended-sediment concentration (SSC) and total suspended solids (TSS) for the data pairs from each State used in the analysis. All SSC and TSS values less than 0.25 mg/L were set equal to 0.25 mg/L to enable plotting the data on logarithmic coordinates.

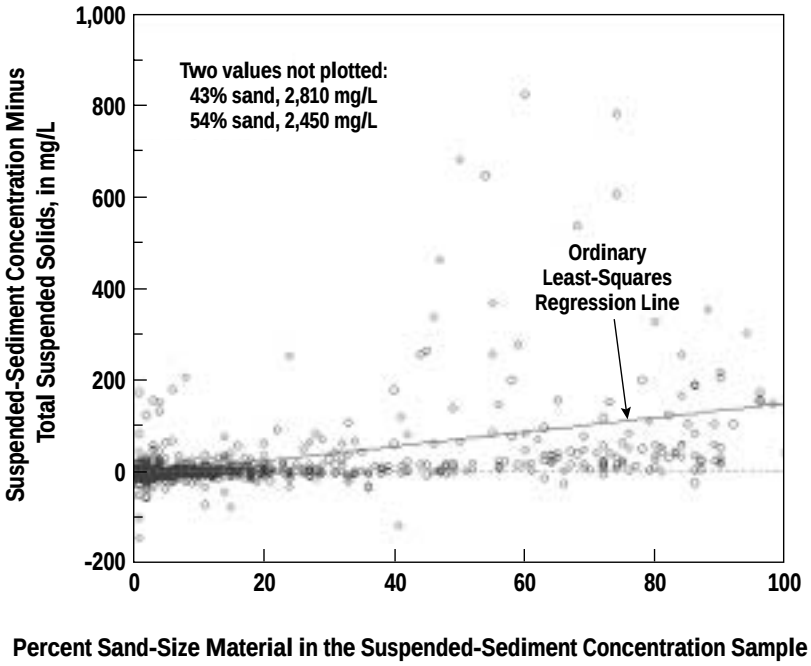

Figure 5. Relation between percent sand-size material in the sample analyzed for suspended-sediment concentration and the remainder of suspended-sediment concentration minus total suspended solids.

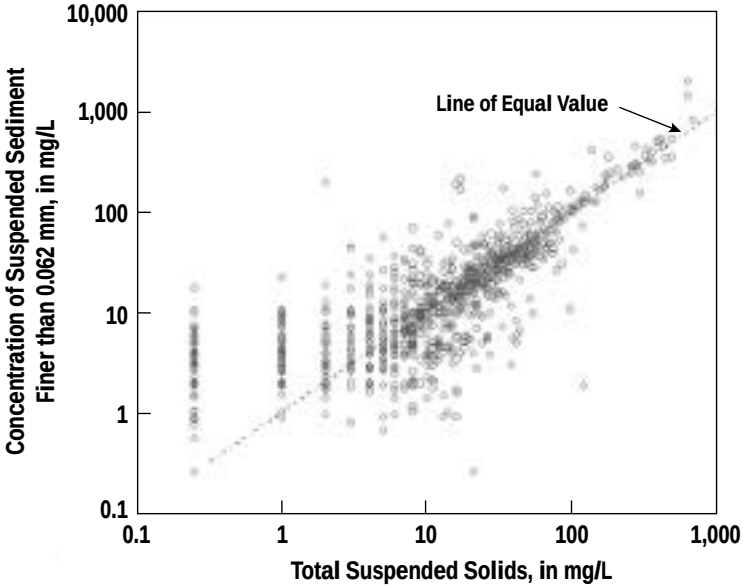

Figure 6. Relationship between total suspended solids and the concentration of suspended sediments finer than 0.062mm in paired suspended-sediment concentration samples. All SSC and TSS values less than 0.25 mg/L were set equal to 0.25 mg/L to enable plotting the data on logarithmic coordinates.

the suspended solid-phase discharges compared to those estimates that are computed from SSC data. TSS discharge underestimates may be negligible for streams conveying a predominantly fine material load over the range of discharges. Substantial underestimates of TSS discharges can be expected for streams conveying sediment loads that exceed about a third sand-size material in composition, and with percentages and concentrations of sand-size material that increase with discharge.

Figure 7 shows an example of the influence of bias resulting from using TSS data to calculate instantaneous sediment discharges for a stream in the northeastern United States. All the TSS and SSC samples used to compute sediment discharges from October 15 through December 24, 1998 were collected by a cooperating agency using an open bottle and analyzed by the cooperator's laboratory. The apparent order-of-magnitude change in sediment discharges between November and December 1998 was not related to any in-stream change in solid-phase transport, but to a change in analytical procedures (Henry Zajd, Jr., U.S. Geological Survey, oral commun., 2000). TSS analyses were performed on all samples collected in October and November 1998, and SSC analyses were used to produce subsequent data. The USGS did not publish daily sediment discharges for the pre-December period shown in figure 7 because the TSS data used in the computations were considered unreliable.

Quality-Control Data

Box plots that show the results of quality-control samples analyzed for SSC and TSS by a cooperating laboratory participating in the USGS National Sediment Laboratory Quality Assurance Program are shown in figure 8. The samples were analyzed in five sample sets. Box plots for sample sets 1997-1, 1997-2, and 1998-1 represent TSS analytical results. Box plots for study sample sets 1998-2 and 1999-1 represent SSC analytical results. This figure illustrates two important characteristics related to sediment-data quality.

First, both the SSC and TSS data tend to be negatively biased. The combined data for all samples analyzed as part of the Sediment Laboratory Quality Assurance Program from 1996 through September 2000 have a median concentration bias of -1.83 percent; the 25th percentile is -4.39 percent; and the 75th percentile is 0.00 percent. The bias primarily reflects a loss of some sediment, such as through a filter, or an inability to weigh accurately very small amounts of fine material in the SSC analytical procedure. The SSC median percent bias values for both study sets are about -2 and -4 percent of the known sediment mass. In contrast, TSS median percent bias values for the three study sets range from -6 to -23 percent from the known sediment mass; the mean difference in TSS median percent bias from the known sediment mass is -16 percent. Only for sample set 1997-2 does any quartile include the TSS value for the known sediment mass. The median percent bias in TSS sample set 1997-1 and in 1998-1 exceeds three F-pseudosigmas[2] from the mean value of all measured sediment mass measurements reported in the USGS National

[2]The F-pseudosigma is a nonparametric statistic analogous to the standard deviation that is calculated by using the 25th and 75th percentiles in a data set. It is resistant to the effect of extreme outliers.

Sediment Laboratory Quality Assurance Program. The analytical method used by the laboratory for determination of TSS in natural-water samples was deemed unacceptable by the U.S. Geological Survey (USGS, 1999b).

Second, the variances associated with the TSS quality-control data are large compared to those for SSC data (figure 8). The least variable data – those from sample set 1997-1 – range from -18 to -32 percent of the known value, and the difference between the 1st and 3rd quartile values is 9 percent. In comparison, the most variable SSC data – those from sample set 1999-1 – range from 0 to -5 percent; the difference in the 1st and 3rd quantile values is 4 percent.

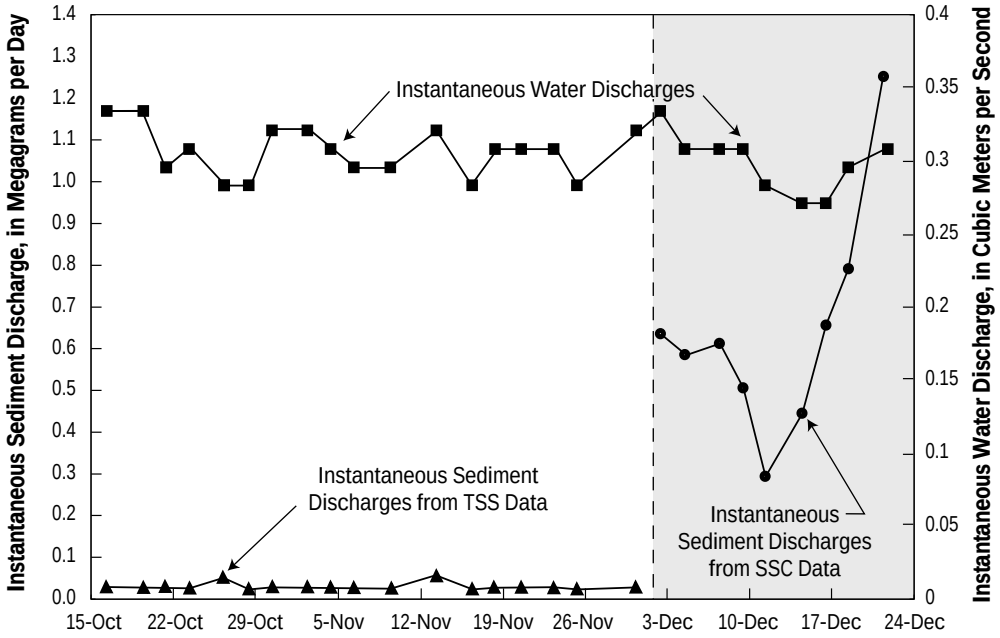

Figure 7. Instantaneous water discharges, and sediment discharges computed from total suspended solids (TSS) and suspended-sediment concentration (SSC) data for a stream in the northeastern United States, 1998.

In terms of bias and variance, the TSS results from two of the first three sample sets – 1997-1 and 1998-1 – were considered unacceptable by the U.S. Geological Survey (U.S. Geological Survey, 1998; 1999a). The SSC results from study sample sets 1998-2 and 1999-1, which were produced by the same laboratory, are considered among the most accurate of all laboratories that participated in the USGS National Sediment Laboratory Quality Assessment Program (John Gordon, U.S. Geological Survey, oral commun., 2000).

CONCLUSIONS

Of the two analytical methods examined for measuring the mass of solid-phase material in natural-water samples — suspended-sediment concentrations (SSC), and total suspended solids (TSS), — data produced by the SSC technique are the more reliable. This is particularly true when the amount of sand in a sample exceeds about a quarter of the dry sediment mass. This conclusion is based on the following observations:

1. The SSC analytical procedure entails measurement of the entire mass of sediment and the net weight for the entire sample. In contrast, only a part of the water-sediment mixture is typically used in the TSS analysis. Difficulties in, and variations for methods associated with obtaining TSS subsamples can result in determinations of solid-phase characteristics that are substantially different from those of the original sample.

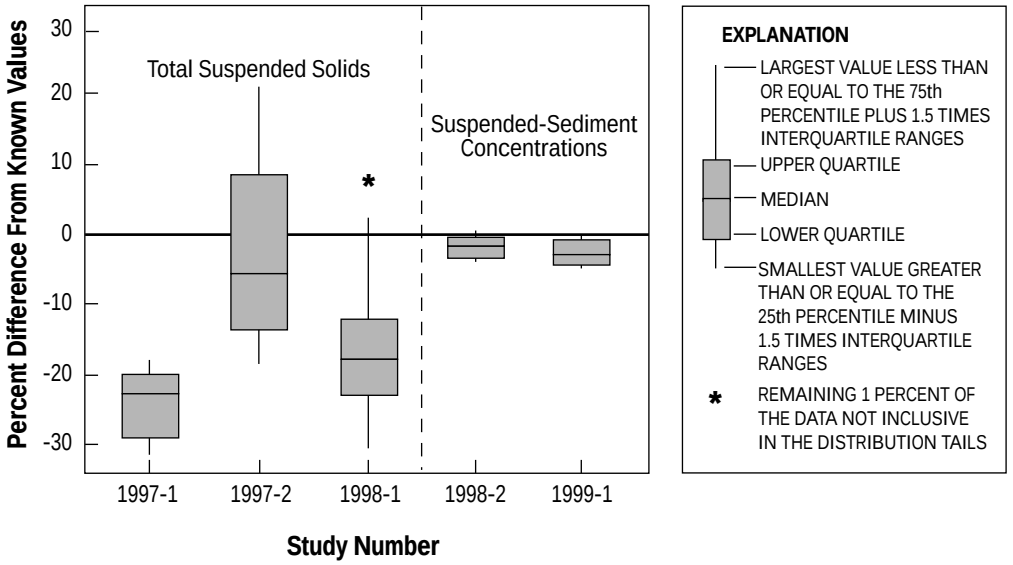

Figure 8. Variability in results of suspended-sediment concentrations and total suspended solids analytical methods in quality-control water samples analyzed by a cooperator laboratory. (John D. Gordon, U.S. Geological Survey, written commun., 2000).

2. Subsampling by pipette or by pouring from an open container will generally result in production of a sediment-deficient subsample. An analysis of 3,235 paired SSC and TSS natural-water samples from eight States showed that SSC values tend to exceed their paired TSS values, particularly at larger values of SSC. This is consistent with the assumption that most subsamples used to determine the TSS data were obtained by pipette or by pouring from an open container.

3. An analysis of 860 paired SSC and TSS natural-water samples for which relative amounts of sand-size and finer material are known for the SSC sample were used to determine the effect of sand-size particles on the TSS analysis. SSC values tend to be larger than their paired TSS values as the percentage of sand-size material exceeds about a quarter of the mass of sediment in the sample. Additionally, a relation between values of TSS and the paired SSC material finer than 0.062 mm showed that for samples with TSS values exceeding about 5 mg/L, the paired SSC and TSS data are more or less evenly distributed around the line of equal value. Sand-size material is more difficult to subsample than finer material due to the large fall velocity of sand-size material as described by Stokes' Law.

The tendency for SSC values to exceed their paired TSS values has important ramifications for computations of suspended solid-phase discharges; those computed using TSS data will often underestimate solid-phase discharges. This is particularly true for sites when the percentages of sand-size material in the water samples exceed about a third and where concentrations and percentages of sand-size material in transport increase with flow.

4. Fifty-three quality-control samples from a cooperator's laboratory — three sample sets totaling 35 TSS analyses of subsamples obtained by pouring from original samples, and two sample sets totaling 18 SSC analyses — were used to compare bias and variance introduced by use of the TSS and SSC analytical methods. Two of the three sample sets analyzed for TSS had unacceptably large mean negative bias. Variances associated with all three TSS sample sets were at least double those associated with the SSC quality-control results from the same laboratory. The two SSC sample sets analyzed by the same laboratory had small variances compared with those for the three TSS sample sets. The slight negative bias values associated with the SSC sample sets were consistent with data analyzed by most laboratories participating in the USGS National Sediment Laboratory Quality Assurance Program.

5. Review of the literature indicates that the TSS method originated as an analytical method for wastewater, presumably for samples collected after a settling step at a wastewater treatment facility. The results of this evaluation do not support use of the TSS method to produce reliable concentrations of solid-phase material in natural-water samples. The TSS method is being misapplied to samples from natural water.

Some SSC and TSS data may be comparable, particularly when the percentage or amount of sand-size material in the sample is less than about 25 percent. TSS values from analyses of samples collected following a settling step for coarser sediments, such as those obtained for compliance purposes at sewage treatment plants and water treatment facilities, may be reliable. However, because relatively few TSS data are associated with the percent sand-size and finer material from SSC samples, it is usually impossible to identify which if any TSS data may be biased. Some of the TSS data may reflect the mass of suspended solids in natural-water samples, but there are currently no absolute means to identify those data, nor a generally reliable procedure to correct biased TSS data.

The TSS method, which was originally designed for analyses of wastewater samples, is shown to be fundamentally unreliable for the analysis of natural-water samples. In contrast, the SSC method produces relatively reliable results for samples of natural water, regardless of the amount or percentage of sand-size material in the samples. SSC and TSS data collected from natural water are not comparable and should not be used interchangeably. The accuracy and comparability of suspended solid-phase concentrations of the Nation's natural waters would be greatly enhanced if all these data were produced by the SSC analytical method.

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U.S. Geological Survey, Reston, Virginia
John R. Gray, Hydrologist/Sediment Specialist
U.S. Geological Survey, Office of Surface Water
415 National Center, 12201 Sunrise Valley Drive
Reston, VA 20192
703/648-5318, FAX 648-5295, jrgray@usgs.gov

EPA Regions 8, 9 and 10 Toxicity Training Tool

January 2010

ACKNOWLEDGMENTS

Principal Authors:

Debra Denton, USEPA Region 9
Jeff Miller, AQUA-Science
Robyn Stuber, USEPA Region 9

Document citation is: Denton DL, Miller JM, Stuber RA. 2007. EPA Regions 8, 9 and 10 toxicity training tool (TTT). January 2010. San Francisco, CA.

PDF files are located at NPDES for EPA R9 Water:
http://www.epa.gov/region09/water/npdes/index.html

Document update on January 2010 revision is solely to incorporate the inclusion of EPA Region 8. No edits have been made in this document.

Contributing Authors and Reviewers:

Rob Burgess, USEPA Office of Research and Development
Kari Fleming, Wisconsin Department of Natural Resources
Stephanie Fong, Central Valley Regional Water Quality Control Board
John Fox, USEPA Office of Research and Development
Kathryn Greenwald, USEPA Office of Enforcement and Compliance Assurance
Karen Larsen, California Central Valley Regional Water Quality Control Board
Regina Linville, California State Water Resources Control Board
VelRey Lozano, USEPA Region 8
Randy Marshall, Washington Department of Environmental Quality
Kimberley Miller, AQUA-Science
Denise Nelson, Arizona Department of Environmental Quality
Bryn Phillips, University of California Davis
Richard Remigio, USEPA Region 9
Patti TenBrook, USEPA Region 9
Amy Wagner, USEPA Region 9
Tom Waller, University of North Texas
Sharon Wilson, USEPA Region 10

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EXECUTIVE SUMMARY

The U.S. Environmental Protection Agency's Pacific Southwest Region and Pacific Northwest Region (EPA Regions 9 and 10) have developed a Whole Effluent Toxicity (WET) Technical Training Tool for implementing WET in National Pollutant Discharge Elimination System (NPDES) permitting programs and Clean Water Act surface water quality monitoring programs. This training tool is designed for use with the EPA regional guidance document, *Regions 9 and 10 Guidance for Implementing Whole Effluent Toxicity* (Denton and Narvaez 1996), and EPA's national guidance document, *Technical Support Document for Water Quality-based Toxics Control* (USEPA 1991a). Since 1996, EPA has issued several important national guidance documents addressing WET implementation, and promulgated updated biological methods for acute and chronic toxicity at 40 CFR 136. These publications are described in the documentation for this training tool in order to provide a concise summary of current EPA program documents and regulations for WET. As such, this tool provides the basis for technical training on the topic of WET for EPA Regions 9 and 10. It is being made available for use by other EPA Regions and States (including Tribes and Territories) seeking basic training on the topic of WET for NPDES permitting and ambient water quality monitoring. This training tool is divided into seven topics with training slides and accompanying documentation (chapters and appendices). The training slides are provided in a Microsoft PowerPoint format. The topics covered are:

- Introduction to WET
- Developing WET Permit Conditions
- Chronic and Acute Toxicity Testing
- Test Review and Evaluation of Test Results
- Toxicity Reduction Evaluations
- Ambient Toxicity Testing and Watershed Assessment
- Enforcement Procedures for WET

This training tool is designed to assist EPA Regions and States implementing existing national policy on WET. It does not substitute for the Clean Water Act, or EPA or State regulations applicable to NPDES permits or WET testing; nor is this document a regulation, itself. This training tool does not impose legally binding requirements on EPA, States, or NPDES permittees, and may not apply in site-specific situations based upon the circumstances. EPA Regions 9 and 10 will change this training tool in the future, as appropriate. Those seeing more information on WET are referred to the documents referenced in this training tool, EPA's webpage at http://www.epa.gov/ (Search: whole effluent toxicity, NPDES, etc.), and EPA's national and regional WET Program staffs.

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LIST OF ACRONYMS AND ABBREVIATIONS

‰	parts per thousand
α	alpha error
β	beta error
AA	atomic absorption
ACR	acute-to-chronic ratio
AML	average monthly limit
ANOVA	analysis of variance
APC	areas of probable concern
APO	administrative penalty order
AO	administrative order
ASTM	American Society for Testing and Materials
AVS	acid volatile sulfide
AWL	average weekly limit
BMP	best management practices
BSAB	Biomonitoring Science Advisory Board
CABW	California aquatic bioassessment workshop
CAMLnet	California Aquatic Macroinvertebrate Laboratory Network
CCC	criteria continuous concentration
CDFG	California Department of Fish and Game
CFR	Code of Federal Regulations
CFS	cubic feet per second
CMC	criteria maximum concentration
CSBP	California stream bioassessment protocol
CV	coefficient of variation
CWA	Clean Water Act
DMR	discharge monitoring report
DO	dissolved oxygen
DOC	dissolved organic carbon
DQO	data quality objective

EC	effect concentration, e.g., EC_{25}, EC_{50}
EDTA	ethylenediamine tetraacetic acid
EMS	Enforcement Management System
EPA	U.S. Environmental Protection Agency (also, the Agency)
FAQ	frequently asked questions
FIFRA	Federal Insecticide, Fungicide, Rodenticide Act
FR	Federal Register
GC	gas chromatography
H_0	null hypothesis
H_a	alternative hypothesis
HPLC	high performance liquid chromatography
IC	inhibition concentration, e.g., IC_{25}, IC_{50}
ICP	ion-coupled plasma
IWC	instream waste concentration (sometimes referred to as receiving water concentration)
IWS	industrial waste surveys
LC	lethal concentration
LOEC	lowest observed effect concentration
LSTE	list of standard taxonomic effort
LOV	letter of violation
LTA	long-term average (LTAa = acute LTA; LTAc = chronic LTA; LTAa,c = acute-to-chronic LTA)
MGD	million gallons per day
MDL	maximum daily limit
MML	median monthly limit
MQO	measurement quality objective
MS	mass spectrometry
MSD	minimum significant difference
MSE	mean square error
NOAEC	no observed adverse effect concentration
NOEC	no observed effect concentration

NOV	notice of violation
NPDES	National Pollutant Discharge Elimination System
OWM	Office of Wastewater Management
OST	Office of Science and Technology
PBO	piperonyl butoxide
PMSD	percent minimum significant difference
POTW	Publicly owned treatment works
QA	quality assurance
QAPP	quality assurance project plan
QC	quality control
RBP	rapid bioassessment protocol
RP	reasonable potential
RWC	receiving water concentration (sometimes referred to as instream waste concentration)
RWQCB	Regional Water Quality Control Board
SEM	simultaneously extracted metals
SETAC	Society of Environmental Toxicology and Chemistry
SFEI	San Francisco Estuary Institute
SI	stressor identification
SMWP	State Mussel Watch Program
SOP	standard operating procedure
SWAMP	Surface Water Ambient Monitoring Program
SWRCB	State Water Resources Control Board
TAC	test acceptability criteria
TIE	toxicity identification evaluation
TDS	total dissolved solids
TMDL	total maximum daily load
TOC	total organic carbon
TRE	toxicity reduction evaluation
TSD	EPA's Technical Support Document for Water Quality-based Toxics Control

TU	toxic unit (TUa = acute toxicity; TUc = chronic toxicity)
USEPA	United States Environmental Protection Agency
USGS	Unites States Geological Survey
WET	whole effluent toxicity
WLA	waste load allocation
WQBEL	water quality based effluent limit
WQC	water quality criteria
WQS	water quality standards

Note: These acronyms and abbreviations may have other meanings in other EPA programs or documents.

DEFINITIONS

Acute-to-Chronic Ratio (ACR) is the ratio of the acute toxicity of an effluent or a toxic to its chronic toxicity. It is used as a factor for estimating chronic toxicity on the basis of acute toxicity data, or for estimating acute toxicity on the basis of chronic toxicity data.

Acute Toxicity Test is a test to determine the concentration of effluent or ambient waters that causes an adverse effect (usually death) on a group of test organisms during a short-term exposure (e.g., 24, 48, or 96 hours). Acute toxicity is measured using statistical procedures (e.g., point estimate techniques or a hypothesis test).

Ambient Toxicity is measured by a toxicity test performed using solely receiving water.

Average Monthly Limit (AML) is the highest allowable average of "daily discharges" over a calendar month, calculated as the sum of all "daily discharges" measured during a calendar month divided by the number of "daily discharges" measured during that month.

Chronic Toxicity Test is a short-term test, usually 96 hours or longer in duration, in which sublethal effects (e.g., significantly reduced growth, reproduction) are usually measured in addition to lethality. Chronic toxicity is defined as TUc = 100/NOEC or TUc = 100/ECp or ICp.

Coefficient of Variation (CV) is a standard statistical measure of the relative variation of a distribution or set of data, defined as the standard deviation divided by the mean. It is also called the relative standard deviation (RSD). The CV can be used as a measure of precision within (within-laboratory) and between (between-laboratory) laboratories, or among replicates for each treatment concentration.

Confidence Interval is the numerical interval constructed around a point estimate of a population parameter.

Criterion Continuous Concentration (CCC) is the highest in-stream concentration of a toxic or an effluent to which organisms can be exposed indefinitely without causing unacceptable effects such as the exceedance of a chronic water quality criterion.

Criterion Maximum Concentration (CMC) is the highest in-stream concentration of a toxic or an effluent to which organisms can be exposed for a brief period of time without causing an acute effect such as the exceedance of an acute water quality criterion.

Daily Discharge is the discharge of a pollutant measured during a calendar day or any 24-hour period that reasonably represents the calendar day for purposes of sampling.

Discharge Monitoring Report (DMR) is EPA's standardized reporting form for the reporting of self-monitoring results by permittees. DMRs must be used by "NPDES-approved States," as well as by EPA. States with NPDES programs may modify the EPA standardized forms to substitute the State agency's name, address, logo, and other similar information, as appropriate, in place of EPA's.

Effect Concentration (EC) is a point estimate of the toxicant concentration that would cause an observable adverse effect (e.g., death, immobilization, or serious incapacitation) in a given percent of the test organisms, calculated from a continuous model (e.g., Probit Model). EC_{25} is a point estimate of the toxicant concentration that would cause an observable adverse effect in 25 percent of the test organisms.

Effluent Flow (Q_e) is the flow (in cubic feet per second or million gallons per day) of a wastewater discharge from a facility expressed in standard NPDES formulas used by permit writers as "Q_e" to calculate water quality based effluent limits.

Endpoint is a biological measurement used to quantify the results obtained from analytical methods such as whole effluent toxicity testing [e.g., lethal concentration (LC_{50}); inhibition concentration (IC_{25}); and no observed effect concentration (NOEC)]. Such endpoints are quantitative measurements of the responses of test organisms (e.g., survival, growth, mobility, reproduction, and weight gain or loss) in response to exposure to a serial dilution of effluent.

Hypothesis Testing is a statistical technique (e.g., Dunnett's test) for determining whether a tested concentration results in a statistically different response from that observed in the control. For the multi-concentration tests, the reported values determined by hypothesis testing are the "no observed effect concentration (NOEC)" and "lowest observed effect concentration (LOEC)." The two hypotheses commonly tested in WET are:

> **Null hypothesis (H_0)**: The effluent is not toxic.
> **Alternative hypothesis (H_a)**: The effluent is toxic.

Inhibition Concentration (IC) is a point estimate of the toxicant concentration that would cause a given percent reduction in a non-lethal biological measurement (e.g., reproduction or growth), calculated from a continuous model (i.e., Interpolation Method). IC_{25} is a point estimate of the toxic concentration that would cause a 25-percent reduction in a non-lethal biological measurement.

Instream Waste Concentration (IWC) is the concentration of a toxicant in the receiving water after mixing. It is also referred to as the receiving water concentration (RWC).

Lethal Concentration, 50 Percent (LC_{50}) is the toxic or effluent concentration that would cause death in 50 percent of the test organisms over a specified period of time.

Long-term Average (LTA) of pollutant concentration or effluent toxicity is calculated from a wasteload allocation (WLA), typically assuming that the WLA is a 99^{th} percentile value (or another upper bound value) based on the lognormal distribution. One LTA is calculated for each WLA (typically an acute LTA and a chronic LTA for aquatic life protection). The LTA represents expected long-term average performance from the permitted facility required to achieve the associated WLA.

Lowest Observed Effect Concentration (LOEC) is the lowest concentration of an effluent or test sample with an effect different from the control effect according to the statistical test used for analysis of toxicity that results in adverse effects on the test organisms (i.e., where the values for the observed endpoints statistically differ from the control).

Maximum Daily Limit (MDL) is the highest allowable discharge measured during a calendar day or 24-hour period representing a calendar day.

Median is the value of the middle score in the distribution.

Median Monthly Limit (MML) is the highest allowable median of "daily discharges" over a calendar month, calculated as the middle value of all "daily dischargers" measured during a calendar month.

Minimum Significant Difference (MSD) is a measure of test sensitivity that establishes the minimum difference required between a control and a test treatment in order for that difference to be considered statistically significant.

Mixing Zone is an area where an effluent discharge undergoes initial dilution with water from upstream and is extended to cover the secondary mixing in the ambient waterbody; an allocated impact zone in which water quality criteria can be exceeded provided that acutely toxic conditions are prevented. States determine whether mixing zones are allowed.

National Pollutant Discharge Elimination System (NPDES) is the EPA program that regulates discharges to the nation's waters. Discharge permits issued under the NPDES program are required by EPA regulation to contain, where necessary, effluent limits based on water quality criteria for the protection of aquatic life and human health.

No Observed Effect Concentration (NOEC) is the highest tested concentration of an effluent or toxicant that causes no observable adverse effect on the test organisms (i.e., the highest concentration of toxicant at which the values for the observed responses are not statistically different from the controls).

Percent Minimum Significant Difference (PMSD) is the minimum significant difference divided by the control mean, expressed as a percent (see minimum significant difference).

Point Estimate is a statistical inference that estimates the true value of a parameter by computing a single value of a statistic from a set of sample data.

Power is the probability of correctly detecting an actual toxic effect (i.e., declaring an effluent toxic when, in fact, it is toxic).

Precision is a measure of reproducibility within a data set. Precision can be measured both within a laboratory (within-laboratory) and between laboratories (between-laboratory) using the same test method and toxicant.

Publicly Owned Treatment Works (POTWs) are facilities, operated by local communities or States or their contractors, that treat domestic wastewater or wastewater from indirect dischargers (e.g., industrial facilities).

Quality Assurance (QA) is a practice in toxicity testing that addresses all activities affecting the quality of the final effluent toxicity data. QA includes evaluation of effluent sampling and handling, source and condition of test organisms, equipment condition, test conditions, instrument calibration, replication, use of reference toxics, record keeping, data, and other aspects of the test and testing procedures.

Quality Control (QC) is the set of focused, routine, day-to-day activities carried out as part of an overall QA program.

Reasonable Potential (RP) is the likelihood that an effluent will cause or contribute to an excursion above a water quality standard based on a number of factors, including the use of data (e.g., whole effluent toxicity test data). In the context of this document, references to RP and WET limits include both lethal and sublethal effects.

Reasonable Potential Multiplier Factor (RPMF) is a numerical value that multiplies the maximum observed effluent value in an effluent data set.

Receiving Water Concentration (RWC) is the concentration of a toxic in the receiving water after mixing, sometimes referred to as the in-stream waste concentration (IWC).

Receiving Water Flow (Q_s) is the flow of the water receiving the discharge expressed in cubic feet per second or millions gallon per day.

Reference Toxicant Test is a check of the sensitivity of the test organisms and the suitability of the test methodology in a toxicity test. Reference toxicant data are part of a routine QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

Significant Difference is defined as a statistically significant difference (e.g., 95 percent confidence level) in the means of two distributions of sampling results.

Standard Deviation is a measure of the variability of a set of data, calculated as the square root of the variance.

Statistic is a computed or estimated quantity, such as the mean, standard deviation, or coefficient of variation.

Test Acceptability Criteria (TAC) are specific criteria for determining whether toxicity test results are acceptable, pursuant to EPA's WET test methods in 40 CFR 136 (additional TAC may be established by a State Permitting Authority). The effluent and reference toxicant must meet specific criteria as defined in the test method (e.g., for the *Ceriodaphnia dubia* survival and reproduction test, the criteria are: 80% or greater survival of all control organisms and an

average of 15 or more young per surviving female in the control solution. Of the surviving control females, 60% must produce three broods.)

Total Maximum Daily Load (TMDL) is the allocation of the pollutant load to each source, which is calculated by estimating the maximum amount of a pollutant that a waterbody can receive and still meet water quality standards.

t-Test (formally Student's t-test) is a statistical analysis comparing two sets of replicate observations, in the case of WET, only two test concentrations (e.g., a control and 100% effluent). The purpose of this test is to determine if the means of the two sets of observations are different [e.g., if the 100% effluent concentration differs from the control (i.e., the test pass or fails)].

Toxicity Test is a procedure using living organisms to determine whether a chemical or an effluent is toxic. A toxicity test measures the degree of the effect of a specific chemical or effluent on exposed test organisms.

Toxic Unit (TU) is a measure of toxicity in an effluent as determined by the acute toxicity units (TUa) or chronic toxicity units (TUc) measured. The larger the TU, the greater the toxicity.

Toxic Unit - Acute (TUa) is 100 times the reciprocal of the effluent concentration that causes 50 percent of the organisms to die in an acute toxicity test ($TUa = 100/LC_{50}$) (see LC_{50}).

Toxic Unit - Chronic (TUc) is 100 times the reciprocal of the effluent concentration that causes no observable effect on the test organisms in a chronic toxicity test ($TUc = 100/NOEC$ or $100/EC_{25}$) (see NOEC).

Toxicity Identification Evaluation (TIE) is a set of site-specific procedures used to identify the specific chemical(s) causing effluent toxicity.

Toxicity Reduction Evaluation (TRE) is a site-specific study conducted in a step-wise process to identify the causative agents of effluent toxicity, isolate the source of toxicity, evaluate the effectiveness of toxicity control options, and then confirm the reduction in effluent toxicity after the control measures are put in place.

Type I Error (alpha) is the rejection of the null hypothesis (H_0) when it is, in fact true (i.e., determining that the effluent is toxic when the effluent is not toxic).

Type II Error (beta) is the acceptance of the null hypothesis (H_0) when it is not true (i.e., determining that the effluent is not toxic when the effluent is toxic). Beta is related to the power of the test.

Variance is a measure of the dispersion in a set of values, defined as the sum of the squared deviations from the mean divided by the total number of values in the set.

Wasteload Allocation (WLA) is the portion of a receiving water's TMDL that is allocated to one of its existing or future point sources of pollution.

Water Quality Criteria are numeric scientifically derived ambient concentrations developed by EPA or States for various pollutants of concern to protect human health and aquatic life. Narrative criteria typically are statements that describe the desired water quality goal.

Water Quality-based Effluent Limit (WQBEL) is a NPDES permit limit established by either an EPA or a State permit writer that is developed to assure protection of aquatic life or human health consistent with applicable State or Tribal water quality standards, including the designated uses for a particular waterbody, the established criteria, and measured analytical data (e.g., chemical, WET or biosurvey), in accordance with the recommendations provided in EPA's 1991 Technical Support Document (TSD).

Water Quality Standard (WQS) Water quality standards are provisions of State or Federal law which consist of a designated use or uses for the waters of the United States and water quality criteria for such waters based upon such uses. Water quality standards are to protect the public health or welfare, enhance the quality of water, and serve the purposes of the Act. States and authorized Tribes are required to develop and adopt a statewide antidegradation policy and identify the methods for implementing the policy.

Whole Effluent Toxicity (WET) is the total toxic effect of an effluent measured directly with a toxicity test.

WET Permit Limit is the water quality-based effluent limit for WET, established by either an EPA or State permit writer, that is used to trigger accelerated WET monitoring and TREs.

WET Permit Trigger is a threshold level for WET in an NPDES permit, established by either an EPA or State permit writer, this is used to trigger accelerated WET monitoring and TREs when there is no reasonable potential for WET and no WET permit limit.

CHAPTER 1. INTRODUCTION

1.1 Overview

This chapter briefly describes the background and history of the whole effluent toxicity (WET) testing program and use of the integrated strategy to achieve and maintain water quality standards required by the U.S. Environmental Protection Agency (EPA).

1.2 Background

The Federal Water Pollution Control Act, commonly known as the Clean Water Act (CWA), was enacted in 1972 with the objective to "*restore and maintain the chemical, physical, and biological integrity of the Nation's waters*." In order to achieve this objective, goals and policies were established in the Act, including:

- Eliminating the discharge of pollutants into navigable waters by 1985;
- Wherever attainable, achieving an interim goal of water quality which provides for the protection and propagation of fish, shellfish, and wildlife, and provides for recreation in and on the water by July 1, 1983; and
- Prohibiting the discharge of toxic pollutants in toxic amounts.

In the 35 years since the CWA was enacted, the EPA and States authorized to administer EPA's National Pollutant Discharge Elimination System (NPDES) permitting program have made significant progress toward achieving these goals and policies. Under the EPA's integrated water quality-based "standards to permits" approach for toxics control, NPDES permits are designed to achieve and maintain water quality standards. A point source that discharges pollutants to surface waters of the United States must do so under the limitations and conditions of an NPDES permit. In setting these limitations and conditions, the EPA and States protect aquatic life using three control approaches discussed in the Technical Support Document for Water Quality-based Toxics Control (USEPA 1991a, referred to as the TSD):

- Chemical-specific control approach,
- WET control approach, and
- Biological criteria/bioassessment and biosurvey approach.

A detailed discussion of the capabilities and limitations of these three approaches is provided in Section 1.5 of the TSD (USEPA 1991a). Since each approach has unique as well as overlapping attributes, sensitivities, and program applications, no single approach for detecting impact should be considered uniformly superior to any other approach. An integrated approach to water quality-based toxics control is essential for a strong toxics control program.

The WET control approach to water quality protection is the primary subject of this document.

EPA defines whole effluent toxicity as "*the aggregate toxic effect of an effluent measured directly by an aquatic toxicity test*" [54 Federal Register (FR) 23868 at 23895, June 2, 1989]. Aquatic toxicity tests are laboratory experiments that measure the biological effect (e.g., survival, growth, and reproduction) of effluents or receiving waters on aquatic organisms. In aquatic toxicity tests, groups of organisms of a particular species are held in test chambers and exposed to different concentrations of an aqueous test sample (e.g., reference toxicant, effluent, or receiving water). Observations are made at predetermined exposure periods. At the end of the test, the responses of test organisms are used to estimate the effects of the aqueous sample.

Beginning in the 1980s, EPA published methods (USEPA 1985a, 1988, 1989a) for estimating the acute and chronic toxicity of effluents and receiving waters to freshwater and marine organisms. WET data gathered in the 1980s indicated that approximately 40 percent of NPDES facilities nationwide discharged effluent with sufficient toxicity to cause water quality problems. Further reductions in the toxicity of NPDES effluents were needed to comply with State narrative "free from toxics in toxic amounts" water quality criteria. Responding to these findings, EPA implemented a national policy for assessing and controlling the discharge of toxic substances to ensure protection of water quality. The *Policy for the Development of Water Quality-Based Permit Limitations for Toxic Pollutants* (49 FR 9016, March 9, 1984) introduced EPA's integrated toxics control program and recommend both chemical-specific analyses and biological techniques to assess effluent discharges and express permit limitations. To support this policy, EPA developed new regulations governing water quality-based permitting in the NPDES program (54 FR 23868, June 2, 1989) and the TSD (USEPA 1991a). Originally published in 1985 and updated in 1991, the TSD provides national guidance to Permitting Authorities implementing WET testing in NPDES permits.

On October 16, 1995, EPA promulgated WET test methods (USEPA 1993a, 1994a, 1994b, 1999a) and added them to the list of EPA methods approved under Section 304(h) of the CWA (40 CFR 136) for use in the NPDES program. These methods were subsequently challenged and under a settlement agreement, EPA conducted a round-robin study which evaluated 12 of the test methods (USEPA 2001a, 2001b). EPA also prepared a WET test methods guidance document (USEPA 2000a) and a WET test method variability guidance document (USEPA 2000b). On November 19, 2002, EPA promulgated revised WET test methods (USEPA 2002a, 2002b, 2002c) [67 FR 69952, November 19, 2002]. These methods were also challenged and ultimately, the U.S. Court of Appeals upheld the validity of the WET test methods against a variety of constitutional, statutory, and administrative law challenges. In *Edison Electric Institute et al. v. EPA*, 391 F.3d 1267 (D.C. Cir. 2004), the Court found that:

- EPA reasonably validated the standardized testing procedures, including their precision and bias, as well as their high rates of successful test completion.
- The methods did not produce unacceptably variable results.
- The method procedures (i.e., replication and comparison to controls) adequately compensated for the inability to determine a method detection limit, and
- The results produced with methods were representative of receiving water toxicity, including receiving waters of the arid West.

It is the position of EPA Regions 9 and 10 that WET test methods yield reproducible and precise results. WET testing plays a vital role in water pollution control programs by regulating complex mixtures of chemicals and helping to identify toxicity in wastewater effluents, stormwater, and ambient waters. We have summarized frequently asked questions (FAQs) to assist Permitting Authorities implementing WET programs (see Appendix A).

1.3 EPA's Integrated Strategy

Based on the stated goals of the CWA, the EPA and individual States implement three approaches to protect water quality. These approaches include chemical-specific control, toxicity testing control, and biological criteria/bioassessments (USEPA 1991a). This document only addresses the protection of aquatic life, not human health. Each of the three control approaches has advantages and limitations.

The chemical-specific approach involves the development of water quality criteria (WQC) for chemicals as expressed in terms of the acute criterion and the chronic criterion. These criteria are developed following EPA water quality guidelines (USEPA 1985b). EPA has developed water quality criteria for the 126 priority pollutants as required under CWA Section 308. These WQC are based on minimum data requirements that include both acute and chronic toxicity tests with the specified numbers and types of aquatic species. WQC are intended to protect most of the tested species, most of the time. The chemical-specific approach can allow prediction of ecological impacts before they occur. It also considers bioaccumulation and human health impacts. A limitation of the chemical-specific approach is that not all toxicants in wastewaters or aqueous samples may be known, and therefore, control requirements can only be established for those that are known. For mixtures of chemicals with unknown interactions or for chemicals having no chemical-specific criteria, sole use of chemical-specific criteria to safeguard aquatic resources would not be protective. Toxicity testing is needed because the chemical-specific approach only addresses individual chemicals and does not address chemical interactions or chemicals that are not known to be in the effluent. In addition, criteria have been developed for only a limited universe of chemicals. This is why the toxicity testing and bioassessment approaches for protecting aquatic life are also critical components for protection of aquatic resources.

The primary advantage of using the toxicity testing approach is that this tool can be used to assess toxic effects (acute and chronic) of all the chemicals in aqueous samples of effluent, receiving water, or stormwater. This allows the effect of the aqueous mixture to be evaluated, rather than the toxic responses to individual chemicals. Some advantages of WET testing include the toxicity of effluent or ambient water is measured directly for the species tested; the aggregate toxicity of all constituents in a complex effluent is measured; and ecological impacts can be predicted before they occur. Toxicity tests can be used to assess ambient waterbodies (i.e., receiving water) making these tools effective in the assessment of small and large watersheds (de Vlaming et al. 2000). This has been demonstrated by the State of California which has successfully used an ambient toxicity testing approach to identify and regulate frequently occurring toxic chemicals. This approach includes pinpointing critical sampling locations for collecting the ambient waters to be assessed using acute and chronic toxicity tests. If toxicity is detected, then additional samples are collected to determine the spatial and temporal

toxicity patterns. Subsequently, EPA's Toxicity Identification Evaluation (TIE) procedures are used to identify the causative toxicant(s). The goal of the TIE is to identify the chemical(s) causing toxicity in an aqueous sample. This ambient toxicity testing approach has led to the 303(d) listing of chemicals beyond the 126 priority pollutants commonly tested; one such listing is the pesticide diazinon, which is not a priority pollutant (SWRCB 2003). In addition, the approach of toxicity testing in conjunction with TIE analysis may be used to determine chemical interactions. These interactions can be additive, synergistic, or antagonistic. Lydy et al. (2004) provides a synthesis review of challenges in regulating pesticide mixtures and pesticide toxicity to aquatic organisms. Limitations of WET are that it directly measures only the immediate bioavailability of a toxicant(s) in the aqueous sample, and the long-term cumulative toxicity of a compound is not measured.

The bioassessment approach can directly assess the status of a waterbody, since biological communities reflect overall ecological integrity; it provides a holistic measure of the aggregate impact of pollutant stressors and can measure historical trends and fluctuating environmental conditions. The primary advantage of the bioassessment approach is that it integrates both the physical and biological stressor effects on aquatic biota. Biological assessments are based on the premise that the structure and function of an aquatic biological community can provide critical information about the quality of the surface water. The waterbodies being evaluated are assessed and compared to predetermined criteria for impairment and non-attainment of a designated use. The stressor identification (SI) process is a method for identifying biological and physical stressors of the impaired waterbody (USEPA 2000c). The bioassessment approach is limited in that bioassessments conducted at critical low flow conditions can be difficult to accomplish; data may not be sufficient to detect impacts without appropriate reference conditions or suitable biocriteria; the methods detect problems after they have occurred; and causes of impairment may not be assigned readily to any one permittee or other source.

Based on the individual strengths of each of the three approaches (chemical-specific, toxicity testing, and biological criteria/bioassessment) protection of aquatic life will be most thorough if all three approaches are used. If a waterbody is impaired, as measured by any of these three approaches (i.e., WQC are not attained) the CWA requires that impaired waterbodies be listed on the State's 303(d) list and that a total maximum daily load (TMDL) be developed to address the pollutant(s) causing the impairment. The TMDL provides the basis for actions to be taken to restore the water to its designated use.

It is EPA's position that the concept of "independent application" be applied to water quality-based situations (USEPA 1991b). One aspect of the policy expresses that water quality standards are to be independently applied. This means that any single assessment method (chemical criteria, toxicity testing, or biocriteria) can provide conclusive evidence that water quality standards are not attained. Since each method has unique, as well as overlapping attributes, sensitivities, and program applications, no single approach for detecting impact should be considered superior to any other approach. The most protective results from each assessment conducted should be used in the effluent characterization process. EPA regulations at 40 CFR 122.44(d)(1), in effect, require independent application of chemical-specific and whole effluent data and criteria when characterizing effluents and making water quality assessments.

1.4 SETAC Technical Workshop

In September 1995, the Office of Wastewater Management (OWM) and Office of Science and Technology (OST) helped fund a Society of Environmental Toxicology and Chemistry (SETAC) technical workshop on WET. The workshop explored the science involved in WET testing and published a peer-reviewed SETAC book, titled "Whole Effluent Toxicity Testing: An Evaluation of Methods and Prediction of Receiving System Impacts" edited by Grothe, Dickson, and Reed-Judkins (1996). The conclusions are highlighted in Attachment 1-1.

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Attachment 1-1. Pellston Workshop

1. WET exposure methods are technically sound and require no immediate modifications.
2. WET testing is an effective tool for predicting impact in lotic receiving systems. Additional laboratory to field validation is not essential for the continued use of WET testing.
3. The guidance provided in the U.S. EPA's *Technical Support Document for Water Quality Based Toxics Control* must be followed closely to meet the objectives of the WET testing program.
4. A number of problems with WET tests are caused by misapplication of the tests, misinterpretation of data, quality of the WET test laboratory, and the lack of training and experience of laboratory personnel, regulators and permittees.
5. Current WET permit limits have sufficient margins of safety so that episodic exceedances should not cause receiving water impacts. The significance of an exceedance of WET limits depends on receiving water conditions, especially dilution at the time of the exceedance, and the duration of the toxic event.
6. Variability in the use of both WET test methods and bioassessment techniques influences test interpretation and acceptability and the extrapolation of WET test results to field impacts.
7. The largest sources of variability in WET testing are the level of analyst expertise and judgment and test organism condition/ health. Deviation from established methods can be controlled by an effective QA/QC program.
8. Currently used statistical methods are widely used and accepted. However, improvements are available that should be considered.
9. Biological assessment approaches, when properly designed, can accurately assess environmental impact to aquatic biota.
10. Bioassessments are needed to compensate for the limitations of WET tests to predict phytotoxicity, sediment toxicity, bioaccumulation, genotoxicity, indirect biotic effects, and effects of persistent chemicals.
11. In addition to WET testing, results from *in situ* testing, ambient toxicity testing, and bioassessments are useful to evaluate WET limits and margins of safety.
12. The relationship between WET tests and receiving water impacts is based largely on animal effects in streams. Minimal data exist describing the effect of effluent toxicity exposure in wetlands, estuaries, and large rivers.
13. Careful thought must be given to selecting appropriate reference conditions for field assessments. Regional reference conditions strengthen assessments of receiving water impacts and facilitate characterization of natural variation.
14. Effluent toxicity is one of several factors that can adversely impact biological communities and is not always the major cause of observed community impacts.

CHAPTER 2. DEVELOPING WET PERMIT CONDITIONS

2.1 Overview

Chapter 2 discusses the development of WET permit conditions. The subjects covered in this chapter include: (1) mixing zones; (2) water quality criteria for WET; (3) reasonable potential determinations with and without facility-specific effluent data; (4) derivation and expression of WET permit limits; and (5) derivation and expression of WET permit triggers (WET permit triggers) used in conjunction with accelerated monitoring and TREs when there is no reasonable potential for WET and no WET permit limits.

When determining reasonable potential and deriving and expressing water quality-based effluent limits (WQBELs) and permit conditions for WET, the Permitting Authority needs to examine the State's water quality criteria for WET, mixing zone policy, and NPDES implementation procedures for determining reasonable potential and calculating WET permit limits or WET permit triggers for accelerated monitoring and TREs. We note that current State practices may differ from EPA's recommended approach for WET implementation outlined in the TSD (USEPA 1991a) and described in this chapter. In all cases, State practices must meet requirements of the Clean Water Act (CWA) and federal NPDES regulations.

Permitting Authorities determining reasonable potential and establishing WQBELs for WET must follow 40 CFR 122.44(d)(1) and should consider EPA guidance for water quality-based permitting in Chapters 3 and 5 of the TSD. For these types of calculations, EPA recommends that WET data be expressed using toxic units (TUs). Section 1.3.1 of the TSD defines TUs as 100 divided by the measured effect concentration expressed as a percentage of whole effluent. Thus, TUa = $100/LC_{50}$ and TUc = 100/NOEC or $100/EC_{25}$. When statistically estimating effluent variability for determining WET reasonable potential procedures following TSD procedures, TUc data for an effluent should be based on point estimate results (e.g., EC_{25}) rather than hypothesis testing results (e.g., NOEC), in order to obtain a better estimate of the effluent coefficient of variations (CV) used for WET permitting (USEPA 2000b). However, WET permit limits or WET permit triggers should continue to be expressed in accordance with State water quality standards and NPDES implementation procedures using either NOEC or EC_{25}.

2.2 Mixing Zones

When deriving WET permit limits, or WET permit triggers for accelerated monitoring and TREs, mixing zones may be considered for an NPDES discharge based on available dilution and assimilative capacity, if authorized and allowed by State water quality standards (WQS). Section 4.3 of the TSD provides background information on mixing zones and discusses EPA's mixing zone policy and how this policy affects the allowable toxic load that can be discharged from a point source. Section 4.4 of the TSD discusses mixing zone analyses for situations in which the discharge does not mix completely with the receiving water within a short distance of the discharge. If complete mixing does not occur near the discharge point and the effluent plume is discernible downstream, then modeling techniques that simulate and predict mixing conditions are more appropriate. Section 4.5 of the TSD discusses the steady-state models most used by States and EPA Regions to calculate wasteload allocations (WLAs) for contaminants. An

example of a steady-state model is the mass balance equation, i.e., the continuity equation, described later in this chapter. Steady-state models assume that the effluent is completely mixed with the receiving water near the discharge point, such as in effluent-dominated streams. Steady-state models require single constant inputs for effluent flow, effluent concentration, receiving water flow, and background receiving water concentration. The critical conditions for receiving stream design flows used in steady-state modeling should reflect water quality criteria durations and frequencies and the hydrologically- and biologically-based design flows generally specified in State WQS. EPA's recommended receiving stream design flows for water quality criteria and their calculation are summarized in Appendix D of the TSD. Critical design flows recommended for use with EPA's acute criterion for WET are the 1Q10 and 1B3. Critical design flows recommended for use with EPA's chronic criterion for WET are the 7Q10 and 4B3. If mixing zones for acute or chronic WET are not authorized for an NPDES discharge, or not allowed by State WQS, then water quality criteria for WET must be applied at the end of the discharge pipe.

2.3 Water Quality Criteria for WET

Water quality standards are provisions of State (or federal) law or regulation which define the water quality goals of a waterbody, or portion thereof, by designating the uses of the waterbody and setting the water quality criteria necessary to protect those uses. States adopt WQS to protect public health or welfare, enhance the quality of water, and serve the purposes of the CWA. Such standards serve the dual purposes of establishing the water quality goals for a specific waterbody and serve as the regulatory basis for the establishment of water quality-based controls and strategies beyond the technology-based levels of treatment required by sections 301(b) and 306 of the CWA. (40 CFR 131.2)

Water quality criteria are elements of State WQS, expressed as constituent concentrations, levels, or narrative statements representing a quality of water that supports a particular use. When these criteria are met, water quality will generally protect the designated use. (40 CFR 131.3) While States have adopted a variety of criteria expressed as constituent concentration levels (or numeric criteria) for various pollutants, all States have adopted criteria expressed as narrative statements (or narrative criteria). These narrative criteria, often referred to as "free-from" criteria (in the case of WET, "no toxics in toxic amounts"), are an effective tool for controlling the discharge of pollutants where numeric criteria are not available. Numeric or narrative criteria for WET serve as the basis for establishing WET controls in NPDES permits. (40 CFR 122.44(d)(1))

EPA's national water quality criteria are developed under the requirements of CWA section 304(a) and are published by EPA in individual criteria documents. The water quality criteria for aquatic life consider a wide range of toxic endpoints, including acute and chronic impacts, and consist of two values—a criterion maximum concentration (CMC) to protect against acute (short-term effects) and a criterion continuous concentration (CCC) to protect against chronic (long-term) effects. At present, EPA has no national criteria developed under CWA section 304(a) for acute and chronic WET. In the absence of such criteria, EPA's recommended magnitudes for WET are as follows. For acute protection, the CMC should be set at 0.3 acute toxic units (TUa) to the most sensitive of at least two test species. For chronic protection, the CCC should be set at 1.0 chronic toxic units (TUc) to the most sensitive of at least three test

species. Also, State procedures for implementing narrative criteria for WET should specify the testing procedure, the duration of the tests (acute or chronic), the test species, and the frequency of testing required. (TSD Section 2.3.3)

2.4 Determining Reasonable Potential for WET

This section follows 40 CFR 122.44(d)(1) and discusses the possible outcomes of a reasonable potential (RP) determination for WET, as described in Chapter 3 of the TSD. Where there is either a numeric or narrative water quality criterion for WET, Permitting Authorities need to characterize WET in NPDES discharges and implement WQBELs for WET, as required by 40 CFR 122.44(d)(1). Following EPA's recommendations in Chapter 3 of the TSD, there are two ways to characterize an effluent to determine the need for WET permit limits. First, an assessment may be conducted using facility-specific effluent data for WET, following procedures outlined in Section 3.3 of the TSD or other State NPDES implementation procedures. Second, an assessment may also be conducted without generating facility-specific effluent data for WET, using the factors described in Section 3.2 of the TSD. Following 40 CFR 122.44(d)(1)(ii), in all situations when determining the need for a WET permit limit, the Permitting Authority is required to consider, at minimum, existing controls on point and nonpoint sources of pollution, the variability of the pollutant or pollutant parameter in the effluent, the sensitivity of the species to toxicity testing and, where appropriate, the dilution of the effluent in the receiving water.

Section 3.3.3 of the TSD describes four possible outcomes of a reasonable potential determination for WET. These are:

- *Outcome 1*. The discharge causes or contributes to an excursion above a numeric or narrative water quality criterion for WET and a WQBEL for WET is required;
- *Outcome 2*. The discharge has the reasonable potential to cause or contribute to an excursion above a numeric or narrative water quality criterion for WET and a WQBEL for WET is required;
- *Outcome 3*. The discharge does not [have the reasonable potential to] cause or contribute to an excursion above a numeric or narrative water quality criterion for WET and a WQBEL for WET is not required; however, WET permit triggers used in conjunction with accelerated monitoring and TREs are recommended by EPA; or
- *Outcome 4*. There is inadequate information to determine whether or not the discharge causes, has the reasonable potential to cause, or contributes to an excursion above a numeric or narrative water quality criterion for WET and a WQBEL for WET is not required; however, WET permit triggers used in conjunction with accelerated monitoring and TREs are recommended by EPA.

When determining the need for WQBELs for WET, Permitting Authorities should use all available effluent data, together with information like that discussed in the following sections, as a basis for this decision. The Permitting Authority may already have facility-specific WET data from NPDES self-monitoring reports, or may decide to require the discharger to generate WET

data prior to permit issuance or as a condition of the permit. NPDES application requirements at 40 CFR 122.21 specify effluent monitoring requirements for WET, based on several factors, including the type of discharge. EPA recommends that WET data be generated prior to permit issuance for the following reasons: (1) the presence or absence of toxicity can be more clearly established or refuted, and (2) where toxicity is shown, effluent variability can be more clearly defined and addressed**.** (TSD Section 3.3.1)

2.4.1 Determining the Need for Permit Limits with Facility WET Data

As described in Section 3.3.2 of the TSD, for facilities with WET data, EPA recommends finding that a discharger has the "reasonable potential" to exceed a water quality criterion for WET if it is demonstrated with a high level of confidence that the upper bound of the lognormal distribution of effluent values for WET are above water quality criteria for WET, at specified critical flow conditions. EPA's recommended statistical approach for determining reasonable potential is a sequential, tiered process that is shown in Box 3-2 of the TSD. First, for each test method and species, effluent data for WET are reviewed to determine the total number of sample observations (n) and identify the maximum observed effluent value. Second, if there is enough sample observations ($n \geq 10$), these data are used to calculate statistics—a mean, standard deviation and coefficient of variation (CV)—which characterize the variability of WET in the effluent. However, if fewer than ten sample observations are available ($n < 10$), then Section 5.5.2 of the TSD recommends using the default CV of 0.6 to characterize the variability of WET in the effluent. Third, following the instructions in Section 3.3.2 of the TSD, the values for "n" and "CV" are used to calculate a reasonable potential multiplier factor. Fourth, the identified maximum observed effluent value for WET is multiplied by the reasonable potential multiplier factor to obtain a probability-based estimated maximum effluent value. Generally for WET, both the identified maximum observed effluent value and the probability-based maximum effluent value are used in the steady-state mass balance equation to project in-stream maximum values for WET, at specified critical flow conditions. Fifth, these projected in-stream maximum values are calculated and compared to the water quality criterion for WET (acute or chronic). If both projected in-stream maximum values are less than, or equal to, the water quality criterion for WET, then the Permitting Authority should exercise judgment as to whether reasonable potential exists. If either of these projected in-stream maximum values is greater than the water quality criterion for WET, then reasonable potential is established for the discharge and the permit must contain WQBELs for WET. Appendix B of this document provides an example of how the steady-state mass balance equation is used by EPA to calculate dilution and establish reasonable potential for acute and chronic WET.

2.4.2 Determining the Need for Permit Limits without Facility WET Data

As described in Section 3.2 of the TSD, the Permitting Authority may choose to develop and require WQBELs to control WET without facility-specify monitoring data, or prior to the generation of effluent data. In doing so, the Permitting Authority needs to follow the requirements in 40 CFR 122.44(d)(1) and clearly document these decisions in the record for the permit. When determining whether or not a discharge causes, has the reasonable potential to cause, or contributes to an excursion above a narrative or numeric water quality criterion for WET, the Permitting Authority can use a variety of factors and information where facility-

specific effluent monitoring data are not available. Also, these factors should be considered when effluent monitoring data are available. Some of the factors described in the TSD include:

- *Dilution*. Toxic impact is directly related to available dilution for the effluent. Dilution is related to the receiving water stream flow, the size of the discharge, whether or not there is an outfall diffuser, etc. The lower the available dilution, the higher the potential is for toxic effects. For example, as discussed in Section 3.3.3 of the TSD, if an effluent's dilution (i.e., in-stream waste concentration; IWC) at the edge of a mixing zone authorized by the Permitting Authority is expected to reach one percent or higher during critical or worst-case design periods, then the effluent may require a WET limit.
- *Type of industry*. Although NPDES discharges should be individually characterized because toxicity problems are site-specific, the "primary" industrial categories are of principal concern. Factors to consider can include the type and efficiency of treatment applied, general materials handling practices, and the functional target of the compound(s) produced.
- *Type of POTW*. POTWs with loadings from indirect dischargers (particularly primary industries) may be candidates for WET limits. However, the absence of industrial input does not guarantee an absence of toxicity problems. Down-the-drain disposal of pesticides, detergents, and other toxicants can result in toxic concentrations in POTW effluents. The types of industrial users, their product lines, raw materials, potential and actual discharges, and control equipment should be evaluated. POTW effluents should be evaluated for potential toxicity due to ammonia and chlorine.
- *Existing data on toxic pollutants*. Discharge monitoring reports (DMRs) and data from NPDES permit application forms may provide some indication of the presence of toxicants. The presence or absence of the 126 priority toxic pollutants (CWA section 307(a) and 40 CFR 131.3(d)) may or may not be an indication of the presence or absence of WET. There are thousands of toxicants not on the list of 126 priority toxic pollutants which are by definition "nonconventional" pollutants that may cause toxicity. Also, combinations of toxicants can produce toxicity where individual toxicants would not. NPDES regulations at 40 CFR 122.21(j)(5) specify that POTWs with design flows equal to or greater than 1 mgd and POTWs required to operate pretreatment programs, must perform specified WET testing and submit these results with their permit applications. Also, for certain types of dischargers, 40 CFR 122.21 allows Permitting Authorities to request additional data, including WET data, at the time of permit application. Also, data may be obtained using CWA section 308, or similar State authority.
- *History of compliance problems and toxic impact*. Permitting Authorities may consider particular dischargers that have had difficulty complying with limits on toxicants or that have a history of known toxicity impacts, as probable candidates for WET limits.
- *Type of receiving water and designated use*. Data on water quality can include reports of fish kills, State lists of priority waterbodies, and State lists of waters that do not meet water quality standards. Sources of this information are the lists of waters generated under CWA section 304(l) and 40 CFR 130.10(d)(6).

The presence of a factor (or combination of factors) described above, such as low available dilution, high quality receiving waters, poor compliance record, and clustered industrial and municipal discharges, could constitute a high priority for WQBELs for WET. If the Permitting Authority chooses to require a WET limit without facility-specific effluent monitoring data, then adequate justification for the limit needs to be provided in the fact sheet or statement of basis for the permit. EPA recommends that the more information the Permitting Authority can acquire to support WET limits, the better a position the authority will be in to defend the limit, if necessary.

2.4.3 Other State Regulations for Determining Reasonable Potential

The Permitting Authorities needs to follow applicable State regulations and policies which govern how reasonable potential for WET is determined. These State requirements must be consistent with the Clean Water Act and EPA's regulations for implementing WET in the NPDES permitting program (e.g., 40 CFR 136, 40 CFR 122.41(j), 40 CFR 122.44(d), 40 CFR 122.21(j)). In the absence of detailed State regulations and policies, EPA recommends that Permitting Authorities follow the approaches and statistical procedure for determining reasonable potential recommended in Chapter 3 of the TSD.

In the *California Ocean Plan* (SWRCB 2005), the California State Water Resources Control Board has adopted general reasonable potential language and specified statistical reasonable potential analysis procedures for both parametric effluent data sets and non-parametric effluent data sets. If there are three or more detected observations for the effluent and these observations are censored by 80% or less, then the parametric reasonable potential analysis procedure is used. This procedure assumes that effluent data are lognormally distributed and calculates an upper confidence bound (i.e., the one-sided, upper 95 percent confidence bound for the 95th percentile of the effluent distribution after complete mixing) for comparison with the water quality criterion. If the upper confidence bound is greater than the water quality criterion, then the California ocean discharge has reasonable potential to cause an excursion above the water quality criterion and a WQBEL is needed. Instructions for conducting a nonparametric reasonable potential analysis or a reasonable potential analysis based on best professional judgment are also given. To support these procedures, State Water Board staff have developed a stand-alone, Windows-based computer program called "RPcalc", the California Ocean Plan Reasonable Potential Analysis Calculator (SWRCB 2005), to assist Permitting Authorities conducting reasonable potential analyses for discharges regulated under the California Ocean Plan. This approach is found at http://www.swrcb.ca.gov/ and an example is provided in Appendix B of this document.

2.4.4 Reasonable Potential Determination Outcomes for WET

Based on Outcomes 1 and 2, described in Section 2.4 of this document, if WET in an NPDES discharge is at levels that cause, have the reasonable potential to cause, or contribute to an excursion above State water quality standards, then the permit must contain WQBELs for WET. This conclusion can be based on one effluent sample observation for WET. Based on Outcome 3, described in Section 2.4 of this document, if WET in an NPDES discharge is below levels that cause, have the reasonable potential to cause, or contribute to an excursion above State water quality standards, then the permit need not contain WQBELs for WET. Based on Outcome 4,

described in Section 2.4 of this document, if there is inadequate information to determine whether WET in an NPDES discharge is below levels that cause, have the reasonable potential to cause, or contribute to an excursion above State water quality standards, then the permit need not contain WQBELs for WET. Although the Permitting Authority does not need to establish WQBELs for WET, there still may be a basis for concern under Outcomes 3 and 4.

Consequently, under each of these four outcomes, EPA recommends that WET monitoring in permits be conducted at frequency sufficient to ascertain discharge compliance with WQBELs for WET, WET permit conditions and, ultimately, State water quality standards. Whether or not WET limits are included in a permit, WET monitoring conditions need to specify: (1) an accelerated monitoring schedule following the exceedance of either a WET permit limit or WET permit trigger; and (2) the number of WET test failures during this schedule that will automatically initiate a TRE. Also, permits should contain a WET reopener condition which allows the Permitting Authority to **"**reopen" the permit and establish additional WET permit conditions or effluent limits based on monitoring results or other factors indicating that the effluent causes, has the reasonable potential to cause, or contributes to an excursion above water quality standards.

2.5 Deriving Permit Limits for WET

When a Permitting Authority determines, using reasonable potential procedures, that a discharge causes, has the reasonable potential to cause, or contributes to an in-stream excursion above State numeric water quality criteria for WET, the permit must contain WQBELs for WET. (40 CFR 122.44(d)(1)(iv)) If State WQS contain only narrative water quality criteria for WET and it is documented in the record for the permit (i.e., fact sheet or statement of basis) that chemical-specific WQBELs are sufficient to attain and maintain the narrative water quality criteria, then WQBELs for WET are not necessary. This is only authorized when the causative toxicant(s) in the effluent have been identified and confirmed. (40 CFR 122.44(d)(1)(v))

As explained in Section 5.1.1 of the TSD, once the decision has been made to develop WQBELs, there is an element of judgment inherent in the specific permit limit derivation procedures used for an individual NPDES discharger. Case-specific considerations will usually dictate the most appropriate conditions in individual situations (e.g., chronic or acute toxicity test, freshwater or marine test organisms, monitoring frequency, etc.); however, the general assumptions used when developing WQBELs should be consistent with the assumptions and principles inherent in effluent characterization and exposure assessment steps preceding the development of WQBELs. The WQBEL derivation procedure used by Permitting Authorities should be fully enforceable and should adequately account for effluent variability, consider available receiving water dilution when appropriate, protect against acute and chronic impacts, account for compliance monitoring sampling frequency, and protect pollutant wasteload allocations (WLAs) and ultimately the WQS.

Chapter 5 of the TSD explains the strengths and weaknesses of different approaches often used by Permitting Authorities to develop WQBELs, including permit limits for WET. This section discusses:

- The development of WLAs for WET using either steady-state models or dynamic models (TSD Chapter 4);
- The "statistical approach" where WQBELs are statistically calculated from the more stringent acute or chronic WLA for WET (TSD Section 5.4.1);
- The "direct application approach" where an acute or chronic WLA for WET is directly applied as a WQBEL (TSD Section 5.4.2); and
- Other approaches used to develop WQBELs for WET based on State WQS and NPDES implementation procedures.

As described in Section 5.2.2 of the TSD, WQBELs for NPDES discharges are established based on the need to maintain effluent quality for a pollutant at a level that will comply with WQS even during critical conditions in the receiving water. This level is determined by the WLA for the pollutant. The WLA, in turn, dictates the necessary level of treatment plant performance for the pollutant—or target long-term average (LTA)—discussed later in this chapter.

2.5.1 Developing Wasteload Allocations for WET

How are wasteload allocations (WLAs) developed for a pollutant in an effluent? There are two major types of water quality models used to develop WLAs for NPDES discharges: dynamic and steady-state. Dynamic models use estimates of effluent variability and the variability of receiving water assimilation factors to develop effluent requirements expressed in terms of the concentration of the pollutant and variability. As a result, the outputs of dynamic models can be used to base WQBELs on probability estimates of receiving water concentrations rather than worst-case assumptions. EPA only recommends using dynamic models to develop WLAs if adequate pollutant data for an effluent and receiving water flow are available to estimate frequency distributions. Traditional steady-state WLA models calculate WLAs at critical conditions, using worst-case assumptions for effluent and receiving water flows and pollutant levels. WQBELs derived from steady-state WLA models are designed to be protective of WQS during critical environmental conditions and all environmental conditions less than critical. Although steady-state WLA models tend to be more conservative than dynamic models because they rely on worst case assumptions, EPA recommends that steady-state WLA models generally be used by Permitting Authorities in most cases and especially where few or no WET data are available, or where daily receiving water flow records are not available. (TSD Section 5.3.2)

When using steady-state models, WLA calculations are always made using critical conditions. To calculate acute and chronic WLAs for WET using a steady-state model, the Permitting Authority needs to choose values for:

- Chronic criterion (CCC) for WET
- Fraction of 7Q10 (or 4B3) receiving water flow available for dilution, as authorized by State mixing zone policy
- Acute criterion for WET (CMC)

- Fraction of 1Q10 (or 1B3) receiving water flow available for dilution, as authorized by State mixing zone policy
- Maximum background level for WET in the receiving water
- Maximum effluent flow

Where receiving water data for WET are not available, EPA recommends assuming a default maximum background value of 0 (zero) TUs when calculating WLAs for acute and chronic WET.

Shown below, the mass balance equation, i.e., the continuity equation, is a simplified steady-state model for calculating WLAs that is generally recommended by EPA for calculating WLAs for acute and chronic WET.

Steady-State Model: Mass Balance Equation

$$Cr\ Qr = Ce\ Qe + Cs\ Qs$$, where

Cr =	Water quality criterion for WET, in TUs
Ce =	Wasteload allocation for WET, in TUs
Qe =	Effluent flow, in million gallons per day (MGD) or cubic feet per second (cfs)
Cs =	Background in-stream value for WET above the discharge point, in TUs
Qs =	Percent (%) in-stream critical flow allowed for dilution, in MGD or cfs

Use of this steady-state mass balance equation for calculating dilution and WLAs for acute and chronic WET assumes that the NPDES discharge achieves complete mixing across the width of the stream near the point of discharge and the effluent plume is not discernible downstream. If this is not the case, then modeling techniques that can simulate and predict mixing conditions are more appropriate for defining the mixing zone and dilution for the discharge. If a mixing zone is allowed and actual background in-stream pollutant levels are considered, then the mass balance equation is: WLA = Ce = (Cr) [(Qe + Qs) / Qe] – [Cs × (Qs / Qe)], and water quality criteria are applied at the edge of the mixing zone. For WET, if a mixing zone is allowed and background in-stream toxicity is set equal to 0 TU, then the mass balance equation reduces to: WLA = Ce = (Cr) [(Qe + Qs) / Qe], and WET criteria are applied at the edge of the mixing zone. If a dilution model is used, e.g., UM3 from Visual Plumes (USEPA 2003a), and it is not necessary to consider the actual, nonzero, ambient concentration of a pollutant in the effluent, then the flux-averaged volumetric dilution factor (Sa) can be used in the mass balance equation: WLA = Ce = Cr × Sa. If a mixing zone is not allowed, then the mass balance equation reduces to: WLA = Ce = Cr, and WET criteria are applied at the end of the pipe. Once both acute and chronic WLAs for

WET have been developed for an NPDES discharge, then WQBELs need to be calculated. Appendix C of this document provide detailed examples of how the steady-state mass balance equation is used by EPA to calculate dilution and WLAs for acute and chronic WET, and WQBELs for acute and chronic WET.

[Note: Where the volumetric dilution factor, Sa = [(Ve + Va) / Ve] = Ce / Cp, in *Dilution Models for Effluent Discharges* (USEPA 1994c). Thus, if Sa = 30 (which means one volume of effluent is diluted with 29 volumes of ambient water), then the concentration of any volumetric tracer or conservative pollutant in the effluent is one thirtieth the concentration in the effluent, only if the ambient concentration is zero. In this definition of Sa, the volumetric dilution factor is very nearly 1 in the region outside the discharge orifice. Following the mass-balance equation, i.e., the continuity equation in Visual Plumes, because the dilution ratio, D = Qs / Qe, then Sa = [(Qe + Qs) / Qe] = 1 + D. In other State documents, e.g., the California Ocean Plan (SWRCB 2005), the volumetric dilution factor, Sa, is considered the dilution ratio, D. In the California Ocean Plan definition, the volumetric dilution factor approaches zero near the discharge orifice. Page 9 of the Visual Plumes manual (USEPA 1994c) notes that above a dilution value of 30, the difference between the two definitions is progressively less than 3%, an inconsequential amount for most regulatory purposes.]

2.5.2 Statistical Approach for Developing WET Permit Limits

The statistical approach for developing WQBELs from WLAs is described in Section 5.4.1 of the TSD. Because effluent quality varies over time, EPA recommends that the Permitting Authority establish WQBELs using this statistical derivation procedure, in conjunction with WLAs, to adequately account for the variability observed in pollutant levels in NPDES discharges. Using this statistical approach, a WLA value is first set at the 99th percentile of necessary treatment plant performance and then translated into the average treatment performance level—long-term average (LTA) and coefficient of variation (CV)—that will ensure the WLA is met under critical conditions over the long-term. When two-value, or three-value steady-state WLAs have been developed for a pollutant (e.g., acute, chronic, and human health), the most stringent LTA is then translated into upper bound percentile values for effluent quality (i.e., 99th percentile and 95th percentile) and expressed as a maximum daily limit (MDL) and an average monthly limit (AML). (TSD Section 5.5.4) In making these translations for WET, the Permitting Authority needs to obtain values for:

- Acute-to-chronic ratio (TSD Section 5.4.1)
- Effluent variability expressed as CV (TSD Section 5.5.2)
- Number of compliance monitoring samples required per month (TSD Section 5.5.3)

Appendix C of this document provides detailed examples of EPA's recommended statistical approach for calculating WET permit limits.

2.5.3 Direct Application Approach for Developing WET Permit Limits

Several direct application approaches are described in Section 5.4.2 of the TSD. One type of direct application is when the Permitting Authority applies the WLA directly as a permit limit, generally a MDL. When a chronic WLA is set as a MDL, the MDL (1-day) should ensure protection of both acute (1-day) and chronic (4-day) water quality criteria. In the absence of additional information, permit writers may sometimes divide the MDL by 1.5 or 2.0 to derive an AML, depending on the expected range of effluent variability. Because this AML is derived without information about the variability of the effluent, this step may not ensure that the AML is protective of water quality criteria. Another type of direct application is when the acute WLA is applied as a MDL and the chronic WLA is applied as the AML; EPA discourages this approach since effluent variability has not been specifically addressed and compliance with the AML (30-day) during critical conditions could exceed the chronic (4-day) water quality criterion.

2.5.4 Other State Regulations

A State may have technology-based permit requirements for WET or use modified versions of the approaches described above to set WQBELs for WET. Permitting Authorities need to follow applicable State regulations and policies which govern how WET is implemented in NPDES permits. State requirements must be consistent with EPA's regulations for implementing WET in the NPDES permitting program (e.g., 40 CFR 136, 40 CFR 122.41(j), 40 CFR 122.44(d), 40 CFR 122.21(j)).

2.6 Permit Limit Expression

NPDES regulations at 40 CFR 122.45(d) require that all permit limits be expressed, unless impracticable, as both a MDL and an AML for all dischargers other than POTWs, and as an average weekly limit (AWL) and AML for POTWs. Following Section 5.2.3 of the TSD, the use of an AWL is not appropriate for WET. In lieu of an AWL for POTWs, EPA recommends establishing an MDL for toxic pollutants and pollutants in water quality permitting, including WET. This is appropriate for two reasons. The basis for the average weekly requirement for POTWs derives from secondary treatment regulations and is not related to the requirement to assure achievement of WQS. Moreover, an average weekly requirement comprising up to seven daily samples could average out daily peak toxic concentrations for WET and therefore, the discharge's potential for causing acute and chronic effects would be missed.

The MDL is the highest allowable value for the discharge measured during a calendar day or 24-hour period representing a calendar day. The permit should contain a condition indicating that the MDL is interpreted as the maximum acute or chronic WET result for that calendar month unless otherwise specified by State requirements. The AML is the highest allowable value for the average of daily discharges obtained over a calendar month. For WET, this is the average of individual WET test results for that calendar month, unless otherwise specified by State requirements.

In cases where an acute mixing zone is either not authorized, or authorized such that a critical instream waste concentration (IWC) for acute WET is set at a percent effluent value greater than 100% effluent, EPA Regions 9 and 10 continue to recommend that the acute WET permit limit should be expressed as a Pass/Fail limit, as described below. In cases where a chronic mixing zone is not authorized, EPA Regions 9 and 10 continue to recommend that the AML for chronic WET should be expressed as a median monthly limit (MML), as described below.

2.6.1 Acute WET Permit Limits for Low-Flow Situations

The following procedure is recommended for monitoring and limiting acute WET in NPDES discharge situations when an acute mixing zone is either not authorized, or authorized such that a critical IWC is set at a percent effluent value greater than 100% effluent. In these situations, where the critical IWC is set at a percent effluent value greater than 100% effluent, calculated WLAs and WQBELs for acute WET—based on EPA's recommended water quality criterion for acute toxicity (CMC) of 0.3 TUa = 100/LC50, and the steady-state mass balance equation—can range from 0.999 TUa down to 0.3 TUa. (TSD Section 5.4.1). For these discharge situations, EPA Regions 9 and 10 continue to recommend hypothesis testing (Denton and Narvaez 1996). This is because the point estimate techniques used to evaluate compliance with EPA's recommended acute toxicity criterion of 0.3 TUa, i.e., "no acute toxicity", cannot be used until the discharge-specific critical percent effluent concentration (LC50) is able to be set at (or below) 100% effluent.

For these discharge situations, the acute WET permit limit should be "Pass" for any one test result. The determination of Pass or Fail from a single-effluent-concentration (paired) acute toxicity test is determined using a one-tailed hypothesis test called a t-test. The objective of a Pass or Fail test is to determine if survival in the single treatment (100% effluent) is significantly different from survival in the control (0% effluent). Following Section 11.3 in the acute test method manuals (USEPA 2002a), the t statistic for the single-effluent-concentration acute toxicity test is calculated and compared with the critical t set at the 5% level of significance. If the calculated t does not exceed the critical t, then the mean responses for the single treatment and control are declared "not statistically different" and the permittee reports "Pass" on the DMR form. If the calculated t does exceed the critical t, then the mean responses for the single treatment and control are declared "statistically different" and the permittee reports "Fail" on the DMR form. The permit should require additional toxicity testing and, ultimately, a TRE, if an acute WET permit limit or trigger is reported as "Fail".

2.6.2 Chronic WET Permit Limits for Low-Flow Situations

When no mixing zone or dilution allowance is authorized, or an NPDES discharge is to a zero flow stream, EPA Regions 9 and 10 continue to recommend that Permitting Authorities establish a monthly median limit (MML) of 1.0 TUc for chronic WET (Denton and Narvaez 1996). Under these discharge situations, chronic WET test results showing no chronic toxicity in 100 percent effluent are reported as censored values at the most hazardous effluent concentration possible to test (i.e., RWC = <1.0 TUc in 100 percent effluent). Such results present unique issues for Permitting Authorities evaluating compliance with average monthly limits for chronic WET that are statistically calculated following EPA's recommendations in Section 5.4.1 of the TSD, as

these calculated values are lower than 1.0 TUc (e.g., 0.8 TUc). While EPA Regions 9 and 10 continue to recommend the use of statistically-calculated maximum daily limits for chronic WET using TSD procedures, discharges without a mixing zone or dilution allowance—where the governing magnitude for the monthly limit is set at 1.0 TUc in 100 percent effluent—differ from discharge situations where the governing magnitudes for WET are set at other effluent dilutions. This is because the 100 percent effluent dilution represents both the censoring level for the toxicity test and the most hazardous effluent concentration possible to test.

Consequently, EPA Regions 9 and 10 continue to recommend direct application of 1.0 TUc as the monthly compliance level for NPDES discharges without a mixing zone or dilution allowance. In conjunction and limited to this discharge situation, because: (1) there are no values below 1.0 TUc and (2) an arithmetic average is sensitive to extremely large and small values, the median is favored as the better measure of central tendency for the monthly compliance level. EPA Regions 9 and 10 continue to believe that setting a median monthly limit at 1.0 TUc, rather than an average monthly limit at either 1.0 TUc or a statistically-calculated value lower than 1.0 TUc, allows Permitting Authorities to: (1) make the best use of all monthly WET test results—including those reported as censored values at the 100 percent effluent concentration—when evaluating compliance with monthly permit limits; and (2) continue to protect against short-term excursions above the 4-day average chronic criterion for WET of 1.0 TUc by establishing the traditional, statistically-calculated maximum daily limit for chronic WET recommended in the TSD.

In summary, use of the MML of 1.0 TUc for chronic WET is recommended only in conjunction with the following permit conditions:

- A statistically calculated MDL for chronic WET (TSD Section 5.4.1); and
- Routine WET monitoring using the most sensitive test species identified through screening using species representing three different phyla (TSD Section 1.3.4).

Appendix C of this document provides an example of EPA Region 9 and 10's recommended approach for calculating chronic WET permit limits for low-flow situations.

While continuing to affirm these recommendations for NPDES discharges when a mixing zone or dilution allowance is not authorized, EPA Regions 9 and 10 recognize that some Permitting Authorities may choose to establish only a maximum daily limit of 1.0 TUc for chronic WET, but no monthly limit. This alternative will protect against short-term excursions above the 4-day average chronic criterion for WET of 1.0 TUc and meet WQS, if used in lieu of the statistical procedure described in this document and in Section 5.4.1 of the TSD.

References

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CHAPTER 3. CHRONIC AND ACUTE TOXICITY TESTING

3.1 Overview

Regardless of whether the permit requirement is a WET permit limit or monitoring trigger (MT), the permit writer will need to develop appropriate testing conditions such as test method/species, testing frequency, and steps to address toxicity (which we have termed "stepwise approach" to addressing toxicity). In the 1996 document (Denton and Narvaez 1996), the EPA Regions 9 and 10 recommended, and continue to recommend the stepwise approach of accelerated testing; if continued toxicity is demonstrated then the permittee needs to conduct a toxicity reduction evaluation (TRE).

The first decision for a permit writer to make in selecting the appropriate toxicity tests is whether to conduct acute and/or chronic tests to address both the acute and chronic criteria. The next question to answer is whether to test with freshwater or marine species. Once these decisions have been made, the following parameters need to be considered when selecting the appropriate test species: taxonomic diversity; type of facility and toxicants; and seasonal and temporal effects. See Appendix D for an example of WET permit language.

3.2 Toxicity Test Methods

3.2.1 Acute Tests

Acute toxicity tests are used to determine the concentration of effluent or ambient water that results in mortality within a group of test organisms during a 24-, 48- or 96-hour exposure. In an acute toxicity test, an effluent sample is collected, diluted, and placed in test chambers with the chosen test species. After 24, 48 or 96 hours, the number of live organisms remaining in each test concentration and in a control is recorded. The acute test methods are listed in Attachment 3-1.

3.2.2 Chronic Tests

A chronic toxicity test is defined as a short-term test in which sublethal effects, such as fertilization, growth or reproduction, are measured in addition to lethality (in some tests). Traditionally, chronic tests are full life-cycle tests or shortened tests (approximately 30 days) known as early life stage tests. Measuring the chronic toxicity of effluents is difficult because of the potential for effluent toxicity to change over time. Thus, even a shortened chronic early life stage test conducted in one month would have to be repeated at intervals to ensure that process or receiving water changes were not altering toxicity in ambient waters. In addition, toxicity spikes occurring during any one portion of a 30-day test could produce a different level of toxic response than an identical spike occurring during a different time of the test. The duration of chronic toxicity tests precludes the use of a single effluent sample due to probable reduction in toxicity with storage and requires extensive logistical arrangements for sampling and handling of effluent. Chronic toxicity test methods of 7 days duration require a minimum of three samples.

As a result of such considerations EPA has developed a suite of shorter toxicity tests (short-term chronic tests) that aim to detect toxicity at chemical concentrations near those that produce chronic toxicity in longer term tests. The short-term chronic tests were developed and selected based on characteristics such as sensitive species, sensitive life-stages and endpoints, taxonomic and ecological diversity, short duration, availability of organisms for testing, and low volume requirements for test solutions. These resulting tests have typical durations of 40 minutes to 7 days, enabling tests to be run with effluent or receiving water samples at lower costs and increased test frequency. The chronic test methods are listed in Attachment 3-2 and 3-3.

EPA standardized the test procedures for conducting the approved acute and chronic WET test methods in the following three method manuals (USEPA 2002a, 2002b, 2002c), which were incorporated by reference into the WET final rule (67 Federal Register 69953). See Attachment 3-4 for a summary of WET method changes. In addition, since first promulgating acute and chronic WET methods in 1995, EPA has continued to recommend that NPDES permitting authorities implement chronic WET in permits for West Coast facilities based on *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (USEPA 1995b; West Coast manual) and other alternative guidance, as directed by State Permitting Authorities. This practice corresponds with the 2002 Final WET Rule (67 Federal Register 69952, 69955). In the preamble to this rulemaking, EPA states: "Because test procedures for measuring toxicity to estuarine and marine organisms of the Pacific Ocean are not listed at 40 CFR part 136, permit writers may include (under 40 CFR 122.41(j)(4) and 122.44[*d*](1)(iv)) requirements for the use of test procedures that are not approved at part 136, such as the *Holmesimysis costata* Acute Test and other West Coast WET methods (USEPA 1995b) on a permit-by-permit basis." Indeed, regulations for POTWs at 40 CFR 122.21(j)(5)(viii) clarify that West Coast NPDES permit applicants, including those in Hawaii, are "exempted" from 40 CFR 136 chronic methods and must use alternative guidance as directed by the Permitting Authority.

3.3 Selection of Freshwater or Estuarine/Marine Test Methods

The decision of whether to use freshwater or estuarine/marine test methods is based on the salinity of the effluent and that of the receiving water. EPA provides technical discussion regarding the selection of test species (see TSD, Section 3.3.6). A summary paper by Goodfellow et al. (2000) provides information on the role of ion imbalance (either excess or deficiency) in aquatic toxicity testing and provides various recommendations that could be considered in addressing these issues. The Goodfellow et al. (2000) paper discusses procedures that use weight-of-evidence approaches to identify ion imbalance toxicity, including direct measurement, predictive toxicity models for freshwater, exchange resins, mock effluents and ion imbalance toxicity with tolerant/susceptible test species. Toxicity associated with ion imbalance of the effluent occurs when the ion concentrations and molar ratios of the effluent exceed or do not meet the physiological tolerance range of the selected test organism. States may have prescribed approaches which require toxicant characterization of the effluent to ascertain whether the toxicity is strictly due to ion imbalance and/or other toxicants within the effluent. If the toxicity is strictly due to ion imbalance and has been demonstrated in the weight-of-evidence approach then, on a site-specific basis, an alternate test species may be substituted.

3.3.1 Freshwater organisms

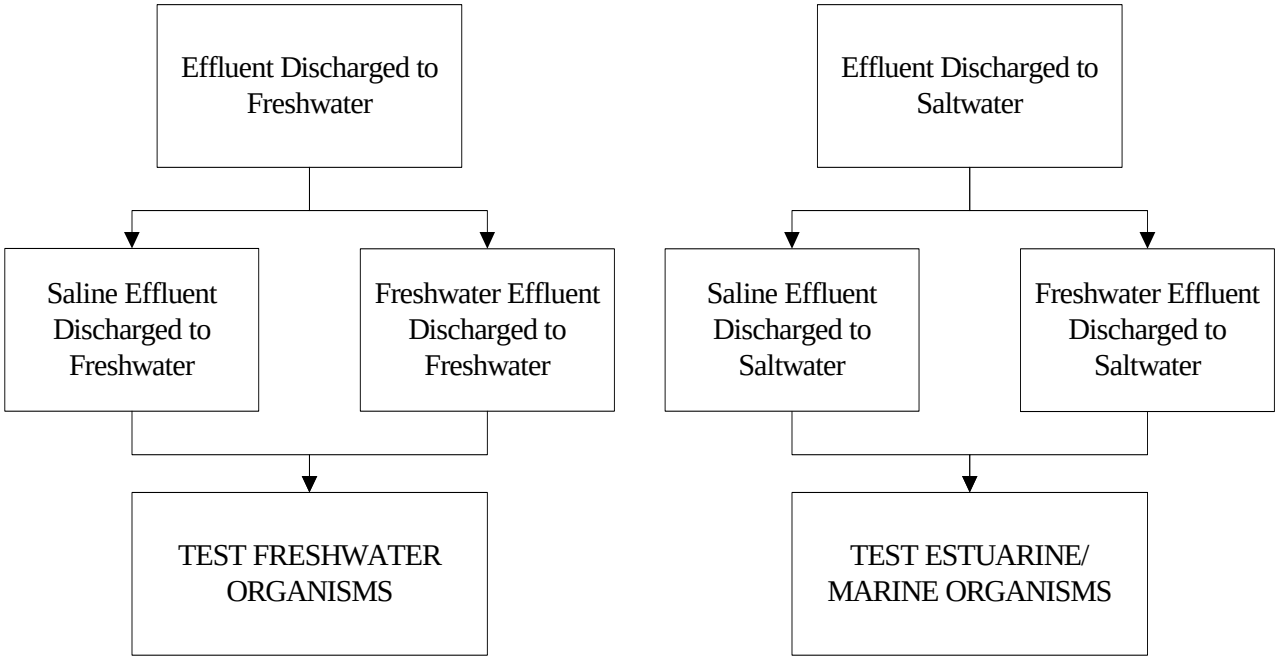
Freshwater organisms are used when the receiving water salinity is <1,000 mg/L (1‰). Species selection for freshwater is straightforward since there are methods for only three species: a fish (fathead minnows), an invertebrate (water flea), and a plant (green algae).

3.3.2 Estuarine/marine organisms

Estuarine/marine test organisms are used when the receiving water salinity is ≥1,000 mg/L (1‰). The EPA has test methods for test species resident to the East Coast and West Coast. There are two fish species (one East Coast and one West Coast), six invertebrate species (one East Coast and five West Coast), and one plant species (West Coast).

The selection of test organism is based on effluent and receiving water characteristics insert, as shown in the decision tree below (Figure 3-1).

Figure 3-1. Selection of Test Species Based on Effluent and Receiving Water Salinity



Saline Effluent Discharged to Estuarine/Marine Waters

The dissolved salts in the effluent are possible toxicants because the type and/or proportion of dissolved salts in the effluent may be different from that of the dissolved salts in the receiving water. The toxicity test should determine if these salts contribute to receiving water toxicity. For this reason, **estuarine/marine organisms are the preferred test species.**

Saline Effluent Discharged to Freshwater

The dissolved salts in the effluent are possible toxicants that are not present in the receiving water. The toxicity test should determine whether the dissolved salts are contributing to receiving water toxicity. For this reason, **freshwater organisms are the preferred test species.**

3.3.3 Freshwater Effluent Discharged to Estuarine/Marine Waters

The lack of dissolved salts in the effluent can affect marine toxicity test organisms. In contrast to the scenarios presented above, the toxicity test does not need to measure this effect since lack of salts is not considered a toxic effect. The estuarine/marine toxicity test methods account for this by requiring the salinity of the effluent be adjusted to the protocol salinity using either dry salts or hypersaline brine. For this reason, **estuarine/marine organisms are the preferred test species.**

3.3.4 Other Considerations

Factors that may be considered in selecting a marine invertebrate are the types of organisms found at the discharge location, types of toxicants discharged by the facility, and the relative sensitivity of the test organisms to known toxicants in the discharge. If the discharge is located near the intertidal zone, then an intertidal test species may be important (e.g., red abalone or bivalves). If the pollutants will be discharged near a kelp forest, where mysids are commonly located, the mysid test method may be more appropriate.

Sometimes, marine test species such as invertebrates and plants may not be amenable for testing at high effluent concentrations such as 100% effluent. For example, if the effluent salinity is 0‰ and hypersaline brine salinity is 100‰, then 66% effluent is the highest concentration that can be attained for tests with a salinity requirement of 34‰ when using only hypersaline brine (USEPA 1995a). **Therefore, a freshwater organism or a marine/estuarine organism that does not require hypersaline brine in the dilution water must be used if the permit limit or trigger is greater than the highest effluent concentration that can be tested.** However, the marine fish test methods, *Menidia* and *Atherinops* can be tested up to 100% effluent. Thus, these fish species can be used for freshwater discharges to saltwater with 100% effluent because dry sea salts (artificial) can be used to attain the method-required salinity (5-36‰).

3.4 Factors to Consider When Selecting Test Species

The Permitting Authority should select the appropriate species to be tested based on taxonomic diversity, type of facility, types of potential toxicants, and effluent seasonal and temporal effects. In addition, the Permitting Authority should evaluate any existing toxicity data provided by the permittee.

3.4.1 Taxonomic Diversity

In the selection of test species, **EPA recommends the use of species from ecologically diverse taxa** (see TSD, Section 1.3.4). The recommendation is to screen an effluent with at least three species (a fish, an invertebrate, and a plant) for chronic testing and two species (a fish and an

invertebrate) for acute testing. This recommendation is based upon the fact that there are species sensitivity differences among different groups of organisms to different toxicants. The initial multiple species screening should be conducted at least three times before selecting the most sensitive species. There are no acute test methods with plant species.

After this screening period, monitoring should be conducted on the most sensitive test species (e.g., the species demonstrating the lowest NOEC or IC_{25} value). It is also recommended in the permit that the permittee shall also re-screen once every year with three species (or two species for acute testing). If the same test species is the most sensitive, then the permittee shall continue to monitor with this test species. It is important to consider re-screening at a different time each year to evaluate effects of potentially different toxicants at different times of the year. For example, POTWs may have pesticide usage from homeowners in the spring and not in the winter months. Other factors to consider are the type of facility and seasonal and temporal effects from a facility.

3.4.2 Type of Facility

It is important to consider the type of toxicants that may be discharged from a facility and which species would be appropriate for such toxicants. For example, if a facility is discharging effluent that primarily consists of herbicides, a plant test method may be more appropriate. Certain species have been found to be sensitive to certain toxicants. Invertebrates are more sensitive to organophosphate pesticides (e.g., diazinon) than fish. Fish are more sensitive to ammonia than invertebrates. In situations where multiple species screening is not practical (such as ambient toxicity testing programs) it may be appropriate to test with the species with known sensitivity to the toxicants of concern.

3.4.3 Seasonal and Temporal Effects

It may be necessary to consider potential seasonal or temporal changes in the effluent when selecting the appropriate testing species. For example, pesticides may be of concern after spring runoff or first fall flush, and typically invertebrates such as water fleas or mysids are typically more sensitive.

Note: The *Selenastrum capricornutum* growth test (USEPA 2002b) now requires the addition of ethylenediamine tetraacetic acid (EDTA) to nutrient stock solutions when conducting this test under NPDES permits; Permitting Authorities are cautioned to consider this possibility when selecting test methods for monitoring effluents that are suspected to contain metals, as EDTA may interfere (i.e., mask) with the potential to ascertain the toxicity of metals.

Note: For controlling pathogen interference in the fathead minnow larval survival and growth test (USEPA 2002b), EPA recommends pathogen control techniques that do not modify the sample, such as the modified test-design technique. Upon approval by the Permitting Authority, however, analysts also may use various sample sterilization techniques that modify the sample to control pathogen interference, provided that parallel testing of unaltered samples further confines the presence of pathogen interference and demonstrates successful pathogen control (See chronic freshwater toxicity test methods manual, Sections 11.3.4.6.1 – 11.3.4.6.4).

3.5 Monitoring Frequency Recommendations

Once the need for a WET limit or monitoring requirement has been determined, the frequency of WET testing needs to be determined. The frequency for monitoring pollutants or pollutant parameters such as WET should be determined on a case-by-case basis, and decisions for setting the monitoring frequency should be set forth in the permit fact sheet. Some states have their own recommended sampling guidelines that can help a permit writer determine an appropriate monitoring frequency. The intent is to establish a frequency of monitoring that will detect most events of noncompliance without requiring needless or burdensome monitoring (Table 3-1).

Table 3-1 Likelihood of Detecting at Least One Toxic Event

Number of Tests (N)	*True Probability of Occurrence* [A]		
	10%	*20%*	*30%*
1	0.10	0.20	0.30
2	0.19	0.36	0.51
3	0.27	0.49	0.66
4	0.34	0.59	0.76
5	0.41	0.67	0.83
6	0.47	0.75	0.88
8	0.57	0.83	0.94
10	0.65	0.89	0.97
12	0.72	0.93	0.99
16	0.81	0.97	0.99
20	0.88	0.99	0.99

a Assumes negligible serial correlation among observations, and true rate of occurrence over time. Probability of occurrence is stated as a percentage of the possible independent sampling events.

3.5.1 Example of Probability of Detecting Toxicity

For example, suppose the (unknown) probability is 0.20 (e.g., probability of occurrence is 20%) that the NOEC for a chronic *Ceriodaphnia* test will occur at or above the permitted TU value. Then, if testing is performed once per quarter (n=4), the probability that, in the course of one year, at least one of the four tests will demonstrate a toxicity at or above the permitted TU value is 0.59. The same would apply to monitoring once per year for four years (n=4). As another example using the same true probability of occurrence (20%), quarterly monitoring for three years (n=12) would be expected to exhibit at least one result exceeding the permitted TU value with high probability (0.93).

EPA recommends that the permit contain a monitoring schedule that increases or decreases in frequency depending on the results of WET testing after at least 20 tests have been completed under consistent treatment operations. **EPA Regions 9 and 10 recommend a minimum of monthly WET testing for majors (>1 MGD) and quarterly for minors (< 1 MGD).** The rationale for this is that majors, given such factors as type, size and variability of the discharge, as well as receiving waters, are generally expected to cause more receiving water impacts than minors. However, a group of minors clustered together could have the same effect as a major. When establishing monitoring frequency for a given facility, the permit writer should consider all available information, and not rely only upon the "major" or "minor" classification.

3.6 Sample Collection

3.6.1 Effluent Sampling

Effluents are usually collected as flow-proportional or time-weighted composite samples, except in instances where the residence time in the treatment plant is very short and the purpose of the sampling is to detect peaks (spikes) in toxicity. **The sampling site should be after the last treatment process (including disinfection and dechlorination) and at a location in the discharge stream as close to the actual discharge point as feasible.** There may be no removal of chlorine or any other constituent by chemical or physical means prior to testing without specific approval from the Permitting Authority. See EPA test method manuals (USEPA 2002a, 2002b, 2002c), Section 8, for discussion on selection of sample types and discussion of sample techniques and equipment. Composite samples should be chilled to the specified temperature in the test method manuals as grab sample is being collected. Grab samples should be chilled immediately following collection.

As recommended in the test method manuals, EPA has not modifed the default maximum 36 hour sample holding time (up to 72 hours with Permitting Authority approval), which must be met for the first use of the sample. However, EPA has provided additional clarification and additional flexibility for use of samples for test renewals when the samples meet the initial sample holding times for first use. Sample holding times apply to "first use of the sample," and samples may be used for renewal at 24, 48, and/or 72 hours after first use. The test method manuals also now provide additional flexibility when shipment of renewal samples is delayed during an ongoing test. If shipping problems (e.g. the unsuccessful Saturday delivery) are encountered with renewal samples after a test has been initiated, the Permitting Authority may allow the continued use of the most recently used sample for test renewal. EPA also clarified that sample collection on days 1, 3, and 5 is the recommended (not required) sample collection scheme. A minimum of three samples are required for seven-day chronic tests, but variations in the sampling scheme (i.e., the days on which new samples are collected) are also allowed.

3.6.2 Sample Collection

Grab samples should be collected beneath the surface in chemically clean, pre-labeled plastic or glass sample containers depending on the physical-chemical properties of the suspected target contaminants. For example, polar constituents and metals can be collected in plastic containers, while non-polar (hydrophilic) constituents such as pesticides must be collected in glass

containers. The container must be filled without head space to avoid loss of volatile constituents.

Composite samples are typically collected using refrigerated programmable electronic samplers that deliver a selected volume of sample to a collection container at predetermined times. Steps must be taken to assure that all collection system components are clean and free from contamination prior to use.

3.6.3 Sample Transport and Storage

Samples should be immediately placed in ice chests and covered with wet ice to assure that samples arrive at the test lab at the recommended range of 0–6 °C. The single allowable exception is when a grab sample is delivered to the test laboratory within 4 hours of collection. Samples must be stored in the dark at 0-6 °C until tested within 36 hours. Note that the composite sample holding time begins when the last volume in the 24-hour sample is collected.

3.7 Dilution Water

3.7.1 Selection of Dilution Water

The use of dilution water is an important part of toxicity testing. Dilution water may be either standard laboratory water and/or receiving water. The type of dilution water used in effluent toxicity tests will depend largely on the objectives of the test.

- If the objective of the test is to estimate the absolute acute or chronic toxicity of the effluent, which is the primary objective of NPDES permit-related toxicity testing, standard laboratory dilution water as defined in each test method is used.
- If the objective of the test is to estimate the toxicity of the effluent in uncontaminated receiving water, the test may be conducted using dilution water consisting of a single grab sample of receiving water (if non-toxic), collected either upstream and outside the influence of the outfall, or with other uncontaminated natural water (ground or surface) or standard dilution water having approximately the same characteristics (hardness and/or salinity) as the receiving water.
- If the objective of the test is to determine the additive or mitigating effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected immediately upstream or outside the influence of the outfall.

Note: If the test organisms have been cultured in water which is different from the test dilution water, a second control, using culture water, should be included in the test.

3.7.2 Criteria for Acceptable Dilution Water

Acceptable dilution water for WET testing has the following properties:

- appropriate for the objectives of the test;
- supports adequate performance of the test organisms with respect to survival, growth, reproduction, or other responses that may be measured in the test (i.e., consistently achieves test acceptability criteria for control responses);
- consistent in quality; and
- does not contain contaminants that could produce toxicity.

In the test method manuals (USEPA 1995a, 2002a, 2002b, 2002c), Section 7 describes the types of dilution water that may be used for WET testing depending upon the objectives of the test.

3.7.3 Selection of Dilution Series

The selection of a dilution series (number and spacing of test concentrations) for WET tests is important in producing reliable and precise results. This is most obvious for effect concentrations such as NOEC and lowest-observable-effect-concentration (LOEC) values generated by hypothesis testing. These values are by definition limited to one of the effluent concentrations selected for the test. The precision of these values also is determined by the distance from the NOEC or LOEC to the next highest or lowest effluent concentration.

The test method manuals (USEPA 1995a, 2002a, 2002b, 2002c) suggest, but do not require, a dilution series of 6.25%, 12.5%, 25%, 50%, and 100% effluent for most effluents. This dilution series should be used as a default when little information is known about the effluent being tested and when initial range finding indicates that the effect concentration of interest is within the 6.25% to 100% effluent range. In many situations, a more appropriate dilution series can be selected based on experience from repeated testing of a given effluent. The WET test method manuals do recommend a dilution factor of 0.5 for preparing test concentrations. This recommendation does not fix the dilution factor, but is provided to establish a lower limit on the dilution factor. The use of dilution factors greater than 0.5 is encouraged when historical testing indicates that an effluent is relatively consistent and effect concentrations generally fall within a given range.

For effluent dominated waters, using a standard dilution series of 6.25%, 12.5%, 25%, 50%, and 100% effluent, a measured NOEC value of 50% indicates that the transition from no observable effects to observable effects occurs somewhere between 50% and 100% effluent concentration (the NOEC-LOEC interval). **Therefore, the following dilution series is recommended for effluent dominated (i.e., low dilution) waters: 12.5%, 25%, 50%, 62.5%, and 100%.**

References

Goodfellow WL, Dorn PB, Ausley LW, Burton DT, Denton DL, Grothe DR, Heber MA, Norberg-King TJ, Rodgers JH. 2000. The role of inorganic ion imbalance in aquatic toxicity testing. *Environ Toxicol Chem* 19(1):175-182.

USEPA. 1995a. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to West Coast marine and estuarine organisms. Chapman GA, Denton DL, and Lazorchak JM, eds. Office of Research and Development. Cincinnati, OH. EPA/600/R-95/136.

USEPA. 2002a. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Fifth Edition. Office of Water, Washington, DC. EPA/821/R-02/012.

USEPA. 2002b. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Fourth Edition. Office of Water, Washington, DC. EPA/821/R-02/013.

USEPA. 2002c. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. Third Edition. Office of Water, Washington, DC. EPA/821/R-02/014.

Attachment 3-1. Acute Test Methods

Species Category	*Receiving Water Type*	*Species*	*Typical Toxicants*	*Salinity Range of Effluent Dilutions*
Fish	Freshwater	Fathead minnow, *Pimephales promelas*	ammonia, chlorine	1-6‰
		Rainbow trout, *Oncorhynchus mykiss*	ammonia, chlorine	1-2‰
	Marine	Silverside, *Menidia beryllina*	ammonia, chlorine	1-36‰ Note: Can be used for end of pipe testing, if the effluent is ≥ 5‰
		Topsmelt, *Atherinops affinis*	ammonia, chlorine	5-36‰ Note: Can be used for end of pipe testing, if the effluent is ≥ 5‰
Invertebrate	Freshwater	Water flea, *Ceriodaphnia dubia*	pesticides	1-3‰
		Water flea, *Daphnia pulex* and *Daphnia magna*	pesticides	1-6‰
	Marine	Atlantic mysid, *Mysidopsis bahia*	metals	15- 36‰
		Pacific mysid, *Holmesimysis costata*	metals, insecticides	32-36‰

Source: USEPA. 2002a. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Fifth Edition. Office of Water, Washington, DC. EPA/821/R-02/012.

Attachment 3-2. Chronic Freshwater Test Methods

Species Category	*Species*	*Test Type*	*Endpoints*	*Type Toxicants*
Fish	Fathead minnow, *Pimephales promelas*	7-day renewal test	survival, growth	surfactants, ammonia
Invertebrate	Water flea, *Ceriodaphnia dubia*	7-day renewal test	Reproduction, survival	pesticides, surfactants
Plant	Green alga, *Selenastrum capricornutum*	96-hour non-renewal	growth	metals, herbicides

Source: USEPA. 2002b. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Fourth Edition. Office of Water, Washington, DC. EPA/ 821/R-02/013.

Attachment 3-3. Chronic East Coast and West Coast Estuarine/Marine Test Methods

Category (Method)	*Species*	*Test Type*	*Test Endpoint*	*Type of Toxicants*	*Salinity Range of Effluent Dilutions*
Fish (West Coast)[a]	Topsmelt, *Atherinops affinis*	7-day renewal	survival, growth	ammonia, chlorine, surfactants	10-36‰
Fish (East Coast)[b]	Inland silverside, *Menidia beryllina*	7-day renewal	survival, growth	surfactants, chlorine, ammonia	5-36‰
Invertebrate (West Coast) [a]	Pacific mysid, *Holmesimysis costata*	7-day renewal	survival, growth, fecundity	metals, insecticides	32-36‰
	Red abalone, *Haliotis rufescens*	48-hr non-renewal	shell development	metals, surfactants	32-36‰
	Mussels, *Mytilus sp.*, Oyster, *Crassostrea gigas*	48-hr non-renewal	larval development	metals, chlorine	28-32‰
	Purple urchin, *S. purpuratus*, Sand dollar, *Dendraster excentricus*	48-hr non-renewal	larval development; fertilization	metals, chlorine	32-36‰
Invertebrate (East Coast) [b]	Atlantic Mysid, *Mysidopsis bahia*	7-day renewal	survival, growth, fecundity	metals, ammonia, insecticides	15-36‰
Plant (West Coast) [a]	Giant kelp, *Macrocystis pyrifera*	48-hr non-renewal	germination, germ-tube length (growth)	metals	32-36‰

a USEPA. 1995a Short-term methods for estimating the chronic toxicity of effluents and receiving waters to West Coast marine and estuarine organisms. Office of Research and Development. Cincinnati, OH. EPA/600/R-95/136.

b USEPA. 2002c. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. Third Edition. Office of Water, Washington, DC. EPA/821/R-02/014.

Attachment 3-4. Wet Test Method Rule

The EPA administrator signed the WET methods rule on November 19, 2002, and promulgated in the FR Notice (67 Federal Register 69952 et seq., November 19, 2002) test methods in 40 CFR Part 136, which are further detailed in USEPA 2002a, 2002b, and 2002c. This FR notice provides the statutory authority, background of WET, summary of final rule, changes from proposed rule, response to major comments, statutory and executive order reviews, and references, all of which are useful to permitting authorities implementing WET. The following is a list of those method changes. This document does not include the WET rule details that are specific to conducting a specific test method (e.g., blocking by known parentage) as that is necessary for the testing laboratory to understand and conduct properly.

Summary of Final Rule

WET Method Changes

- Minor corrections and clarifications,
- Incorporation of updated method precision data,
- Requirement for "blocking" by known parentage in the *Ceriodaphnia dubia* Survival and Reproduction test,
- Specification of procedures to control pH drift that may occur during testing,
- Review procedures for the evaluation of concentrations-response relationships,
- Clarification of limitations in the generation of confidence intervals,
- Guidance on dilution series selection,
- Clarification of requirements regarding acceptable dilution waters,
- Procedures for determining and minimizing the adverse impact of pathogens in the Fathead Minnow Survival and Growth Test,
- Requirement for the use of ethylenediaminetetraacetic acid (EDTA) in the *Selenastrum capricornutum* Growth Test.

Additional Revisions to WET Test Methods

- Requirement to meet specific variability criteria when NPDES permits require sublethal WET testing endpoints expressed using hypothesis testing,
- Increases in the required minimum number of replicates for several tests,
- Clarification of required and recommended test conditions for the purposes of reviewing WET test data submitted under NPDES permits,
- Additional clarification of sample holding times,

- Clarification of requirements for reference toxicant testing and additional guidance on evaluating reference toxicant test results,
- Clarification of allowable sample holding temperatures,
- Clarification of biomass as the measured endpoint in survival and growth tests,
- Clarification of requirements for measuring total residual chlorine in WET samples,
- Modification of the test terminations criteria for the *Ceriodaphnia dubia* Survival and Reproduction Test to exclude the counting of fourth brood neonates,
- Additional minor corrections identified by commenters.

References

USEPA. 2002a. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Fifth Edition. Office of Water, Washington, DC. EPA/821/R-02/012.

USEPA. 2002b. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Fourth Edition. Office of Water, Washington, DC. EPA/821/R-02/013.

USEPA. 2002c. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. Third Edition. Office of Water, Washington, DC. EPA/821/R-02/014.

CHAPTER 4. TEST REVIEW AND EVALUATION OF TEST RESULTS

4.1 Overview

This chapter is designed to provide the permit writer a background for evaluating and reviewing WET test results. The statistics used to analyze WET test results are discussed, as well as the quality assurance procedures necessary to implement a successful WET testing program. Test review is an important part of an overall quality assurance program (see Section 4 of test method manuals) and is necessary for ensuring that all test results are reported accurately**. Test review should be conducted on each test by both the testing laboratory and the Permitting Authority.**

This chapter will describe the two statistical approaches typically used to generate the toxicity test effect concentrations. Effect concentrations are concentrations of a test material (i.e., effluent, reference toxicant, receiving or stormwater) associated with the observed biological endpoints (e.g., mortality, growth) followed by data for which is analyzed using either hypothesis testing procedures or point estimate techniques. This chapter will also discuss the test review process for Permitting Authorities which includes:

- examination of the sample handling and collection,
- review of test conditions,
- review of test acceptability criteria (TAC),
- review of concentration-responses and
- evaluation of percent minimum significant differences (PMSDs; test variability)

4.2 Terms and Definitions

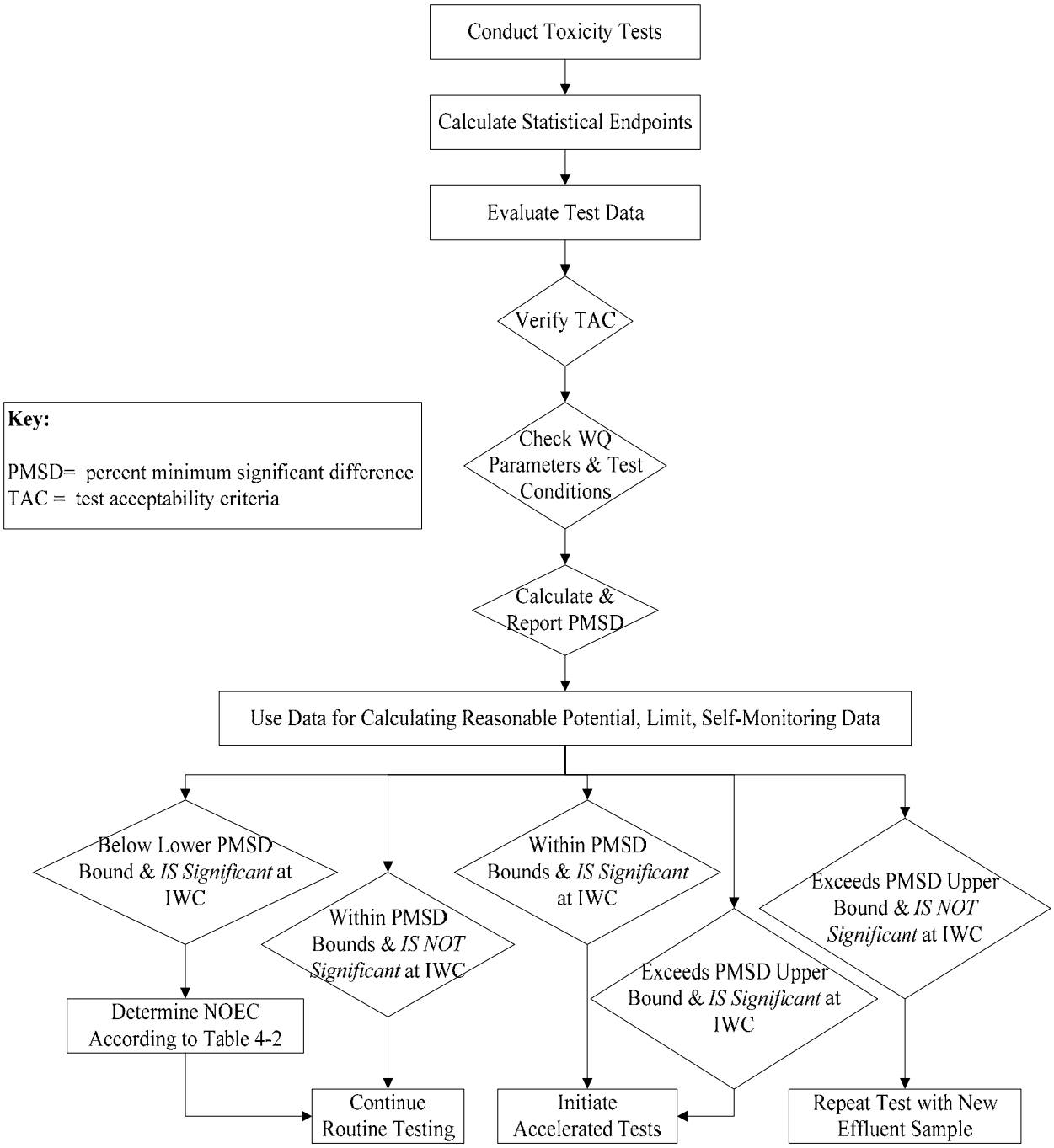
Effect concentrations are concentrations of the test material (e.g., effluent) that produce a specified degree of toxic response or effect (e g., the LC_{50} is the concentration that produces 50% mortality). Effect concentrations are derived from the observed biological endpoints using either hypothesis testing procedures or point estimate techniques.

Hypothesis testing is a statistical procedure (e.g., Dunnett's test) for determining whether a test concentration is statistically different from the control. Endpoints determined from hypothesis testing in aquatic toxicity methods are NOECs and LOECs.

Point estimate procedures are used to determine the toxic concentration that would cause an observable adverse effect (e.g., reduced growth, expressed as EC_{25}) in a given percent of test organisms, calculated from a continuous model (e.g., Probit model). Endpoints determined from point estimates include LC_{50} for acute and EC_{25} for chronic methods. EC_{25} is a point estimate of the toxicant concentration that would cause an observable adverse effect in 25 percent of the test organisms.

A flow chart of the test review and evaluation process is shown in Figure 4-1 below.

Figure 4-1. Test Review and Evaluation Process Flow Chart



4.3 Statistical Approaches to Evaluate Multiple-Concentration Test Designs

This section will highlight some of the statistical discussions covered in the EPA acute (USEPA 2002a) and chronic test methods (USEPA 1995a, 2002b, 2002c). The objective of a toxicity test

is to estimate the highest "safe" or "no-effect concentration" of an effluent, stormwater or ambient water. When a single WET test is conducted, the observed toxicological measured biological endpoints (e.g., survival, reproduction, growth) are recorded. At the end of a test, the data are subjected to an array of statistical analyses to quantify the effects observed during the test. EPA test methods currently recommend two statistical approaches to estimate effect concentrations either hypothesis testing approaches and point estimate techniques both of which are applicable for acute and chronic testing. A good review and discussion of pros and cons of these two statistical approaches is highlighted in Fox and Denton (2002).

The statistical methods used for analyzing test data should be reviewed to verify that the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report. **In all cases (flowchart recommended statistical approaches or flowchart deviations), the data reviewers should verify that necessary assumptions are met for the statistical approach used.**

4.3.1 Hypothesis testing procedures

Hypothesis testing procedures, such as the Dunnett's test, are used to determine the NOEC. The NOEC is the highest concentration of toxicant to which organisms are exposed in a toxicity test that causes no observable adverse effects on the test organisms (i.e., the highest concentration of toxicant in which the values for the observed responses are not statistically significantly different from the controls). Determining the NOEC does not mean, though, that there was "no toxic effect", only that no statistically significant effect was observed.

The procedures consist of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison procedure for comparing each of the treatment means with the control mean, in a series of paired tests. The assumptions when using ANOVA are that the data are distributed normally when tested by Shapiro-Wilk's Test and that the group variances are homogenous when tested by Bartlett's Test. In cases where the number of replicates for each concentration is not equal, a test may be performed with Bonferroni's adjustment for multiple comparisons, instead of using Dunnett's procedure. If either of the two statistical assumptions (normality or homogeneity of variance) fails, then one of the two non parametric tests should be used. The Steel's Many-One Rank Test should be used if there are four replicates per test concentration. If the number of replicates is not equal, then Wilcoxon Rank Sum Test with Bonferroni's adjustment should be used. (See EPA test method manuals, Chapter on Data Analysis, USEPA 1995a, 2002a, 2002b, 2002c).

Hypothesis tests provide comparisons between one or more effluent concentrations and an appropriate dilution water control. The benefits of hypothesis testing include the following:

- the results can provide statistical information regarding test variability (e.g., minimum significant difference (MSD)
- the results inform the regulator of the NOEC

- the researcher can use the same statistical methods for many different test methods and endpoints;
- the researcher can test just the instream waste concentration (IWC) vs. the control (by using a standard t-test); and,
- the researcher can use routine statistical analyses (USEPA 1995a, 2002a, 2002b).

An important criticism of hypothesis tests is that they might have either poor or excessive statistical power since the majority of analyses do not constrain statistical beta error (see Attachment 4-1 for a discussion on defining false positives and false negatives). In one case, a large effect size (e.g., significant biological effect) might not be statistically significant, but in another case a small effect size (e.g., small biological effect) might be statistically significant. Another criticism of hypothesis testing is that no true dose-response relationship can be derived using the hypothesis test, since the NOEC is dependent upon the selection of the dilution series. The true effect level might lie somewhere in between the NOEC and the LOEC. For example, with an NOEC of 25% and an LOEC of 50%, the actual NOEC might lie somewhere between these values. The inability to generate precision estimates with NOECs is also a criticism.

To alleviate some of these concerns, the spacing of the dilution series should be carefully selected. Ideally the concentrations should bracket the IWC and include the IWC as one of the test concentrations. In addition, the within-test variability of individual tests should be reviewed (see discussion on PMSDs). When NPDES permits require sublethal hypothesis testing endpoints, the within-test variability must be reviewed and variability criteria must be applied (see Attachment 4-2 on defining test precision).

4.3.2 Point estimate techniques

Point estimation technique is used to determine the toxicant concentration that would cause an observable adverse effect in a given percent "p" of the organisms. For point estimates, typically the results are reported as the effective concentration (EC) or the inhibition concentration (IC). ECp is generally used with quantal endpoints (e.g., survival or fertilization). When survival is the quantal endpoint, the ECp is typically expressed as the LCp (lethal concentration). The inhibition concentration, ICp, is generally used for tests where a nonquantal continuous endpoints (e.g., length, weight, or reproduction) are measured.

Most point estimate endpoints, such as the LC, EC, or IC are derived from a mathematical model that assumes a continuous concentration-response relationship. By definition, any LC, EC, or IC value is an estimate of some amount of adverse effect. Thus the assessment of a "safe" concentration must be made from a biological standpoint rather than with a statistical test. The biologist must determine some amount of adverse effect that is deemed to be "safe," in the sense that from a practical biological viewpoint it will not affect the normal propagation of fish and other aquatic life in receiving waters.

The statistical models are highlighted in the EPA test method manuals flowchart. Probit analysis is used to estimate LC or EC values from 1 to 50 percent effect of the test organisms measuring quantal endpoints (e.g., survival, fertilization, germination, larval development). The analysis

consists of adjusting the data for mortality in the control, and then using a maximum likelihood technique to estimate the parameters of the underlying log tolerance distribution, which is assumed to have a particular shape.

Probit analysis is contingent on the assumption of a normal distribution of log tolerances. If the normality assumption is not met, and at least two partial mortalities are not obtained, Probit analysis should not be used. It is important to check the results of the Probit analysis to determine if use of this analysis is appropriate. The chi-square test for heterogeneity provides a good test of appropriateness of the analysis. The computer program checks the chi-square statistic calculated for the data set against the tabular value, and provides an error message if the calculated value exceeds the tabular value.

If an acute toxicity data does not fit the Probit model, then LC_{50} may be estimated by Spearman-Karber method or the trimmed Spearman-Karber for acute toxicity only. If a test results in 100% survival and 100% mortality in adjacent treatments (all or nothing effect), the LC_{50} may be estimated using the Graphical method. **If chronic toxicity endpoints, the Linear Interpolation method should be used when Probit analysis is not appropriate, since the effect concentration needed to be observed is less than a 25% effect.**

The Linear Interpolation method is a procedure to calculate a point estimate of the effluent or other toxicant concentration that causes a given percent reduction of the test organisms (e.g., ≤25% effect) in continuous endpoints (e.g., reproduction or growth). Use of the Linear Interpolation method is based on the assumptions:

- the responses are monotonically non-increasing (the mean response for each higher concentration is less than or equal to the mean response for the previous concentration)
- the responses follow a piece-wise linear response function, and
- the responses are from a random, independent, and representative sample of test data.

4.3.3 Point estimate confidence intervals

EPA acknowledges that some point estimation techniques do not generate confidence intervals, but this does not preclude the use of point estimates in compliance determinations. Confidence intervals are not currently reported in the Permit Compliance System (the national database tracking compliance with NPDES permits) nor are they used in compliance determinations. Compliance with permit requirements is based on the point estimate itself and not confidence intervals surrounding the estimate. This approach is no different in WET testing than in chemical testing, where compliance is also based on the analytical result itself.

4.4 Statistical Approaches to Evaluate 2 Sample-Concentration Test Designs

Often in ambient and stormwater toxicity testing design, a laboratory control and a single concentration (e.g., 100% stormwater or ambient water) is tested. In these pass/fail tests, the objective is to determine if the survival in the single treatment (e.g., effluent, ambient, stormwater) is significantly different from the control survival. In this testing design the determination of pass or fail from a single aqueous concentration is ascertained with a standard t-

test (USEPA 2002a, see figure 12 of the acute toxicity "Data analysis section" or in the chronic test method manuals, the appendix on "Single-concentration toxicity test - comparison of control with 100% effluent or receiving water"). First, after the data have been transformed, a test of the assumption of normality is conducted with the Shapiro Wilk's test. The F test for equality of variances is used to test the homogeneity of variance assumption. To perform the t-test, obtain values for the means and variances and use the one-tailed test at the 0.05 level of significance. If the calculated t is greater than the critical t, the conclusion is that the survival in the 100% ambient or stormwater test concentration is significantly less than the survival in the control (i.e., the sample is toxic). **EPA Regions 9 and 10 recommend that the statistical significance (i.e., pass/fail) of a two-sample test design be determined with either a modified t-test (if homogeneity of variance is not achieved) or a standard t-test (if homogeneity of variance is achieved).**

4.5 Test Review Considerations

Test review is an important part of an overall quality assurance program (see QA/QC chapter in the test methods manual). It is necessary to ensure that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the Permitting Authority. The components of test review include:

- review of sample handling and collection,
- review of test acceptability criteria,
- review of test conditions,
- review of concentration-response relationships,
- review of reference toxicant tests, and
- review of test variability (i.e., examination of PMSD values).

4.6 Review of Sampling and Handling

The collection and handling of samples are reviewed to verify that the sampling and handling procedures (see Section 8 of the test method manuals) were followed. Chain-of-custody forms are reviewed to verify that samples were tested within allowable sample holding times. Any deviations from the procedures given in Section 8 of the test method manuals should be documented and described in the data report.

4.7 Review of Test Acceptability Criteria

Test acceptability criteria (TAC) set minimum requirements for performing toxicity tests. These minimum requirements are clearly identified in the test method manuals. Both effluent and reference toxicant tests must meet these TAC. As should be stated in the NPDES permit, if a test fails either the effluent or reference toxicant TAC, then the permittee must repeat the test as soon as possible. For example, the control for both the effluent test and the reference toxicant test must achieve 80% or greater survival and produce an average of 15 young per female for the chronic water flea survival and reproduction test method. These requirements are stated in the

summary of test conditions and test acceptability criteria table in each chapter for the test method manuals. Note, for each test method there is a table in the manuals titled, "Summary of test conditions and test acceptability criteria" for each test species. The Permitting Authority should be familiar with these summary test conditions and TAC.

Test data are reviewed to verify that TAC requirements for a valid test have been met. **Any test not meeting the minimum TAC is considered invalid. All invalid tests must be repeated with a newly collected sample, as soon as possible, but no later than 14 days.**

4.8 Review of Test Conditions

Test conditions are reviewed and compared to the specifications listed in the summary of test condition tables provided for each method. Physical and chemical measurements taken during the test (e.g., temperature, pH, and dissolved oxygen) also are reviewed and compared to specified ranges. Any deviations from specifications should be documented and described in the data report.

The summary of test condition tables presented for each method identifies test conditions as required or recommended. For WET test data submitted under NPDES permits, all "required" test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample. Deviations from "recommended" test conditions must be evaluated on a case-by-case basis to determine the validity of test results. Deviations from recommended test conditions may or may not invalidate a test result depending on the degree of the departure and the objective of the test. The reviewer should consider the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or accepting a test result as valid. For example, if dissolved oxygen is measured below 4.0 mg/L in one test chamber, the reviewer should consider whether any observed mortality in that test chamber corresponded with the drop in dissolved oxygen.

Also, an individual test may be conditionally acceptable if temperature, dissolved oxygen and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test conditions and test acceptability criteria specified for each test method). The acceptability of the test will depend on the experience and professional judgment of the laboratory investigator and the Permitting Authority (see section on data evaluation in the test method manuals). Whereas slight deviations in test conditions may not invalidate an individual test result, test condition deviations that continue to occur frequently in a given laboratory may indicate the need for improved quality control in that laboratory.

4.9 Review of Reference Toxicants

The purpose of generating reference toxicant data is (1) to asses the health and sensitivity of test organisms over time, and 2) to document and demonstrate initially and ongoing acceptable laboratory performance. Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month with a reference toxicant for each toxicity test method conducted in the laboratory during a month. **For a given test method, successive tests must be performed with the same reference toxicant, at the same concentrations in the same dilution water, using the same data analysis methods.** Regardless of the source of test

organisms (in-house cultures or purchased from external suppliers), the testing laboratory must perform at least one acceptable reference toxicant test per month for each type of toxicity test method conducted in that month. If a test method is conducted only monthly, or less frequently, a reference toxicant test must be performed concurrently with each effluent toxicity test. This requirement will document ongoing laboratory performance and assess organism sensitivity and consistency when organisms are cultured in-house. When organisms are obtained from external suppliers, concurrent reference toxicant test must be performed with each effluent sample, unless the test organism supplier provides control chart data from at least the last five months of reference toxicant testing. This requirement assesses organism sensitivity and health when organisms are obtained from external vendors.

The test review of a given effluent or receiving water should include review of the associated reference toxicant test and current control chart. The test reviewer should verify that a quality control reference toxicant test was conducted according to the specified frequency required by the Permitting Authority or recommended by the method. The TAC, test conditions, concentration-response relationship, and test sensitivity of the reference toxicant tests are reviewed to verify that the reference toxicant tests conducted were valid. The results of the reference toxicant tests are then plotted on a control chart and compared to the current control chart limits. Reference toxicant tests that fall outside of the recommended control chart limits are evaluated to determine the validity of associated effluent and receiving water tests (see chapter on quality assurance of test method manuals). Reference toxicant tests should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. An out of control reference toxicant test does not necessarily invalidate the associated test results. The reviewer should consider the degree to which the reference toxicant test fell outside of the control chart limits, the width of the limits, the direction of the deviation (toward increasing test organism sensitivity or toward decreasing test organism sensitivity), the test conditions of both the effluent and the reference toxicant tests, and the objective of the test. More frequent and/or concurrent reference toxicant testing may be advantageous if recent problems (e.g. invalid tests, reference toxicant test results outside of control chart limits, reduced health of organism cultures, or increased within-test variability) have been identified in testing.

4.10 Review of Concentration-Response Relationships

In toxicology, it is conventional to plot the data in the form of a curve relating the dose of the chemical to cumulative percentage of test organism demonstrating a response such as death or reduced growth. Typically, as the toxicant increases in concentration a greater biological response is measured (e.g., increase in lethality, or decrease in growth or reproduction).

The concept of a concentration-response or a dose-response relationship is “the most fundamental and pervasive one in toxicology” (Casarett and Doull 1975). Note, a concentration-response relationship is analogous to the dose-response relationship employed in mammalian toxicity testing. This concept assumes that there is a causal relationship between the dose of a toxicant (or concentration for toxicants in solution) and a measured response. A response may be any measurable biochemical or biological parameter that is correlated with exposure to the toxicant. The classical concentration-response relationship is depicted as a sigmoidal-shaped curve, however, the particular shape of the concentration-response curve may differ for each

coupled toxicant and response pair. In general, more severe responses (such as acute effects) occur at higher concentrations of the toxicant, and less severe responses (such as chronic effects) occur at lower concentrations. A single toxicant also may produce multiple responses, each characterized by a concentration-response relationship. A corollary of the concentration-response concept is that every toxicant should exhibit a concentration-response relationship, given that the appropriate response is measured and given that the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent is toxic and in identifying anomalous test results.

The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. EPA's document (USEPA 2000a) provides guidance on evaluating concentration-response relationships to assist in determining the validity of WET test results. **All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to EPA guidance on the evaluation of concentration-response relationships (USEPA 2000a).** The EPA guidance (2000a) provides review steps for 10 different concentration-response patterns that may be encountered in WET test data. Based on the review, the guidance provides one of three determinations:

- that calculated effect concentrations are reliable and should be reported,
- that calculated effect concentrations are anomalous and should be explained, or
- that the test was inconclusive and should be repeated with a newly collected sample.

It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Data from some tests may suggest consultation with professional toxicologists and/or regulatory officials. Tests that exhibit unexpected concentration-response relationships also may indicate a need for further investigation and possible retesting.

4.11 Review of Test Variability

When NPDES permits require sublethal hypothesis testing endpoints for the chronic test methods USEPA 2002b, 2002c (e.g., growth or reproduction NOECs and LOECs), the within-test variability must be reviewed and variability criteria must be applied as described in this section. When the methods are used for non-regulatory purposes, the variability criteria are recommended but are not required, and their use (or the use of alternative variability criteria) may depend upon the intended uses of the test results and the requirements of any applicable data quality objectives and quality assurance plan. Good test precision or low within-test variability is a general measure of test quality (see Attachment 4-2). Note: the Permitting Authority may always be more stringent than specified as above.

To measure test variability, calculate the PMSD achieved in the test. The PMSD is the smallest percentage decrease in growth or reproduction from the control that could be determined as statistically significant in the test. The PMSD is calculated as 100 times the MSD divided by the control mean. The MSD equation is shown in Attachment 4-1. PMSD may be calculated

legitimately as a descriptive statistic for within-test variability, even when the hypothesis test is conducted using a non-parametric method. The PMSD bounds were based on a representative set of tests, including tests for which a non-parametric method was required for determining the NOEC or LOEC. The hypothesis testing procedures to determine test results should follow the statistical flow charts provided for each method. That is, when test data fail to meet assumptions of normality or heterogeneity of variance, a nonparametric method (determined following the statistical flowchart for the method) should be used to calculate test results, but the PMSD may be calculated as described above (using parametric methods) to provide a measure of test variability.

Compare the PMSD measured in the test with the upper PMSD bound variability criterion listed in Table 4-1. When the test PMSD exceeds the upper bound, the variability among replicates is unusually large for the test method. Such a test should be considered insufficiently sensitive to detect toxic effects on growth or reproduction of substantial magnitude. A finding of toxicity at a particular concentration may be regarded as trustworthy, but a finding of "no toxicity" or "no statistically significant toxicity" at a particular concentration should not be regarded as a reliable indication that there is no substantial toxic effect on growth or reproduction at that concentration.

If the PMSD measured for the test is less than or equal to the upper PMSD bound variability criterion in Table 4-1, then the test's variability measure lies within normal bounds and the effect concentration estimate (e.g., NOEC or LOEC) would normally be accepted unless other test review steps raise serious doubts about its validity. If the PMSD measured for the test exceeds the upper PMSD bound variability criterion in Table 4-1, then one of the following two cases applies.

If toxicity is found at the permitted receiving water concentration (RWC) based upon the value of the effect concentration estimate (NOEC or LOEC), then the test shall be accepted and the effect concentration estimate may be reported, unless other test review steps raise serious doubts about its validity.

If toxicity is not found at the permitted RWC based upon the value of the effect concentration estimate (NOEC or LOEC) and the PMSD measured for the test exceeds the upper PMSD bound, then the test shall not be accepted, and a new test must be conducted promptly on a newly collected sample (preferably within 14 days).

To avoid penalizing laboratories that achieve unusually high precision, lower PMSD bounds shall also be applied when a hypothesis test result (e.g., NOEC or LOEC) is reported. Lower PMSD bounds, which are based on the 10th percentiles of national PMSD data, are presented in Table 4-1. The 10th percentile PMSD represents a practical limit to the sensitivity of the test method because few laboratories are able to achieve such precision on a regular basis and most do not achieve it even occasionally. In determining hypothesis test results (e.g., NOEC or LOEC), a test concentration shall not be considered toxic (i.e., significantly different from the control) if the relative difference from the control is less than the lower PMSD bounds in Table 4-1.

If the permit specifies that self-monitoring data are to be generated using hypothesis testing approaches, then the analyst should report the NOEC as follows. Find the smallest concentration for which (a) the treatment mean differs significantly from the control mean and (b) the relative difference (see example below) is not smaller than the 10th percentile in Table 4-2. Therefore, the NOEC is the next smaller test concentration. In other words, concentrations having a very small relative difference from the control (smaller than the lower PMSD bound) would be treated as if they do not differ significantly from control (even if they do so), for the purpose of determining the NOEC.

Table 4-1 Variability Criteria (Upper and Lower PMSD Bounds) for Sublethal Hypothesis Testing Endpoints Submitted Under NPDES Permits[a]

Test Method	*Endpoint*	*Lower PMSD Bound*	*Upper PMSD Bound*
Method 1000.0 Fathead Minnow Larval Survival and Growth Test[b]	Growth	12	30
Method 1002.0 *Ceriodaphnia dubia* Survival and Reproduction Test[b]	Reproduction	13	47
Method 1003.0 *Selenastrum capricornutum* Growth Test[b]	Growth	9.1	29
Method 1006.0, Inland Silverside Larval Survival and Growth Test[c]	Growth	11	28
Method 1007.0, *Mysidopsis bahia* Survival, Growth and Fecundity Test[c]	Growth	11	37

a Lower and upper PMSD bounds were determined from the 10th and 90th percentile, respectively, of PMSD data from EPA's WET Interlaboratory Variability Study (USEPA 2001a; USEPA 2001b)
b USEPA 2002b
c USEPA 2002c

Table 4-2 illustrates the application of the lower PMSD bound for the reproduction endpoint of a *Ceriodaphnia* chronic test. In this example, the effluent test's PMSD was 9.9, smaller than the 10th percentile value of 13 (USEPA 2002b). The IWC concentration differed significantly from the control. The test falls under outcome number 5, a significant but very small difference at the IWC. The first step is to calculate the relative differences from control (Table 4-1) as [(control mean - treatment mean) divided by (control mean)] × 100. The next step is to determine which relative differences exceed the PMSD lower bound, 13 in this case (see the 3rd column of Table 4-1). Finally, the NOEC is determined as described above. The NOEC is 12.5 percent effluent for this example.

Table 4-2 Example of Applying the Lower PMSD Bound for the Chronic *Ceriodaphnia dubia* Test with the Reproduction Endpoint

Concentration	*Reproduction*	*Relative Difference*	*Does Relative Difference*

(Percent Effluent)	*(Mean Of Ten Replicates)*	*From Control*	*Exceed 13?*
100%	5.08 *	82	Yes
50%	12.4 *	56	Yes
25%	23.4 *	17	Yes
IWC = 12.5%	25.3 *	10	No
6.25%	26.1	7.4	No
Control	28.2	0	No

* Differs statistically from the control as determined by MSD = 2.8 neonates. Thus, treatment means that are less than 28.2 - 2.8 = 25.4 would be statistically significant. These correspond to relative differences greater than 100 (2.8 / 28.2) = 9.9 percent.

NOTE: The lower PMSD bound for this method and endpoint is 13. In this example, the statistically determined NOEC is 6.25 percent effluent using the test's (very small) PMSD of 9.9. Therefore, the reported NOEC should be 12.5 percent effluent after applying the lower PMSD bound.

To assist in reviewing within-test variability, EPA recommends maintaining control charts of PMSDs calculated for successive effluent tests (USEPA 2000b). A control chart of PMSD values characterizes the range of variability observed within a given laboratory, and allows comparison of individual test PMSDs with the laboratory's typical range of variability. Control charts of other variability and test performance measures, such as the MSD, standard deviation or CV of control responses, or average control response, also may be useful for reviewing tests and minimizing variability.

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Attachment 4-1. Background Statistics: Hypothesis Testing

One objective of a toxicity test is to determine if the toxicological measurement endpoint in one treatment (an effluent dilution) differs from the endpoint in another treatment (a control). The null hypothesis (H_0) is that there is no difference between the two treatments (i.e., the effluent or ambient water is not toxic). The alternative hypothesis (H_a) is that there is a statistical difference between the control and the treatment (i.e., the effluent or ambient water is toxic). The table below presents the possible outcomes and decisions that can be reached in hypothesis testing.

Comparison of Type I and Type II Statistical Decision Errors[a]

Decision	***True Condition Treatment = Control***	***True Condition Treatment > Control***
Treatment = Control	Correct Decision (1 – α)	False Negative Type II error (β)
Treatment > Control	False Positive Type I error (α)	Correct Decision (1- β) (power)

a The alpha, α, represents the probability of a type I statistical error (i.e., false positive) and beta, β, is the probability of making a type II statistical error (i.e., false negative).

Hypothesis tests can be designed to control (minimize) the chances of making incorrect decisions. A Type I error (alpha, α) results in the false conclusion that an effluent is toxic when the effluent is not toxic. A Type II error (beta, β) results in the false conclusion that the effluent is not toxic, when the effluent is actually toxic. Traditionally, acceptable values for α have ranged from 1 to 10% with 5% used most commonly. This choice should depend upon the consequences of making a Type I error. Historically, having chosen α, environmental researchers have ignored β and the associated power of the test (l- β). Power is the probability of correctly detecting a true toxic effect (i.e., declaring an effluent toxic when in fact it is toxic).

α and β are dependent on each other (as α increases, β decreases), assuming that sample size (number of treatments, number of replicates), the amount of difference to detect and the variability are held fixed. Increasing α level of a statistical test increases the power of the test, if all other factors are held constant. Selection of the appropriate α level of a test is a function of the costs associated with making Type I errors. For a given α, β decreases (power increases) as the sample size increases and the variance decreases. The desired power of the statistical analysis should be considered in the study plan development.

The use of the statistical tests can protect regulators from concluding the effluent is toxic when it is not. The statistical tests can control the risk of a Type I error, which is important when the results are shown to be toxic. Without a power analysis, the assurance that a sample is not toxic is questioned, and the possibility exists that a false negative has occurred.

Although the EPA test method manuals (USEPA 1995a, 2002a, 2002b, 2002c) require an α of 0.05 (5%), a level of β is not specified. If β is not specified, then we might not detect toxicity when, in fact, an effluent is toxic. Without specifying the level of β, there is little incentive for a

testing laboratory to produce precise test results (i.e., limit test variability). Therefore, EPA requires the review of percent minimum significant difference (PMSDs) by testing laboratories and Permitting Authorities. Note, the EPA (2000b) discussed using an α level of 0.01 under specific conditions. **However, in the final WET methods rule, EPA recommended that only an α rate of 0.05 is to be used.**

Test sensitivity and minimum significant difference

The MSD is a measure of the within-test variability and represents the amount of difference from the control that can be detected statistically.

The following formula is used to calculate MSD (described by USEPA 1995a, 2002a, 2002b, 2002c):

$$MSD = d\, s_w \sqrt{(1/n_1) + (1/n)}$$

Where

d = critical value for the Dunnett's procedure.
s_w = the square root of the within mean square error (MSE).
n_1 = number of experimental units in the control treatment.
n = the number of experimental units per treatment, assuming an equal number at all other treatment.

The MSD is often expressed as a percentage of the toxicological endpoint in the control response (%MSD = 100 x MSD/control mean). The MSD, though, incorporates alpha (type I error) and experimental design (number replicates, treatments), in addition to an estimate of test variability (i.e., MSE). Distributions of the MSD values of multiple tests for a specific reference toxicant and test method can be used to determine the level of sensitivity that can be achieved by a certain percentage of the tests. The MSD should increase as the MSE increases when the number of replicates and treatments and alpha are constant.

To summarize, the sensitivity of the toxicity test will depend in part on the number of replicates of experimental units per treatment, the alpha and beta (provided beta is used to determine the effect size desired), and the variability (e.g., MSE). The power to detect differences increases (i.e., MSD decreases) as the variability decreases and the effect size increases. These discussions demonstrate the importance of measuring test sensitivity and setting the power for toxicity test methods. The issue of false positive and false negative errors needs to be evaluated along with test power and sensitivity to decide the appropriate testing frequency for compliance purposes.

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Attachment 4-2. Importance of Quality Control Procedures and Defining Test Precision

Quality Control Procedures

This quality assurance (QA) section will only highlight the general discussions from the test method manuals, such as the use of reference toxicants, and defining test precision with reference toxicants. Development and maintenance of a toxicity test laboratory QA program requires an ongoing commitment by laboratory management. As stated in the toxicity test method manuals each toxicity test laboratory should:

- Appoint a QA officer with the responsibility and authority to develop and maintain a QA program;
- Prepare a quality assurance plan with stated data quality objectives;
- Prepare written descriptions of laboratory standard operating procedures for culturing, toxicity testing, instrument calibration, sample chain-of-custody procedures, laboratory sample tracking system, glassware cleaning, etc.; and
- Provide an adequate, qualified technical staff for culturing and testing organisms, and suitable space and equipment to assure reliable data.

The EPA acute and chronic toxicity test method manuals each contain a chapter on QA procedures. Topics covered in the chapter include handling of effluents and receiving waters, quality of test organisms, food quality, calibration and standardization, reference toxicant testing, and record keeping. Of particular importance is the requirement to conduct satisfactory reference toxicant tests in conjunction with effluent or ambient water tests. Reference toxicant tests confirm the sensitivity of the test organisms and demonstrate a laboratory's ability to obtain consistent results with WET test methods. Appropriate laboratory practices are essential in obtaining quality test data. QA practices for toxicity tests include all aspects of the test that affect the quality of the data such as:

- Effluent/ambient water sampling and handling
- source and condition of the test organisms
- condition of equipment
- test conditions
- instrument calibration
- replication
- use of reference toxicants
- record keeping
- data evaluation

Test Precision

Precision is a measure of test consistency or repeatability both within a laboratory (intralaboratory) and among several laboratories (interlaboratory). Precision is quantified by a variety of measures including the CV of point estimates (e.g., LC_{50} for acute endpoints and EC/IC_{25} for chronic endpoints) from multiple tests conducted with the same test method and reference toxicant. EPA (2000b) analysis demonstrated and concluded that comparisons of WET method precision with method precision for analytes commonly limited in NPDES permits clearly demonstrate that the variability of the WET methods is within the range of variability experienced in other types of analyses. In addition, several researchers (Grothe et al. 1996, Burton et al. 1996, DeGraeve et al. 1998) noted that method performance improves when prescribed methods are followed closely by experienced analysts.

Test results will depend upon the species tested, source of the test organisms, water quality parameters (e.g., use of temperature as specified in the test method manuals) and food and dilution water quality. The repeatability or precision of toxicity tests is also a function of the number of test organisms used in each test concentration.

Factors which can affect test success and precision include:

- the experience and skill of the laboratory analyst
- test organism condition and sensitivity;
- dilution water quality;
- chemical and physical water quality parameters (e.g., temperature, DO); and
- quality and quantity of food provided.

The EPA TSD (USEPA 1991a) contains the summarized intra- and interlaboratory precision data for the freshwater and east coast marine test methods. Grothe and Kimerle (1985), Rue et al. (1988), Morrison et al. (1989), Grothe et al. (1990) discussed the precision of select toxicity test methodologies and found them to be comparable to commonly accepted chemical analytical methodologies. Grothe and Kimerle (1985) concluded that the reproducibility of the *D. magna* toxicity test was as good as, if not better than, commonly accepted analytical methods. They postulated that one of the main reasons those low CVs were obtained in their study was because the method was clearly defined and uniformly followed by all laboratories. Anderson (1991) and the Biomonitoring Science Advisory Board (BSAB 1994) have examined the precision of test methods used on the west coast and generally found the tests had very good precision. Denton et al. (1992) also found the overall interlaboratory CVs for four west coast marine species ranged from 11.5% for *Haliotis rufescens*, the red abalone larval development test to 38.7% for *Strongylocentrotus purpuratus*, the purple urchin fertilization test. The BSAB report (1994) also concluded that toxicity tests should not be gauged by variability alone. The report also concluded that other factors at least as important as precision included sensitivity, accuracy and ecological relevance. WET testing can be improved most usefully by decreasing intra-test variability.

Specific factors that affect variability in WET analyses have been described in several papers (Burton et al. 1996; Ausley 1996; Erickson et al. 1998; Davis et al. 1998). The most important initial consideration in developing precise data is a laboratory's experience and success in performing a specific analysis. Most critical reviews of WET data precision emphasize this initial consideration. Experienced professionals most likely will be able to develop the most consistent and reliable information and can interpret anomalous conditions in the testing or results.

An additional factor in considering WET test method variability is whether the prescribed methods (e.g., see chapter on test review of the EPA test method manuals) are being followed appropriately. Both the Permitting Authority and permittee should strive to ensure that such practices are in place for any program developing WET data, whether by national laboratory accreditation, State regulatory certification, direct permittee oversight, or specific contractual agreement with the laboratory.

When the variability of WET analyses is viewed in the context of the NPDES program, these techniques produce data that are as precise as those from chemical analyses (USEPA 2000b). As with any other analytical system, lack of experience in performing the analyses, lack of adherence to prescribed QA practices or failure to follow good laboratory practices will reduce the precision of the results. Studies of these factors by independent researchers from both the regulatory and regulated communities support these conclusions. While examples of poor-quality, highly variable results from chemical analyses have also been publicized, these results are frequently influenced by the shortcomings mentioned above. **Permittees who must generate and use WET data should become well-educated in data quality interpretation, and permittees should require that QC practices be followed by laboratories generating the data.** See "Conclusions and Guidance to Laboratories, Permittees, and Regulatory Authorities" chapter of EPA (2000b) for more detailed discussion and approaches to address to minimize test method variability.

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Attachment 4-3 Evaluation of Toxicity Data

Permit Review

1. Examine the test results to verify that the laboratory is using the test method and dilution series as required in the NPDES permit. The dilution series being tested should always include the receiving water concentration (RWC). Note: This may need to be performed only after a permit has been first issued.

2. Evaluate the test results against the permit requirements for WET to assess whether the limit or numeric monitoring trigger is being achieved. For example, where a WET limit or numeric monitoring trigger is expressed in terms of TUs then the value is expressed as a value "not to be exceeded." Where a WET limit or numeric monitoring trigger is expressed in terms of "% effluent at the RWC," the value is expressed as a value that the % effluent must be at or above.

Test Review

Test review is an important part of the overall quality assurance program and is necessary for ensuring that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the Permitting Authority. Note, see the chapter on Test Review of the specified toxicity test methods manual.

1. Examine the results to verify the sample was maintained at the proper temperature from time of collection to arrival at the testing laboratory. Also, does the sample meet the test initiation and renewal holding time requirements?

2. Evaluate the test results for the effluent to verify that the laboratory met the TAC as specified in the test method. See the individual "Summary of Test Conditions and TAC" section for each test method (USEPA 1995a, 2002a, 2002b, 2002c). All invalid tests must be repeated with a newly collected sample, as specified in permit.

3. Examine the "Summary of Test Conditions and TAC" section for the specific method to determine whether the required and recommended test conditions were met. Below is a single example for a required test condition and a recommended test condition that would be specific to the particular toxicity test method listed in the permit.

    a. Did the laboratory conduct the test using the required test conditions? Some of the test conditions listed which are specified as "required" and therefore the condition must be met. For example, did the test use the required minimum number of replicates, number of test organisms, test type, etc.? All required test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample.

    b. Did the laboratory conduct the test using the recommended test conditions? Some of the listed test conditions are specified as "recommended" and therefore the

range should be obtained. For example, when the test method specifies number of test organisms per test chamber, the test condition will provide a recommended number of test organisms (e.g., 10 larvae) per test chamber. A testing laboratory can use more than the recommended number of test organisms per chamber as long as the loading capacity is maintained.

4. Examine the statistical results to verify the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report.

5. Examine the concentration-response relationships as these must be reviewed to ensure that calculated test results are interpreted appropriately. All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to EPA guidance on the evaluation of concentration-response relationship (USEPA 2000a).

6. Test review of a given effluent or receiving water test should include review of the associated reference toxicant test and current control chart. Were out-of-control reference toxicant test results evaluated to determine appropriate corrective action?

7. The within-test variability of individual tests should be reviewed. When NPDES permits require sublethal hypothesis testing endpoints from Methods 1000.0, 1002.0, 1003.0, 1006.0, and 1007.0 (e.g., growth or reproduction NOECs and LOECs), within-test variability must be reviewed and variability criteria must be applied as described in the Method Manuals Section on Test Review. See “Conclusions and Guidance to Laboratories, Permittees, and Regulatory Authorities” chapter of EPA (2000b) for more detailed discussion and approaches to address to minimize test method variability.

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CHAPTER 5. TOXICITY REDUCTION EVALUATIONS

5.1 Overview

When WET testing demonstrates that effluent toxicity exceeds the NPDES permit limit or monitoring trigger, the principal mechanism for bringing a permittee into compliance is a Toxicity Reduction Evaluation (TRE). The TRE is a methodical, stepwise and iterative process that uses information generated in each step to identify the causative toxicant(s) of WET and either remove them at the source or implement in-plant treatment to reduce their concentration(s) below toxic levels, and then confirm the reduction in effluent toxicity through WET monitoring. Ultimately, the goal of the TRE is to achieve compliance with permit WET requirements. TREs can vary widely in complexity and expense, ranging from simply improving housekeeping procedures to conducting intensive Toxicity Identification Evaluations (TIEs). EPA and others have published extensive TRE/TIE technical guidance that is referenced at the end of this chapter. In addition, numerous TIE papers and case studies have been published, which demonstrate the efficacy of the TIE process in identifying the cause(s) of WET.

5.2 Approaches for Reducing Toxicity

Toxicity may be reduced by implementing one of two approaches within the TRE: (1) a TIE, or (2) treatability studies. The decision to pursue either the TIE or treatability approach depends on a number of site-specific and cost considerations. Generally, the TIE approach is favored because it results in control of toxics at the source rather than modifying plant operations to treat or degrade the toxicity with subsequent discharge to the environment. In practice, the TIE approach is usually implemented first, with the treatability option applied if the TIE approach is unsuccessful in identifying and controlling toxicity. Identification of the toxicant(s) and reduction at the source would likely lead to chemical-specific limits, whereas toxicity reduction using a treatability approach would generally result in a WET limit. Regardless of which approach is used, toxicity must be reduced to levels that ensure compliance with permit requirements and attainment water quality standards as demonstrated by continued WET monitoring.

5.3 TRE/TIE Work Plan

EPA Regions 9 and 10 recommend that an initial TRE/TIE Work Plan be developed by the permittee within 60-90 days of the effective date of the permit. The TRE Plan developed by the permittee is intended to be a written description of activities that will take place in the event of a WET exceedence. The TRE Work Plan, at a minimum, has the following characteristics (Norberg-King et al. 2005, Chapter 3):

- Identify the roles and responsibilities of the TRE team
- Describe a complete list of data types to be reviewed
- Provide an overview of proposed steps to address and resolve toxicity. The plan should be detailed yet allow flexibility for inclusion of other approaches as additional TRE information is obtained.

- Include a schedule for conducting the TRE and reporting progress to the Permitting Authority.

Because most TRE/TIE work plans are developed before any permit violation or monitoring trigger occurs, they initially must be fairly generic in nature. **However, the work plan should be updated with an implementation time schedule, as the TRE progresses, to incorporate site-specific information and altered TIE approaches suggested by results of the initial TIE activities.** Any alterations to the approaches or implementation schedule should be thoroughly justified and a rationale for the proposed course of action must be presented. Reasonable time should be allowed for each aspect of the study. The time it takes to conduct a TRE can vary considerably depending on the facility type, and complexity and characteristics of the effluent toxicity. For example, an industrial facility with limited processes and waste streams should be easier to characterize than a large POTW with multiple influents from industrial and urban sources. Other factors, such as multiple toxicants, qualitative and quantitative changes in toxicity, intermittent and/or non-persistent toxicity all tend to increase the time it takes to complete a TRE. EPA indicates that most TREs are resolved within 28 months (Norberg-King et al. 2005). Ausley et al. (2005) has suggested the following time frames for the various TRE components.

Time Frame for Conducting a TRE

Task	*Time Frame (Months)*
Data, process and housekeeping review	1 – 3
Phase I TIE	1 – 6
Phase II TIE	1 – 6
Phase III TIE	1 – 3
Toxicity Source Tracking	1 – 3
Toxicity Treatability	1 – 3
Total	**6 – 30 months**

Source: Adapted from Ausley et al. 2005

Permittees should seek technical review and comment from their Permitting Authority when developing TRE plans that outline investigative and problem resolution techniques, including reasonable timelines and milestones, in order to avoid delays and maximize consideration of relevant factors that may affect toxicity. The Permitting Authority should then approve the TRE schedule and completion date. The Permitting Authority should either concur with the technical merit of the plan or recommend modifications that would improve its technical merit. A close cooperative relationship should be established among the permittee (and, if applicable, the permittee's technical consultant) and the Permitting Authority early in the TRE process. This relationship should be maintained until the TRE is successfully completed and any controls necessary to prevent unacceptable levels of toxicity are fully implemented. This process allows

all parties to understand the requirements and expectations, and encourages evolution of the plan toward the most effective resolution. Collaboration among the parties throughout the TRE process will add to its effectiveness. EPA describes a 7-step TRE process, which is shown in Figure 5-2 and briefly described below.

Step 1: Accelerated WET Monitoring

The Permitting Authority should establish in the permit conditions under which the permittee must initiate accelerated monitoring and the TRE. Generally, this will be when WET testing results obtained during routine WET monitoring indicate toxicity above either WET permit limit or monitoring trigger. **This document recommends that accelerated monitoring consist of six WET tests conducted at approximately 2-week intervals over a 12-week period.** During this accelerated monitoring phase, if more than one sample demonstrates an unacceptable level of toxicity, the permittee must initiate the TRE work plan. When intermittent toxicity is found (i.e., when toxicity is not detected in every test event with each subsequent sampling event) the permittee should alter sampling procedures to obtain and store adequate sample volume such that WET testing and subsequent TIE procedures can be conducted on the same sample (if the WET testing indicates toxicity).

Step 2: Information and Data Acquisition

The first step in the TRE is the collection of information and analytical data pertaining to effluent toxicity. This information includes data on the operation and performance of the treatment plant, including:

- Industrial waste surveys (IWS)
- Permit applications
- Industrial user compliance reports
- Plant design criteria

The importance of this initial information gathering phase cannot be overstated in terms of optimizing a successful outcome of the TRE. By carefully reviewing the information collected and comparing trends in flow patterns, treatment efficiency, wastewater loading and effluent constituents with toxicity patterns over time, permittees may be able to narrow the scope of further investigations and possibly even identify problem constituents.

Step 3: Facility Performance Evaluation

POTW treatment deficiencies that cause poor pollutant removal can have an adverse effect in toxicity reduction. As an initial step, effluent toxicity, operations and performance data should be carefully examined to identify treatment deficiencies or in-plant sources of toxicity. In addition, the POTW pretreatment program data should be reviewed to indicate possible sources of toxicity. The municipal TRE manual (USEPA 1999b) provides in-depth discussion of parameters to be evaluated in this part of the TRE.

If a treatment deficiency is identified, studies should be conducted to evaluate treatment modifications before proceeding with the TRE. If plant performance is not a cause of toxicity, or treatment modifications do not reduce toxicity, the permittee should proceed with the TIE.

Step 4: Toxicity Identification Evaluation

EPA has published TIE procedures to determine the causes of acute and chronic effluent toxicity to freshwater species (USEPA 1989b, 1989c, 1991c, 1992, 1993b, 1993c) and to estuarine/marine organisms (USEPA 1996a). The generic TIE protocols are performed in three phases: toxicity characterization (Phase I), toxicant identification (Phase II), and toxicant confirmation (Phase III). A flow diagram of the TIE process is shown in Figure 5-2.

The Phase I TIE manipulations are designed to sequester or remove toxicity caused by specific classes of chemicals, as shown in Table 5-1.

Table 5-1. Purpose of Phase I TIE Treatments

TIE Treatment	*Treatment Identifies*
Initial toxicity (unaltered effluent)	Initial toxicity test demonstrating toxicity of sample
Baseline toxicity (unaltered effluent tested simultaneously with other TIE manipulations)	Results compared to TIE manipulations to assess effectiveness of TIE manipulations
pH adjustment/filtration (pH 3 and pH 11)	Particulate-bound toxicants
pH adjustment/aeration (pH 3 and pH 11)	Ammonia and volatile, oxidizable toxicant
C18 (or C8) solid-phase extraction (SPE) at pH 3, pH 9, and pH i	Polar and non-polar organic chemicals
Sodium thiosulfate addition	Oxidants and some cationic metals[a]
Ethylene diaminetetraacetic acid (EDTA) addition	Cationic metals
Graduated pH adjustments	Ammonia and pH-sensitive toxicants
Piperonyl butoxide (PBO)	Organophosphate insecticides (decreases toxicity) Pyrethroid insecticides (increases toxicity)

a Copper, silver and selenium

Each of these TIE treatments are applied to the test sample and comparison of the level of baseline toxicity with the TIE treatments identify the physical/chemical characteristics of the

toxicants. It is essential that proper controls and blanks be used with each TIE treatment, and that a high level of QA/QC is maintained throughout the TIE process. EPA cautions that the investigator should approach the TIE without a preconceived notion as to the cause of toxicity and therefore all treatments should be conducted. On the other hand, if one wants to know the role of a single chemical or class of chemicals, ammonia for example, the TIE can be designed to accomplish that goal. If the standard suite of Phase I treatments are ineffective in identifying cause(s) of toxicity, other techniques can be used, including anion and cation ion exchange resins and activated charcoal molecular sieves (Burgess et al. 1997).

Application of the TIE process over the years has demonstrated its applicability to virtually every test species used in WET, including marine species (Burgess et al. 1995), although the use of marine species require that test samples be adjusted (after the TIE treatment). Note, the samples need adjustment of the salinity before the TIE manipulations to insure salinity consistency between treatments and because some of the manipulations must be performed with salinity adjusted samples; for example, the graduated pH manipulation) to the appropriate salinity using dry sea salts or hypersaline brine (Ho et al. 1995; USEPA 1996a). One other caveat in marine TIEs is that due to the strong carbonate buffering capacity of sea water, the only effective means of controlling pH is to use controlled atmospheric chambers.

The EPA Phase I TIE manuals (USEPA 1991c, 1992, 1996a) describe a process where the sample is split into aliquots, each of which is subjected to a single TIE manipulation concurrently with the other treatments ("parallel" treatment approach). However, EPA points out that the Phase I TIE characterization procedures are relatively broad and can indicate more than one class of toxicant. Additional tests or an altered approach may be needed to delineate/confirm the role of a particular chemical class in the effluent toxicity, especially when multiple toxicants are present (USEPA 1993b, 1993c). For example, when the primary toxicant is present in high concentrations, it may mask the other potential toxicant(s) in the sample – ammonia is a common example. In these cases, sequential treatments ("stacked" treatment approach) can be used to evaluate the role of secondary toxicants; for example, removal of ammonia by zeolite followed by removal of non-polar organics by SPE treatment in cases where multiple toxicants are present at toxic concentrations.

Results of Phase I can be compared with pretreatment program data and chemical-specific effluent data to identify potential toxicants. However, chemical analysis conducted in the absence of Phase I TIE information (i.e., chemical class of toxicant(s), to guide the type of analysis) are usually wasted expenditures. For this reason, EPA cautions that chemical-specific tracking should be conducted after the toxicant(s) are identified and confirmed in Phase II and Phase III TIEs, respectively (USEPA 1993b, 1993c).

The Phase I TIE process should be repeated with multiple samples until a clear pattern of toxicity emerges.

5.4 Interpretation of Phase I TIE Results

Phase I characterization provides information on the chemical class(es) responsible for the effluent toxicity, and is applicable to both acute and chronic endpoints. The following guidance

is given by EPA for interpreting Phase I TIE results for various classes of toxicants. Note that the toxic response is assessed by comparing the results for each of the TIE treatments, against the toxicity measured in the baseline (pre-treatment) test.

Ammonia

- Ammonia toxicity can be assessed by zeolite removal or the graduated pH test.
- Toxicity increases in the graduated pH test at higher pH
- Toxicity decreases after zeolite treatment. If the zeolite removal procedure is used, an ammonia add-back step (spiking the zeolite-treated sample with ammonia at the original concentration in the sample) is essential to ensure that the zeolite has not removed other constituents.
- The effluent is more toxic to *P. promelas* than to *C. dubia*.

  Note: If the concentration of total ammonia (as nitrogen) is 5 mg/L or more and chronic toxicity is a concern, the potential for ammonia toxicity should be evaluated.

Drawing conclusions about ammonia toxicity based solely on observed concentrations can be misleading. Ammonia is an example of a toxicant that acts independently of other toxicants in effluents. Even through ammonia concentrations may appear to be sufficient to cause all of the effluent toxicity, other toxicants may be present and may contribute to toxicity when ammonia is removed. Thus, it is important to ascertain if ammonia is masking other potential toxicant(s) in the test sample using the sequential TIE approach previously described.

Oxidants

- Toxicity is reduced or removed in the sodium thiosulfate addition test.
- Toxicity is reduced or removed in the aeration test.
- The sample is less toxic over time when held at 4 °C (and the type of container does not affect toxicity).
- *C. dubia* are more sensitive to the effluent than *P. promelas*.

Non-polar organic toxicants

- Toxicity in the post C18 SPE column test is absent or reduced
- Toxicity was recovered in the methanol eluate test (‘stronger’ solvents may be required to elute highly lipophilic chemicals from SPE columns)
- Toxicity was dramatically changed by piperonyl butoxide (PBO) addition (PBO decreases toxicity of organophosphate insecticides and increases toxicity of pyrethroid insecticides)

Cationic metals

- Toxicity is removed or reduced in the EDTA addition test

- Toxicity is removed or reduced in the post-C18 SPE column test
- Toxicity is removed or reduced in the filtration test, especially when pH adjustments are coupled with filtration
- Toxicity is removed or reduced in the sodium thiosulfate addition test
- Erratic dose response curves are observed

None of these characteristics is definitive, with the possible exception of EDTA. In addition, toxicity may be pH sensitive in the range at which the graduated pH test is performed, but may become more or less toxic at lower or higher pH depending on the particular metal involved.

Surfactants

- Toxicity is removed or reduced in the filtration test.
- Toxicity is removed or reduced by the aeration test. In some cases, toxicity may be recovered from the walls of the aeration vessel using a dilution water or methanol rinse.
- Toxicity is removed or reduced in the post-C18 SPE column test. The toxicity may or may not be recovered in the methanol eluate test. If a series of methanol concentrations (e.g., 25, 50, 75, 80, 85, 90, 95 and 100% in water) is used to elute the column, toxicity may be observed in multiple fractions.
- Toxicity is removed or reduced in the post-C18 SPE column test using unfiltered effluent. Toxicity reduction/removal is similar to that observed in the filtration test and toxicity may or may not be recovered in the methanol eluate test or by extraction from the glass fiber filter used in the filtration test.
- Toxicity degrades over time as the effluent sample is held in cold storage (4 °C). Degradation is slower when the effluent sample is stored in glass containers instead of plastic containers.

Total Dissolved Solids (TDS)

- pH adjustments do not remove or reduce toxicity and a precipitate is not visible in the pH adjustment test, pH adjustment and filtration test, or pH adjustment and aeration test.
- There is no loss of toxicity in the post-C18 SPE column tests, or a partial loss of toxicity, but no change in conductivity measurement.
- There is no change in toxicity with the EDTA addition test, sodium thiosulfate addition test, or the graduated pH test.
- There is a greater sensitivity by *C. dubia* and *D. pulex* compared to *D. magna*, together with high conductivity readings.
- A mock effluent prepared with the same ions as the effluent exhibits similar toxicity as the effluent (Goodfellow et al. 2000).
- Toxicity is removed or reduced by ion exchange resin.

- Toxicity is not removed or reduced by passing the effluent over activated carbon.

A list of toxicants identified in the TIE process is provided in Table 5-2 at the end of this chapter.

5.5 Phase II TIE Procedures

The Phase II guidance manual (USEPA 1993a) describes procedures for use in identification of specific classes of toxicants, including:

- Ammonia
- Cationic metals
- Polar and non-polar organic chemicals
- Chlorine
- Filterable toxicants

Phase II treatment techniques are similar to Phase I and are applicable to acute and chronic test methods with most WET test species. Phase II incorporates chemical-specific analytical procedures, including gas-chromatography (GC), GC/mass spectrometry (GC/MS), high-performance liquid chromatography (HPLC)/MS, atomic absorption (AA), and/or ion-coupled plasma (ICP)/MS to identify toxicants. The reader is referred to the EPA Phase II manual (USEPA 1993b), Municipal TIE Guidance (USEPA 1999b) and the SETAC TRE/TIE book (Norberg-King et al. 2005) for a detailed description of Phase II TIE procedures and examples of TIE case studies.

5.6 Phase III TIE Procedures

A thorough confirmation of the cause(s) of toxicity is a key part of the TIE process, although it is often the most laborious and difficult aspect. This confirmation must be performed over a considerable period to be certain that the cause(s) of toxicity is not changing over time. This is particularly true for POTWs, where control over influent is not complete. USEPA (1993c) emphasizes that sample integrity is particularly important in Phase III. Field replicates, system blanks and controls should be used as appropriate to prevent interferences and toxicity artifacts.

Suspect toxicant(s) identified in Phases I and Phase II are confirmed through application of one or more Phase III steps, including:

- Correlation approach
- Symptom approach
- Species sensitivity approach
- Spiking approach

- Mass balance approach

These approaches are not discussed in detail here, but are fully explained along with specific examples in EPA TIE manuals (USEPA 1991c, 1992, 1993b, 1993c). The reader is encouraged to review this material before reviewing TIE reports.

Step 5: Toxicity Source Evaluation

Once the TIE has identified and confirmed the chemical(s) responsible for the toxicity, efforts are initiated to identify the source(s) of the chemical(s). This process entails sampling of influent trunk lines from residential and industrial dischargers. Two types of source identification studies may be performed: chemical tracking or toxicity-based tracking. In some circumstances, both approaches have been conducted concurrently.

Chemical-specific tracking is recommended when the POTW effluent toxicant(s) have been identified and confirmed in the TIE, and can be readily traced to the responsible sewer dischargers. Toxicity tracking is used when TIE data indicate the type of effluent toxicant, but the specific toxicant(s) is not identified. Toxicity tracking involves treating the sewer samples in a bench-scale treatment simulation prior to toxicity measurements to account for the toxicity removal that is provided by the POTW.

The sampling strategy for toxicity source evaluations involves two tiers. Tier I focuses on sampling and analysis of the main sewer lines in the collection system. Tier II involves testing sewer lines and indirect dischargers upstream of the main lines identified as being toxic in Tier I. This tiered approach can be used to identify the contributors of toxicity and/or toxicants by eliminating segments of the collection system that do not contribute toxicity/toxicants.

Step 6: Toxicity Control Evaluation

Using the results of each of the above TRE elements, alternatives for effluent toxicity reduction are evaluated and the most feasible option(s) is selected for implementation. Effluent toxicity may be controlled either through pretreatment regulations or in-plant treatment modifications or additions. In some cases, several control methods may be required to achieve the desired toxicity reduction. Selection of control options is usually based on technical and cost criteria.

If the toxicity source evaluation is successful in locating the sources that are contributing to the POTW effluent toxicants, local limits can be developed and implemented. If in-plant control appears to be a feasible approach, treatability testing may be used to evaluate methods for optimizing existing treatment processes and to assess options for additional treatment. A description of treatability approaches can be found in the municipal TRE manual (USEPA 1999b).

Step 7: Toxicity Control Implementation and Follow-Up Monitoring

The toxicity control method or technology is implemented and follow-up WET testing is conducted at increased frequency to ensure that the control method achieves the TRE objectives and meets permit limits.

5.7 Inconclusive TRE/TIEs

TIEs that fail to characterize toxicants effectively frequently do so for one of two reasons. The first is the inability of inexperienced individuals to properly conduct and/or interpret results of TIE steps and obtain unambiguous identification of the toxicant(s). The second is difficulty in applying TIE techniques to samples with intermittent toxicity, toxicity caused by changing toxicants, and/or unstable effluent toxicity (Ausley et al. 1998; USEPA 2001c; SETAC 2005). Effluents with one or more of these characteristics pose significant challenges for even experienced analysts. EPA (USEPA 2001c) states that under conditions where the permittee has implemented an exhaustive TRE plan and all other permit requirements, but is still unable to attain or maintain compliance with toxicity-based limits, special technical evaluation may be warranted and civil relief granted. Solutions to these cases are pursued jointly with TIE experts at Regional or State offices or the EPA Duluth laboratory.

5.8 Conclusions

- The TRE/TIE process has been well described and updated in numerous documents published by EPA and others.
- A key aspect to successful TRE/TIEs is the development of a detailed TRE study plan early in the process.
- Application of these methods has generally resulted in toxicant(s) identification and mitigation of effluent toxicity problems, allowing the permittee to return to compliance.
- Enforcement decisions should be guided on a case- and site-specific basis considering existing and historical toxicity, including magnitude, frequency and duration of toxicity and importantly, the permittee’s diligence in resolving and preventing WET non-compliance.
- Finally, permittees and regulatory authorities should establish early in the TRE process a cooperative and communicative relationship that would be maintained until the TRE is successfully completed and controls fully implemented. Good communication and a well-conceived TRE plan will assure that all parties understand the requirements and expectations that will result in a more effective and faster resolution of the effluent toxicity.

Figure 5-1. Summary of the TRE Process

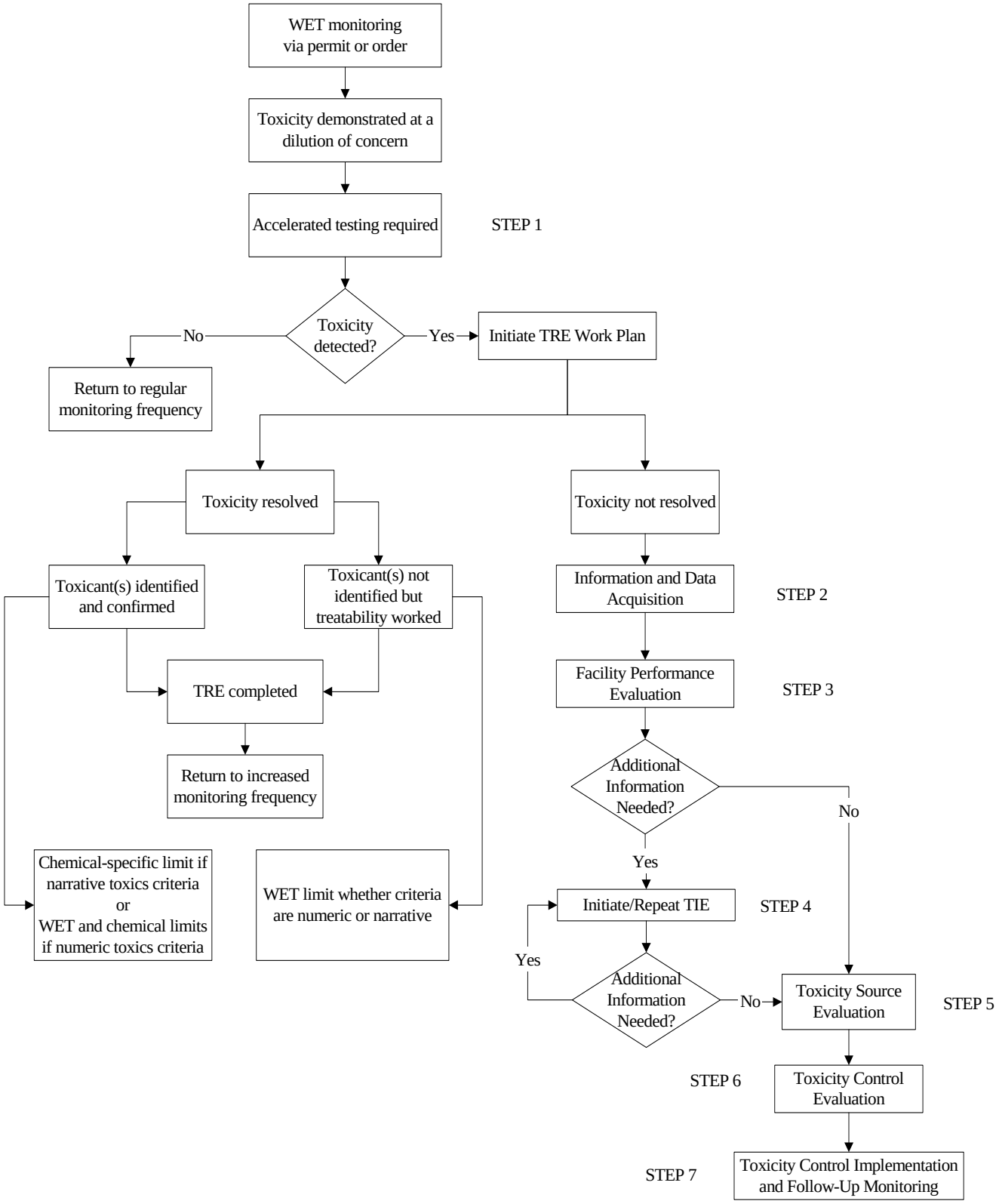

Figure 5-2. Flow Diagram of a Toxicity Identification Evaluation

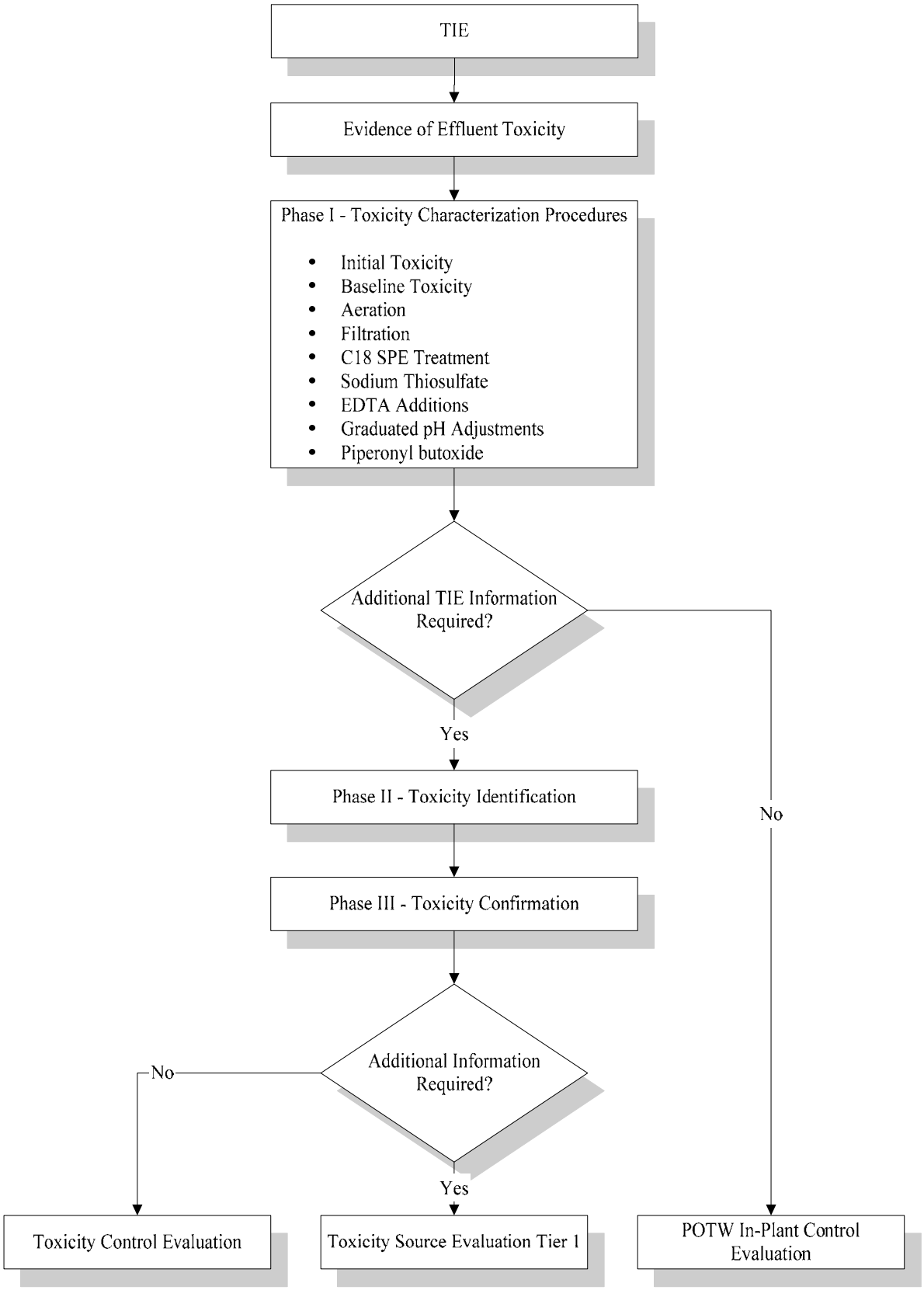

Table 5-2 Toxicants Identified in POTW Effluents

Toxicant Type	*Level of Concern*	*Potential Source*	*Information Need*	*References and Case Studies*
Chlorine	0.5-1 mg/L	POTW disinfection	Temp, pH during test; Cl^- conc. Phase I TIE oxidant test	
Ammonia	5 mg/L as NH_3-N	Domestic and industrial sources; POTW sludges; dewatering streams	NH_3-N conc, pH, temp, salinity, cond, at receipt and during the test. Phase I TIE graduated pH and zeolite treatment	USEPA 1999, Appendix G SETAC Case Study 6.5
Non-Polar Organics; OP insecticides (e.g., diazinon and chlorpyrifos)	Effluent concentrations $\geq EC_{25}$; Diazinon 0.12-0.58 µg/L; Chlorpyrifos 0.03 µg/L	Lawn pest control, pet care, veterinary, food vendors	High resolution analysis (GC/MS). Phase I TIE SPE test and SPE eluate add-back	USEPA 1999, Appendices A,B,F,G and H SETAC Case Studies 6.10, 6.11, 6.13, 6.14, 6.16, 6.17, 6.25, 6.29, 6.35, 6.36, 6.37
Metals: cadmium, copper, lead, nickel, zinc	Various depending on water quality parameters and test species	POTW treatment additives; industrial users	Dissolved metals, hardness, alkalinity, and pH. Phase I TIE EDTA test	USEPA 1999, Appendix G SETAC Case Studies 6.6 and 6.22
Treatment additives; dechlorination chemicals; polymers, biocides	Varies	Disinfection, dechlorination, sludge processing solid clarification	Information on toxicity of products; Use rates Phase I and II TIE results	SETAC Case studies 6.15, 6.18
Surfactants	Varies	Industrial users	Methylene blue active substances (MBAS) Phase I and II TIE results	SETAC Case Study 6.19
Ions and Total Dissolved Solids (TDS)	1,000-6,000 µmhos depending on test species, endpoint and TDS constituents	Industrial users; sludge processors; reverse-osmosis dischargers	TDS, ion analysis, anion, cation balance Phase I and II TIE results	SETAC Case studies 6.4, 6.5, 6.7, 6.8, 6.24, 6.28

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CHAPTER 6. AMBIENT TOXICITY TESTING AND WATERSHED ASSESSMENT

6.1 Overview

This chapter provides guidance to permit writers who are including stormwater or ambient conditions in permits. Although, WET tests are used as the primary tool for stormwater and ambient monitoring, the conditions under which they are used are generally different from monitoring continuous effluent discharges. Procedures which should be considered include:

- Experimental design – sample collection location, single vs. multiple concentrations
- Sampling – frequency, volume, container material, holding time
- Toxicity test method – organism selection, renewal frequency

Additionally, this chapter provides a broad overview of tools to be considered for stormwater and ambient monitoring, and provide examples of programs that have utilized tools including sediment toxicity testing, bioassessments, and in situ testing.

6.2 Introduction

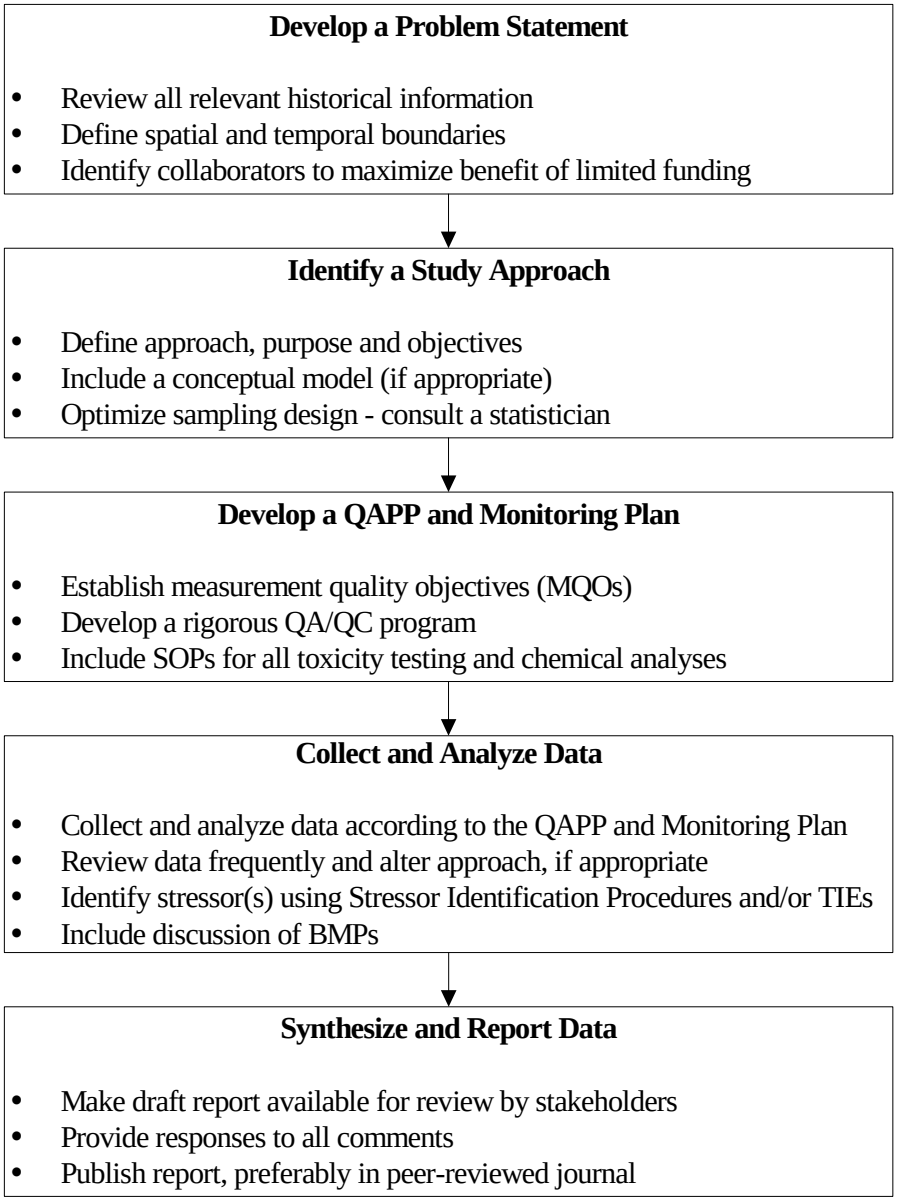
Permitting authorities are, by the very nature of what they do, stewards of the nation's water resources. As such, their ultimate goal is to maintain those resources in a condition that, "meets the needs of the present without compromising the ability of future generations to meet their needs" (Bruntland 1987). The Clean Water Act (CWA) states, "The objective of this act is to restore, and maintain the chemical, physical, and biological integrity of the nation's waters." It is no longer sufficient to think about aquatic ecosystems from a single perspective like point sources, non-point sources, sediment, stormwater, or the air/water interface. A holistic approach, using the watershed as the integrating unit, has clearly been recognized by EPA as the focal point for measuring how well the objectives of the CWA are being met.

According to the Watershed Information Network (www.epa.gov/owow/watershed/), a watershed is an area of land that drains to a common place, such as a stream, lake, estuary, wetland, aquifer, or the ocean. Since the goal of permitting authorities is to maintain healthy water resources, they are increasingly not only required to monitor effluent discharges, but potential watershed pollution in the form of stormwater discharges and non-point source toxicity to receiving waters, or ambient waters. Much like effluent outfalls are monitored with toxicity and chemistry, stormwater outfalls and receiving waters can be monitored with similar tools, but with specific considerations for their use.

Once, the Permitting Authority identifies the questions to be addressed, the development of the Quality Assurance Project Plan (QAPP) integrates all technical and data quality aspects of a project including planning, implementation, and assessment. EPA requires that all environmental data used in decision-making be supported by an approved QAPP. EPA requirements for QAPPs can be found at http://www.epa.gov/quality/qs-docs/rsfinal.pdf. Ambient water quality monitoring conducted in California using state funds must be compatible with the State's Surface Water Ambient Monitoring Program (SWAMP). The objective of

SWAMP is to provide high quality data that is comparable and accessible. The current requirements necessary to be considered SWAMP-compatible are detailed in the links found at www.swrcb.ca.gov/swamp. Before any study is undertaken, there are certain common steps, regardless of the study, that should be performed. These steps are outlined in Figure 6-1.

Figure 6-1. Recommended Steps in Development and Implementation of Environmental Monitoring Studies



6.3 Use of WET Testing in Stormwater and Ambient Monitoring

Toxicity testing procedures that are typically used in WET testing compliance, coupled with other biological assessments, have become increasingly important tools for identification of waterbodies which fail to meet goals of the CWA. In general the same organisms, testing protocols and sampling methods used in WET testing can be used in stormwater and ambient water monitoring. However, stormwater and ambient water study designs may need to incorporate different test organisms and sampling strategies to meet the goals of the study.

Monitoring in freshwater ecosystems typically employs EPA three-species toxicity tests with freshwater algae (*Selenastrum capricornutum*), the cladoceran (*Ceriodaphnia dubia*), and the fathead minnow (*Pimephales promelas*) (USEPA 2002a, 2002b). There are numerous advantages in using established WET test species for ambient monitoring including well understood life history and husbandry of the test organism, and established test protocols with a robust statistical basis for endpoint interpretation. Depending on site-specific water quality conditions, it may be appropriate to utilize other species. For example, standard WET species may not tolerate high TDS waters characteristic of some ambient and storm waters. In cases where water quality characteristics are not compatible with standard test species, the permitting authority should use best scientific judgment within local and state agencies and EPA to select alternate species and/or testing approaches.

For testing of estuarine environments, EPA has published short-term chronic toxicity test methods for several West Coast species which could be used for environmental monitoring in estuarine and marine environments (USEPA 1995a). The estuarine species include topsmelt (*Atherinops affinis*) and mysid (*Holmesimysis costata*). For testing marine waters, protocols for Pacific oyster (*Crassostrea gigas*), mussel (*Mytilus* sp.), red abalone (*Haliotis rufescens*), giant kelp (*Macrocystis pyrifera*), sea urchin (*Strongylocentrotus purpuratus*), and sand dollar (*Dendraster excentricus*) are available. Monitoring programs may be conducted in areas that contain species of special concern. EPA has provided guidance on selection of standard test organisms that would predict responses of species that are threatened or endangered (USEPA 2003b).

6.4 Stormwater Monitoring

Stormwater monitoring for toxicity is really a special case of effluent monitoring, the main difference being that stormwater is episodic. There are special conditions associated with stormwater monitoring in cities and towns where collected stormwater is conveyed through separate storm sewer systems or through combined sewers to a treatment plant prior to discharge. In most cases, stormwater is directly discharged to the receiving system without treatment. Ultimately, a successful stormwater program minimizes the level of contaminants in the stormwater. The most severe receiving water problems due to wet weather flows are likely associated with chronic exposures to contaminated sediment and to habitat destruction.

Since 1990, EPA has developed Phase I of the NPDES Stormwater Program (http://cfpub.epa.gov/npdes/home.cfm?program_id=6). Most stormwater discharges are considered point sources and require coverage by an NPDES permit. The Phase I program

addressed sources of stormwater runoff that had the greatest potential to negatively impact water quality. Under Phase I, EPA required NPDES permit coverage for stormwater discharges from medium and large separate stormwater systems, eleven categories of industrial activity, and construction activity that disturb five or more acres of land. Phase II of the program requires NPDES coverage for stormwater from certain regulated small municipal separate storm sewer systems and construction activity disturbing between 1 and 5 acres of land.

The "quality" of the wet weather flow is dependent in large part on the use designations of the land it flows over. There are differences between constituents in wet weather flows originating in high mountain forested areas and those originating in fully developed urban areas. According to Pitt (2003) urban receiving waters may have many beneficial goals, including:

- stormwater conveyance (flood protection),
- biological uses (warm water fishery, biological integrity, etc.),
- noncontact recreation (linear parks, aesthetics, boating, etc.),
- contact recreation (swimming), and
- water supply.

However, with full-scale development and lack of stormwater controls, severely degraded streams will be commonplace in highly urbanized areas. Some studies have shown significant aquatic life impacts even in watersheds that are less than 10% urbanized (Pitt 2003; Booth and Jackson 1997). In the Pacific Northwest, Horner et al. (1997) found that when imperviousness reached about 8% in the watershed, there was a rapid decline in the biological conditions in the receiving water. Severe problems were found when imperviousness reached 30%. Claytor (1996) found that when only conventional water quality measures are used to evaluate the status of non-tidal streams, 87% supported their designated biological uses. However, when biological assessments were included, only 13% of the streams supported their designated biological uses. According to the EPA Stormwater website designed to provide guidance for reducing contaminant input into receiving waters, the primary method to control stormwater discharges is through the use of Best Management Practices (BMPs). EPA maintains a web site http://www.bmpdatabase.org/index.htm that contains a database of roughly 200 BMPs.

6.5 Ambient Monitoring

The receiving waters of either an effluent or stormwater discharge are monitored to achieve a greater understanding of the potential effects of the discharge. Standard effluent monitoring tools, such as toxicity testing and water chemistry are used gather data on receiving water impacts, but other tools include in situ toxicity tests, bioassessments, and sediment toxicity testing. The experimental design of the ambient monitoring study will be based on the study questions and the tools that are chosen. Water column toxicity tests will pick up more ephemeral toxicity, and therefore should be used in fewer places, but perhaps more often. In situ water column toxicity tests can integrate toxicity over time, and could probably be used more sparingly, at least temporally. Sediment acts as a sink for many chemicals, particularly hydrophobic contaminants, and sediment toxicity testing tends to monitor the potential for longer

term effects. Sediment toxicity tests could be used less often temporally, but over a wider spatial range. Bioassessment also monitors long term trends, and is not generally considered a diagnostic tool, but could be used to assess long term impacts.

Several studies in California have successfully used ambient toxicity testing to identify and regulate frequently occurring toxic chemicals (Foe and Sheipline 1993; Kuivila and Foe 1995; de Vlaming et al. 2000). In these studies integral sampling locations were selected and ambient waters were collected to be assessed acute and chronic toxicity If toxicity was detected, additional samples were collected for testing to determine spatial and temporal patterns, as well as for conducting toxicity identification evaluations (TIEs) to identify the causative agents. This approach has led to the listing of chemicals broader than the 126 priority pollutants commonly tested. For example, diazinon was identified as causing water quality impairments and lead to 303(d) listings in several watersheds in California.

6.6 Special Considerations

Unlike effluents, where the constituents in the discharge remain fairly consistent, the constituents in stormwater and ambient samples can be ephemeral. Storm events are episodic, and depending on land use, a variety of contaminants can be present in the runoff. Receiving waters are similarly dynamic depending on inputs from point and non-point sources. Because of their inherent differences from effluents, toxicity testing of stormwater and receiving water have some specific method considerations. Areas which need to be considered differently for stormwater or ambient testing than the effluent testing program include: (1) sampling location and sample type, (2) sample containers, (3) sample initiation test, (4) sample renewals, and (5) experimental test design (single vs. multiple concentration testing).

6.6.1 Sampling Location

Selection of appropriate sample sites and sampling regimes are critical to the success of environmental monitoring studies. Sampling design in environmental monitoring programs is inevitably a compromise between cost/effort and accurately reproducing the regimen to which the organisms are actually exposed in the environment. Many sampling scenarios involve the use of integrator sites where multiple discharges and/or tributary flows combine. The United States Geological Survey's (USGS) "Seamless Data Distribution System" (http://seamless.usgs.gov) enables a user to view and download many geospatial layers, such as the National Evaluation Data set, National Land Cover Data set, and High Resolution Orthoimagery. If toxicity is detected at the integrator site, each of the contributing sources is tested to determine the source of the toxicity. Although this seems intuitive, care must be taken to assure that the samples are taken in such a way that takes into account the hydrology of the system being studied. USGS maintains a web site (http://water.usgs.gov/waterwatch) that reports in real time flows in mainstem rivers and major tributaries. In addition, real-time stream flows for California are posted the California Department of Water Resources website (http://cdec.water.ca.gov), which is useful in developing sampling plans. Land use information is critical for designing monitoring studies when it is important to know the contribution of flows from agricultural and urban areas. In addition, for agricultural areas, knowledge of crop type (http://gis.ca.gov) and pesticide use in

specific areas (http://calpip.cdpr.ca.gov) can be useful in tracing sources of toxicity from agricultural chemicals.

6.6.2 Timing of Sample Collection

Monitoring stormwater for toxicity requires a special understanding of what needs to be monitored (Herricks and Milone 1998) although the methods used to test stormwater may not be any different from those used to test ambient/receiving waters. The challenge associated with stormwater testing is in developing sampling strategies that incorporate realistic exposure scenarios. Routine stormwater monitoring can differ from a "first flush" event that is generally more toxic because of contaminant buildup on impervious surfaces during the dry season. Similarly, first flush events from agricultural settings can occur after winter dormant spraying and pesticide applications in the spring. The greater the period between rainfall events, the greater is the potential for build-up of contaminants.

Timing of sampling of stormwater discharge depend on the intensity of the storm as well as preexisting conditions surrounding the site such as amount of impervious surfaces, characteristics of the collection system and soil saturation. The effect of these factors on discharge volume can be monitored using a hydrograph plot (flow vs. time). Contaminants will usually move into the receiving water as the storm hydrograph increases (Burton and Pitt 2001). Depending on the purpose of the study, multiple samples can be collected and tested throughout the runoff event to assess short-term effects and contaminant loading.

If a study objective was to monitor the toxicity associated with a particular storm event in a particular watershed at a particular site, or multiple sites, then samples collected over the period of the storm, based on the watershed characteristics and hydrograph would provide the most realistic time-scale for exposure. Herricks and Milone (1998) discuss a variety of approaches for determining the appropriate time scale of exposure for a given watershed. Miller et al. 2005 present results of flow-through toxicity studies for studying stormwater in an urban creek using *C. dubia.*

At the other extreme of exposure would be water column organisms that are picked up and carried for an extended, but unknown, period of time with the first flush of water that enters the receiving system. In this case, samples of the first flush of water can be used to expose organisms in the laboratory using WET test methods with or without renewals, depending on what the investigator is attempting to mimic.

For ambient sampling, knowledge of land use, pesticide application patterns and timing, and system hydrology is required to select sample site locations and timing. For both stormwater and ambient samples, sites that demonstrate adverse effects, timely collection of additional site samples is essential to establish the frequency, magnitude, and duration of the toxicity at the site.

6.6.3 Sample Collection

Effluent monitoring generally utilizes composite sampling to collect water during a discreet period of discharge. Depending on the objectives of the study, composite sampling can also be

used for stormwater and ambient monitoring, but grab samples are used most often. The use of grab samples, the episodic nature of storm events, and the level of effort involved in the collection of receiving water samples can often lead to difficulties in adhering to a 36-hour sample holding time and the ability to collect multiple samples for renewals in an individual test. All tests should be conducted as soon as possible following sample collection. EPA has allowed exceptions to the 36-hour holding time, for example, when effluents are shipped overseas for testing (Denton and Narvaez 1996). The primary reason for an extension of the holding time would be the consideration of the sampling and laboratory technicians safety (Burton and Pitt 2001; see page 255), and logistics of coordinating collection and transport of multiple samples within a short period. Since, storm events are not pre-determined and typically are occurring rapidly throughout a watershed; therefore, many site samples must be coordinated with short notification. The 36-hour holding time for test initiation should be targeted, but no more than 72 hours should elapse before initial use of a sample. Typically, environmental monitoring programs use a single sample for all toxicity test renewals. For acute studies (typically 96 hours), a single test sample is usually collected and used to renew test solutions daily or at 48 hrs. EPA specifies the use of a minimum of 3 samples for chronic toxicity studies with fish and invertebrates (USEPA 1995a, 2002b, 2002c), but depending on the study question, sampling for storm events, might occur only once, or several times throughout the hydrograph. Another solution is to renew the test solutions with a mixture of ambient waters and stormwaters, if such waters could be collected following test initiation while meeting WET test holding time specifications (Katznelson and Mumley 1997).

During sample collection, it is critical to confirm and record the site location using GPS coordinates, note site characteristics, measure basic water chemistry (temperature, dissolved oxygen, conductivity), and estimate flow velocity and volume. The latter information may be challenging to obtain but is critical for estimating toxicant loading. Generally, glass sample containers are recommended for ambient and stormwater samples. Samples must be immediately placed on wet ice and transported to the testing lab, where testing should be initiated as soon as possible. Even assuming that all conditions of sample holding (36 hrs maximum at $\leq 6°$ C) are met, significant quantities of some chemical classes of constituents (e.g., organophosphates, pyrethroid insecticides and surfactants) may sorb to sample containers during the holding period. Vigorous shaking of sample containers prior to distributing to test containers to re-dissolve sorbed constituents is recommended (Wheelock et al. 2005).

6.6.4 Data Analysis

Initially, samples are tested at without dilution such as 100% concentration. The test endpoint data is analyzed using a standard t-test approach as described in the test methods manual (see USEPA 2002a, page 86). Many sampling plans specify that if toxicity is detected, the site shall be re-sampled and retested using a dilution series to determine the duration, frequency and magnitude of the toxicity. Toxic samples should immediately be subjected to TIE procedures to attempt to identify the toxic chemical(s).

6.6.5 Stormwater *In Situ* vs. First Flush

There are potentially two entirely different kinds of exposure from stormwater events. For sessile organisms (e.g., organisms which do not move with the discharge flow), the exposure is the culmination of all the water and constituents that pass over them during an event. In this case, *in situ* monitoring, using methods that can withstand the changes in the flow regime, can characterize that exposure. The effects of that exposure may be more difficult to predict, as they may not occur until some time after the exposure. One way to address this is to remove the *in situ* systems after the storm event and monitor their responses in clean water. Herricks and Milone (1998) studied time-varying exposures in the laboratory using the cladoceran *C. dubia*, the fish *P. promelas*, and the amphipod *H. azteca*. Their work showed the need for appropriate time-scales of exposure. Organisms that reside in the water column would move with the stormwater flows. Therefore, exposing *C. dubia* to the first flush sample in a storm event would probably not represent the exposure most of these sorts of organisms would receive.

6.7 Additional Monitoring Tools

There are additional tools that can be utilized for monitoring of stormwater and ambient water. Three of these tools are discussed below: in situ toxicity testing, sediment toxicity testing, and bioassessments. The use of these tools, and others, can either lead to the identification of an impairment, or monitor a currently impaired waterbody. Once impairment has been identified, identification of the primary stressors is pursued through the EPA stressor identification process (USEPA 2000c). This process was developed to identify any type of stressor or combination of stressors that cause biological impairment. The Stressor Identification (SI) process entails critically reviewing the available environmental information, analyzing potential exposure scenarios, and developing monitoring programs to fill in data gaps. The reader is encouraged to review the SI document prior to developing or reviewing environmental monitoring programs. Some types of monitoring approaches and their applications are shown in Table 6-1.

Table 6-1. Types of Monitoring Approaches and Their Applications

Type	*Approach*	*Applications*
Chemical Condition	Water quality sampling	Screen for impairment; identify specific pollutants of concern; identify water quality trends; determine support of designated contact recreation uses; identify potential pollution sources.
Physical Condition	Watershed survey	Determine land use patterns; determine presence of current and historical pollution sources; identify gross pollution problems; identify water uses, users, diversions, and stream obstructions
	Habitat assessment	Determine and isolate impacts of pollution sources, particularly land use activities; interpret biological data; screen for impairments
Biological Condition	Macroinvertebrate sampling	Screen for impairment; identify impacts of pollution and pollution control activities; determine the severity of the pollution problem and rank stream sites; identify water quality trends; determine support of designated aquatic life uses.

Source: USEPA 1997a.

6.7.1 *In-Situ* Testing

Toxicity tests using standard WET organisms and performed on ambient water samples are considered surrogate exposures for environmental realism. Exposing these organisms *in situ* can increase the environmental relevance. The test organisms used for *in situ* biomonitoring range from the same organisms used in WET toxicity testing to a wide array of other organisms. The list of references that follow are only a small number of articles on *in situ* toxicity testing: WET test organisms (Anderson 2002; Dickson et al. 1996; Hemming et al. 2001) amphipods (Maltby et al. 2003; Rainbow and Kwan 1995; Gerhardt et al. 1998); algae (Twist et al. 1997); real-time biomonitors (Allen et al. 1996; Waller et al. 1995; Kuster et al. 2004; Gerhardt et al. 1998; Kieu et al. 2001; Charoy et al. 1995).

Organisms can also be exposed in situ for bioaccumulation studies. Freshwater and marine mussels bioaccumulate both metals and organics and have been used extensively to evaluate sources of environmental pollution . Mussels can be placed in the field for varying periods and have the additional endpoints of growth and survival. Strategically located mussels can identify chemical inputs.

Several large monitoring programs have used mussels to monitor contaminants and determine contaminant bioavailability in the water column. The San Francisco Estuary Institute (SFEI) has a long history of using bivalves (resident clams and transplanted oysters and mussels) as sentinel species. Davis and Taberski (2002) reported on the use of mussels as part of a regional monitoring program of water quality in San Francisco Bay. California's Department of Fish and Game State Mussel Watch Program (SMWP) has been in effect since 1976. The Mussel Watch program is part of a worldwide monitoring effort designed to detect the presence and concentration of toxic pollutants in estuarine and marine waters (Martin and Severeid 1984). California has also employed mussels in the freshwater toxic substances monitoring program (SWRCB 1990).

6.7.2 Sediments

Because sediments can be sinks for many contaminants, they are potentially impacted by discharges to a receiving waters. The Contaminated Sediment Management Plan (USEPA 1998) has as its goal, “to reduce fragmentation, duplication, and increase more holistic approaches to pollution prevention.” For example, NPDES permitted facilities may be meeting all their chemical-specific, parameter-specific, and WET requirements and yet sediment contamination could result from releases from these facilities. There are more than ten Federal statutes that provide authority to EPA program offices to address the problem of contaminated sediment. The EPA (1998) studied data from 1,372 of 2,111 watersheds in the continental United States and, based on the approach discussed below, identified 96 watersheds that contain “areas of probable concern” (APC). Four goals have been established to address the problem of contaminated sediment (USEPA 1998). These goals are:

- prevent the volume of sediment from increasing,
- reduce the volume of existing contaminated sediment,

- ensure that sediment dredging and dredged material disposal are managed in an environmentally sound manner, and
- develop scientifically sound sediment management tools for use in pollution prevention, source control, and dredged material management.

It is important to note that these 96 watersheds have been identified from existing databases and do not represent all the watersheds or portions of watersheds that might meet the criteria for APCs. A complete inventory of contaminated sediments in the United States has not as yet been established (USEPA 1997b). It is also important to note that the time span covered by the database from which these 96 APC watersheds were developed was 1980 to 1993. An updated report that is in draft form (USEPA 2001d) will provide new estimates for data up to and including 1999 as to the number and distribution of APCs.

Through the Federal Insecticide, Fungicide, Rodenticide Act (FIFRA), EPA has the authority to ban or restrict the use of pesticides that have the potential to contaminate sediments if the risk is judged to be unreasonable. However, sediment toxicity has not been a part of routine test procedures and risk assessments for pesticide registration, re-registration or special review, even though prevention is clearly a better strategy than remediation.

Sediment has been functionally defined as all of the detrital, organic and inorganic particles that settle to the bottom of a body of water. In many sediment types (depositional sediments), water is found between the particles in the sediment and is termed interstitial or porewater. This water becomes very important in consideration of toxicity of contaminated sediment. Power and Chapman et al. (1992) divide sediment into four main compartments: interstitial water, organic, inorganic, and anthropogenically derived materials, including contaminants and eroded topsoil. According to their classification scheme, the largest volume is occupied by interstitial water that may occupy over 50% by volume of surface sediments. The inorganic phase includes the rock and shell fragments and mineral grains that originate from natural erosion of terrestrial materials. Organic matter is a variable, but small, fraction that occupies a low volume but is an important component because it can regulate the sorption and bioavailability of many contaminants.

Sediment toxicity tests are utilized much like WET tests, but their focus is on evaluating ambient sediment conditions. Freshwater and marine sediment testing protocols are described fully by the EPA (USEPA 1994d, 1994e, 2000d). The objective of sediment toxicity testing is to determine if chemicals in the sediment are harmful to, or accumulated by, benthic organisms. Sediment toxicity tests can be used to (1) determine the relationship between toxic effects and bioavailability, (2) investigate interactions among chemicals, (3) compare the sensitivities of different organisms, (4) determine spatial and temporal distribution of contamination, (5) evaluate dredge material, (6) measure toxicity as part of product licensing or safety testing or chemical approval, (7) rank areas for cleanup, and (8) set cleanup goals and estimate the effectiveness of remediation or management practices (USEPA 2000d). In addition to the methods in EPA 2000d, standard methods for assessing the toxicity of contaminants associated with sediments have been developed using amphipods, midges, polychaetes, oligochaetes, mayflies, and cladocerans (ASTM 1999a, 1999b, ASTM 1999c; USEPA 1994d, 1994e; Environment Canada 1997a, 1997b).

The sediment quality triad is an integrative approach for evaluating sediments (Chapman et al. 1992). This process is defined as any three-component integrative assessment that includes sediment toxicity, sediment chemistry and some measure of *in situ* bioeffects (often benthic infaunal community structure). The sediment quality triad is based on a ***weight of evidence*** approach for determining impact. For example, if chemistry indicates a potential impact, and toxicity tests show adverse effects, then the weight of evidence is strong that contaminants are impacting the sediment. Multiple toxicity tests on a variety of species do not substitute for other part of the triad, but do increase the strength of the toxicity leg. Detection of resident community alterations through bioassessments also reinforces the possibility of an impact.

Often, when information is gathered for assessing impacts, a tiered approach is used. By starting with the least complex and least expensive testing methodologies, a weight-of-evidence can be built over multiple metrics. If the metrics of the triad provide mixed results, then additional information may be needed to resolve the conflicts. However, some conclusions from mixed effect results can guide additional studies (Table 6-2). As with most assessments of environmental quality, the more quality information that is available, the greater is the likelihood that the assessment will be accurate.

Table 6-2. Information Provided by Differential Triad Response

Contamination	*Toxicity*	*Alteration*	*Possible Conclusions*
+	+	+	Strong evidence for pollution-induced degradation
-	-	-	Strong evidence that there is no pollution-induced degradation
+	-	-	Contaminants are not bioavailable
-	+	-	Unmeasured chemicals or conditions exist with the potential to cause degradation
-	-	+	Alteration is not due to toxic chemicals
+	+	-	Toxic chemicals are stressing the system
-	+	+	Unmeasured toxic chemicals are causing degradation
+	-	+	Chemicals are not bioavailable or alteration is not due to toxic chemicals

Source: Chapman et al. 1992

6.7.3 Sediment Collection

Procedures for collecting, storing, and manipulating sediments for chemical and toxicological analyses are well documented (USEPA 2001d; ASTM 2000). The EPA test methods manual represents a compilation of information from governmental documents to peer-reviewed literature and is an important source of information regarding the sediment phase of the aqueous

environment. ASTM has also published a guide for the collection, storage, characterization and manipulation of sediments for toxicological testing (ASTM 2000).

The goal of any sediment sampling program should be to collect sediment in a manner that produces minimally disturbed sediment. The methods used to sample, transport, handle, and store and manipulate sediments and interstitial waters can influence the physicochemical properties and the results of chemical, toxicity and bioaccumulation analyses (USEPA 2001d). Many of the areas covered in EPA's technical manual are subjects of active research programs and, while the intent of the manual is to provide methodologies that minimize sampling impact, the authors recognize that methods are likely to evolve and that new additions of the technical guidance will reflect those advances. To keep pace with the changes visit www.epa.gov and search on sediment sampling and sediment testing.

There are many devices that have been used to collect whole sediments. The choice of sampling method is dependent to a large degree on what the sample is to be used for. The EPA sediment technical manual (USEPA 2001d) has a good discussion of the various collection methods and their strengths and weaknesses. Sampling sediments to determine the average concentration of chemical contaminants can be problematic. For monitoring and assessment studies, the upper 10-15 cm of sediment is normally collected because this is the area where most of the epibenthic and benthic organisms and the most recently deposited sediments are found. These samples can be used for physical and chemical analyses, benthic community analysis, and toxicity tests. In many instances, sub-samples of equal size from sediment samples can eliminate or reduce the influence of unequal sized grab samples.

Interstitial water, or pore water, is the liquid contained within every sediment sample. This water is may occupy up to 50% by volume in silt and depositional sediments (Sarda and Burton 1995; USEPA 2001d). Because interstitial water is in intimate contact with the sediment, it is assumed to be in thermodynamic equilibrium with contaminants in the sediment, and is generally to be considered the route of exposure for many sediment contaminants. In addition, contaminants in interstitial water can be transported to overlying waters through diffusion, bioturbation and re-suspension (Sarda and Burton 1995).

Interstitial water can be used to evaluate sediment toxicity with organisms that are normally used in aquatic toxicity tests (Carr and Nipper 2003). To evaluate interstitial water it must be separated from the sediment matrix. It should be noted that extraction of interstitial water can alter the chemistry of the sample (Sarda and Burton 1995). There are several methods used to isolate interstitial water from sediment including centrifugation, pressurization, or suction. *In situ* sampling devices for interstitial water have also been used. The most commonly used methods are "peepers" and suction devices. Peepers are samplers that have a rigid body with openings covered with permeable membranes. Prior to deployment, the openings are filled with a medium consistent with sample objectives. The peeper is then placed in the sediment and the medium in the openings is allowed to come into equilibrium with the surrounding interstitial water. The equilibration time varies, but multiple-week exposures are not unusual (USEPA 2001d; Sarda and Burton 1995). These methods generally produce smaller volumes of water (<500 mL) compared to centrifugation and pressurization and are often limited to shallower

water depths. A variety of peeper designs along with diffusion samplers, vapor diffusion samplers, and semi-permeable membrane devices are discussed on the EPA website (http://clu-in.org/programs/21m2/sediment/). Regardless of the method of collection porewater samples should be processed as soon as possible after collection.

6.7.4 Freshwater Sediment Test Organisms

The EPA sediment test methods manual (USEPA 2000d) describes five methods for three organisms to measure the toxicity and bioaccumulation of contaminants from freshwater sediments. Two of the methods, one for the amphipod *Hyalella azteca* and one for the insect *Chironomus tentans*, measure survival and growth over a 10-day exposure period. One of the methods measures survival, growth and reproduction of *H. azteca* over a 42-day test, and one measures effects on *C. tentans* over the life-cycle of the insect. A bioaccumulation test with *Lumbriculus variegatus* is also presented.

Recently, sediment toxicity has been documented in urban waterways (Amweg et al. 2006) and agriculturally dominated waterways (Weston et al. 2004). The reader is encouraged to consult these published studies prior to designing or reviewing sediment toxicity. Phillips et al. (2006) and Anderson et al. (2006) describe TIE procedures for identification of the causes of toxicity in sediments from agriculturally dominated watersheds in California.

6.7.5 Bioassessments

Benthic infauna surveys can be accurate indicators of ecosystem health, and benthic surveys are frequently used as biocriteria to assess ecological integrity (Gibson et al. 2000; Borja 2005). Benthic data can be evaluated against historical data, reference conditions, models and indices, and with consensus professional judgment. Although standard benthic evaluation tools exist, the interpretation of benthic data is often subjective and based on best professional judgment (SCCWRP 2006). Moreover, because the presence of resident biota is region-specific, interpretation of bioassessment data must be based on the ecoregion.

Rapid Bioassessment Protocols (RBPs) were developed for freshwater environments as inexpensive screening tools for determining if a stream was supporting its designated aquatic life use (Plafkin et al. 1989). EPA guidance for marine bioassessments is provided in Gibson et al. (2000), but there are also a number of published marine bioassessment studies (e.g. Thompson and Lowe 2004; Weisberg et al. 1997; Smith et al. 2001). As these protocols were applied and modified, the areas in which the protocols provided useful information expanded to include:

- Characterizing the existence and severity of impairment to the water resource
- Helping to identify sources and causes of impairment
- Evaluating the effectiveness of control actions and restoration activities
- Supporting use attainability studies and cumulative impact assessments
- Characterizing regional biotic attributes of reference conditions.

The revised RBPs have been adopted and modified by various states to meet their monitoring needs (Barbour et al. 1999). Once adapted to the characteristics of a state, consistent reproducible procedures can be used to evaluate the status of a wadeable river or stream. One of the goals of the application of RBPs is to develop ***biocriteria*** that can be tailored to reflect the kind of biological system that should be found in waters that have a particular designated use (public water supply, for protection of fish, shellfish, and wildlife, and for recreational, agricultural, industrial, and navigational purposes). Once biocriteria are developed, a biosurvey of a receiving system with a particular designated use can be performed to determine if that system meets the requirements for that designated use. There are only a few places in the country that have developed biocriteria.

Implementing biocriteria in California is the responsibility of the State Water Resources Control Board and the Regional Water Quality Control Boards. In California, there is not one single entity responsible for developing statewide bioassessment protocols. As a consequence, five candidate programs exist in California that could provide the framework for the implementation of statewide bioassessment methods (SWRCB 2003). Bioassessments have been conducted at over 3000 sites in California by a variety of agencies. The California Department of Fish and Game (CDFG) bioassessment methodology has been used the most, with over 2500 sites sampled (SWRCB 2003). The more recent organization of California's Surface Water Ambient Monitoring Program (SWAMP) should provide the impetus to implement a better organized and standardized biological and assessment program (SWRCB 2003).

The California DFG is a leader in establishing taxonomic standards for statewide bioassessment efforts, an immense undertaking, given the size and diversity of ecoregions in California. The CABW was established as a forum for researchers, agency personnel and private consultants working in the field of freshwater biology. In 1995 the California Aquatic Macroinvertebrate Laboratory Network (CAMLnet http://www.dfg.ca.gov/cabw/camlnetste.pdf) workgroup was started to develop consistent, sound methodological approaches to aquatic bioassessment, to provide mentoring and support, and to facilitate communication by promoting discussion of findings and bioassessment programs.

In 1999, CAMLnet produced the first edition of the CAMLnet List of Standard Taxonomic Effort (LSTE). This document defines the basic level of taxonomic resolution to be used by all CSBP data analyses. To conform to the CSBP standard effort levels, taxa may be identified to more, but not less precise, levels than those listed in the LSTE. The latest version (2003) of the list can be found at www.dfg.ca.gov/cabw/camlnetste.pdf. These protocols fit the essentials of the wadeable protocol to these specialized habitats.

An important and difficult step that is being pursued is the establishment of reference conditions for each of the types of waterbodies. The reference sites are, in theory, pristine sites for that waterbody type. Once the bioassessments of the reference conditions are in place, all streams of the same physical attributes (e.g., wadeable steams in a particular hydrologic unit) should have conditions equal to the reference site's conditions. In practice, it is difficult to find pristine sites for any given waterbody type, so the use of "least impacted" sites are often used instead. Regardless of the final choice of bioassessment protocols chosen for use, they will become an important tool in the arsenal of tools water quality managers have at their disposal.

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CHAPTER 7. ENFORCEMENT PROCEDURES FOR WET

7.1 Overview

The following discussion provides guidance on determining appropriate enforcement responses to violations of WET limits and conditions. This guidance incorporates the two main goals of EPA's NPDES compliance and enforcement program which are (1) to compel or require the permittee to expeditiously achieve and maintain compliance, and (2) to serve as a deterrent.

7.2 Background

CWA Section 309(a) states that any violation of a permit condition or limitation is subject to enforcement. Through EPA's 1989 national NPDES enforcement guidance, Enforcement Management System (EMS) guidance, the EPA Regional or State enforcement authority is encouraged to initiate an appropriate enforcement response to all permit violations. EPA's overall approach to enforcement applies to all parameters, including WET. Once a facility has been identified as having an apparent permit violation(s), the Permitting Authority reviews all available data on the seriousness of the violation, the compliance history of the facility, and other relevant facts to determine whether to initiate an enforcement action and the type of action that is appropriate. **The EMS recommends an escalating response to continuing violations of any parameter. Regions 9 and 10's enforcement follows the EMS.**

In a joint memorandum issued by EPA Headquarters Office of Regulatory Enforcement and Office of Wastewater Management (USEPA 1995b) EPA clarified National policy with regard to the two most common issues raised by the regulated community involving the enforcement of WET requirements in NPDES permits: 1) single exceedance of WET limits, and 2) inconclusive toxicity reduction evaluations (TREs).

EPA does not recommend that the initial response to a single exceedance of a WET limit, causing no known harm, be a formal enforcement action with a civil penalty. The regulated community has expressed concern about the potential for third party lawsuits for single exceedance of WET. Citizens cannot sue a permittee on the basis of a single violation of a permit limit. Under section 505(a) of the CWA, citizens are allowed to take a civil action against anyone who is alleged to "be in violation" of any standard or limit under the CWA. In Gwaltney of Smithfield, Ltd., v. Chesapeake Bay Foundation, Inc., 484 U.S. 49, 1008 S.Ct. 376, 98 L.Ed.2d 306 (1987), the Supreme Court held that the most natural reading of "to be in violation" is "a requirement that citizen-plaintiffs allege a state of either continuous or intermittent violation--that is, a likelihood that a past polluter will continue to pollute in the future." A State may have its own enforcement policy which may be more stringent.

In the case of inconclusive TREs, EPA recommends that solutions in these cases be pursued jointly with expertise from EPA and/or the States as well as the permittee. Solutions may involve special technical evaluation, as well as relief of civil penalties. The primary corrective action required for violations of WET limits is completion of a TRE, including, if necessary, a TIE. This requirement is incorporated into the Regions' NPDES permits. The permit language addressed in this document contains provisions requiring the permittee to: implement the generic

TRE plan; increase the testing frequency following a violation or monitoring trigger if necessary; and, if also necessary, initiate a facility-specific TRE and a TIE following additional toxic sample(s) during the accelerated monitoring period. The permits require permittees to develop and submit a generic TRE workplan within 90 days of permit issuance.

Table 7-1 summarizes the Regions' WET enforcement responses. The following sections discuss the types of noncompliance and the appropriate enforcement responses in more detail. Appropriate federal or State laws, policy and enforcement personnel need to be consulted prior to a determination of noncompliance or initiation of enforcement actions.

Table 7-1. Enforcement Response Summary

Noncompliance	*Initial Response*	*Elevated Response Following Repeated or Sustained Violations*
Limit Violations	Phone call, LOV or NOV	NOV/AO; AO/APO; judicial referral
Failure to Conduct TRE, TIE, or Accelerate Testing	NOV/AO	AO/APO; judicial referral
Failure to Test	NOV/AO	AO/APO
Invalid Results - Good Faith Effort - Lack of Good Faith - Failure to Re-Test	 Tech. Assist. NOV/AO NOV/AO	 NOV/AO; APO AO/APO AO/APO
Failure to Comply with Narrative Conditions of	NOV/AO	AO/APO

LOV = Letter of Violation NOV = Notice of Violation
AO = Administrative Order APO = Administrative Penalty Order

7.3 Types of Noncompliance

Noncompliance with the NPDES permit and the CWA includes:

(a) violation of WET permit limit(s),

(b) failure to conduct WET tests,

(c) failure to provide valid test results (i.e., meet all test acceptability criteria) or otherwise comply with the permit's test and quality assurance procedures, including failure to re-test within 14 days following the failure to meet test acceptability criteria,

(d) failure to comply with any other WET NPDES permit conditions, including the conditions requiring:

(1) an increase in the testing frequency following a violation or monitoring trigger requirement,

(2) an initiation of a TRE within 15 days of a violation as described in AO or permit

(3) initiation of a TIE following a subsequent violation during the accelerated monitoring period,

(4) a submittal of a generic TRE work plan within 90 days of permit issuance,

(5) initial screening, or annual re-screening, for the most sensitive species,

(e) failure to comply with the permit's reporting requirements and,

(f) failure to comply with the terms and conditions of an Administrative Order (AO) or consent decree.

7.4 Types of Enforcement Actions

EPA or an appropriate State has discretion to determine that enforcement action is warranted and the type of action that is appropriate. EPA's EMS recommends an escalating response to continuing violations. There are three major categories of potential responses: no action, informal enforcement action and formal enforcement action. In ascending order of severity, the enforcement actions available to EPA include Notice of Violations (NOVs) and Administrative Orders (AOs), Administrative Penalty Orders (APOs), Civil judicial action, and criminal prosecution. EPA Region 9 generally issues an AO along with all NOVs (with the exception of NOVs issued to Federal Facilities). Other EPA Regions and States may issue NOVs without an accompanying AO. Similar State actions are available to each authorized State. Determination of the appropriate enforcement response for WET violations will be based on the same factors used to determine the appropriate response for chemical-specific violations, that is, the need to compel or expedite a permittee's return to compliance, and the deterrent value of a particular enforcement response. EPA/State should consider such factors as:

(a) the duration of noncompliance or number of violations;

(b) the severity or significance of the violations, and the resultant environmental harm;

(c) the cause or source of the violations and a permittee's degree of control over the causative agent of toxicity;

(d) a permittee's history of violations/recalcitrance; and,

(e) the economic benefit gained from noncompliance.

7.4.1 Notice of Violation and Administrative Order for Compliance

An AO, or its equivalent, issued in conjunction with a NOV, should require the permittee to comply with WET limits and conditions by specified dates. Required compliance with most narrative permit conditions should be immediate. The AO should specify the required corrective actions, or require the permittee to develop, submit for approval, and implement a corrective action plan. Generally, EPA/State should issue an NOV/AO or the equivalent under the following scenarios:

(a) a permittee failed to conduct the required WET tests on one or more occasions;

(b) after a WET limit violation, a permittee failed to initiate a TRE and/or TIE, or failed to increase the testing frequency;

(c) a permittee failed to comply with any narrative WET permit condition on one or more occasions including conditions addressing reporting requirements, species screening requirements, or submittal of a TRE workplan;

(d) a permittee failed to provide valid test results, or otherwise failed to comply with permit conditions regarding test procedures or quality assurance, including the requirement to re-test within 14 days following the failure to meet test acceptability criteria;

(e) a permittee's TRE efforts are inadequate, the corrective actions are inadequate, or the time frames for completing corrective actions are unacceptable;

(f) a permittee may need some additional incentive to complete the necessary corrective actions (e.g., when corrective actions require long construction schedules, or are expensive, or a permittee has a history of recalcitrance);

(g) WET violations resulted in documented environmental impacts;

(h) the permittee has not eliminated or reduced the toxicity within a reasonable amount of time, and the violations are ongoing, whether continuously or sporadically.

7.4.2 Administrative Penalty Order (APO)

Issuance of an APO would be appropriate if the permittee has demonstrated recalcitrance; if violations have continued over an extended time period or have repeatedly reoccurred; if the violations are especially serious; or if the violations could have reasonably been avoided. APOs only penalize permittees for past violations. Therefore, if additional corrective action is necessary, an AO should also be issued, or a civil judicial referral should be considered. EPA/State should consider issuing an APO, or its equivalent, for the following situations:

(a) a permittee failed to initiate a TRE and/or TIE, or failed to increase the testing frequency, on several occasions or after an extended period of noncompliance;

(b) a permittee repeatedly failed to comply with any narrative WET condition or repeatedly failed to provide valid test results;

(c) a permittee repeatedly failed to conduct WET tests;

(d) the WET limit violation(s) was caused by negligence, poor operation and maintenance practices, a poor pretreatment program, or other circumstances within the control of the permittee which could have reasonably been avoided. [Note: Certain types of negligence may be dealt with more appropriately through criminal prosecution. These cases should be referred to EPA's criminal investigations division, or to the appropriate State agency.];

(e) the WET violation(s) resulted in, or contributed to, significant adverse environmental impacts;

(f) the permittee gained significant economic benefit from noncompliance;

(g) the permittee demonstrated recalcitrance in initiating or completing corrective actions; and,

(h) the penalty calculation, which is based on economic benefit and gravity, is less than $157,000.

7.4.3 Civil Judicial Action

A civil judicial action is appropriate under circumstances similar to an AO with an APO, but where the severity of violations or degree of recalcitrance is greater; additional corrective actions are required; or the economic benefit derived from noncompliance is greater. EPA and the State should consider a civil referral in response to the following:

(a) a permittee's repeated failure to conduct a TRE or increase the testing frequency during an extended period of noncompliance or recurring periods of noncompliance despite previous enforcement actions or other direction from EPA or the State;

(b) a permittee's repeated failure to conduct a TRE in an aggressive or good faith manner, or to otherwise eliminate or reduce toxicity;

(c) a permittee's failure to adequately comply with an AO;

(d) situations where extensive corrective action is required, especially extensive construction, or where a permittee may need extra incentive to complete corrective actions due to time, cost or potential recalcitrance;

(e) situations where corrective actions are costly and allowed the permittee to gain significant economic benefit from delayed compliance;

(f) situations where the violations resulted in or contributed to significant environmental impacts; and

(g) the penalty calculation, based on economic benefit and gravity, exceeds $157,000.

7.4.4 Criminal Prosecution

For willful, knowing, or negligent violations of the NPDES permit or CWA, the permittee can be subject to criminal penalties. These cases should be referred to the Criminal Investigations Division of EPA, or the appropriate State office.

7.5 Other Factors to Consider When Deciding an Appropriate Response:

In comparison to chemical-specific effluent limit violations, it can be more difficult to identify the causative agents of WET violations and to isolate the sources of toxicity. In addition, once the toxic agents and sources are identified, it can be more difficult to control these sources, especially without costly technological solutions. This is especially true for municipal treatment facilities where the public, commercial establishments and industry can all contribute to toxicity. Although these factors should not deter EPA or the State from taking enforcement action, they

should be considered when assessing the appropriate enforcement response and determining reasonable compliance dates.

In general, the EPA Regions or the State may decide enforcement action is not necessary following a violation of WET limitations if the permittee adequately complies with its NPDES permit requirements for accelerating testing and conducting a TRE. Enforcement action would be appropriate if the permittee failed to aggressively conduct a TRE or was otherwise recalcitrant in addressing the toxicity.

Exceptions to this general guideline include situations where the WET violation(s) are of large magnitude, or contributed to significant environmental impacts (there may be violations of chemical-specific effluent limits as well); the permittee may need additional incentive to complete corrective actions identified by the TRE; the permittee failed to eliminate/reduce toxicity within a reasonable time frame; or, the WET violations were caused by circumstances within the control of the permittee and could have been reasonably avoided. In cases like these, EPA/State should consider enforcement action even if the permittee did initiate a timely TRE.

7.6 Invalid Test Results

When a permittee is experiencing difficulty in meeting test acceptability criteria, EPA/State's initial response should be technical assistance (provided the permittee is making a good faith effort). If this proves unsuccessful, or the permittee is not making a good faith effort, EPA/State should then consider enforcement action. The initial enforcement action will typically be a Notice of Violation and Administrative Order (NOV/AO), or its equivalent, which would require the permittee to take appropriate measures to ensure the tests are properly conducted, such as finding a contract lab that is able to conduct the tests. In addition, if the permittee fails to re-test within 14 days following one or more failures to meet test acceptability criteria, EPA/State should issue an enforcement order.

7.7 Noncompliance with Other Narrative WET Permit Conditions

A permittee's failure to comply with any other narrative WET permit condition, such as the requirement to develop a TRE workplan, screen for the most sensitive species, or comply with reporting requirements, should also result in enforcement action. Initially, EPA or the State should issue an NOV/AO (or its equivalent) which requires immediate compliance. An exception could be made for first time or infrequent offenders who generally appear to be acting in good faith. In these cases, EPA/State could resolve issues of noncompliance through a verbal notice of violation, or a simple written NOV without an AO.

References

USEPA. 1995b. National policy regarding whole effluent toxicity enforcement. July 6, 1995. Memorandum from Brian Maas. Office of Enforcement and Compliance Assurance. Washington, DC.

APPENDIX A

FREQUENTLY ASKED QUESTIONS

Permitting:

Q: Are WET tests reliable and accurate to be used in the NPDES permitting program?

A: While some permittees may still contend that WET tests are inherently unreliable and inaccurate, the U.S. Court of Appeals recently rejected arguments that the variability observed in WET test methods (i.e., method variability) is excessive, concluding ". . . EPA has demonstrated that it is not." (See Edison Electric Institute, et al., v. Environmental Protection Agency, et al., 391 F. 3d 1267, 1272 (D.C. Cir. 2004)). In this case, the Court determined that EPA had "gone far enough" to minimize the effect of organic idiosyncrasy (the use of living specimens) by taking experimental and statistical precautions in designing and refining the WET test methods, denying the petitioners' complaint that EPA had not gone far enough to minimize the potential for variability between and within-tests.

Q: Can a State use either point estimate or hypothesis testing techniques for analyzing toxicity test data?

A: EPA allows State Permitting Authorities the choice of either hypothesis testing or point-estimation techniques for developing permit conditions and determining compliance. While several important drawbacks of the NOEC have been identified, hypothesis testing, per se, with safeguards is acceptable (Fox and Denton 2002). Such safeguards can include: testing a series of concentrations to verify and quantify a concentration-response relationship; increasing power (i.e., decreasing the type II error rate); closely bracketing the IWC by adjacent concentrations; applying an percent minimum significant difference (PMSD) as a test sensitivity criterion. Note, that for reasonable potential determination EPA has recommended using point estimate procedures in NPDES testing even when NPDES self-monitoring data are required to be determined using hypothesis testing techniques (USEPA 2000b). However, the permit limit can be still expressed and reported using hypothesis testing techniques, while also requiring reporting of specified point estimates for calculating facility-specific CVs for determing reasonable potential of toxicity.

Q: Should detection or quantitation limits be set if toxicity limits are established?

A: EPA has stated that method detection limit concepts are not applicable to WET test methods and have not been applied historically to toxicity testing methods developed by EPA or other scientific entities. EPA also believes that the test design employed in WET testing including controls, replication, and hypothesis testing or point estimation techniques, all provide an adequate protection from false positives. Detection limits are applicable only to tests that rely on instrumental measurements; the detection limits represent the sensitivity thresholds of the technology below which measurements become unreliable or impossible. Because WET testing is a biological and experimental method, rather than an instrumental method,

detection limit concepts are not applicable. In the Edison electric Institute et al v EPA (D.C. Cir. 2004, pg 10 - 11), it was decided that the described safeguards in EPA's WET methods addressed the petitioners' concerns and that EPA had offered a reasoned, thorough explanation of its decision on this subject without further requirement by law.

Q: Is the use of a numeric limit justifiable?

A: Yes, EPA emphasizes that the Clean Water Act (CWA), NPDES regulations, EPA's Technical Support Document for Water Quality-based Toxics Control (TSD, USEPA 1991a) all clearly envision that effluent limits should be expressed numerically. (See CWA 301(b)(1)(C) and 502(11); 40 CFR 122.44(d)(1)(iv) and (k) and 122.2).

a. By definition, 40 CFR 122.2 describes an effluent limitation as a restriction imposed . . . on quantities, discharge rates, and concentrations of 'pollutants';

b. According to 40 CFR 122.44(d)(v), limits on whole effluent toxicity are necessary when chemical-specific limits are not sufficient to attain and maintain applicable numeric or narrative water quality;

c. See chapter 5 of the TSD (USEPA 1991a), which describes the methodology to be used for calculating a statistical numeric limit for pollutants, including chronic toxicity;

d. Appendix B of the TSD, Basic Principals for Whole Effluent Toxicity, describes EPA's intent to have numeric limits for chronic toxicity, "Final whole effluent toxicity limits must be included in permits where necessary to ensure that State Water Quality standards are met. These limits must properly account for effluent variability, available dilution, and species sensitivity." This does not fit the description of a numeric effluent limit, because a narrative effluent limit cannot account for variability or available dilution. A numeric limit, on the other hand, can be calculated in such a way as to account for variability or available dilution; and,

e. In this document and (Denton and Narvaez 1996), both describe establishing limitations for chronic toxicity in the form of a daily maximum and a monthly median.

Q: When writing a permit for a discharge which only occurs intermittently throughout the year. What type of WET requirements should be incorporated into the permit?

A: Permit conditions describing the appropriate limits and monitoring triggers, test methods and species should clearly be defined in any permit. However in the situations of intermittent discharges it would be identified in the permit that during those periods of no discharge, the monitoring and testing conditions would not apply.

Q: What does that mean when it says that "permittees must certify on DMR statements that these are accurate"?

A: EPA clarified in its March 3, 2000 memorandum to EPA Regional Water Management Division Directors and Enforcement Division Directors that the purpose and meaning of the DMR certification was to certify only that all WET test results had been submitted and not tampered with or inappropriately modified prior to reporting on the DMR. The memorandum sought to resolve the confusion over the term accuracy which is sometimes used as a term to describe performance characteristic of a measurement system. In the context of DMR certification, the term accuracy is a certification of information submission, namely that information provided is accurate as a layperson uses the term, rather than accurate as the term is used to describe quantifiable performance of a measurement system. Therefore, the DMR certification is not intended to certify the WET test results are accurate including whether or not the WET test results are valid from a toxicity test standpoint (e.g., quality assurance/quality control on the tests was done properly by the analytical laboratory). Rather when a person certifies that the submission of WET testing information is accurate to the best of his/her knowledge and belief, the person certifies that the results obtained using the WET test procedures are faithfully and truthfully transcribed on the information submission, and the results were in fact results obtained using the specified test procedures.

Q: Is WET appropriate for circumstances of effluent dominated streams, and storm and ambient water applications?

A: The Permitting Authority, at the time of permit issuance, makes a determination as to whether WET testing, permit limitations for WET, or other requirements are appropriate and necessary to protect the receiving stream from potential toxic impacts from the permit to discharges. This determination is made on a case-by-case basis after considering the existing controls on points and non-point sources of pollution, the variability of the pollutant or plume parameters in the effluent, the sensitivity of the species to toxicity testing, and the dilution of the effluent in the receiving water (40 CFR 122.44(d)(1)(ii)).

Q: What is EPA's guidance to States in regards to a single exceedance of a WET limit?

A: EPA points to the August 15, 1995 national policy memo regarding WET enforcement and it specifies that the initial enforcement response to a single exceedance of a WET limit, causing no known harm, should not be a formal enforcement action with a civil penalty, but that any violation of a WET limit is of concern and should receive an immediate, professional review by the Permitting Authority (USEPA 1995b). EPA's recommended response to an isolated or infrequent violation of a WET limit, causing no known harm, is issuance of a letter of violation or Administrative Order which does not include a penalty. EPA policy suggests that additional testing is an appropriate initial response to a single WET limit violation and an escalated enforcement response to repeated violations.

Q: Are mixing zones applicable for WET permit limits?

A: This depends on the authorization of mixing zones for toxicity under a States' water quality standards. However, mixing zones even if allowable in a State's water standards plan may not be appropriate for a specific discharge location. This depends on the receiving water habitat. For example, if there are threatened and endangered species to be protected or sensitive spawning grounds, then a mixing zone may not be appropriate.

Q: Do Permitting Authorities have discretion to evaluate and, if necessary, reject unrepresentative or invalid WET data before use them in making a reasonable potential determination?

A: Yes, however this does not mean that Permitting Authorities have the right to determine that valid and representative WET data that demonstrate effluent toxicity are to be considered irrelevant and disregarded when determining whether a WET limit is needed in a NPDES permit.

Q: How should potential ionic imbalance toxicity to be evaluated?

A: Ion imbalances can cause toxicity in effluents. When toxicity effluent limits or monitoring triggers are exceeded, the permittee shall implement a TRE as described in their TRE work plan. Where TDS is a suspected toxicant, the permittee should utilize EPA's TIE procedures, in conjunction with recommendations prescribed by Goodfellow et al. (2000) to identify the specific ions contributing to TDS toxicity; regulatory or technical solutions may be possible if ions are identified as the only responsible effluent toxicant. In situations where ions and another toxicant are identified, the initial responsibility is to effectively address the other toxicant. After that toxicant is dealt with, then ion-specific toxicity in the discharge can be appropriately addressed and potential management and regulatory options considered by the Permitting Authority.

Testing Issues:

Q: Are toxicity test methods as precise as analytical test methods?

A: EPA found that WET test methods are as precise as chemical methods (USEPA 2000b). WET test method variability can be minimized by focusing on strict adherence to the EPA WET test method procedures; by using additional test acceptability criteria (TAC); by ensuring that laboratory personnel are properly trained to perform the tests correctly; and other preventive measures such as proper sample collection and storage.

Q: How is precision calculated for a test method?

A: Coefficient of variation (CV) is the descriptive statistics for quantifying test method precision. CV is the ratio of the standard derivation to mean. The precision of the effect concentrations is quantified by obtaining multiple test results under similar test conditions using the same test material. For example, the standard deviation and mean for EC_{25}

obtained for a specific test method from multiple monthly reference toxicant tests conducted at one laboratory would quantify “within-laboratory” precision for that laboratory.

Q: How are the types of test method variability defined?

A: There are several measures of variability related to WET tests included are within-test variability, within-laboratory variability, and between-laboratory variability. Within-test (intra-test) variability is the variability in test organism response within a concentration averaged across all concentrations of the test material in a single test. Within-laboratory (intra-laboratory) variability is the variability that is measured when reference toxicant tests are conducted using specific methods under reasonably constant conditions in the same laboratory. Within-laboratory variability includes within-test variability. Between-laboratory (inter-laboratory) variability is the variability between laboratories. It is measured by obtaining results from different laboratories using the same test method and the same test material (e.g., reference toxicant).

Q: Define the applicability of method detection limit to WET test methods?

A: EPA established the method detection limit concept specifically for chemical methods, where results generally consist of a single measurement of the pollutant of interest by an analytical instrument. The method detection limit concept uses information about the variability of the measurement system to determine a response level at which the measurement can be reliably distinguished from background noise, thus providing protection from false positive results. In WET testing, the final result is not based on a single measurement, but is the product of a series of replicated measurements on a range of effluent concentrations. The additional measurements, controls, replication, and statistical approaches included in WET test method measurement system ensure that measured responses can be reliably distinguished from background noise.

Q: Are laboratories routinely able to achieve the required test acceptability criteria on a routine basis?

A: EPA conducted a national interlaboratory study (USEPA 2001a, 2001b) of toxicity test methods; EPA confirmed that the methods are adaptable to a wide variety of laboratories and that the methods generate reproducible results in laboratories.

Q: Are the chronic tests for NPDES effluent testing required to use multi-concentrations rather than a single concentration vs control?

A: The decision to use a multi-concentration or single-concentration tests approach is typically defined in a State’s water quality standard control plan or policy. The November 2002 WET methods rule did not address or change any EPA policy concerning multi-concentration versus single concentration testing. With regards to the method manuals, they do not definitively say that multi-concentration testing is required for NPDES effluent tests. In fact, Section 8.10.1 says "the tests recommended for use in determining discharge permit compliance in the NPDES program are multi-concentration, or definitive, tests which

provide...". Often a State will utilize the single-concentration testing approach for assessing the toxicity of ambient or stormwaters. EPA recommends using multi-concentration testing for NPDES testing of effluents, but it is not required.

Q: Does EPA or its test method manuals require a specific dilution series to be used?

A: EPA has not required a specific dilution series or procedure for selecting dilution series. EPA recommends that test concentrations be selected independently for each test based on the objective of the study, the expected range of toxicity, the receiving water concentration, and any available historical testing information on the effluent.

Q: When there is no mixing zone allowed for toxicity, what dilution series should be used?

A: The following is suggested 100%, 62.5%, 50%, 25%, and 12.5%. This is following the basic suggestion of using the 0.5 dilution series. However, it is encouraged to include an additional concentration between 50 and 100% effluent especially since compliance would be based at the 100 percent effluent. The 62.5% effluent concentration is suggested especially if compliance is assessed at both 1.0 TU as a monthly median and 1.6 TU (e.g., 62.5% effluent) as a monthly average.

Q: What is the interpretation of the terms of “required” (using the term “must”) and those that are “recommended” (using the term “should”) when following the EPA test method manuals?

A: When EPA promulgated the 2002 WET methods (USEPA 2002a, 2002b, 2002c), these test method manuals clearly distinguish between required and recommended test conditions for the purposes of reviewing WET test data submitted under NPDES permits. EPA defined in the manual tables on summary of test conditions and test acceptability criteria for each method, such that each test condition is identified as required or recommended to be clear. In addition, EPA clarified the section on test review to each test method manuals. This section of the test methods manual provide technical guidance on the review of sampling and handling procedures, test acceptability criteria, test conditions, statistical methods, concentration-response relationships, reference toxicant testing, and within-test variability. This section clarifies that for WET test data submitted under NPDES permits, all required test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample. Deviations from recommended test conditions must be evaluated on a case-by-case basis to determine the validity of test results. Deviations from recommended test conditions may or may not invalidate the test results depending on the degree of the departure and the objective of the test. The data reviewer should consider the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or excepting a test results. For example, if dissolved oxygen is measured below 4.0 mg/L in one test chamber, the reviewer should consider whether any observed mortality in that test chamber corresponded with the drop in dissolved oxygen.

Q: What effluent pH should be used?

A: If the objective of the WET test is to determine the toxicity of the effluent and the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET tests is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample on completion of collection (for freshwater testing) or after adjusting the sample salinity (for marine testing). The objective of testing is to determine the absolute toxicity of the effluent, so the effluent pH should be the target. The target pH should not be an average or median of the effluent pH. The target for each WET test should be the pH of the individual sample when sample collection is complete (e.g., if the sample is a 24 hr composite, use the measured pH of the composite after the compositing is completed). An average or median effluent pH should not be used, because WET test results are snapshots and should not try to be otherwise.

Reference Toxicant Testing:

Q: What is the purpose of reference toxicant testing?

A: The purpose of reference toxicant testing is: 1) to assess the health and sensitivity of test organisms over time, and 2) to document and demonstrate initially and ongoing acceptable laboratory performance. These purposes of reference toxicant testing are reflected in the reference toxicant testing requirements under quality of test organisms in the test method manuals. For a given test method, successive tests must be performed with the same reference toxicant, at the same test conditions, in the same dilution water type, using the same data analysis methods.

Q: At what frequency should reference toxicant tests be required?

A: The test method manuals specify that, regardless of the source of test organisms (in-house cultures or purchased from external suppliers), the testing laboratory must perform at least one acceptable reference toxicant test per month for each toxicity test method conducted in that month. If the test method is conducted only monthly, or less frequently, a reference toxicant test must be performed concurrently with each effluent toxicity test. Reference toxicant tests performed by organism suppliers cannot substitute for this requirement, because in addition to assessing the sensitivity of test organisms, reference toxicant testing is used to document ongoing laboratory performance. The manuals do allow reference toxicant control charts from organism suppliers as a substitute for concurrent reference toxicant testing with each effluent test. While the method manuals require the conduct of reference toxicant tests, it is the responsibility of the Permitting Authority to determine the requirements for reporting test results and associated data and a State can always be more stringent than minimum requirements of the test method manuals.

Q: How should a failed reference toxicant test result be evaluated and how should it impact effluent testing?

A: EPA test method manuals include the added a caution that “reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving

water tests. Reference toxicant tests do provide information on trends in organism sensitivity and laboratory performance that can be useful in evaluating and interpreting effluent and receiving water tests results. For this reason, EPA has recommended evaluating the following elements of reference toxicant test results in the review of effluent and receiving water test data: the degree to which the reference toxicant tests result is outside of control chart limits, the width of the limits, the direction of a deviation (toward increased test organism sensitivity or toward decreased test organism sensitivity), the test conditions of both the effluent tests and the reference toxicant tests, and the objective of the test. ." In addition, EPA added recommendations to track the ongoing performance of individual QC measures such as PMSD, average control response, and CV of control response.

Sample Handling, Collection Issues:

Q: Should effluent samples only be collected as composite samples?

A: Composite and grab samples are allowed for WET testing depending on the objectives of the tests. Both sampling techniques have advantages and disadvantages that are described in the manuals. Permitting Authority should evaluate these advantages and disadvantages in light of the test objectives when selecting a sample type.

Q: Are the stipulations on filtration of effluents described in Section 9.1.2 of EPA/821/R-02/012 (USEPA 2002a) recommendations or requirements?

A: According to Section 9.1.2 of the acute test methods manual, filtering the sample through a 60 um mesh is only a requirement when the sample contains indigenous organisms that will interfere with the test. For example, some predatory invertebrates could eat the test organisms. If these interfering organisms are not present, the sample does not have to be filtered.

Q: What is the sample temperature range that is specified in the test methods manual?

A: According to the test method manuals the storage and shipping temperature of samples is in the range of 0-6 degree C. This modification provides greater consistency with national environmental laboratory accreditation conference (NELAC) standards.

Q: When should total residual chlorine be measured in the test methods?

A: If total residual chlorine is not detected in effluent or dilution water at test initiation, it is unnecessary to measure total residual chlorine at test solution renewal or at test termination. If total residual chlorine is detected at test initiation, then measurement of total residual chlorine at test solution renewal and test termination would continue to be required. It is not necessary to measure total residual chlorine in the laboratory prepared synthetic dilution water.

Q: Should treatment plant effluents be dechlorinated prior to toxicity testing?

A: The goal of the WET test is to determine the potential toxicity of the final effluent; therefore, if the final effluent has been treated with chlorine and dechlorinated, then that is what is to be tested. Dechlorination using anhydrous sodium thiosulfate to reduce chlorine would only be allowed at the discretion of the Permitting Authority. For example, if the effluent is toxic and the suspected toxicant is total residual chlorine (TRC), then the Permitting Authority could suggest having the permittee conduct a side by side test (minimum of three tests) of final effluent (w/o dechlorination) and with dechlorination to assist in determining whether TRC is causing toxicity. In addition, the permittee would need to conduct a definitive TIEs to determine that TRC is the sole toxicant. If so, then the Permitting Authority needs to address whether there is an appropriate TRC limit for the effluent and facility.

Note language in paragraph, 8.8.5 the chronic toxicity test method manuals, states, “At a minimum, pH, conductivity, and TRC are measured in the undiluted effluent or receiving water, and pH, and conductivity are measured in the dilution water. Therefore, an effluent sample at test initiation that has TRC concentrations above the toxic effect level (see TIE procedures to obtain toxic effect levels) would be causing toxicity to the test species, which is not allowed.

Q: What is the holding time requirement for first-use effluent sample?

A: The holding time requirements for first use of a sample have not changed. This requirement continues to state that the lapsed time from collection to first use of the sample must not exceed 36 hours. The allowance for Permitting Authorities to issue a variance for up to 72 hours also remains in the test method manuals. However, EPA clarified in the 2002 test method manuals, that samples can be used for test renewal at 24, 48, and/or 72 hours after first use. In the previous version of the freshwater chronic test methods manual, it stated that samples can be used for renewal at "24 and 48 hours after test initiation." This statement was modified to add "72 hours" based on comments that were received on the proposed rule. For example, when conducting the chronic *Ceriodaphnia* test over the duration of six to eight days (the maximum test duration is 8 days) with samples collected as recommended on days one, three, and five, the third sample must be used at 72 hours after first use in order to make the final renewal of the test. Otherwise, a fourth sample would need to be collected for renewal on the final day. This fourth sample would also need to be collected whether it was used or not because the decision to extend the test to an eighth day is not made until renewal on the seventh day, when it is generally too late to collect and ship another sample. For this reason, the holding time requirement was modified to allow use of samples at 72 hours after first use.

The second modification that was made (to both the freshwater chronic and marine chronic manuals) was to add an allowance for the use of existing samples for renewal when shipping problems are encountered. This allowance states: "If shipping problems (e.g., unsuccessful Saturday delivery) are encountered with renewal samples after a test has been initiated, the Permitting Authority may allow the continued use of the most recently used sample for test renewal." This modification was also added in response to comments on the proposed rule.

Based on these holding time requirements, a sample could be 108 hours old when last used (36 hours from collection to first use plus 72 hours from first use to last use). It should be noted, however, that these represent maximum allowable times and should not represent standard practice. EPA still recommends the collection of three samples on days 1, 3, and 5. Using this regime, sample holding times will be well below the maximums unless test durations are extended to 8 days or shipping problems are encountered.

Q: Do the test method manuals allow flexibility in determining the sample renewal collection schedule?

A: Yes, the test method manuals specify that sample collection on days 1, 3, and 5 is an example and not required sample collection scheme. For example, if shipping problems (e.g., unsuccessful Saturday delivery) are encountered with renewal samples after a test has been initiated, the Permitting Authority may allow the continued use of the most recently used sample for test renewal." This means that if the shipment of a renewal effluent sample is not received on the precise day, this does not necessitate the termination of the test.

pH Adjustments:

Q: What is pH shock and is it real?

A: EPA believes that pH drift alone is not considered a test interference if pH stays within the organism's tolerance range. The degree of pH drift typically observed in effluent samples should generally only interfere with test results if the sample contains a compound with toxicity that is pH dependent and at a concentration that is nearer the toxicity threshold. EPA does not have evidence to suggest that pH shock resulting from transferring organisms from culture water pH to test solution pH produces toxicity, provided the changes in pH or within the organism tolerance range (pH 6-9). Belanger and Cherry (1990) showed that *Ceriodaphnia dubia* survival and reproduction did not differ significantly in tests conducted at pH values ranging from 6 to 9, regardless of pH acclimation history. Acclimating organisms to test pH (for four weeks) only affected test performance when testing at pH 5.0 and 10.0 (beyond the normal organism tolerance range

Q: Are pH adjustments allowed for the chronic test methods?

A: The use of pH control is a modification to the tests and procedures that affects the measure toxicity of the sample, so Permitting Approval of this modification is required. The procedure is intended to control for pH drift that could produce artifactual toxicity, however, the procedure could be misused to artificially reduce sample toxicity when pH control is unwarranted. Approval of the procedure by the Permitting Authority will ensure that pH control is warranted in the test procedure. Permitting Authority approval in this instance is consistent with other method modifications, such as modification of sample holding times. The issue is not pH adjustment; it is control of pH drift during the test when the drift itself (not adjustments) is responsible for artifactual toxicity. There needs to be more side-by-side testing (minimum of 3 side-side tests) to confirm that pH drift is responsible for artifactual toxicity. This side-by-side testing does not mean testing the effluent at two different pHs. It

means testing a split sample, where pH is uncontrolled in one treatment and controlled (avoiding drift) in the other treatment. For example, if the collected sample is pH 6.5, then both of the side-by-side treatments start the test at 6.5. The uncontrolled treatment may drift to 7.5 during the test, but the controlled treatment is maintained at 6.5. Then, assess whether the tests differ in toxicity, and if so what is causing toxicity?

Q: Is pH adjustments allowed for the acute test methods?

A: EPA has not provided additional techniques that include modification of the sample to control pH drift in acute test methods because the current acute methods provide adequate remedies for pH drift without modifying the sample. pH drift in acute tests may be remedied by more frequent test renewals or the use of flow-through testing.

Q: What is the optional treatment for controlling pH drift?

A: EPA believes that the CO2 controlled atmosphere technique provides the best pH control with least amount of sample modification. This technique uses the existing carbonate buffering system in the sample to control pH. While the method modification provides guidance on using this technique, a particular method for ministering the technique is not prescribed. The manual describes two methods for using the CO2 controlled atmosphere technique: injecting a predetermined volume of CO2 into closed test containers, and; flushing a chamber containing the test vessels with a mixture of CO2 and air. Another technique for pH control is to eliminate airspace in the test vessel with a lid. This is effective when the partial pressure of CO2 in the test solution is higher than that in the atmosphere, since it prevents CO2 from escaping and allowing pH to rise. This technique would be allowable provided that is capable of adequately controlling pH.

Q: Is the use of pH buffers or addition of chemical acceptable?

A: EPA has not recommended the use of organic buffers for controlling test pH because this technique represents a greater modification of the sample than the CO2 controlled atmosphere technique. The use of organic buffers means adding a foreign substance to the sample that could potentially produce unknown interactions that may modify sample toxicity. EPA agrees that the addition of any foreign chemical to the sample is not ideal; however, atmospheric CO2 alone is not always sufficient to adjust and maintain pH. EPA adds in the manual the following caution: “the addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample.”

Specific Method Issues:

Acute Test Methods:

Q: Are the rainbow and brook trout approved test methods in Part 136?

A: Yes, these methods are approved in Part 136, and included as summary of test conditions and TAC as Table 15 of EPA (2002a).

Q: When conducting a 96-hour acute test method are renewal(s) required?

A: At 48-hour a renewal is required minimum for the 96-hour acute toxicity test methods.

Q: Explain the change in the growth endpoints (dividing by the number of surviving organisms) to the biomass endpoint (dividing by the number of original organisms) used in the fish test methods.

A: In the 1995 WET final rule, EPA changed the test endpoint from the growth endpoint that was based on the number of surviving organisms, to the biomass endpoint that combines growth and survival and is based on the number of initial organisms. EPA made this change: 1) to provide consistency with other methods (e.g., *C dubia* survival and reproduction tests) that incorporate survival along with sublethal effects, and; 2) because the combined survival and growth (or biomass) endpoint is a more sensitive measure than the growth endpoint alone. Data from Markle et al. (2000) support this conclusion by showing that point estimates calculated using the biomass endpoint were always lower (i.e., more biologically sensitive) than point estimates calculated using the growth endpoint. While the 1995 WET final rule changed the test endpoint to a combined survival and growth endpoint, test method manuals continue to refer to the endpoint as a "growth" endpoint. In fact, a combined survival and growth endpoint is more accurately termed biomass.

Q: What is blocking by known parentage?

A: It is a block randomization procedure that distributes offspring from a single parent evenly among the test treatments. For a given replicate, one neonate from the same parent is distributed to each test treatment. Process is repeated for each replicate using a new parent.

Q: Are 4th brood neonates for the chronic *Ceriodaphnia dubia* test method in the 4th edition manual?

A: In the *C. dubia* test, offspring from 4th or higher broods should not be counted and should not be included in the total number of neonates produced during the test.

Q: When using the *Mysidopsis bahia* chronic toxicity test method is the fecundity endpoint required (i.e., mandatory)?

A: The WET methods clearly state that achievement of the fecundity endpoint is not required for an acceptable *Mysidopsis bahia* chronic test. The tests acceptability criteria for this method state that "the minimum requirements for an acceptable test are 80% survival and an average weight of the lease 0.20 mg/mysid in the controls. If fecundity in the controls is adequate (egg production by 50% of the females), fecundity should be used as the criterion of effect in addition to survival and growth." The fecundity endpoint therefore is an optional endpoint in this test method, and the failure to generate this endpoint does not affect the validity or acceptability of the test.

Stormwater and Ambient Testing Issues:

Q: Have the toxicity test methods been used to assess agricultural, urban, and industrial stormwater runoff toxicity? If so, what toxicant(s) have been identified?

A: Toxicity testing of stormwaters has been used as a monitoring tool for urban and agricultural stormwater assessments in California. For example, researchers have identified the pesticides diazinon and chlorpyrifos in urban stormwaters (Katznelson and Mumley 1997; Bailey et al. 2000; Fong et al. 2000; Larsen et al. 2000; Larsen and List 2002; SRWP 2000). Toxicity testing of stormwaters from agricultural settings has identified rice pesticides, diazinon, chlorpyrifos, carbofuran, and carbaryl as toxicants (SRWP 1998; Foe et al. 1998; Reyes et al. 2000; Werner et al. 2000).

Q: Are acute and/or chronic test method(s) used to assess storm and ambient waters?

A: Typically, acute tests (96 hours or less) are primarily being used to initially assess the toxicity of storm and ambient waters. This is for several reasons, including the short-term nature of most storm events, the fact that renewals may not be necessary (except for the 96-hour test with a renewal at 48-hours), and the need to target and prioritize survival impacts first.

Q: What testing factors may need to be considered differently for stormwater testing compared to testing effluent from a continuous discharge?

A: The main factors include (1) sample collection and sample initiation holding time, (2) sample renewals, and (3) test design - single vs. multiple concentration testing (see below).

Q: Can an exception to the 36-hour holding time for initiation of the test be allowed for storm and ambient water testing?

A: All tests should be conducted as soon as possible following sample collection. EPA has allowed exceptions to the 36-hour holding time, for example, when effluents are shipped overseas for testing (Denton and Narvaez 1996). The primary reason for an extension of the holding time would be the consideration of the sampling, laboratory technician safety (Burton and Pitt 2001; see page 255), and logistics of coordinating collection and transport of multiple stormwater samples within a short period of time. Storm events are not pre-determined events and typically occur rapidly throughout a watershed; therefore, many site

samples must be coordinated and processed with short notification to the toxicity testing laboratories. It is encouraged that the 36-hour holding time for test initiation be targeted; however, the Permitting Authorities may allow an exception beyond the 36-hours. However, no more than 72 hours should elapse before initial use of a sample.

Q: How is the standard test renewal practices specified in the test method manuals followed, given that storm events may be of short duration?

A: EPA 5th edition acute test methods specify that test solutions be renewed after 48 hours for a 96-hour test. However, for storm events in short duration, this is not always feasible. A more realistic option, in cases when a second stormwater sample may not be available, would be to renew the test solutions with a mixture of ambient waters and stormwaters if such waters could be collected following test initiation while meeting WET test holding time specifications (Katznelson and Mumley 1997). Another option would be to collect sufficient volume during the storm event to use for the start of the test and at the 48-hour renewal.

Q: Are single concentrations (100% storm or ambient water) compared to a control in WET stormwater tests or are multiple dilutions of the stormwater or ambient water being tested?

A: Either testing approach may be applied, depending on the purpose of the testing and the discharge setting. For example, if the receiving stream is small and stormwater-dominated during storm events, "screening" tests of undiluted stormwater (100% stormwater or ambient water) discharges may be appropriate. Multiple-dilution WET tests would be needed to determine the magnitude of effect and to generate LC_{50}s (acute) or NOECs (chronic).

Q: When would a multiple dilution test be performed if a single concentration test is initially conducted?

A: A single concentration is typically compared to a control to determine the effect in 100% stormwater and ambient water exposures as a first tier to assess stormwaters and ambient water with a standard t-test approach as described in the test methods manual (see USEPA 2002a, page 86). A multiple concentration test could be considered for the next sampling event if toxicity is of significant magnitude in the 100% stormwater (e.g., 100% mortality within 24 to 48 hours). The testing facility may consider testing the original sample (assuming sufficient volume collected) with a dilution series to more fully characterize the sample, for those samples which demonstrate high mortality within a short timeframe.

Q: What is meant by the term "first flush" when referring to collection of stormwater samples?

A: "First flush" refers to the first waters released from a discharge point as a result of a storm event or runoff associated with ice and snow melt. Typically, constituent concentrations are highest in this "first flush" sample. "First flush" is operationally defined by a time-period in some states (e.g., waters discharged within the first 15 or first 30 minutes of a discharge event). However, the "first flush" may not always contain the highest concentrations of pollutants as this depends on the rain intensity, type of pollutant, and size of the watershed. The first flush phenomenon is more prevalent for rains with relatively constant intensities

and small watershed size (Burton and Pitt 2001). Therefore, it is important to understand the watershed in order to determine if sampling of first flush in a storm event is critical. Another consideration is to capture the first seasonal flush (e.g., after an extended dry period) in arid areas.

Q: Is capturing the first flush important?

A: The precedent has been established for chemical-specific stormwater sampling to sample first-flush discharges suggests the potential for higher chemical-specific toxicity in first-flush samples. This "first flush" effect depends on the nature and form of the pollutant (Ward and Elliot 1995). The chemograph peak slightly precedes that of the hydrograph for sediments or sediment-bound pollutants (e.g., chlorpyrifos, phosphorus) entrained in the water column. However, for dissolved pollutants like diazinon, the chemograph peak follows that of the hydrograph.

Q: Is timing of sample collection to a flow measurement important?

A: A measurement of flow should coincide with the collection of stormwater samples for WET testing. This typically entails measuring flow discharge from the site, in addition to the amount of rainfall causing the discharge event. It is important to establish when sampling occurred relative to the streamflow hydrograph (and subsequent chemograph) (Ward and Elliot 1995). Scientists must consider the magnitude of a toxic response in relation to flow of receiving waters when making chemical or toxicity assessments of receiving or stormwaters in the regulatory arena (permitting and TMDL development) and when developing study designs. Therefore, if assessment and quantification of the mass loadings are of interest, then concurrent flow measurements from a US Geological Survey gauging station located near the point of interest and within the same watershed should be collected (USGS 1999, 2000). Measurement of flow concurrent with sample collection should be considered if a nearby and representative gauging station is not available.

Test Review and Data Analysis:

Q: What steps should the Permitting Authority take to review the test result in determining whether the test is reliable?

A: See Attachment 4-3, Evaluation of Toxicity Data of this document and the test method manuals chapter on "Report Preparation and Test Review".

Q: Should concentration-response curves be evaluated?

A: Yes, the test method manuals (see chapter on test review) requires the laboratory and the Permitting Authority to review concentration-response curves. The EPA guidance (USEPA 2000a) assists the data reviewer through a stepwise process to determine the cause for non-ideal concentration-response relationships, and determine whether the test result is reliable, anomalous, and/or whether a new sample and toxicity test is required. This standardization

of concentration-response relationship review will decrease discrepancies in data interpretation amongst Permitting Authorities.

Q: How should Permitting Authorities address variability?

A: EPA is aware that there has been concern about the variability of the WET test method. EPA undertook an evaluation of an extensive toxicity dataset and published the document that examines the issue of test variability entitled "Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications under the National Pollutant Discharge Elimination System Program" (USEPA 2000b). This document outlines approaches for Permitting Authorities to consider in the context of within-test variability.

Q: What is percent minimum significant difference (PMSD)?

A: PMSD is the minimum significant difference (MSD) divided by the control mean, expressed as a percent. MSD is a measure of test sensitivity that establishes the minimum difference required between a control and a test treatment in order for that difference to be considered statistically significant.

Q: How should a test with a PMSD greater than the upper PMSD bound (according to the chapter on test review in the test methods manual) be evaluated?

A: According to the chapter on test review, which includes a discussion on test variability, "The within-test variability of individual test should be reviewed." Excessive within-test variability may invalidate a test result and warrant further testing. For additional guidance on evaluating within-test variability as measured by PMSD, reviewer should consult EPA (2000b). If the PMSD measured for the test exceeds the upper PMSD bound variability criterion as defined in the test methods manual, then one of the two following cases applies: 1) if toxicity is found at the permitted receiving water concentration (RWC) based upon the value of the effect concentration estimate (NOEC), then the test should be accepted and the effect concentration estimate may be reported, unless other test review steps raise serious doubts about its validity, or 2) if toxicity is not found at the permitted RWC based upon the value of the effect concentration estimate (NOEC) and the PMSD measured for the test exceeds the upper PMSD bound, then the test shall not be accepted, and a new test must be conducted promptly on a newly collected sample.

Q: How should a test with a PMSD less than the upper PMSD bound (according to the chapter on test review of the test methods manual) to be evaluated?

A: Lower PMSD bounds shall also be applied when a hypothesis test result (NOEC) is reported. In determining hypothesis test results (NOEC), a test concentration shall not be considered toxic (i.e., significantly different from the control) if the relative difference from the control is less than the lower PMSD bound (see pertinent table for values in test methods manual, chapter on report preparation and test review). See EPA (2000b) for specific examples of implementing lower PMSD bounds and Table 4-2 of this document.

Q: What is the advantage to implementing a PMSD bound versus a control CV that should be achieved?

A: In the chronic test method manuals, EPA has required variability criteria (i.e., PMSD) when NPDES permits require sublethal endpoints expressed using hypothesis testing. EPA chose to implement variability criteria based on the PMSD, rather than control CV, because the PMSD is most directly applied to the determination of hypothesis testing results. The PMSD includes exactly the variability affecting the NOEC determination, while the CV for the control or any one treatment represents only a portion of the variability affecting the NOEC determination. Permitting Authorities are free to continue the use of variability control strategies adopted within their jurisdiction, but when NPDES permits require sublethal WET testing endpoints expressed using hypothesis testing, the variability criteria must be implemented as well.

Q: Should the laboratory maintain control charts for PMSDs?

A: EPA recommends that laboratories track PMSD values over time so that the testing laboratory may assess the normal operating ranges of this parameter in the laboratory and identify periods of decreased consistency. This information is useful in quickly identifying and correcting potential problems and sources of variability. The tracking of PMSD values also is useful for evaluating whether a laboratory needs to increase test replication to consistently achieve the variability criteria.

Q: Can chronic tests be used to determine acute toxicity without needing to conduct a separate acute toxicity test? For example, if permit requires acute testing (48 or 96 hour) for the water flea, and the fathead minnow and chronic testing for the water flea, fathead minnow, and green algae can the chronic tests, be assessed for both acute and chronic toxicity results?

A: Currently, the acute test methods manual does not include an acute toxicity test method for algae or plants. For example, the 7-day chronic fathead minnow survival and growth test method both acute and chronic toxicity can be assessed concurrently. At the end of the toxicity test, statistical values can be determined for 7-day survival and growth with either NOECs or EC/IC_{25}s. In addition, any of the following acute assessments can be determined: 24-hr, 48-hr, 72-hr, or 96-hr for either LC_{50}s or NOAEC (no observed adverse effect concentrations) values. For the 7-day chronic *C. dubia* survival and reproduction test method both acute and chronic toxicity can be assessed concurrently. At the end of the toxicity test, statistical values can be determined for the 7-day survival with NOEC only (i.e., no LC_{50}s can be assessed based on experimental design) and reproduction with either NOECs or IC_{25}s. In addition, any of the following acute assessments can be determined: 24-hr, 48-hr, 72-hr, or 96-hr NOAEC (NOEC) values. Since, the experimental design for the chronic C. dubia is 10 replicates of one water flea/replicate, no point estimates can be determined. So, if the acute toxicity standard is based on LC_{50} determination, then a separate acute *C. dubia* test would need to be conducted.

Q. What is the measured rate of false positives for WET test methods?

A: EPA evaluated and assessed the false positive rate in their WET interlaboratory variability study and conclusively showed that measured false positive rates were below the theoretical rate of 5% estimated for the methods.

Q: What are alpha and beta errors?

A: A type I (alpha) error (i.e., false positive) results in the false conclusion that an effluent is toxic when it is not toxic. A type II (beta) error (i.e., false negative) results in the false conclusion that an effluent is not toxic when it actually is toxic. Power (1 - beta) is the probability of correctly detecting a true toxic effect (i.e., declaring an effluent toxic when it is in fact toxic). The EPA test method manuals recommend an alpha rate of 0.05 or 5 percent in the toxicity test method manuals. The risks of a high rate of type II errors is the risk to the environment that toxicity is occurring however it is not detected for various reasons, such as infrequent sampling, and/or lack of test sensitivity (high within-test variability). The risk of a test producing a false positive result is that the permittee may need to conduct additional tests (i.e., accelerated testing).

Q: How are alpha and beta related?

A: Alpha and beta are related (i.e., as alpha increases, beta decreases), assuming that the sample size (number of treatments, number of replicates), size of difference to be detected, and variance are held constant.

Q: What alpha rate should be used for data analysis?

A: The recommended alpha rate to be used according to the test method manuals is 0.05. Note, the WET Methods Guidance document (USEPA 2000a) does discuss using an alternate alpha rate of 0.10 under very specific conditions, however this was not recommended in EPA's final WET methods rule action. Therefore, the alpha rate to be used is the rate of 0.05.

Q: Can a sample be deemed a false positive?

A: No. If a test is properly conducted and correctly interpreted, identifying any particular outcome as a "false positive" is impossible. An effluent that is deemed toxic should require that the permittee conduct additional toxicity tests to determine if toxicity is reoccurring. Even if no toxicity is demonstrated in follow-up test result, this does not rule out that the original toxic event was a true toxic spike in the effluent.

Chapter 5 of the variability document (USEPA 2000b) specifically addresses “false positives.” The hypothesis test procedures prescribed in the WET methods should provide adequate protection against incorrectly concluding that an effluent is toxic when it is not. EPA strongly recommends that WET testing laboratories carefully review the statistical procedures used to produce WET test results and other factors (i.e., biological and statistical quality assurance), and verify that test conditions and test acceptability criteria were achieved. If a test is properly conducted and correctly interpreted, identifying any particular outcome as a “false positive” should not happen.

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APPENDIX B

DETERMINING REASONABLE POTENTIAL FOR WET

Table B-1. Effluent-specific Coefficient of Variation (CV) Equations for Lognormal Distribution

x_i	=	daily pollutant measurement i (in effluent)	
y_i	=	$\ln (x_i)$	
n	=	sample size of effluent data set	
μ_y *	=	$\Sigma (y_i) / n$	$1 \leq i \leq n$
σ_y^2 *	=	$\Sigma [(y_i - \mu_y)^2] / (n - 1)$	$1 \leq i \leq n$
σ_y *	=	$\sqrt{\sigma_y^2}$ *	$1 \leq i \leq n$
E(X) *	=	$\exp (\mu_y + 0.5 \sigma_y^2)$	
V(X) *	=	$[\exp (2 \mu_y + \sigma_y^2)] [\exp (\sigma_y^2) - 1]$	
CV(X) *	=	$[\exp (\sigma_y^2) - 1]^{½}$	

Note: Formulas are based on the lognormal distribution. “*” means “estimator”.

Table B-2. Reasonable Potential Multiplier Factor Equations

n = sample size of effluent data set

P_n = $(1 - \text{confidence level})^{1/n}$
= $(1 - 0.99)^{1/n}$

$$\frac{C_{95\ (\text{or } 99)}}{C_{Pn}} = \frac{\exp [z_{95\ (\text{or } 99)}\ \sigma_y * - 0.5\ \sigma_y^2 *]}{\exp (z_{Pn}\ \sigma_y * - 0.5\ \sigma_y^2 *)}$$

$$= \exp [(z_{95\ (\text{or } 99)} - z_{Pn})\ \sigma_y *]$$

= reasonable potential multiplier factor (RPMF),
where $\sigma_y^2 * = \ln (CV^2 + 1)$

Note: Formulas are based on the lognormal distribution. "*" means "estimator".

z_{95} = 1.645
z_{99} = 2.326
z_{Pn} = $4.91 [P_n^{0.14} - (1 - P_n)^{0.14}]$

"z_{Pn}" can be obtained from this formula, for $3 < n < 50$, with relative error less than 0.5%. "z_{Pn}" can also be obtained from the table of the Standard Normal distribution by linear interpolation, and it can be obtained from any statistical program, spreadsheet, or calculator that reports quantiles for the Standard Normal distribution.

Table B-3. Reasonable Potential Multiplier Factors: 0.95 "confidence level" and 95% percentile (rounded to one digit after the decimal)

	CV																			
n	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0
1	1.4	1.9	2.6	3.6	4.7	6.2	8.0	10.1	12.6	15.5	18.7	22.4	26.4	30.8	35.6	40.7	46.3	52.1	58.4	64.9
2	1.3	1.6	2.0	2.5	3.1	3.8	4.6	5.4	6.4	7.4	8.5	9.7	10.9	12.2	13.6	15.0	16.5	18.0	19.6	21.1
3	1.2	1.5	1.8	2.1	2.5	3.0	3.5	4.0	4.6	5.2	5.8	6.5	7.2	7.9	8.6	9.3	10.1	10.8	11.6	12.3
4	1.2	1.4	1.7	1.9	2.2	2.6	2.9	3.3	3.7	4.2	4.6	5.0	5.5	6.0	6.4	6.9	7.4	7.8	8.3	8.8
5	1.2	1.4	1.6	1.8	2.1	2.3	2.6	2.9	3.2	3.5	3.9	4.2	4.5	4.9	5.2	5.6	5.9	6.2	6.6	6.9
6	1.1	1.3	1.5	1.7	1.9	2.1	2.4	2.6	2.9	3.1	3.4	3.7	3.9	4.2	4.4	4.7	5.0	5.2	5.5	5.7
7	1.1	1.3	1.4	1.6	1.8	2.0	2.2	2.4	2.6	2.8	3.1	3.3	3.5	3.7	3.9	4.1	4.3	4.5	4.7	4.9
8	1.1	1.3	1.4	1.6	1.7	1.9	2.1	2.3	2.4	2.6	2.8	3.0	3.2	3.3	3.5	3.7	3.8	4.0	4.2	4.3
9	1.1	1.2	1.4	1.5	1.7	1.8	2.0	2.1	2.3	2.4	2.6	2.8	2.9	3.1	3.2	3.3	3.5	3.6	3.8	3.9
10	1.1	1.2	1.3	1.5	1.6	1.7	1.9	2.0	2.2	2.3	2.4	2.6	2.7	2.8	3.0	3.1	3.2	3.3	3.4	3.5
11	1.1	1.2	1.3	1.4	1.6	1.7	1.8	1.9	2.1	2.2	2.3	2.4	2.5	2.6	2.8	2.9	3.0	3.1	3.2	3.3
12	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.9	2.0	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	3.0	3.0
13	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0	2.1	2.2	2.3	2.4	2.4	2.5	2.6	2.7	2.8	2.8
14	1.1	1.2	1.3	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0	2.1	2.2	2.2	2.3	2.4	2.5	2.5	2.6	2.7
15	1.1	1.2	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.8	1.9	2.0	2.1	2.1	2.2	2.3	2.4	2.4	2.5	2.5
16	1.1	1.1	1.2	1.3	1.4	1.5	1.5	1.6	1.7	1.8	1.9	1.9	2.0	2.1	2.1	2.2	2.2	2.3	2.4	2.4
17	1.1	1.1	1.2	1.3	1.4	1.4	1.5	1.6	1.7	1.7	1.8	1.9	1.9	2.0	2.0	2.1	2.2	2.2	2.3	2.3
18	1.1	1.1	1.2	1.3	1.3	1.4	1.5	1.5	1.6	1.7	1.7	1.8	1.9	1.9	2.0	2.0	2.1	2.1	2.2	2.2
19	1.1	1.1	1.2	1.3	1.3	1.4	1.5	1.5	1.6	1.6	1.7	1.7	1.8	1.8	1.9	1.9	2.0	2.0	2.1	2.1
20	1.1	1.1	1.2	1.2	1.3	1.4	1.4	1.5	1.5	1.6	1.6	1.7	1.7	1.8	1.8	1.9	1.9	2.0	2.0	2.0

Table B-4. Reasonable Potential Multiplier Factors: 0.99 "confidence level" and 99% percentile (rounded to one digit after the decimal)

	CV																			
n	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0
1	1.6	2.5	3.9	6.0	9.0	13.2	18.9	26.4	36.0	48.1	63.0	81.0	102.3	127.3	156.2	189.2	226.5	268.3	314.7	366.0
2	1.4	2.0	2.9	4.0	5.5	7.4	9.8	12.6	16.1	20.2	24.8	30.2	36.2	42.9	50.2	58.3	67.0	76.4	86.5	97.2
3	1.4	1.9	2.5	3.3	4.4	5.6	7.1	8.9	11.0	13.4	16.0	18.9	22.1	25.6	29.4	33.4	37.7	42.2	47.0	52.0
4	1.3	1.7	2.3	2.9	3.8	4.7	5.9	7.2	8.7	10.3	12.2	14.1	16.3	18.6	21.0	23.6	26.3	29.1	32.0	35.1
5	1.3	1.7	2.1	2.7	3.4	4.2	5.1	6.2	7.3	8.6	10.0	11.5	13.1	14.8	16.5	18.4	20.3	22.3	24.4	26.5
6	1.3	1.6	2.0	2.5	3.1	3.8	4.6	5.5	6.4	7.5	8.6	9.8	11.1	12.4	13.8	15.2	16.7	18.2	19.8	21.4
7	1.3	1.6	2.0	2.4	2.9	3.	4.2	5.0	5.8	6.7	7.6	8.6	9.7	10.8	11.9	13.1	14.3	15.5	16.8	18.1
8	1.2	1.5	1.9	2.3	2.8	3.3	3.9	4.6	5.3	6.1	6.9	7.8	8.7	9.6	10.5	11.5	12.5	13.6	14.6	15.7
9	1.2	1.5	1.8	2.2	2.7	3.2	3.7	4.3	4.9	5.6	6.3	7.1	7.9	8.7	9.5	10.4	11.2	12.1	13.0	13.9
10	1.2	1.5	1.8	2.2	2.6	3.0	3.5	4.1	4.6	5.3	5.9	6.6	7.3	8.0	8.7	9.4	10.2	11.0	11.7	12.5
11	1.2	1.5	1.8	2.1	2.5	2.9	3.4	3.9	4.4	4.9	5.5	6.1	6.7	7.4	8.0	8.7	9.4	10.0	10.7	11.4
12	1.2	1.4	1.7	2.0	2.4	2.8	3.2	3.7	4.2	4.7	5.2	5.8	6.3	6.9	7.5	8.1	8.7	9.3	9.9	10.5
13	1.2	1.4	1.7	2.0	2.3	2.7	3.1	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.6	8.1	8.7	9.2	9.8
14	1.2	1.4	1.7	2.0	2.3	2.6	3.0	3.4	3.8	4.3	4.7	5.2	5.7	6.2	6.6	7.1	7.6	8.1	8.6	9.1
15	1.2	1.4	1.6	1.9	2.2	2.6	2.9	3.3	3.7	4.1	4.5	5.0	5.4	5.9	6.3	6.8	7.2	7.7	8.1	8.6
16	1.2	1.4	1.6	1.9	2.2	2.5	2.8	3.2	3.6	4.0	4.4	4.8	5.2	5.6	6.0	6.4	6.9	7.3	7.7	8.1
17	1.2	1.4	1.6	1.9	2.1	2.4	2.8	3.1	3.5	3.8	4.2	4.6	5.0	5.4	5.8	6.1	6.5	6.9	7.3	7.7
18	1.2	1.4	1.6	1.8	2.1	2.4	2.7	3.0	3.4	3.7	4.1	4.4	4.8	5.2	5.5	5.9	6.3	6.6	7.0	7.4
19	1.2	1.4	1.6	1.8	2.1	2.3	2.6	2.9	3.3	3.6	3.9	4.3	4.6	5.0	5.3	5.7	6.0	6.4	6.7	7.0
20	1.2	1.3	1.6	1.8	2.0	2.3	2.6	2.9	3.2	3.5	3.8	4.1	4.5	4.8	5.1	5.5	5.8	6.1	6.4	6.7

Table B-5. Example Numerical Calculation for Reasonable Potential Determination

The following equations are recommended in Chapter 3 and Appendix E of the TSD (USEPA 1991a) for determining reasonable potential in accordance with 40 CFR 122.44(d)(1). These equations are based on the lognormal distribution and are suitable for WET data expressed in units of TUc = 100 / NOEC (or IC25), or units of TUa = 100 / LC50. Note that "*" means "estimator". This example uses three significant figures and the final result is expressed using two significant figures. The example assumes that WET data (in TUc) have, at most, two significant figures.

Step 1

In this example, chronic toxicity effluent data for compliance monitoring are reported in units of TUc = 100 / NOEC. No acute toxicity effluent data are available. The chronic WET data are reviewed and both the observed maximum effluent value (maxCe) and total sample size of effluent data set (n) are identified.

TUc	=	{ 8, 4, 4, 4, 2, >16, 8, 8, 16, >16, 8, 4, >16, 2, 16, 8, 4, 2, >16, 2 } in units of TUc = 100 / NOEC
maxCe	=	observed maximum effluent value
	=	>16 TUc
n	=	total sample size of effluent data set
	=	20

Following Section 5.5.2 of the TSD, if n is <10, then effluent variability (CV) is estimated using 0.6; proceed to Step 4. If n is ≥10, then proceed to Step 2 in order to estimate effluent-specific variability. In this example, n is ≥10; proceed to Step 2.

Step 2

In order to better estimate effluent variability, chronic toxicity effluent data are also reported in units of TUc = 100 / IC25. These data are used to calculate estimates for sigma (σ_y *) and CV.

TUc	=	{ 4.3, 1.9, 2.0, 1.9, 1.9, 33.2, 5.6, 5.2, 9.1, 29.4, 9.3, 2.0, 38.5, 1.7, 6.4, 6.1, 2, 1.5, 28.6, 2.4 } in units of TUc = 100 / IC_{25}	
x_i	=	{ 4.3, 1.9, 2.0, 1.9, 1.9, 33.2, 5.6, 5.2, 9.1, 29.4, 9.3, 2.0, 38.5, 1.7, 6.4, 6.1, 2, 1.5, 28.6, 2.4 } in units of TUc = 100 / IC_{25}	
y_i	=	log base *e* of daily pollutant measurement i	
	=	$\ln (x_i)$	
	=	{ 1.45, 0.641, 0.693, 0.641, 0.641, 3.50, 1.72, 1.64, 2.20, 3.38, 2.23, 0.693, 3.65, 0.530, 1.85, 1.80, 0.693, 0.405, 3.35, 0.875 }	
n	=	sample size of effluent data set	
	=	20	
μ_y *	=	mean of logarithms	
	=	$\Sigma (y_i) / n$	$1 \leq i \leq n$
	=	32.5 / 20	
	=	1.62	
σ_y^2 *	=	variance of logarithms	
	=	$\Sigma [(y_i - \mu_y)^2] / (n - 1)$	$1 \leq i \leq n$
	=	23.2 / 19	
	=	1.22	
σ_y *	=	standard deviation of logarithms	$1 \leq i \leq n$
	=	$\sqrt{\sigma_y^2}$ *	
	=	$\sqrt{1.22}$	
	=	1.10	
E(X) *	=	$\exp (\mu_y + 0.5 \sigma_y^2)$	
	=	exp [1.62 + (0.5) (1.22)]	
	=	9.29	

$V(X)^* = [\exp(2\mu_y + \sigma_y^2)][\exp(\sigma_y^2) - 1]$
$= \{\exp[(2)(1.62) + 1.22]\}[\exp(1.22) - 1]$
$= [86.4][2.38]$
$= 205$

$CV(X)^* = [\exp(\sigma_y^2) - 1]^{1/2}$
$= [\exp(1.22) - 1]^{1/2}$
$= 1.54$

Proceed to Step 3.

Step 3

The reasonable potential multiplier factor is calculated using estimates for sigma (σ_y *) and CV from Step 2.

$P_n = (1 - \text{confidence level})^{1/n}$
$= (1 - 0.99)^{1/20}$
$= 0.794$

$z_{Pn} = 0.8205$ "z_{Pn}" is found from a table of the Standard Normal distribution, by linear interpolation between tabled values $z_{0.7939} = +0.82$ and $z_{0.7967} = +0.83$.

Reasonable potential multiplier factor (RPMF) $= \dfrac{C_{95\ (or\ 99)}}{C_{Pn}}$

$= \dfrac{\exp[z_{95\ (or\ 99)}\ \sigma_y^* - 0.5\ \sigma_y^{2*}]}{\exp(z_{Pn}\ \sigma_y^* - 0.5\ \sigma_y^{2*})}$

$= \exp[(z_{95\ (or\ 99)} - z_{Pn})\ \sigma_y^*]$
$= \exp[(2.326 - 0.8205)(1.10)]$
$= \exp(1.66)$
$= 5.26$,

where $\sigma_y^* = \sqrt{\sigma_y^{2*}}$
$= \sqrt{1.22}$
$= 1.10$

Note: Using Table B-4 for the reasonable potential multiplier factor, at n = 20 for a CV = 1.5, the reasonable potential multiplier factor is 5.1; at n = 20 for a CV = 1.6, the reasonable potential multiplier factor is 5.5. Using linear interpolation to the

CV = 1.54 gives a reasonable potential multiplier factor of 5.26. This sometimes differs from the exact reasonable potential multiplier factor calculated above because the numbers in Table B-4 have been rounded.

Proceed to Step 4.

Step 4

The statistically estimated maximum effluent value ($maxCe_{RP}$) is calculated using the reasonable potential multiplier factor (RPMF) from Step 3 and the observed maximum effluent value (maxCe) from Step 1.

maxCe	=	observed maximum effluent value
	=	{ 8, 4, 4, 4, 2, >16, 8, 8, 16, >16, 8, 4, >16, 2, 16, 8, 4, 2, >16, 2 } in units of TUc = 100 / NOEC
	=	>16 TUc
$maxCe_{RP}$	=	statistically estimated maximum effluent value
	=	(RPMF) (maxCe)
	=	(5.26) (>16)
	=	84 TUc

In addition, because no acute toxicity effluent data are available to evaluate the reasonable potential to exceed the water quality criterion for acute toxicity, a default acute-to-chronic ratio is recommended in Section 1.3.4 of the TSD in order to estimate effluent levels for acute toxicity.

ACR	=	acute-to-chronic ratio in TSD Section 1.3.4
	=	LC_{50} / NOEC
	=	TUc / TUa
	=	10
TUc,a	=	TUc / 10 , where chronic toxicity is expressed in acute toxic units (TUc,a)
maxCe	=	observed maximum effluent value in units of TUc,a
	=	(maxCe in units of TUc) / 10
	=	>16 / 10
	=	>1.6 TUc,a
$maxCe_{RP}$	=	statistically estimated maximum effluent value in units of TUc,a
	=	(RPMF) (maxCe in units of TUc,a)
	=	(5.26) (>1.6)
	=	8.4 TUc,a

Proceed to Step 5.

Step 5

The resultant magnitudes of chronic and acute toxicity in the receiving water after effluent discharge (Cr) are calculated using the mass balance equation, a steady-state model, and compared with water quality criteria for chronic and acute toxicity. If a resultant magnitude for toxicity (Cr) is greater than a water quality criterion for toxicity, then reasonable potential is established and a WQBEL is needed, in accordance with 40 CFR 122.44(d)(1).

$$Cr\ Qr = Ce\ Qe + Cs\ Qs \quad ,$$

where C = critical value for WET (in units of TUc, TUa)
Q = critical value for flow (in units of cfs or MGD)
r = effluent plus upstream after discharge
e = effluent discharge
s = upstream before discharge

Sa = critical dilution factor authorized by Permitting Authority
= (1 + Qs / Qe) or output from dilution model

Cr = resultant magnitude for toxicity in the receiving water after effluent discharge

$$= \frac{Ce + [\ Cs\ (\ Qs / Qe\ )\ ]}{1 + (\ Qs / Qe\ )}$$

$$= \frac{Ce + [\ Cs\ (\ Sa - 1\ )\ ]}{Sa}$$

In this example, the resultant magnitude of chronic WET in the receiving water after effluent discharge (Cr) to compare with water quality criterion for chronic toxicity (CCC).

CCC = criterion continuous concentration to protect against chronic effects
= 1.0 TUc

Sa_c = chronic critical dilution factor
= (1 + $Qs_{7Q10\ (or\ 4B3)}$ / Qe)
= 8

Cs = critical value for WET upstream before discharge
= 0 TU

Ce	=	most critical value for WET in the effluent discharge in units of TUc
	=	maximum (maxCe or $maxCe_{RP}$)
	=	maximum (>16 or 84)
	=	84 TUc
Cr	=	$\frac{Ce + [Cs (Sa - 1)]}{Sa}$
	=	$\frac{84 + [0 (8 - 1)]}{8}$
	=	11 TUc
Reasonable Potential	=	Cr > CCC
	=	11 TUc > 1 TUc
	=	Yes, permit needs a WQBEL.

In this example, the resultant magnitude of acute WET in the receiving water after effluent discharge (Cr) to compare with water quality criterion for acute toxicity (CMC).

CMC	=	criterion maximum concentration to protect against acute effects
	=	0.3 TUa
Sa_a	=	acute critical dilution factor
	=	(1 + $Qs_{1Q10 \text{ (or } 1B3)}$ / Qe)
	=	1
Cs	=	critical value for WET upstream before discharge
	=	0 TU
Ce	=	most critical value for WET in the effluent discharge in units of TUc,a
	=	maximum (maxCe or $maxCe_{RP}$)
	=	maximum (>1.6 or 8.4)
	=	8.4 TUc,a

$$Cr = \frac{Ce + [\, Cs\, (\, Sa - 1\,)\,]}{Sa}$$

$$= \frac{8.4 + [\, 0\, (\, 1 - 1\,)\,]}{1}$$

$$= 8.4 \text{ TUc,a}$$

Reasonable Potential = $Cr > CMC$
= $8.4 \text{ TUc,a} > 0.3 \text{ TUa}$
= Yes, permit needs a WQBEL.

References

USEPA. 1991a. Technical support document for water quality-based toxics control. Office of Water. Washington, DC. EPA/505/2-90/001.

APPENDIX C

DERIVING PERMIT LIMITS FOR WET

Table C-1. Example Calculations for Developing Permit Limits from Two-value, Steady-state Wasteload Allocations for WET

The following equations are recommended in Chapter 5 and Appendix E of the TSD (USEPA 1991a) for calculating water quality-based effluent limits (WQBELs) for WET. These equations are based on the lognormal distribution and are suitable for WET data expressed in units of TUc = 100 / NOEC (or IC25), or units of TUa = 100 / LC50. Note that "*" means "estimator". This example uses three significant figures and the final result is expressed using two significant figures. The example assumes that WET data (in TUc) have, at most, two significant figures.

Generally, wasteload allocations for WET in effluent discharge are calculated using the mass balance equation, a steady-state model.

$$C_r Q_r = C_e Q_e + C_s Q_s \quad ,$$

where C = critical value for WET (in units of TUc or TUa)
Q = critical value for flow (in units of cfs or MGD)
r = effluent plus upstream after discharge
e = effluent discharge
s = upstream before discharge

S_a = critical dilution factor authorized by Permitting Authority
= $(1 + Q_s / Q_e)$ or output from dilution model

C_e = wasteload allocation (WLA) in units of TUc, TUa, or TUa,c
= $C_r + [(Q_s / Q_e) (C_r – C_s)]$
= $C_r + [(S_a – 1) (C_r – C_s)]$

The wasteload allocation (WLAc) for chronic toxicity in the effluent discharge is calculated using the mass-balance equation.

Cr	=	criterion continuous concentration (CCC) to protect against chronic effects
	=	1.0 TUc
Cs	=	critical value for WET upstream before discharge
	=	0 TU
Sa_c	=	chronic critical dilution factor
	=	(1 + $Qs_{7Q10\ (or\ 4B3)}$ / Qe)
	=	8
Ce	=	WLA in units of TUc
	=	Cr + (Sa – 1) (Cr – Cs)
	=	1 + (8 – 1) (1 – 0)
	=	8 TUc

The wasteload allocation for acute toxicity in the effluent discharge is expressed in chronic toxic units (WLAa,c) and calculated using the mass-balance equation and an acute-to-chronic ratio.

ACR	=	acute-to-chronic ratio in TSD Section 1.3.4
	=	LC_{50} / NOEC
	=	TUc / TUa
	=	10
TUa,c	=	10 × TUa , where acute toxicity is expressed in chronic toxic units (TUa,c)
Cr	=	criterion maximum concentration (CMC) to protect against acute effects
	=	0.3 TUa
Cs	=	critical value for WET upstream before discharge
	=	0 TU
Sa_a	=	acute critical dilution factor
	=	(1 + $Qs_{1Q10\ (or\ 1B3)}$ / Qe)
	=	1
Ce	=	WLA in units of TUa,c
	=	[Cr + (Sa – 1) (Cr – Cs)] × ACR
	=	[0.3 + (1 – 1) (1 – 0)] × 10
	=	3 TUa,c

After both acute and chronic wasteload allocations are determined, the critical treatment performance level (coefficient of variation, CV, and long term average, LTA) that will allow the effluent to meet the wasteload allocations is calculated. Following Section 5.5.2 of the TSD, if k is <10, then effluent variability (CV) is estimated using 0.6. If k is ≥10, then the following equations are used to estimate effluent-specific variability.

x_i = daily pollutant measurement i (in effluent) in units of TUc = 100 / IC25, or TUa = 100 / LC50
= { 4.3, 1.9, 2.0, 1.9, 1.9, 33.2, 5.6, 5.2, 9.1, 29.4, 9.3, 2.0, 38.5, 1.7, 6.4, 6.1, 2, 1.5, 28.6, 2.4 } in units of TUc = 100 / IC25

y_i = log base *e* of daily pollutant measurement i
= $\ln (x_i)$
= { 1.45, 0.641, 0.693, 0.641, 0.641, 3.50, 1.72, 1.64, 2.20, 3.38, 2.23, 0.693, 3.65, 0.530, 1.85, 1.80, 0.693, 0.405, 3.35, 0.875 }

k = sample size of effluent data set
= 20

μ_y * = mean of logarithms
= $\Sigma (y_i) / k$ $\quad 1 \leq i \leq k$
= 32.5 / 20
= 1.62

σ_y^2 * = variance of logarithms
= $\Sigma [(y_i - \mu_y)^2] / (k - 1)$ $\quad 1 \leq i \leq k$
= 23.2 / 19
= 1.22

σ_y * = standard deviation of logarithms $\quad 1 \leq i \leq k$
= $\sqrt{\sigma_y^2}$ *
= $\sqrt{1.22}$
= 1.10

E(X) * = $\exp (\mu_y + 0.5 \sigma_y^2)$
= $\exp [1.62 + (0.5) (1.22)]$
= 9.29

V(X) * = $[\exp (2 \mu_y + \sigma_y^2)] [\exp (\sigma_y^2) - 1]$
= $\{ \exp [(2) (1.62) + 1.22] \} [\exp (1.22) - 1]$
= [86.4] [2.38]
= 205

CV(X) * = $[\exp (\sigma_y^2) - 1]^{½}$

$= [\exp (1.22) - 1]^{½}$
$= 1.54$

The long-term average for chronic toxicity (LTAc) and the long-term average for acute toxicity (LTAa,c) in the effluent discharge are calculated using the following equations. Knowing CV(X) *, these long-term average values may be determined using the pre-calculated "WLA multipliers" in TSD Table 5-1.

LTAc = chronic (4-day average) long term average in units of TUc
$= WLAc \times \exp (0.5 \sigma_4^2 - z_{0.99} \sigma_4)$
$= 8 \times \exp [(0.5) (0.466) - (2.326) (0.683)]$
$= 8 \times 0.258$
$= 2.06$, or

= WLAc × chronic WLA multiplier from TSD Table 5-1 for CV and 99th percentile
$= 8 \times 0.258$
$= 2.06$

LTAa,c = acute (1-day average) long term average in units of TUa,c
$= WLAa,c \times \exp (0.5 \sigma^2 - z_{0.99} \sigma)$
$= 3 \times \exp [(0.5) (1.22) - (2.326) (1.10)]$
$= 3 \times 0.142$
$= 0.426$, or

= WLAc × acute WLA multiplier from TSD Table 5-1 for CV and 99th percentile
$= 3 \times 0.141$
$= 0.423$

where CV = CV(X) *
$= 1.54$
$\sigma_4^2 = \ln [(CV^2 / 4) + 1]$
$= 0.466$
$\sigma^2 = \ln (CV^2 + 1)$
$= 1.22$
$z_{0.99}$ = 2.326 is recommended for WLA in TSD Section 5.5.4

Permit limits are calculated using the lower (more limiting) LTA discharge condition.

LTA = minimum (LTAc or LTA,a,c)
= minimum (2.06 or 0.426)
= 0.426

A maximum daily limit (MDL) and average monthly limit (AML) are calculated using the more limiting discharge condition—defined by the LTA and CV—using the following equations. Knowing CV(X) *, the maximum daily limit and average monthly limit may be determined using the pre-calculated "LTA multipliers" in TSD Table 5-2.

MDL		=	maximum daily limit
		=	$LTA \times \exp (z_{0.99} \sigma - 0.5 \sigma^2)$
		=	$0.426 \times \exp [(2.326) (1.10) - (0.5) (1.22)]$
		=	0.426×7.02
		=	3.0 TUa,c , or
		=	LTA × maximum daily limit LTA multiplier from TSD Table 5-2 for CV and 99th percentile
		=	0.426×7.07
		=	3.0 TUa,c
AML		=	average monthly limit
		=	$LTA \times \exp (z_{0.95} \sigma_n - 0.5 \sigma_n^2)$
		=	$LTA \times \exp (z_{0.95} \sigma_4 - 0.5 \sigma_4^2)$
		=	$0.426 \times \exp [(1.645) (0.683) - (0.5) (0.466)]$
		=	0.426×2.44
		=	1.0 TUa,c , or
		=	LTA × average monthly limit LTA multiplier from TSD Table 5-2 for CV, 95th percentile, and $n \geq 4$
		=	0.426×2.43
		=	1.0 TUa,c
where	CV	=	CV(X) *
		=	1.54
	σ^2	=	$\ln (CV^2 + 1)$
		=	1.22
	$z_{0.99}$	=	2.326 is recommended for MDL in TSD Section 5.5.4
	n	=	number of samples per month ≥ 4
		=	4
	σ_n^2	=	$\ln [(CV^2 / n) + 1]$
	σ_4^2	=	$\ln [(CV^2 / 4) + 1]$
		=	0.466
	$z_{0.95}$	=	1.645 is recommended for AML in TSD Section 5.5.4
MDL		=	3.0 TUa,c
AML		=	1.0 TUa,c

Following Section 2.6.2 in Chapter 2 of this document, EPA Regions 9 and 10 continue recommend that Permitting Authorities establish a monthly median limit (MML) of 1.0 TUc for chronic WET, when the statistically-calculated AML is at or less than 1.0 TUc. As a result, in this example where the acute-to-chronic ratio is 10, the recommended permit limits for chronic WET are:

MDL	=	maximum daily limit 3.0 TUa,c
MML	=	median monthly limit 1.0 TUa,c

In addition, because these permit limits have been developed using a default acute-to-chronic ratio of 10, the permit should include: (1) side-by-side acute and chronic WET monitoring in order to develop an effluent-specific acute-to-chronic ratio, and (2) a permit reopener condition authorizing revisions to these WQBELs, if appropriate, based on this new information.

Table C-2. Example Calculations for Developing Permit Limits from Two-value, Steady-state Wasteload Allocations for WET under Low Flow Conditions

The following equations are recommended in Chapter 5 and Appendix E of the TSD (USEPA 1991a) for calculating water quality-based effluent limits (WQBELs) for WET. These equations are based on the lognormal distribution and are suitable for WET data expressed in units of TUc = 100 / NOEC (or IC25), or units of TUa = 100 / LC50. Note that "*" means "estimator". This example uses three significant figures and the final result is expressed using two significant figures. The example assumes that WET data (in TUc) have, at most, two significant figures.

Generally, wasteload allocations for WET in effluent discharge are calculated using the mass balance equation, a steady-state model.

$$C_r Q_r = C_e Q_e + C_s Q_s \quad ,$$

where C = critical value for WET (in units of TUc or TUa)
Q = critical value for flow (in units of cfs or MGD)
r = effluent plus upstream after discharge
e = effluent discharge
s = upstream before discharge

Sa = critical dilution factor authorized by Permitting Authority
= (1 + Qs / Qe) or output from dilution model

Ce = wasteload allocation (WLA) in units of TUc, TUa, or TUa,c
= Cr + [(Qs / Qe) (Cr – Cs)]
= Cr + [(Sa – 1) (Cr – Cs)]

The wasteload allocation (WLAc) for chronic toxicity in the effluent discharge is calculated using the mass-balance equation.

Cr = criterion continuous concentration (CCC) to protect against chronic effects
= 1.0 TUc

Cs = critical value for WET upstream before discharge
= 0 TU

Sa_c = chronic critical dilution factor
= (1 + $Qs_{7Q10\ (or\ 4B3)}$ / Qe)
= 1

Ce = WLA in units of TUc
= Cr + (Sa – 1) (Cr – Cs)

= 1 + (1 – 1) (1 – 0)
= 1 TUc

The wasteload allocation for acute toxicity in the effluent discharge is expressed in chronic toxic units (WLAa,c) and calculated using the mass-balance equation and an acute-to-chronic ratio.

ACR = acute-to-chronic ratio in TSD Section 1.3.4
= LC50 / NOEC
= TUc / TUa
= 10

TUa,c = 10 × TUa , where acute toxicity is expressed in chronic toxic units (TUa,c)

Cr = criterion maximum concentration (CMC) to protect against acute effects
= 0.3 TUa

Cs = critical value for WET upstream before discharge
= 0 TU

Sa_a = acute critical dilution factor
= (1 + $Qs_{1Q10\ (or\ 1B3)}$ / Qe)
= 1

Ce = WLA in units of TUa,c
= [Cr + (Sa – 1) (Cr – Cs)] × ACR
= [0.3 + (1 – 1) (1 – 0)] × 10
= 3 TUa,c

After both acute and chronic wasteload allocations are determined, the critical treatment performance level (coefficient of variation, CV, and long term average, LTA) that will allow the effluent to meet the wasteload allocations is calculated. If k is ≥10, then the equations in Table C-1 are used to estimate effluent-specific variability. In this example, following Section 5.5.2 of the TSD, k is <10 and effluent variability (CV) is estimated using 0.6.

x_i = daily pollutant measurement i (in effluent) in units of TUc = 100 / IC25, or TUa = 100 / LC50
= { 1, 1, 1, 1.8, <1 } in units of TUc = 100 / IC25

k = sample size of effluent data set
= 5

CV(X) * = $[\exp (\sigma_y^2) - 1]^{½}$
= 0.6

The long-term average for chronic toxicity (LTAc) and the long-term average for acute toxicity (LTAa,c) in the effluent discharge are calculated using the following equations. Knowing CV(X) *, these long-term average values may be determined using the pre-calculated "WLA multipliers" in TSD Table 5-1.

LTAc = chronic (4-day average) long term average in units of TUc
= $WLAc \times \exp (0.5 \sigma_4^2 - z_{0.99} \sigma_4)$
= $1 \times \exp [(0.5) (0.0862) - (2.326) (0.294)]$
= 1×0.527
= 0.527 , or

= WLAc × chronic WLA multiplier from TSD Table 5-1 for CV and 99th percentile
= 1×0.527
= 0.527

LTAa,c = acute (1-day average) long term average in units of TUa,c
= $WLAa,c \times \exp (0.5 \sigma^2 - z_{0.99} \sigma)$
= $3 \times \exp [(0.5) (0.307) - (2.326) (0.554)]$
= 3×0.321
= 0.963 , or

= WLAc × acute WLA multiplier from TSD Table 5-1 for CV and 99th percentile
= 3×0.321
= 0.963

where CV = CV(X) *
= 0.6
σ_4^2 = $\ln [(CV^2 / 4) + 1]$
= 0.0862
σ^2 = $\ln (CV^2 + 1)$
= 0.307
$z_{0.99}$ = 2.326 is recommended for WLA in TSD Section 5.5.4

Permit limits are calculated using the lower (more limiting) LTA discharge condition.

LTA = minimum (LTAc or LTA,a,c)
= minimum (0.527 or 0.963)
= 0.527

A maximum daily limit (MDL) and average monthly limit (AML) are calculated using the more limiting discharge condition—defined by the LTA and CV—using the following equations. Knowing CV(X) *, the maximum daily limit and average monthly limit may be determined using the pre-calculated "LTA multipliers" in TSD Table 5-2.

MDL = maximum daily limit
= $LTA \times \exp (z_{0.99} \sigma - 0.5 \sigma^2)$
= $0.527 \times \exp [(2.326) (0.554) - (0.5) (0.307)]$
= 0.527×3.11
= 1.6 TUc , or

= LTA × maximum daily limit LTA multiplier from TSD Table 5-2 for CV and 99th percentile
= 0.527×3.11
= 1.6 TUc

AML = average monthly limit
= $LTA \times \exp (z_{0.95} \sigma_n - 0.5 \sigma_n^2)$
= $LTA \times \exp (z_{0.95} \sigma_4 - 0.5 \sigma_4^2)$
= $0.527 \times \exp [(1.645) (0.294) - (0.5) (0.0862)]$
= 0.527×1.55
= 0.82 TUc , or

= LTA × average monthly limit LTA multiplier from TSD Table 5-2 for CV, 95th percentile, and $n \geq 4$
= 0.527×1.55
= 0.82 TUc

where	CV	=	CV(X) *
		=	0.6
	σ^2	=	ln (CV^2 + 1)
		=	0.307
	$z_{0.99}$	=	2.326 is recommended for MDL in TSD Section 5.5.4
	n	=	number of samples per month ≥ 4
		=	4
	σ_n^2	=	ln [(CV^2 / n) + 1]
	σ_4^2	=	ln [(CV^2 / 4) + 1]
		=	0.0862
	$z_{0.95}$	=	1.645 is recommended for AML in TSD Section 5.5.4

Following Section 2.6.2 in Chapter 2 of this document, EPA Regions 9 and 10 continue recommend that Permitting Authorities establish a monthly median limit (MML) of 1.0 TUc for chronic WET, when no mixing zone is authorized or an NPDES discharge is to a zero flow stream and the statistically-calculated AML is at or less than 1.0 TUc. As a result, in this example, the recommended permit limits for chronic WET are:

MDL	=	maximum daily limit
		1.6 TUc
MML	=	median monthly limit
		1.0 TUc

References:

USEPA. 1991a. Technical support document for water quality-based toxics control. Office of Water. Washington, DC. EPA/505/2-90/001.

APPENDIX D

ACUTE WET PERMIT LANGUAGE

xx. Acute Whole Effluent Toxicity Requirements

For routine monitoring frequency (i.e., monthly, quarterly, semi-annual or annual), and permit years for split sampling of WET and other monitored parameters (i.e., 1, 2, 3, 4 and 5), select <u>proper</u> paragraph 1, as described in Chapter 3 of this document.

1. Monitoring Frequency

   The permittee shall conduct ***monthly/quarterly/semi-annual*** acute toxicity tests on 24-hour composite effluent samples. Once each calendar year, at a different time of year from the previous years, the permittee shall split a 24-hour composite effluent sample and concurrently conduct two toxicity tests using a fish and an invertebrate species; the permittee shall then continue to conduct routine ***monthly/quarterly/semi-annual*** toxicity testing using the single, most sensitive species.

   Acute toxicity test samples shall be collected for each point of discharge at the designated NPDES sampling station for the effluent (i.e., downstream from the last treatment process and any in-plant return flows where a representative effluent sample can be obtained). During years ***1, 2, 3, 4 and 5*** of the permit, a split of each sample shall be analyzed for all other monitored parameters at the minimum frequency of analysis specified by the effluent monitoring program.

1. Monitoring Frequency

   The permittee shall conduct annual acute toxicity tests on 24-hour composite effluent samples. Each calendar year, at a different time of year from the previous years, the permittee shall split a 24-hour composite effluent sample and concurrently conduct two toxicity tests using a fish and an invertebrate species; the permittee shall then continue to conduct routine annual toxicity testing using the single, most sensitive species.

   Acute toxicity test samples shall be collected for each point of discharge at the designated NPDES sampling station for the effluent (i.e., downstream from the last treatment process and any in-plant return flows where a representative effluent sample can be obtained). During years ***1, 2, 3, 4 and 5*** of the permit, a split of each sample shall be analyzed for all other monitored parameters at the minimum frequency of analysis specified by the effluent monitoring program.

To monitor acute whole effluent toxicity with proper species and test methods select <u>proper</u> paragraph 2, as described in Chapter 3 of this document. Please note that freshwater discharges to marine or estuarine receiving water bodies are monitored using either freshwater species and test methods or saltwater species and test methods, based on the magnitude of the discharge specific mixing zone or dilution allowance authorized by the permitting authority. Choose <u>one</u> vertebrate species and <u>one</u> invertebrate species.

2. Freshwater Species and Test Methods

   Species and short-term test methods for estimating the acute toxicity of NPDES effluents are found in the fifth edition of *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA/821/R-02/012, 2002; Table IA, 40 CFR Part 136). The permittee shall conduct 96-hour static renewal toxicity tests with the following vertebrate species:

   - The fathead minnow, *Pimephales promelas* (Acute Toxicity Test Method 2000.0);
   - The rainbow trout, *Oncorhynchus mykiss*, or brook trout, *Salvelinus fontinalis* (Acute Toxicity Test Method 2019.0);

   And the following invertebrate species:

   - The daphnid, *Ceriodaphnia dubia* (Acute Toxicity Test Method 2002.0);
   - The daphnid, *Daphnia pulex*, or daphnid, *Daphnia magna* (Acute Toxicity Test Method 2021.0).

2. Marine and Estuarine Species and Test Methods

   Generally, species and short-term test methods for estimating the acute toxicity of NPDES effluents are found in the fifth edition of *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA/821/R-02/012, 2002; Table IA, 40 CFR Part 136). The permittee shall conduct 96-hour static renewal toxicity tests with the following vertebrate species:

   - The topsmelt, *Atherinops affinis* (Larval Survival and Growth Test Method 1006.0[1] in the first edition of *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA/600/R-95/136, 1995) (specific to Pacific Coast waters);
   - The Inland silverside, *Menidia beryllina*; Atlantic silverside, *Menidia menidia*; or Tidewater silverside, *Menidia peninsulae* (Acute Toxicity Test Method 2006.0);
   - The sheepshead minnow, *Cyprinodon variegates* (Acute Toxicity Test Method 2004.0);

[1] Daily observations for mortality make it possible to calculate acute toxicity for desired exposure periods (i.e., 96-hour LC50, etc.).

And the following invertebrate species:

- The West Coast mysid, *Holmesimysis costata* (Table 19 in the acute test methods manual) (specific to Pacific Coast waters);
- The mysid, *Americamysis bahia* (Acute Toxicity Test Method 2007.0).

Select proper paragraph 3, as described in Chapter 2 of this document. Acute WET permit limits or triggers established by the permitting authority must follow applicable water quality standards and NPDES regulations and are discharge specific. 40 CFR Part 122.44(d)(1). Note that WET permit limits or triggers specified in paragraph 3 are based on EPA's recommendations in Technical Support Document for Water Quality-based Toxics Control (EPA/505/2-90-001, 1991; TSD) and EPA's Regions 9 and 10 Guidance for Implementing Whole Effluent Toxicity Testing Programs (Denton and Narvaez, 1996).

If a mixing zone or dilution allowance for an effluent discharge is either not authorized or authorized such that a critical IWC is set at a % effluent value greater than 100% effluent, then a Pass or Fail test is recommended.

When a mixing zone or dilution allowance for an effluent discharge is authorized such that a critical IWC is set at a % effluent value at or lower than 100% effluent, then EPA's recommended procedures for calculating acute WET permit limits or triggers are found in Box 5-2 and Tables 5-1 and 5-2 of the TSD.

3. Acute WET Permit Trigger

   There is no acute toxicity effluent limit for this discharge. The acute WET permit trigger for this discharge is "Pass" for any one test result. For this permit, the determination of Pass or Fail from a single-effluent-concentration (paired) acute toxicity test is determined using a one-tailed hypothesis test called a t-test. The objective of a Pass or Fail test is to determine if survival in the single treatment (100% effluent) is significantly different from survival in the control (0% effluent). Following Section 11.3 in the acute test methods manual (EPA/821/R-02/012, 2002), the t statistic for the single-effluent-concentration acute toxicity test shall be calculated and compared with the critical t set at the 5% level of significance. If the calculated t does not exceed the critical t, then the mean responses for the single treatment and control are declared "not statistically different" and the permittee shall report "Pass" on the DMR form. If the calculated t does exceed the critical t, then the mean responses for the single treatment and control are declared "statistically different" and the permittee shall report "Fail" on the DMR form. This permit requires additional toxicity testing if the acute WET permit trigger is reported as "Fail".

3. Acute WET Permit Triggers

   There are no acute toxicity effluent limits for this discharge. The acute WET permit triggers are any one test result greater than ***xxx*** TUa (during the monthly reporting period), or any one or more test results with a calculated average value greater than ***yyy***

TUa (during the monthly reporting period). Results shall be reported in TUa, where TUa = 100/LC50. The Lethal Concentration, 50 Percent (LC50) is the toxic or effluent concentration that would cause death in 50 percent of the test organisms over a specified period of time. This permit requires additional toxicity testing if an acute WET permit trigger is exceeded.

3. Acute WET Permit Limit

   There is an acute toxicity effluent limit for this discharge. The acute WET permit limit for this discharge is "Pass" for any one test result. For this permit, the determination of Pass or Fail from a single-effluent-concentration (paired) acute toxicity test is determined using a one-tailed hypothesis test called a t-test. The objective of a Pass or Fail test is to determine if survival in the single treatment (100% effluent) is significantly different from survival in the control (0% effluent). Following Section 11.3 in the acute test methods manual (EPA/821/R-02/012, 2002), the t statistic for the single-effluent-concentration acute toxicity test shall be calculated and compared with the critical t set at the 5% level of significance. If the calculated t does not exceed the critical t, then the mean responses for the single treatment and control are declared "not statistically different" and the permittee shall report "Pass" on the DMR form. If the calculated t does exceed the critical t, then the mean responses for the single treatment and control are declared "statistically different" and the permittee shall report "Fail" on the DMR form. This permit requires additional toxicity testing if the acute WET permit limit is reported as "Fail".

3. Acute WET Permit Limits

   There are acute toxicity effluent limits for this discharge. The acute WET permit limits are any one test result greater than ***xxx*** TUa (during the monthly reporting period), or any one or more test results with a calculated average value greater than ***yyy*** TUa (during the monthly reporting period). Results shall be reported in TUa, where TUa = 100/LC50. The Lethal Concentration, 50 Percent (LC50) is the toxic or effluent concentration that would cause death in 50 percent of the test organisms over a specified period of time. This permit requires additional toxicity testing if an acute WET permit limits is exceeded.

4. Quality Assurance

   a. Quality assurance measures, instructions, and other recommendations and requirements are found in the test methods manual previously referenced. Additional requirements are specified, below.

The acute instream waste concentrations and effluent dilution series specified by the permitting authority are discharge specific and are determined based on applicable water quality standards, NPDES regulations, and requirements and recommendations in the test methods manuals. Note that the instream waste concentrations and dilution series specified in paragraph 4.b are based on EPA's recommendations in Technical Support Document for Water Quality-based Toxics Control (EPA/505/2-90-001, 1991; TSD), EPA's Regions 9 and 10 Guidance for Implementing Whole Effluent Toxicity Testing Programs (Denton and Narvaez, 1996), test method manuals previously referenced, and on Chapters 2 and 3 of this document.

b. This discharge is subject to a determination of Pass or Fail from a single-effluent-concentration (paired) acute toxicity test using a one-tailed hypothesis test called a t-test. The acute instream waste concentration (IWC) for this discharge is 100% effluent. The 100% effluent concentration and a control shall be tested.

b. For this discharge, a mixing zone or dilution allowance is authorized such that critical IWCs are set at % effluent values at or lower than 100% effluent. The acute instream waste concentrations (IWCs) for this discharge are ***XXX***% effluent and ***YYY***% effluent. A series of at least five effluent dilutions and a control shall be tested. At minimum, the dilution series shall include and bracket the IWCs.

Select for dilution water based on test methods required in paragraph 2.

c. Effluent dilution water and control water should be prepared and used as specified in the test methods manual *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA/821/R-02/012, 2002); and/or, for *Atherinops affinis, Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA/600/R-95/136, 1995). If the dilution water is different from test organism culture water, then a second control using culture water shall also be used. If the use of artificial sea salts is considered provisional in the test method, then artificial sea salts shall not be used to increase the salinity of the effluent sample prior to toxicity testing without written approval by the permitting authority.

d. If organisms are not cultured in-house, then concurrent testing with a reference toxicant shall be conducted. If organisms are cultured in-house, then monthly reference toxicant testing is sufficient. Reference toxicant tests and effluent toxicity tests shall be conducted using the same test conditions (e.g., same test duration, etc.).

e. If either the reference toxicant or effluent toxicity tests do not meet all test acceptability criteria in the test methods manual, then the permittee must resample and retest within 14 days.

f. Following Paragraph 12.2.6.2 of the test methods manual, all acute toxicity test results from the multi-concentration tests required by this permit must be reviewed and reported according to EPA guidance on the evaluation of concentration-response

relationships found in *Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing (40 CFR 136)* (EPA/821/B-00/004, 2000).

Select <u>proper</u> paragraph 4.g for review of with-in test variability based on test methods required in paragraph 2.

g. Within-test variability of individual toxicity tests should be reviewed for acceptability and variability criteria (upper and lower PMSD bounds) should be applied, as directed under Section 12.2.8 - *Test Variability* of the test methods manual, *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*. Under Section 12.2.8, the calculated percent minimum significant difference (PMSD) for both reference toxicant test and effluent toxicity test results must be compared with the upper and lower PMSD bounds variability criteria specified in Table 3-6 - *Range of Relative Variability for Endpoints of Promulgated WET Methods, Defined by the 10th and 90th Percentiles from the Data Set of Reference Toxicant Tests*, taken from *Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System Program* (EPA/833/R-00/003, 2000), following the review criteria in Paragraphs 12.2.8.2.1 and 12.2.8.2 of the test methods manual. Based on this review, only accepted effluent toxicity test results shall be reported on the DMR form. If excessive within-test variability invalidates a test result, then the permittee must resample and retest within 14 days.

g. Because this permit provides for a 96-hour LC50 endpoint from Method 1006.0 in *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA/600/R-95/136, 1995), with-in test variability must be reviewed for acceptability and a variability criterion (upper %MSD bound) must be applied, as directed under the test method. Based on this review, only accepted effluent toxicity test results shall be reported on the DMR form. If excessive within-test variability invalidates a test result, then the permittee must resample and retest within 14 days.

h. If the discharged effluent is chlorinated, then chlorine shall not be removed from the effluent sample prior to toxicity testing without written approval by the permitting authority.

i. Where total ammonia concentrations in the effluent are <u>≥</u>5 mg/L, toxicity may be contributed by unionized ammonia. pH drift during the toxicity test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (e.g., metals) are present. This problem is minimized by conducting toxicity tests in a static-renewal or flow-through mode, as outlined in Paragraph 9.5.9 of the test methods manual.

5. Initial Investigation TRE Workplan

   Within 90 days of the permit effective date, the permittee shall prepare and submit a copy of their Initial Investigation Toxicity Reduction Evaluation (TRE) Workplan (1-2 pages) to the permitting authority for review. This plan shall include steps the permittee intends to follow if toxicity is measured above an acute WET permit limit or trigger and should include, at minimum:

   a. A description of the investigation and evaluation techniques that would be used to identify potential causes and sources of toxicity, effluent variability, and treatment system efficiency.

   b. A description of methods for maximizing in-house treatment system efficiency, good housekeeping practices, and a list of all chemicals used in operations at the facility.

   c. If a Toxicity Identification Evaluation (TIE) is necessary, an indication of who would conduct the TIEs (i.e., an in-house expert or outside contractor).

6. Accelerated Toxicity Testing and TRE/TIE Process

   a. If an acute WET permit limit or trigger is exceeded and the source of toxicity is known (e.g., a temporary plant upset), then the permittee shall conduct one additional toxicity test using the same species and test method. This test shall begin within 14 days of receipt of test results exceeding an acute WET permit limit or trigger. If the additional toxicity test does not exceed an acute WET permit limit or trigger, then the permittee may return to their regular testing frequency.

   b. If an acute WET permit limit or trigger is exceeded and the source of toxicity is not known, then the permittee shall conduct six additional toxicity tests using the same species and test method, approximately every two weeks, over a 12 week period. This testing shall begin within 14 days of receipt of test results exceeding an acute WET permit limit or trigger. If none of the additional toxicity tests exceed an acute WET permit limit or trigger, then the permittee may return to their regular testing frequency.

   c. If one of the additional toxicity tests (in paragraphs 6.a or 6.b) exceeds an acute WET permit limit or trigger, then, within 14 days of receipt of this test result, the permittee shall initiate a TRE using, based on the type of treatment facility, EPA manual *Toxicity Reduction Evaluation Guidance for Municipal Wastewater Treatment Plants* (EPA/833/B-99/002, 1999) or EPA manual *Generalized Methodology for Conducting Industrial Toxicity Reduction Evaluations* (EPA/600/2-88/070, 1989). In conjunction, the permittee shall develop and implement a Detailed TRE Workplan which shall include: further actions undertaken by the permittee to investigate, identify, and correct the causes of toxicity; actions the permittee will take to mitigate the impact of the discharge and prevent the recurrence of toxicity; and a schedule for these actions.

d. The permittee may initiate a Toxicity Identification Evaluation (TIE) as part of a TRE to identify the causes of toxicity using the same species and test method and, as guidance, EPA test method manuals: *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures* (EPA/600/6-91/003, 1991); *Methods for Aquatic Toxicity Identification Evaluations, Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA/600/R-92/080, 1993); *Methods for Aquatic Toxicity Identification Evaluations, Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA/600/R-92/081, 1993); and *Marine Toxicity Identification Evaluation (TIE): Phase I Guidance Document* (EPA/600/R-96-054, 1996).

7. Reporting of Acute Toxicity Monitoring Results

a. A full laboratory report for all toxicity testing shall be submitted as an attachment to the DMR for the month in which the toxicity test was conducted and shall also include: the toxicity test results—for determination of Pass/Fail; LC50; TUa = 100/LC50; NOAEC; TUa = 100/NOAEC—reported according to the test methods manual chapter on report preparation and test review; the dates of sample collection and initiation of each toxicity test; all results for effluent parameters monitored concurrently with the toxicity test(s); and progress reports on TRE/TIE investigations.

b. The permittee shall notify the permitting authority in writing within 14 days of exceedance of an acute WET permit limit or trigger. This notification shall describe actions the permittee has taken or will take to investigate, identify, and correct the causes of toxicity; the status of actions required by this permit; and schedule for actions not yet completed; or reason(s) that no action has been taken.

8. Permit Reopener for Acute Toxicity

In accordance with 40 CFR Parts 122 and 124, this permit may be modified to include effluent limitations or permit conditions to address acute toxicity in the effluent or receiving waterbody, as a result of the discharge; or to implement new, revised, or newly interpreted water quality standards applicable to acute toxicity.

APPENDIX D

CHRONIC WET PERMIT LANGUAGE

xx. Chronic Whole Effluent Toxicity Requirements

For routine monitoring frequency (i.e., monthly, quarterly, semi-annual or annual), yearly determination of test species sensitivity (i.e., fish, invertebrate, or alga), and permit years for split sampling of WET and other monitored parameters (i.e., 1, 2, 3, 4 and 5), select <u>proper</u> paragraph 1, as described in Chapter 3 of this document.

1. Monitoring Frequency

   The permittee shall conduct ***monthly/quarterly/semi-annual*** chronic toxicity tests on 24-hour composite effluent samples. Once each calendar year, at a different time of year from the previous years, the permittee shall split a 24-hour composite effluent sample and concurrently conduct three toxicity tests using a fish, an invertebrate, and an alga species; the permittee shall then continue to conduct routine ***monthly/quarterly/semi-annual*** toxicity testing using the single, most sensitive species.

   Chronic toxicity test samples shall be collected for each point of discharge at the designated NPDES sampling station for the effluent (i.e., downstream from the last treatment process and any in-plant return flows where a representative effluent sample can be obtained). During years ***1, 2, 3, 4 and 5*** of the permit, a split of each sample shall be analyzed for all other monitored parameters at the minimum frequency of analysis specified by the effluent monitoring program.

1. Monitoring Frequency

   The permittee shall conduct annual chronic toxicity tests on 24-hour composite effluent samples. Each calendar year, at a different time of year from the previous years, the permittee shall split a 24-hour composite effluent sample and concurrently conduct three toxicity tests using a fish, an invertebrate, and an alga species.

   Chronic toxicity test samples shall be collected for each point of discharge at the designated NPDES sampling station for the effluent (i.e., downstream from the last treatment process and any in-plant return flows where a representative effluent sample can be obtained). During years ***1, 2, 3, 4 and 5*** of the permit, a split of each sample shall be analyzed for all other monitored parameters at the minimum frequency of analysis specified by the effluent monitoring program.

To monitor chronic whole effluent toxicity with proper species and test methods select <u>proper</u> paragraph 2, as described in Chapter 3 of this document. Please note that freshwater discharges to marine or estuarine receiving water bodies are monitored using either freshwater species and test methods or saltwater species and test methods, based on the magnitude of the discharge specific mixing zone or dilution allowance authorized by the permitting authority.

3. Freshwater Species and Test Methods

   Species and short-term test methods for estimating the chronic toxicity of NPDES effluents are found in the fourth edition of *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* (EPA/821/R-02/013, 2002; Table IA, 40 CFR Part 136). The permittee shall conduct static renewal toxicity tests with the fathead minnow, *Pimephales promelas* (Larval Survival and Growth Test Method 1000.0[1]); the daphnid, *Ceriodaphnia dubia* (Survival and Reproduction Test Method 1002.0[1]); and the green alga, *Selenastrum capricornutum* (also named *Raphidocelis subcapitata*) (Growth Test Method 1003.0).

3. Marine and Estuarine Species and Test Methods

   Species and short-term test methods for estimating the chronic toxicity of NPDES effluents are found in the first edition of *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA/600/R-95/136, 1995) and applicable water quality standards; also see 40 CFR Parts 122.41(j)(4) and 122.44(d)(1)(iv) and 40 CFR Part 122.21(j)(5)(viii) for POTWs. The permittee shall conduct a static renewal toxicity test with the topsmelt, *Atherinops affinis* (Larval Survival and Growth Test Method 1006.0[1]); a static non-renewal toxicity test with the giant kelp, *Macrocystis pyrifera* (Germination and Growth Test Method 1009.0); and a toxicity test with one of the following invertebrate species:

   - Static renewal toxicity test with the mysid, *Holmesimysis costata* (Survival and Growth Test Method 1007.0[1]);
   - Static non-renewal toxicity test with the Pacific oyster, *Crassostrea gigas*, or the mussel, *Mytilus* spp., (Embryo-larval Shell Development Test Method 1005.0);
   - Static non-renewal toxicity test with the red abalone, *Haliotis rufescens* (Larval Shell Development Test Method);
   - Static non-renewal toxicity test with the purple sea urchin, *Strongylocentrotus purpuratus*, or the sand dollar, *Dentraster excentricus* (Embryo-larval Development Test Method); or
   - Static non-renewal toxicity test with the purple sea urchin, *Strongylocentrotus purpuratus*, or the sand dollar, *Dendraster excentricus* (Fertilization Test Method 1008.0).

[1] Daily observations for mortality make it possible to calculate acute toxicity for desired exposure periods (i.e., 7-day LC50, 96-hour LC50, etc.).

If laboratory-held cultures of the topsmelt, *Atherinops affinis*, are not available for testing, then the permittee shall conduct a static renewal toxicity test with the inland silverside, *Menidia beryllina* (Larval Survival and Growth Test Method 1006.0[1]), found in the third edition of *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms* (EPA/821/R-02/014, 2002; Table IA, 40 CFR Part 136).

Select proper paragraph 3, as described in Chapter 2 of this document. Chronic WET permit limits or triggers established by the permitting authority must follow applicable water quality standards and NPDES regulations and are discharge specific. 40 CFR Part 122.44(d)(1). Note that the median monthly chronic WET permit limit or trigger specified in paragraph 3 is based on EPA's recommendations in Technical Support Document for Water Quality-based Toxics Control (EPA/505/2-90-001, 1991; TSD) and EPA's Regions 9 and 10 Guidance for Implementing Whole Effluent Toxicity Testing Programs (Denton and Narvaez, 1996) when a mixing zone or dilution allowance for an effluent discharge is not authorized by the permitting authority. EPA's recommended procedures for calculating chronic WET permit limits or triggers when a mixing zone or dilution allowance for an effluent discharge is authorized by the permitting authority are found in Box 5-2 and Tables 5-1 and 5-2 of the TSD.

3. Chronic WET Permit Triggers

   There are no chronic toxicity effluent limits for this discharge. For this discharge, a mixing zone or dilution allowance is not authorized and the chronic WET permit triggers are any one test result greater than 1.6 TUc (during the monthly reporting period), or any one or more test results with a calculated median value greater than 1.0 TUc (during the monthly reporting period). Results shall be reported in TUc, where TUc = 100/NOEC. The No Observed Effect Concentration (NOEC) is the highest concentration of toxicant to which organisms are exposed in a short-term chronic test that causes no observable adverse effects on the test organisms (e.g., the highest concentration of toxicant in which the values for the observed responses are not statistically significantly different from the controls). This permit requires additional toxicity testing if a chronic WET permit trigger is exceeded.

3. Chronic WET Permit Triggers

   There are no chronic toxicity effluent limits for this discharge. For this discharge, a mixing zone or dilution allowance is authorized and the chronic WET permit triggers are any one test result greater than ***xxx*** TUc (during the monthly reporting period), or any one or more test results with a calculated average value greater than ***yyy*** TUc (during the monthly reporting period). Results shall be reported in TUc, where TUc = 100/NOEC. The No Observed Effect Concentration (NOEC) is the highest concentration of toxicant to which organisms are exposed in a short-term chronic test that causes no observable adverse effects on the test organisms (e.g., the highest concentration of toxicant in which the values for the observed responses are not statistically significantly different from the

controls). This permit requires additional toxicity testing if a chronic WET permit trigger is exceeded.

3. Chronic WET Permit Limits

There are chronic toxicity effluent limits for this discharge. For this discharge, a mixing zone or dilution allowance is not authorized and the chronic WET permit limits are any one test result greater than 1.6 TUc (during the monthly reporting period), or any one or more test results with a calculated median value greater than 1.0 TUc (during the monthly reporting period). Results shall be reported in TUc, where TUc = 100/NOEC. The No Observed Effect Concentration (NOEC) is the highest concentration of toxicant to which organisms are exposed in a short-term chronic test that causes no observable adverse effects on the test organisms (e.g., the highest concentration of toxicant in which the values for the observed responses are not statistically significantly different from the controls). This permit requires additional toxicity testing if a chronic WET permit limit is exceeded.

3. Chronic WET Permit Limits

There are chronic toxicity effluent limits for this discharge. For this discharge, a mixing zone or dilution allowance is authorized and the chronic WET permit limits are any one test result greater than ***xxx*** TUc (during the monthly reporting period), or any one or more test results with a calculated average value greater than ***yyy*** TUc (during the monthly reporting period). Results shall be reported in TUc, where TUc = 100/NOEC. The No Observed Effect Concentration (NOEC) is the highest concentration of toxicant to which organisms are exposed in a short-term chronic test that causes no observable adverse effects on the test organisms (e.g., the highest concentration of toxicant in which the values for the observed responses are not statistically significantly different from the controls). This permit requires additional toxicity testing if a chronic WET permit limit is exceeded.

4. Quality Assurance

   a. Quality assurance measures, instructions, and other recommendations and requirements are found in the test methods manual previously referenced. Additional requirements are specified, below.

The chronic instream waste concentrations and effluent dilution series specified by the permitting authority are discharge specific and are determined based on applicable water quality standards, NPDES regulations, and requirements and recommendations in the test method manuals. Note that the instream waste concentrations and dilution series specified in paragraph 4.b are based on EPA's recommendations in Technical Support Document for Water Quality-based Toxics Control (EPA/505/2-90-001, 1991; TSD) and EPA's Regions 9 and 10 Guidance for Implementing Whole Effluent Toxicity Testing Programs (Denton and Narvaez, 1996) when a mixing zone or dilution allowance for an effluent discharge is not authorized by the permitting authority. EPA's recommended procedures for specifying instream waste concentrations and a dilution series when a mixing zone or dilution allowance for an effluent discharge is authorized by the permitting authority are found in the test method manuals previously referenced and in Chapters 2 and 3 of this document.

b. For this discharge, a mixing zone or dilution allowance is not authorized. The chronic instream waste concentrations (IWCs) for this discharge are 100% effluent and 62.5% effluent. A series of at least five effluent dilutions and a control shall be tested. At minimum, the dilution series shall include the IWCs and three dilutions below the IWCs (e.g., 100%, 62.5%, 50%, 25% and 12.5%).

b. For this discharge, a mixing zone or dilution allowance is authorized. The chronic instream waste concentrations (IWCs) for this discharge are ***XXX*** % effluent and ***YYY*** % effluent. A series of at least five effluent dilutions and a control shall be tested. At minimum, the dilution series shall include and bracket the IWCs.

Select proper paragraph 4.c for dilution water based on test methods required in paragraph 2.

c. Effluent dilution water and control water should be standard synthetic dilution water, as described in the test methods manual *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* (EPA/821/R-02/013, 2002). If the dilution water is different from test organism culture water, then a second control using culture water shall also be used.

c. Effluent dilution water and control water should be prepared and used as specified in the test methods manual *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA/600/R-95/136, 1995) and/or *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms* (EPA/821/R-02/014, 2002). If the dilution water is different from test organism culture water, then a second control using culture water shall also be used. If the use of artificial sea salts is considered provisional in the test method, then artificial sea salts shall not be used to increase the salinity of the effluent sample prior to toxicity testing without written approval by the permitting authority.

d. If organisms are not cultured in-house, then concurrent testing with a reference toxicant shall be conducted. If organisms are cultured in-house, then monthly

reference toxicant testing is sufficient. Reference toxicant tests and effluent toxicity tests shall be conducted using the same test conditions (e.g., same test duration, etc.).

e. If either the reference toxicant or effluent toxicity tests do not meet all test acceptability criteria in the test methods manual, then the permittee must resample and retest within 14 days.

f. Following Paragraph 10.2.6.2 of the freshwater test methods manual, all chronic toxicity test results from the multi-concentration tests required by this permit must be reviewed and reported according to EPA guidance on the evaluation of concentration-response relationships found in *Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing (40 CFR 136)* (EPA/821/B-00-004, 2000).

Select <u>proper</u> paragraph 4.g for review of with-in test variability based on test methods required in paragraph 2.

g. Because this permit requires sublethal hypothesis testing endpoints from Methods 1000.0, 1002.0, and 1003.0 in *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* (EPA/821/R-02/013, 2002), with-in test variability must be reviewed for acceptability and variability criteria (upper and lower PMSD bounds) must be applied, as directed under Section 10.2.8 - *Test Variability* of the test methods manual *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*. Under Section 10.2.8, the calculated percent minimum significant difference (PMSD) for both reference toxicant test and effluent toxicity test results must be compared with the upper and lower PMSD bounds variability criteria specified in Table 6 - *Variability Criteria (Upper and Lower PMSD Bounds) for Sublethal Hypothesis Testing Endpoints Submitted Under NPDES Permits*, following the review criteria in Paragraphs 10.2.8.2.1 through 10.2.8.2.5 of the test methods manual. Based on this review, only accepted effluent toxicity test results shall be reported on the DMR form. If excessive within-test variability invalidates a test result, then the permittee must resample and retest within 14 days.

g. Because this permit requires sublethal hypothesis testing endpoints from test methods in *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA/600/R-95/136, 1995), within-test variability must be reviewed for acceptability and a variability criterion (upper %MSD bound) must be applied, as directed under each test method. Based on this review, only accepted effluent toxicity test results shall be reported on the DMR form. If excessive within-test variability invalidates a test result, then the permittee must resample and retest within 14 days.

g. Because this permit provides for a sublethal hypothesis testing endpoint from Method 1006.0 in *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms* (EPA/821/R-02/014, 2002), within-test variability must be reviewed for acceptability and variability criteria

(upper and lower PMSD bounds) must be applied, as directed under Section 10.2.8 - *Test Variability* of the test methods manual *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms*. Under Section 10.2.8, the calculated percent minimum significant difference (PMSD) for both reference toxicant test and effluent toxicity test results must be compared with the upper and lower PMSD bounds variability criteria specified in Table 6 - *Variability Criteria (Upper and Lower PMSD Bounds) for Sublethal Hypothesis Testing Endpoints Submitted Under NPDES Permits*, following the review criteria in Paragraphs 10.2.8.2.1 through 10.2.8.2.5 of the test methods manual. Based on this review, only accepted effluent toxicity test results shall be reported on the DMR form. If excessive within-test variability invalidates a test result, then the permittee must resample and retest within 14 days.

h. If the discharged effluent is chlorinated, then chlorine shall not be removed from the effluent sample prior to toxicity testing without written approval by the permitting authority.

i. pH drift during the toxicity test may contribute to artifactual toxicity when pH-dependent toxicants (e.g., ammonia, metals) are present in an effluent. To determine whether or not pH drift during the toxicity test is contributing to artifactual toxicity, the permittee shall conduct three sets of parallel toxicity tests, in which the pH of one treatment is controlled at the pH of the effluent and the pH of the other treatment is not controlled, as described in Section 11.3.6.1 of the test methods manual, *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* (EPA/821/R-02/013, 2002). Toxicity is confirmed to be artifactual and due to pH drift when no toxicity above the chronic WET permit limit or trigger is observed in the treatments controlled at the pH of the effluent. If toxicity is confirmed to be artifactual and due to pH drift, then, following written approval by the permitting authority, the permittee may use the procedures outlined in Section 11.3.6.2 of the test methods manual to control sample pH during the toxicity test.

5. Initial Investigation TRE Workplan

Within 90 days of the permit effective date, the permittee shall prepare and submit a copy of their Initial Investigation Toxicity Reduction Evaluation (TRE) Workplan (1-2 pages) to the permitting authority for review. This plan shall include steps the permittee intends to follow if toxicity is measured above a chronic WET permit limit or trigger and should include, at minimum:

a. A description of the investigation and evaluation techniques that would be used to identify potential causes and sources of toxicity, effluent variability, and treatment system efficiency.

b. A description of methods for maximizing in-house treatment system efficiency, good housekeeping practices, and a list of all chemicals used in operations at the facility.

c. If a Toxicity Identification Evaluation (TIE) is necessary, an indication of who would conduct the TIEs (i.e., an in-house expert or outside contractor).

6. Accelerated Toxicity Testing and TRE/TIE Process
   a. If a chronic WET permit limit or trigger is exceeded and the source of toxicity is known (e.g., a temporary plant upset), then the permittee shall conduct one additional toxicity test using the same species and test method. This test shall begin within 14 days of receipt of test results exceeding a chronic WET permit limit or trigger. If the additional toxicity test does not exceed a chronic WET permit limit or trigger trigger, then the permittee may return to their regular testing frequency.

   b. If a chronic WET permit limit or trigger is exceeded and the source of toxicity is not known, then the permittee shall conduct six additional toxicity tests using the same species and test method, approximately every two weeks, over a 12 week period. This testing shall begin within 14 days of receipt of test results exceeding a chronic WET permit limit or trigger. If none of the additional toxicity tests exceed a chronic WET permit limit or trigger, then the permittee may return to their regular testing frequency.

   c. If one of the additional toxicity tests (in paragraphs 6.a or 6.b) exceeds a chronic WET permit limit or trigger, then, within 14 days of receipt of this test result, the permittee shall initiate a TRE using as guidance, based on the type of treatment facility, EPA manual *Toxicity Reduction Evaluation Guidance for Municipal Wastewater Treatment Plants* (EPA/ 833/B-99/002, 1999) or EPA manual *Generalized Methodology for Conducting Industrial Toxicity Reduction Evaluations* (EPA/600/2-88/070, 1989). In conjunction, the permittee shall develop and implement a Detailed TRE Workplan which shall include: further actions undertaken by the permittee to investigate, identify, and correct the causes of toxicity; actions the permittee will take to mitigate the impact of the discharge and prevent the recurrence of toxicity; and a schedule for these actions.

   d. The permittee may initiate a Toxicity Identification Evaluation (TIE) as part of a TRE to identify the causes of toxicity using the same species and test method and, as guidance, EPA test method manuals: *Toxicity Identification Evaluation: Characterization of Chronically Toxic Effluents, Phase I* (EPA/600/6-91/005F, 1992); *Methods for Aquatic Toxicity Identification Evaluations, Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA/600/R-92/080, 1993); *Methods for Aquatic Toxicity Identification Evaluations, Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA/600/R-92/081, 1993); and *Marine Toxicity Identification Evaluation (TIE): Phase I Guidance Document* (EPA/600/R-96-054, 1996).

7. Reporting of Chronic Toxicity Monitoring Results

   a. A full laboratory report for all toxicity testing shall be submitted as an attachment to the DMR for the month in which the toxicity test was conducted and shall also include: the toxicity test results—in NOEC; TUc = 100/NOEC; EC25 (or IC25); and TUc = 100/EC25 (or IC25)—reported according to the test methods manual chapter on report preparation and test review; the dates of sample collection and initiation of each toxicity test; all results for effluent parameters monitored concurrently with the toxicity test(s); and progress reports on TRE/TIE investigations.

   b. The permittee shall notify the permitting authority in writing within 14 days of exceedance of a chronic WET permit limit or trigger. This notification shall describe actions the permittee has taken or will take to investigate, identify, and correct the causes of toxicity; the status of actions required by this permit; and schedule for actions not yet completed; or reason(s) that no action has been taken.

8. Permit Reopener for Chronic Toxicity

   In accordance with 40 CFR Parts 122 and 124, this permit may be modified to include effluent limitations or permit conditions to address chronic toxicity in the effluent or receiving waterbody, as a result of the discharge; or to implement new, revised, or newly interpreted water quality standards applicable to chronic toxicity.

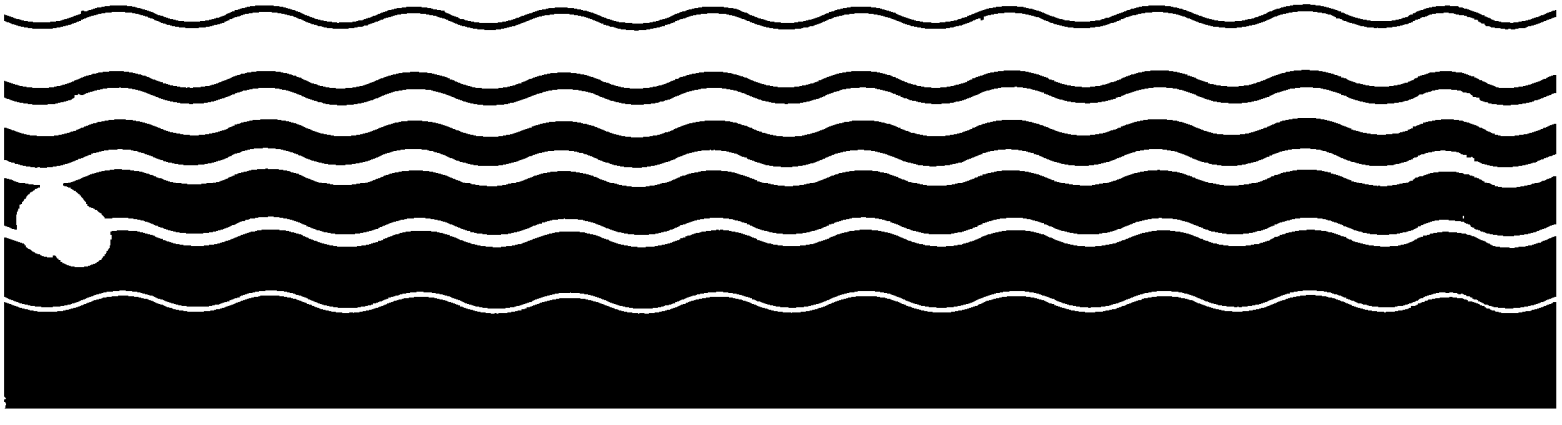
United States
Environmental Protection
Agency

Office Of Water
(EN-336)

EPA 833-B-92-001
November 1992

EPA

Guidance Manual For The Preparation Of Part 2 Of The NPDES Permit Applications For Discharges From Municipal Separate Storm Sewer Systems



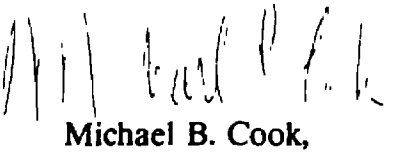
FOREWORD

This manual provides detailed guidance on the development of Part 2 permit applications for municipal separate storm sewer systems. It provides technical assistance and support for all municipal separate storm sewer systems subject to regulatory requirements under the National Pollutant Discharge Elimination System (NPDES) program for storm water point source discharges. This manual also emphasizes the application of pollution prevention measures and implementation of Best Management Practices (BMPs) to reduce pollutant loadings and improve water quality.

The control of pollution from urban and industrial storm water discharges is critical in maintaining and improving the quality of the Nation's waters. Pollutants in storm water discharges from many sources are largely uncontrolled. The *National Water Quality Inventory, 1990 Report to Congress*, provides a general assessment of water quality based on biennial reports submitted by the States under Section 305(b) of the Clean Water Act (CWA). The report indicates that roughly one third of the impairment in assessed waters is due to storm water runoff.

This document was issued in support of Environmental Protection Agency (EPA) regulations and policy initiatives involving the development and implementation of a national storm water program. This document is Agency guidance only. It does not establish or affect legal rights or obligations. Agency decisions in any particular case will be made applying the laws and regulations on the basis of specific facts when permits are issued or regulations promulgated.

This document will be revised and expanded periodically to reflect additional guidance. Comments from users are welcomed. Send comments to U.S. EPA, Office of Wastewater Enforcement and Compliance, 401 M Street, SW, Mail Code EN-336, Washington, D.C. 20460.



Michael B. Cook,
Director
Office of Wastewater Enforcement
and Compliance

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CHAPTER 1
INTRODUCTION

1.0 INTRODUCTION

1.1 OVERVIEW

Control of pollution from urban and industrial storm water discharges is an important factor in maintaining and improving the quality of the Nation's waters. To help improve the quality of storm water discharges, Congress passed the Water Quality Act (WQA) in 1987. The WQA added to the Clean Water Act (CWA) a provision [Section 402(p)] that directed the U.S. Environmental Protection Agency (EPA) to establish final regulations governing storm water discharges under the National Pollutant Discharge Elimination System (NPDES) program.

In response, EPA published regulations in the November 16, 1990, Federal Register (55 FR 47990) that established NPDES permit application requirements for storm water point source discharges As part of these regulations, municipal separate storm sewer systems (MS4s) that serve populations greater than 250,000 ("large MS4s"), MS4s that serve populations between 100,000 and 250,000 ("medium MS4s"), and other MS4s identified by the permitting authority must be covered by NPDES permits. The regulations establish a two-part application process for these MS4s In April 1991, EPA issued guidance on the preparation of Part 1 of the NPDES permit application for discharges from MS4s (EPA, 1991b). The present manual provides guidance on the preparation of Part 2 applications. The information in this manual should help municipalities focus their efforts on activities that meet the application requirements.

1.2 SUMMARY OF THE CLEAN WATER ACT REQUIREMENTS

Section 402 of the CWA prohibits the discharge of any pollutant to waters of the United States from a point source, unless that discharge is authorized by a NPDES permit. Efforts to improve water quality under the NPDES program have traditionally focused on reducing pollutants in discharges of industrial process wastewater and municipal sewage. As pollution control measures have been implemented for these discharges, it has become evident that diffuse sources of water pollution (those occurring over a wide area) are also major contributors to water quality degradation. Recent studies, including the Nationwide Urban Runoff Program (NURP) study (EPA, 1983), have shown that storm water runoff from urban and industrial areas typically contains the same general types of pollutants that are often found in wastewater in industrial discharges. Pollutants commonly found in storm water runoff include heavy metals, pesticides, herbicides, and synthetic organic compounds such as fuels, waste oils, solvents, lubricants, and grease. These compounds can have damaging effect on both human health and aquatic ecosystems. In addition to pollutants, the high volumes of storm water discharged from MS4s in areas of rapid urbanization have had significant impacts on aquatic ecosystems due to physical modifications such as bank erosion and widening of channels.

The statutory provisions governing discharges from MS4s are contained in CWA Section 402(p)(3)(B). In general, Congress provided that permits for discharges from MS4s:

- May be issued on either a system- or jurisdiction-wide basis;
- Shall effectively prohibit non-storm water discharges into the MS4, and
- Shall require controls to reduce the discharge of pollutants to the maximum extent practicable (MEP).

Under the storm water program, the initial round of NPDES permits will emphasize the use of Best Management Practices (BMPs) to reduce pollutant loadings from MS4s. These BMPs include pollution prevention measures, management practices, control techniques, and design and engineering practices. As with any discharger subject to the NPDES program, MS4s must meet technology-based requirements [in this case, the "maximum extent practicable" standard of Section 402(p)] as well as applicable water quality standards.

1.3 THE PERMIT APPLICATION PROCESS

The goal of the NPDES program for municipal storm water is the reduction and elimination of pollutants in storm water discharges from large and medium MS4s. The permit application process in 40 CFR 122.26(d) is designed to meet this goal by developing site-specific NPDES permits containing storm water management programs for individual MS4s. Site-specific permitting is crucial given the differing nature of discharges from MS4s in different parts of the country and the varying impacts of these discharges on receiving waters. To facilitate this process, the regulations specify a two-part permit application.

Part 1 of the permit application initiates the process through which municipalities began to identify sources of pollutants to the municipal storm sewer system. Part 1 also requires municipalities to propose strategies to characterize storm water discharges from their municipal separate storm sewer systems. *Guidance for the Preparation of Part 1 of The NPDES Permit Applications for Discharges From Municipal Separate Storm Sewer Systems* was issued in April 1991, and is available through EPA's Storm Water Hotline [(703) 821-4823].

The present manual describes how to meet the Part 2 permit application requirements for storm water discharges from large and medium MS4s Part 2 of the permit application builds upon the foundation established in Part 1 and provides for the development of comprehensive storm water management programs. Part 2 requires particular information that MS4s must have developed to have an effective storm water control plan. However, each applicant is given flexibility on how to present and organize this information in a way which best suits the MS4's needs and is most consistent with its overall storm water management strategy. This guidance presents examples which illustrate some alternative ways to present information that will fulfill the Part 2 permit application requirements.

1.4 WHO MUST SUBMIT A PART 2 APPLICATION

Municipalities, incorporated places, and counties with unincorporated urban areas that own or operate a large or medium MS4 that discharges to waters of the United States are required to obtain a NPDES storm water permit. In addition, small MS4s (less than 100,000) that are owned or operated by a municipality other than those identified in the NPDES regulation can be designated by the permitting authority as part of the large or medium municipal separate storm sewer system due to the interrelationship between the discharges of the designated storm sewer and the discharges from municipal separate storm sewers.

Under EPA's definition of MS4, "large" MS4s serve populations greater than 250,000, and "medium" MS4s serve populations of at least 100,000, but less than 250,000. Population is determined by the most recent Decennial Census by the Bureau of the Census. A list of large and medium municipalities identified in the November 16, 1990, rule is contained in Exhibit 1-1, in which population was based on the 1980 Census. **After the publication of the November 16, 1990, rule, the Bureau of the Census released data for 1990, and, as a result, some additional municipalities may be required to submit applications, while others may fall below 100,000. These changes are not reflected in Exhibit 1-1.**

Exhibit 1-1: Large and Medium MS4s
(Based on 1980 Census Data)

Municipalities, Counties, and Incorporated Areas With Populations greater than 250,000 which Must Submit NPDES Storm Water Applications

State	Entity
Alabama	Birmingham
Arizona	Phoenix
	Tucson
California	Long Beach
	Los Angeles
	Los Angeles County
	Oakland
	Sacramento
	Sacramento County
	San Diego
	San Diego County
	San Francisco
	San Jose
Colorado	Denver
Delaware	New Castle County
District of Columbia	
Florida	Dade County
	Jacksonville
	Miami
	Tampa
Georgia	Atlanta
	DeKalb County
Hawaii	Honolulu County
Illinois	Chicago
Indiana	Indianapolis
Kansas	Wichita
Kentucky	Louisville
Louisiana	New Orleans
Maryland	Anne Arundel County
	Baltimore County
	Baltimore
	Montgomery County
	Prince George's County
Massachusetts	Boston
Michigan	Detroit
Minnesota	Minneapolis
	St Paul
Missouri	Kansas City
	St. Louis
Nebraska	Omaha
New Jersey	Newark
New Mexico	Albuquerque
New York	Buffalo
	Bronx Borough
	Brooklyn Borough
	Manhattan Borough
	Queens Borough
	Staten Island Borough
North Carolina	Charlotte
Ohio	Cincinnati
	Cleveland
	Columbus
	Toledo
Oklahoma	Oklahoma City
	Tulsa
Oregon	Portland
Pennsylvania	Philadelphia
	Pittsburgh
Tennessee	Memphis
	Nashville/Davidson
Texas	Austin
	Dallas
	El Paso
	Fort Worth
	Harris County
	Houston
	San Antonio
Utah	Salt Lake County
Virginia	Fairfax County
	Norfolk
	Virginia Beach
Washington	King County
	Seattle
Wisconsin	Milwaukee

Municipalities, Counties, and Incorporated Areas with Populations between 100,000 and 250,000 which Must Submit NPDES Storm Water Applications.

State	Entity
Alabama	Huntsville
	Jefferson County
	Mobile
	Montgomery
Alaska	Anchorage
Arizona	Mesa
	Pima County
	Tempe
Arkansas	Little Rock
California	Alameda County
	Anaheim
	Bakersfield
	Berkeley
	Concord
	Contra Costa County
	Fremont
	Fresno
	Fullerton
	Garden Grove
	Glendale
	Huntington Beach
	Kern County
	Modesto
California, cont	Orange County
	Oxnard
	Pasadena
	Riverside
	Riverside County
	San Bernardino
	San Bernardino County
	Santa Ana
	Stockton
	Sunnyvale
	Torrance
Colorado	Aurora
	Colorado Springs
	Lakewood
	Pueblo
Connecticut	Bridgeport
	Hartford
	New Haven
	Stamford
	Waterbury
Florida	Broward County
	Escambia County
	Fort Lauderdale
	Hialeah
	Hillsborough County
	Hollywood
	Orange County
	Orlando
	Palm Beach County
	Pinellas County
	Polk County
	Sarasota County
	St Petersburg
Georgia	Clayton County
	Cobb County
	Columbus
	Macon
	Richmond County
	Savannah
Idaho	Boise City
Illinois	Peoria
	Rockford
Indiana	Evansville
	Fort Wayne
	Gary
	South Bend
Iowa	Cedar Rapids
	Davenport
	Des Moines
Kansas	Kansas City
	Topeka
Kentucky	Jefferson County
	Lexington-Fayette
Louisiana	Baton Rouge
	Jefferson Parish
	Shreveport

(continued)

Exhibit 1-1: Large and Medium MS4s (cont.)
(Based on 1980 Census Data)

Massachusetts	Springfield	North Carolina	Durham	Texas, cont'd	Corpus Christi
	Worcester		Greensboro		Garland
Michigan	Ann Arbor		Raleigh		Irving
	Flint		Winston-Salem		Lubbock
	Grand Rapids		Cumberland County		Pasadena
	Lansing	Ohio	Akron		Waco
	Livonia		Dayton	Utah	Salt Lake City
	Sterling Heights		Youngstown	Virginia	Alexandria
	Warren	Oregon	Eugene		Arlington County
Mississippi	Jackson		Multnomah County		Chesapeake
Missouri	Independence		Washington County		Chesterfield County
	Springfield	Pennsylvania	Allentown		Hampton
Nebraska	Lincoln		Erie		Henrico County
Nevada	Clark County	Rhode Island	Providence		Newport News
	Las Vegas	South Carolina	Columbia		Portsmouth
	Reno		Greenville County		Richmond
New Jersey	Elizabeth		Richland County		Roanoke
	Jersey City	Tennessee	Chattanooga	Washington	Snohomish County
	Paterson		Knoxville		Spokane
New York	Albany	Texas	Amarillo		Pierce County
	Rochester		Arlington		Tacoma
	Syracuse		Beaumont	Wisconsin	Madison
	Yonkers				

Source. 55 FR 48073, November 16, 1990.

The definition of MS4 excludes those conveyances that are designed to discharge storm water runoff combined with municipal sanitary sewers ("combined sewer systems"). Therefore, municipalities that own or operate combined sewer systems may petition to have their population, based on Bureau of the Census figures, reduced by the number of people served by the combined sewer system. If the total population served by the separate storm sewer system alone is less than 100,000, the municipality may be eligible for an exemption from NPDES storm water permit requirements. Municipalities should contact their permitting authority for additional information. **Exhibit 1-1 does not reflect any modifications in the application requirements for cities with combined sewer systems.**

1.5 SUBMITTING THE PART 2 APPLICATION

Completed Part 2 applications should be submitted to the appropriate permitting authority listed in Exhibit 1-2. For municipalities in States with authorized NPDES programs, the permitting authority is the State office listed in Exhibit 1-2. Because some of these States may have application requirements in addition to EPA's, municipalities in States with authorized NPDES programs should contact their States for guidance. For municipalities in States without approved NPDES programs, the permitting authority is the EPA Regional Office listed in Exhibit 1-2.

Municipalities with populations greater than 250,000 (large MS4s) were to submit their Part 2 applications by November 16, 1992. Municipalities with populations greater than 100,000, but less than 250,000 (medium MS4s), must submit Part 2 applications by May 17, 1993. Inquiries regarding Part 2 applications or the permitting process should be directed to the appropriate permitting authority.

Exhibit 1-2: NPDES Storm Water Program Permitting Authorities

State	Permit Auth	Contact
Alabama	State	Aubrey White Water Division 1751 Dickinson Dr Montgomery, AL 36130 (205) 271-7811
Alaska	EPA	Steve Bubnick U S EPA Region 10 WD-134 1200 6th Ave. Seattle, WA 98101 (206) 553-8399
Arizona	EPA	Eugene Bromley U S EPA Region 9 W-5-1 75 Hawthorne St. San Francisco, CA 94105 (415) 744-1906
Arkansas	State	Mark Bradley Permitting Section Chief 8001 National Dr. P O Box 8913 Little Rock, AR 72219-8913
California	State	Archie Matthews Div of Water Qual Control Dept. of State Water Res Bd. Mail Code G8 901 P Street Sacramento, CA 95814 (916) 657-0525
Colorado	State	Patricia Nelson Dept. of Health Water Quality Control Div WPCD-PE-B2 4300 Cherry Drive South Denver, CO 80222-1530 (303) 692-3590
Connect-icut	State	Permit Coordinator Dept of Envir Protection Water Management Bureau 165 Capitol Ave. Hartford, CT 06106 (203) 566-7167
Delaware	State	Chuck Schadel Dept of Natural Resources Surface Water Management 89 Kings Hwy , P O Box 1401 Dover, DE 19903 (302) 739-5731
District of Columbia	EPA	Kevin Magerr U S EPA Region 3 3WM53 841 Chestnut Bldg Philadelphia, PA 19107 (215) 597-1651
Florida	EPA	Chris Thomas U.S. EPA Region 4 4WM-FP 345 Courtland St. N E. Atlanta, GA 30365 (404) 347-2391
Georgia	State	Allen Hallum Municipal Permitting Prog Ga. Env Protection Div 4244 International Pkwy Suite 110 Atlanta, GA 30354 (404) 362-2680
Hawaii	State	Steve Chang Dept of Health Clean Water Branch Five Water Front Plaza #500 Ala Moana Blvd. Honolulu, HI 96813 (808) 586-4309
Idaho	EPA	Steve Bubnick U S EPA Region 10 WD-134 1200 6th Avenue Seattle, WA 98101 (206) 553-8399
Illinois	State	Sue Epperson EPA Water Poll. Control Permits Section #15 P O Box 19276 Springfield, IL 62794-9276 (217) 782-0610
Indiana	State	Catherine Hess Dept. of Env Mgmt. NPDES Permits Group Room #718 105 S Meridian St. P O Box 6015 Indianapolis, IN 46206-6015 (317) 232-8704

(Continued)

Exhibit 1-2: NPDES Storm Water Program Permitting Authorities (cont.)

State	Permit Auth	Contact
Iowa	State	Monica Wnuck Dept of Natural Resources Wallace State Building 900 E Grand Street Des Moines, IA 50319-0034 (515) 281-7017
Kansas	State	Don Carlson Dept. of Health and Env Bureau of Water Ind. or Mun. Progs. Section Forbes Field, Building 740 Topeka, KS 66620 (913) 296-5555
Kentucky	State	Douglas Allgeier Dept. of Env Protection Water Division 14 Reilly Road Frankfort, KY 40601 (502) 564-3410
Louisiana	EPA	Brent Larsen U S EPA Region 6 6W-PM 1455 Ross Ave Dallas, TX 75202 (214) 655-7175
Maine	EPA	Shelley Puleo U S EPA Region 1 JFK Building/WCP Boston, MA 02203 (617) 565-3525
Maryland	State	Brian Clevenger MD Dept. of Environment Sed. & Storm Water Admin. 2500 Broening Hwy Baltimore, MD 21224 (410) 631-3545
Mass-achusetts	EPA	Shelley Puleo U S EPA Region 1 WCP JFK Building Boston, MA 02203 (617) 565-3525
Michigan	State	Gary Boersen Dept of Natural Resources Surf Wtr Qual Div -Permits P O Box 30028 Lansing, MI 48909 (517) 373-1982
Minnesota	State	Scott Thompson Pollution Control Agency 520 Lafayette Rd St. Paul, MN 55155-3898 (612) 296-7203
Miss-issippi	State	Louis Lavalee Dept. of Env Quality Office of Pollution Control Ind. Wastewater Branch P O Box 10385 Jackson, MS 39289-0385 (601) 961-5074
Missouri	State	Karl Fett Dept. of Natural Resources Water Poll Control Program 205 Jefferson St P O Box 176 Jefferson City, MO 65102 (314) 526-2928
Montana	State	Fred Shewman Water Quality Bureau Cogswell Building Helena, MT 59620 (406) 444-2406
Nebraska	State	Clark Smith Environmental Quality P O Box 98922 Lincoln, NE 68509 (402) 471-4239
Nevada	State	Rob Saunders Conserv & Natural Res Environmental Protection 333 W Nye Lane Carson City, NV 89710 (702) 687-5870
New Hampshire	EPA	Shelley Puleo U S EPA Region 1 WCP JFK Building Boston, MA 02203 (617) 565-3525
New Jersey	State	Barry Chalofsky NJ DEPE Office of Regulatory Policy CN423 Trenton, NJ 08625-0423 (609) 633-7021

(Continued)

Exhibit 1-2: NPDES Storm Water Program Permitting Authorities (cont.)

State	Permit Auth	Contact
New Mexico	EPA	Brent Larsen U S EPA Region 6 6W-PM 1445 Ross Ave Dallas, TX 75202 (214) 655-7175
New York	State	Ken Stevens Wastewater Facilities Design NY State Dept. of Env Cons 50 Wolf Road Albany, NY 12233 (518) 457-1157
North Carolina	State	Colleen Sullins Environmental Management Water Permits & Eng P O Box 29535 Raleigh, NC 27626-0535 (919) 733-5083
North Dakota	State	Shelia McClenathan Dept of Health Water Quality Div 1200 Missouri Ave. P O Box 5520 Bismarck ND 585202-5520 (701) 221-5210
Ohio	State	John Morrison OEPA Water Pollution Control 1800 Watermark P O Box 1049 Columbus, OH 43266 (614) 644-2017
Oklahoma	EPA	Brent Larsen U S EPA Region 6 6W-PM 1445 Ross Avenue Dallas, TX 75202 (214) 655-7175
		Ted Williamson Discharge Permits Division Oklahoma Dept of Health 1000 N E. 10th Oklahoma City, OK 73117
Oregon	State	Ranei Nomura DEQ-Water Quality 811 SW 6th Ave Portland, OR 97204 (503) 229 5256
Pennsylvania	State	R B Patel Environmental Resources Water Quality Management P O Box 2063 Harrisburg, PA 17120 (717) 787-8184
Puerto Rico	EPA	Jose Rivera U S EPA Region 2 Wtr Permits & Compl. Br 26 Federal Plaza, Room 845 New York, NY 10278 (212) 264-2911
Rhode Island	State	Peter Duhamel Division of Water Resources 291 Promenade St Providence, RI 02908 (401) 277-6519
South Carolina	State	Arturo Ovalles DHEC Industry and Agriculture Wastewater Division 2600 Bull St Columbia, SC 29201 (803) 734-5241
South Dakota	EPA	Vern Berry U S EPA Region 8 8-WM-C Suite 500 999 18th St Denver, CO 80202 2466 (303) 293-1630
Tennessee	State	Robert Haley Dept of Env Wtr Poll Ctrl 401 Church St 6th Floor L & C Annex Nashville, TN 37243-1534 (615) 532 0625
Texas	EPA	Brent Larsen U S EPA Region 6 6W-PM 1445 Ross Ave. Dallas, TX 37243-1534
Utah	State	Harry Campbell Div of Water Qual P O Box 144870 Salt Lake City, UT 84114-4870 (801) 538-6146

(Continued)

Exhibit 1-2: NPDES Storm Water Program Permitting Authorities (cont.)

State	Permit Auth	Contact
Vermont	State	Brian Kooker Env Conserv Permits Compliance & Protection 103 S Main St. Annex Building Waterbury, VT 05671-0405 (802) 244-5674
Virgin Islands	State	Marc Pacifico Dept. of Planning & Nat Resources Div of Env Protection 1118 Watergut Project Box 1118 Christiansted St. Croix, VI 00820-5065 (809) 773-0565
Virginia	State	Burton Tuxford VA Water Control Board 4900 Cox Road Glen Allen, VA 23060 (804) 527-5000
Wash-ington	State	Ed O'Brien Dept. of Ecology Industrial Storm Water Unit Water Quality Div P O. Box 47696 Olympia, WA 98504-7696 (206) 438-7614
West Virginia	State	Jerry Ray Office of Water Resources 1201 Greenbriar St. Charleston, WV 25311 1088 (304) 558-0375
Wisconsin	State	Anne Manuel Dept. of Natural Resources Wastewater Management P.O Box 7921 Madison, WI 53707 (608) 267-7694
Wyoming	State	John Wagner Dept. of Envir Quality Herschler Building 4th Floor Cheyenne, WY 82002 (307) 777-7082

Source Poll of Regional and State offices

1.6 USE OF INFORMATION IN PART 1 AND PART 2 APPLICATIONS

The information submitted in the Part 1 and Part 2 permit applications provides applicants with a starting point for developing comprehensive storm water management programs. For example, the field screening data submitted with the Part 1 application provides a basis for a program to control illicit discharges. Also, the application information may assist in prioritizing controls and in long-term tracking of program effectiveness.

Permitting authorities will use the information from each municipality's Part 1 and 2 applications as the basis for establishing conditions in that municipality's NPDES storm water permit. For example, if a municipality submits a satisfactory application, all or part of its proposed storm water management program is likely to become an integral part of its permit

1.7 ORGANIZATION OF THIS MANUAL

Chapter 1, *Introduction*, provides a brief overview of the Part 2 permit application process It discusses who must submit a Part 2 application and how the information in the applications will be used It also contains a summary of the statutory and regulatory basis for the NPDES storm water program.

Chapter 2, *The Part 2 Application*, describes the statutory and regulatory requirements of municipal NPDES storm water permit applications in more detail. Chapter 2 outlines the specific requirements of the Part 1 and Part 2 applications, explains how Part 2 builds on the Part 1 application, and describes the interconnection among the various components of the Part 2 application.

Chapter 3, *Adequate Legal Authority*, describes how municipalities must demonstrate that they have adequate legal authority to carry out the program requirements [§122.26(d)(2)(i)]

Chapter 4, *Source Identification*, provides guidance on identifying major outfalls and inventorying dischargers to the MS4 [§122 26(d)(2)(ii)]

Chapter 5, *Discharge Characterization*, provides guidance for submitting quantitative data on the MS4 and developing a proposed monitoring program [§122 26(d)(2)(iii)].

Chapter 6, *Proposed Management Program*, describes the steps municipalities must take when they develop site-specific storm water management programs [§122.26(d)(2)(iv)]. These plans are the heart of the municipal permit application, and the permitting authority will probably incorporate all or part of the municipality's proposed management program into their NPDES storm water permit. In their proposed management programs, municipalities must describe management practices, control techniques and systems, design and engineering methods, and other provisions that are aimed at reducing the discharge of pollutants to the "maximum extent practicable"

Chapter 7, *Assessment of Controls*, explains how a municipality can assess the effectiveness of its storm water management program and target priorities through the use of direct and indirect measures [§122 26(d)(2)(v)]

Chapter 8, *Fiscal Analysis*, provides guidance on estimating necessary capital and operation and maintenance expenditures, and financing these expenditures [§122 26(d)(2)(vi)].

1.8 OTHER GUIDANCE AVAILABLE

Municipalities should use this guidance document together with the Part 1 guidance (EPA, 1991b). Exhibit 1-3 lists other sources of guidance available from EPA's Storm Water Hotline [(703) 821-4823] In addition, applicants may wish to obtain further information from the documents identified in the bibliography at the end of this guidance (Appendix A).

Exhibit 1-3
Documents Available from the EPA Storm Water Hotline
[(703) 821-4823]

November 16, 1990, Federal Register - 55 FR 47990 National Pollutant Discharge Elimination System (NPDES) Permit Application Requirements for Storm Water Discharges - Final Rule

March 21, 1991, Federal Register - 56 FR 12098 Application Deadline for Group Applications Final Rule; Application Deadline for Individual Applications - Proposed Rule

August 16, 1991, Federal Register - 56 FR 40948 NPDES General Permits and Reporting Requirements for Storm Water Discharges Associated with Industrial Activity - Proposed Rule

November 5, 1991, Federal Register - 56 FR 50548 Application Deadlines, Final Rule and Proposed Rule

April 2, 1992, Federal Register - 57 FR 11394 Application Deadlines, General Permit Requirements and Reporting Requirements, Final Rule

Summary of November 16, 1990, Storm Water Application Rule

Summary of August 16, 1991, Proposed Storm Water Implementation Rule

August 16, 1991, Proposed Storm Water Implementation Rule Package Fact Sheet

April 2, 1992, Storm Water Program Rule Fact Sheet

Guidance Manual for the Preparation of NPDES Permit Applications for Storm Water Discharges Associated with Industrial Activity (EPA 505/8-91-002, April 1991)

Guidance Manual for the Preparation of Part 1 of the NPDES Permit Applications for Discharges From Municipal Separate Storm Water Systems (EPA 505/8-91-003A, April 1991)

Typical Values of Annual Storm Events Statistics for Rain Zones of the United States ("Urban Targeting and BMP Selection", EPA Region V, November 1990)

List of EPCRA (SARA Title III) Section 313 Water Priority Chemicals (Draft)

List of State and EPA Regional Storm Water Contacts

State NPDES Program Status

Question and Answer Document

List of Reportable Quantities for Hazardous Substances Under CERCLA

NPDES Storm Water Sampling Guidance Document (EPA 833-B-92-001, July 1992)

(Continued)

Exhibit 1-3
Documents Available from the Storm Water Hotline (cont.)

September 9, 1992, Federal Register - 57 FR 41176 Final NPDES General Permits for Storm Water Discharges from Construction Sites - Notice
September 9, 1992, Federal Register - 57 FR 41236 Final NPDES General Permits for Storm Water Discharges Associated with Industrial Activity - Notice
September 9, 1992 Federal Register - 57 FR 41344 National Pollutant Discharge Elimination System, Request for Comment on Alternative Approaches for Phase II Storm Water Program - Proposed Rule

* The following documents are available from the National Technical Information Service (NTIS) (1) *Storm Water Management for Industrial Activities, Developing Pollution Prevention Plans and Best Management Practices* (EPA 832-R-92-006, September 1992), (2) *Storm Water Management for Construction Activities, Developing Pollution Prevention Plans and Best Management Practices* (EPA 832-R-92-005, September 1992)

CHAPTER 2
THE PART 2 APPLICATION

2.0 THE PART 2 APPLICATION

2.1 BACKGROUND

The NPDES permit application requirements for MS4s [40 CFR 122.26(d)] establish a two-part application designed to meet the goal of developing comprehensive site-specific storm water quality management programs for MS4s.

The purpose of the two-part application process is to develop information, in a reasonable time frame, that will build successful storm water management programs and allow permitting authorities to make informed decisions about permit conditions. The application process is designed to focus the efforts of municipalities in two areas: prohibiting non-storm water discharges into storm sewers, and implementing controls that reduce the discharge of pollutants from MS4s to the maximum extent practicable.

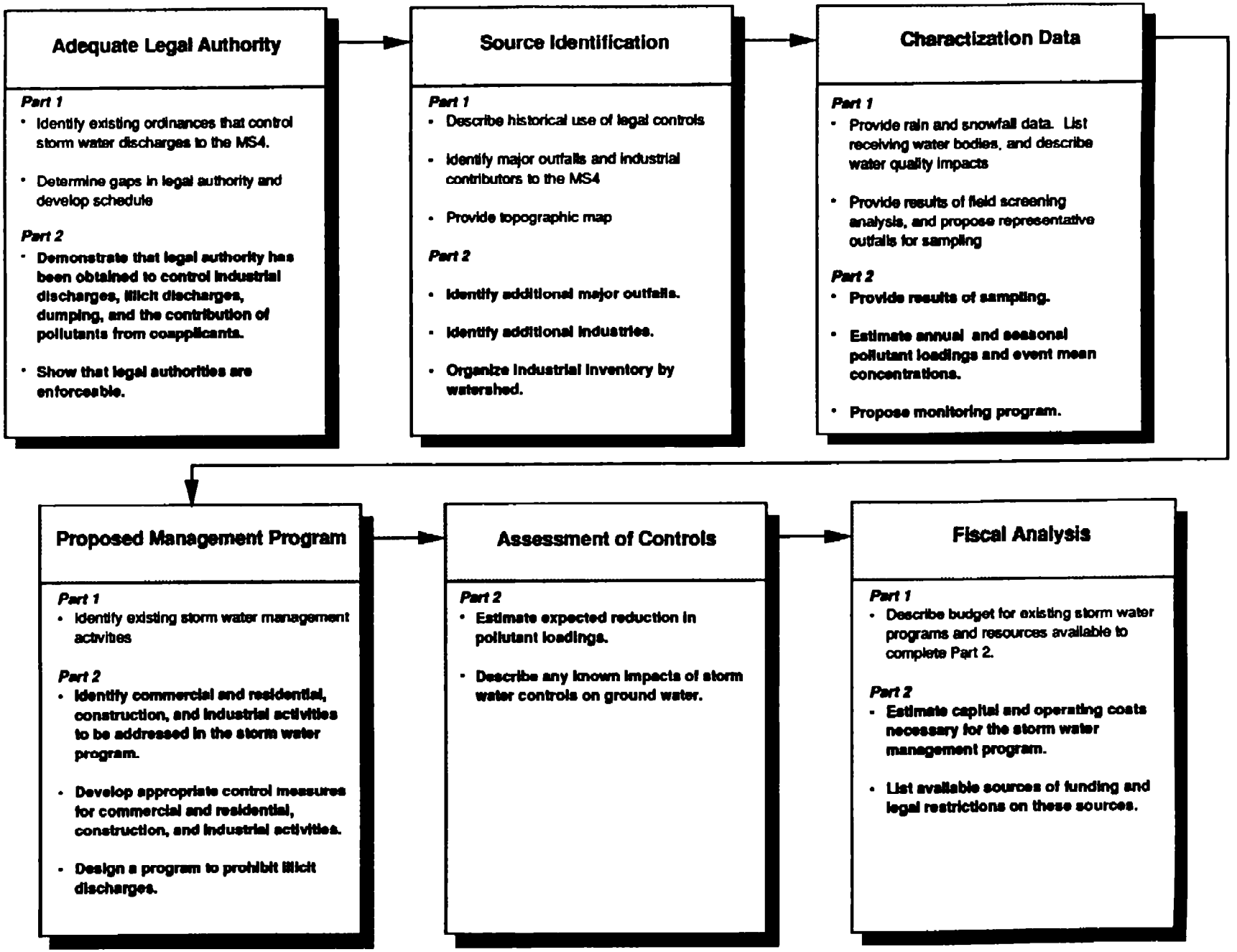
Part 1 of the application requires information on existing programs and legal authority. In addition, Part 1 requires the results from field screening of major outfalls to detect illicit connections. The **Part 2** application requirements are intended to build upon the information submitted with the Part 1 application Each part has virtually the same major areas of concern, but the Part 2 application requires a greater level of detail. Part 2 of the permit application requires a demonstration of adequate legal authority, additional information on pollutant sources and outfalls, a limited amount of representative quantitative sampling data, a proposed monitoring program, a proposed storm water management program, an estimate of the effectiveness of storm water controls, and a fiscal analysis. The requirements for the Part 1 and Part 2 applications are summarized briefly in Exhibit 2-1, and described in more detail in Section 2 2 The storm water regulations underlying this guidance can be found in Appendix B

Before applicants proceed with the detailed development of their permit applications; they **should recognize the fundamental** requirements:

- Who or what are the primary contributors of pollutants in storm water discharges from MS4s?
- Where are these sources of pollutants located in relation to receiving water resources?
- What is the magnitude of these pollutant sources and their potential impact on receiving waters?
- How does the municipality plan to reduce or eliminate the contribution of pollutants in storm water discharges or prevent the damaging influences of these discharges?
- Why did the municipality select the activities or best management practices (BMPs) it proposes?
- When will the municipality implement its proposed program?
- How will the applicant assess the effectiveness of the program? What criteria or measures will apply?
- How will the municipality fund proposed program activities?

Wherever appropriate, the applicant must also show that it has adequate legal authority to implement, enforce, or mandate compliance with applicable ordinances, statutes, contracts, or other similar vehicles as required by the storm water regulation.

Exhibit 2-1: Part 1 and Part 2 Storm Water Application Requirements.



These questions (described above) that an applicant must address follow a natural progression or development. For example, before applicants can identify how they will reduce the contribution of pollutants in storm water discharges (the fourth bullet point above), they must identify pollutant sources and estimate the magnitude of pollutant loads (bullet points 1-3 above).

2.2 PART 1 APPLICATIONS

Sections 2.2.1 and 2 2 2 provide overviews of the regulatory requirements of §122 26(d). Section 2 2 3 describes the relationship among the various application provisions.

2.2.1 Overview of the Part 1 Application

Part 1 applications consist of the following six elements

- **General information.** The applicant's name, address, telephone number of contact person, ownership status and status as a State or local government entity

- **Legal authority.** A description of existing legal authority to control discharges to the MS4, and if this authority does not meet the required criteria, a list of additional authority needed and a schedule and commitment to seek such authority.

- **Source identification.** A description of the historic use of ordinances, guidance, or other controls that limit non-storm water discharges to any publicly owned treatment works (POTW), and a topographic map covering an area one mile beyond the service boundaries of the MS4 showing:

  - the location of known municipal sewer system outfalls;

  - a description of all land use activities;

  - the location and activities of landfills;

  - the location and permit number of any known discharge to the MS4;

  - the location of major structural controls for storm water discharges (such as retention basins, or major infiltration devices); and

  - identification of publicly owned parks, recreational areas, and other open lands.

- **Discharge characterization.** A summary of the types and characteristics of storm water discharges, including:

  - monthly mean rain and snowfall estimates and the average number of storm events per month;

  - existing quantitative data describing the volume and quality of discharges from the MS4, including a description of the outfalls and sampling methods used;

  - a list of "downstream" water bodies receiving discharge from the MS4, and a description of the impact of outfall upon them;

  - the results of field screening analysis for illicit discharges at either selected field screening points or major outfalls covered in the permit application; and

  - a proposed characterization plan for conducting sampling and obtaining the quantitative data necessary to complete Part 2 of the application.

- **Management programs.** A description of existing management programs to control pollutants from the municipal separate storm sewer system. For example, what procedures are in place to control pollution from construction activities, and how do they work? What is the program (such as investigation procedures and how they operate) for identifying illicit connections to the municipal storm sewer system?

- **Fiscal resources** A presentation of the municipality's budget for existing storm water programs and for completing Part 2 of the permit application.

2.2.2 Overview of the Part 2 Application

The Part 2 application must include the following elements:

- **Adequate legal authority.** A demonstration that the municipality can operate according to the legal authority established by ordinance, statute, or series of contracts. The municipality also must demonstrate that its authority is enforceable. A discussion of how adequate legal authority may be demonstrated appears in Chapter 3 of this guidance.

- **Source identification.** An inventory, organized by watershed, of the facilities that may discharge storm water associated with industrial activity to the MS4. The applicant also must identify the location of any major outfall that discharges to waters of the United States that was not reported in Part 1. A discussion of the information to be submitted for each such facility in the inventory appears in Chapter 4 of this guidance.

- **Characterization data.** Sampling results for 5-10 outfalls designated by the permitting authority, estimates of cumulative annual pollutant loadings and event mean concentrations, and a proposed schedule to submit estimates of seasonal pollutant loadings and event mean concentrations for each major outfall identified in the source identification sections of Part 1 and 2. The *Characterization Data* provision of the Part 2 application also requires the development of an on-going monitoring program covering the term of the permit. Procedures for meeting the requirements of this section appear in Chapter 5.

- **Proposed management program.** A program that shows the municipality's comprehensive planning process for the reduction and control of pollutants, the staff and equipment available to implement the program, and a full description of how controls will be implemented to reduce pollutants from all sources of storm water. Municipalities must also describe how the program will be implemented and maintained. The Part 2 requirements for a proposed management program are described in Chapter 6.

- **Assessment of controls.** An estimate of the projected effectiveness of the municipal storm water management program, and an identification of the known impacts of storm water controls on ground water. The assessment of controls is discussed in Chapter 7.

- **Fiscal analysis.** A fiscal analysis of the capital and operation and maintenance expenditures needed to accomplish the activities (including implementation) required by the characterization data and proposed management program sections of the Part 2 application. This fiscal analysis must include projected expenses for each fiscal year of the permit term. A discussion of the fiscal analysis is included in Chapter 8.

2.2.3 Relationship Among Application Requirements

The required elements of the Part 2 application are related to each other. As a result, this guidance addresses how the application elements are related, and how information gathered for one requirement will assist the applicant in meeting other requirements. For example, the information gathered for the *Industrial Source Identification* provision of the Part 2 application will assist the municipality in.

- Targeting monitoring goals to potential pollutant sources, which may include selecting monitoring locations and chemical specific sampling frequencies (a requirement of the *Characterization Data* provision);

- Identifying illicit discharges (a requirement of the *Proposed Management Program's* illicit connection provision);

- Identifying facilities with the greatest potential for degrading receiving water quality (a requirement of the *Proposed Management Program's* industrial program provision), and

- Targeting sites that handle, store, or transport toxic or hazardous materials for on-site inspections (another requirement of the *Proposed Management Program's* industrial program provision).

As another example, the information that the applicant must prepare for the *Characterization Data* provision (e.g., the results of the sampling requirement and the estimated event mean concentrations and annual pollutant loads) may help the municipality:

- Evaluate the contribution of pollutants in storm water discharges from individual sources and determine which sources may require inspections or controls (a requirement of the *Proposed Management Program's* industrial program provision);

- Predict the impact of storm water discharges on receiving waters known to be impacted. (In the *Proposed Management Program*, additional controls may be warranted for construction sites or other industrial activities that discharge to these waters); and

- Determine what BMPs may be appropriate for given areas (another requirement of the *Proposed Management Program*)

Exhibit 2-2 summarizes some of these key interrelationships, although many other interrelationships exist. A more detailed discussion of specific information requirements and interrelationships among provisions is provided in subsequent chapters As municipalities prepare their permit applications, they should coordinate all program requirements.

Exhibit 2-2
Examples of Relationship Among Part 2 Requirements

					Fiscal Analysis
				Assessment of Controls	Cost/benefit analysis identifies the most cost-effective BMPs
			Proposed Management Program	Estimates of reductions in pollutant loadings predicts impact of storm water management activities	Fiscal analysis considers costs of controls, maintenance, and capital improvements Management program may include feasibility analyses that consider cost.
		Characterization Data	Annual pollutant loads help prioritize areas for BMPS On-going monitoring indicates success of BMPs and need to re-prioritize	On-going monitoring program verifies program effectiveness. Instream monitoring verifies biological recovery.	Fiscal analysis considers cost of on-going monitoring
	Source Identification	Land use information and organization of industry by watershed defines representative sampling points	Inventory of industrial users helps the city target facilities for inspections and control measures	Estimates of pollutant load reductions depend on land use	Industrial inventory identifies potential sources of storm water utility fees
Adequate Legal Authority	Some sources or outfalls may be outside a city's jurisdiction. Interjurisdictional agreements may be necessary.	Authority to require sampling and obtain information for industries and dischargers outside of the MS4's jurisdiction at sampling points	Legal authority needed to implement BMPS, control and inspect industry, and prohibit dumping and illicit discharge	Need information gathering and inspection authority where it is necessary to inspect, monitor, and enter the facility or the site	Legal authority is required for some financing plans, such as a storm water utility

2.3 ADDITIONAL FACTORS TO BE CONSIDERED IN DEVELOPING THE PART 2 APPLICATION

As discussed in the previous section, the various provisions of the Part 2 application process are interconnected

All municipalities covered by §122 26(d) must submit a Part 2 permit application that meets the requirements of the storm water permit application regulations. However, each MS4 is unique, and each Part 2 submission will be different. Municipal separate storm sewer systems differ in many ways, including population served, geologic and climatologic settings, density of development, and form of government These underlying factors make each applicant unique

The major factors that applicants should consider are

- Population and projected growth rate;
- Zoning and existing land use patterns;
- Nature of watershed and receiving waters;
- Climatic conditions, soil types, and watershed delineations,
- Existing municipal functions and municipal lands,
- Other environmental impacts;
- Public involvement; and
- Intergovernmental coordination.

In addition, municipalities must implement their storm water management programs in a manner that is consistent with other applicable Federal, State, and local environmental laws.

Population and Projected Growth Rates

Some storm water BMPs are more appropriate for densely developed areas, while other methods may be more useful in developing areas Consequently, defining current population densities and projecting future areas of population growth provides the basic information that can assist in the evaluation and prioritization of appropriate storm water control strategies

Zoning and Existing Land Use Patterns

Through ordinances, permits, or contracts, municipalities may mandate storm water controls for new residential, commercial, or industrial developments in order to improve or assure maintenance of the quality of receiving waters at or near pre-development levels. The Nationwide Urban Runoff Program (NURP) study (EPA, 1983), pointed out that some of the best opportunities for implementing cost effective measures to prevent or reduce pollutants in storm water occur during new development. These measures may include structural controls, such as storm water detention basins or constructed storm water wetlands, or nonstructural alternatives such as cluster development and buffer zones Sections 122.26(d)(1)(iii)(B)(2) and 122 26(d)(2)(ii) require the municipality to establish comprehensive management plans for new development (see Chapter 6)

Nature of Watershed and Receiving Waters

The types of storm water controls appropriate for a MS4 depend on the nature of the watershed and the receiving waters. This includes geologic and hydrologic features such as slope drainage patterns and stream size For example, roadside swales may not be practical in areas with steep terrain, but can be very useful in flat areas In addition, structural BMPs or other management measures that control the volume and timing of release are appropriate where uncontrolled storm water may cause physical impacts to receiving waters (especially small streams, rivers, and wetlands).

Information on the watershed and the receiving waters is required in the Part 1 permit application [§122 26(d)(1)(iv)(C)]. In Part 1, applicants are required to list water bodies that receive discharges from the MS4. The list of water bodies includes downstream segments, lakes, and estuaries where pollutants from the system discharges may accumulate and result in non-attainment of State water quality standards Part 1 also requires a description of known water quality impacts. Applicants must include a discussion of water bodies that were cited in:

- State reports required by CWA Sections 305(b), 304(l), and 314(a);
- The State Nonpoint Source Report; and
- Other reports identifying sensitive watersheds

Part 1 applicants should also include in this discussion a description of impacts caused by dissolved oxygen depression, bioaccumulation of toxics, excessive sedimentation, hydrologic modification, habitat destruction, etc.

Municipalities are expected to give priority consideration to those classes of pollutant sources that contribute significant loadings or pose a significant impact on receiving waters. Applicants must consider control methods that address storm water discharges from commercial and residential areas; illicit discharges and illegal disposal, storm water discharges from industrial areas; and storm water runoff from construction sites. Municipalities' permits will differ substantially in the emphasis placed on controlling various sources of pollutants in discharges from the MS4. Permits for older municipalities may emphasize control of cross-connections, while permits for municipalities with large areas of new development may emphasize the installation of permanent structural controls during construction

The Part 2 storm water permit application requires descriptions of management programs to address sources of pollutants discharged to separate storm sewer systems. For management strategies to be effective, municipalities must give prior consideration to the nature (e g., physical and biological parameters) and the designated uses of receiving waters such as streams, tributaries, and natural wetlands For example, a storm water management program for a newly developing area with an existing shallow, slow-moving stream could include provisions to ensure that the post-development peak discharge flow rate for the stream is held to a certain percentage of its historical or pre-development peak discharge flow rate

Climatic Conditions, Soil Types, and Watershed Delineations

Seasonal variations in precipitation can have a significant impact on storm water quality For example, extended dry seasons in areas such as the southwestern United States result in pollutant loads distinctly higher than in other parts of the country during the first several storms of the wet season. Areas with more frequent rain and snowfall throughout the year may have more storm water discharges, but the discharges may have consistently lower pollutant concentrations than those in the Southwest. In addition, areas with significant snowfall may experience a peak in storm water discharge volume and pollutant concentration during the spring thaw.

Natural soil conditions affect the potential for storm water to recharge ground water. Porosity and permeability are properties of the soil that govern the size and number of the interstitial spaces through which water may flow. Compaction (e.g , compression of the soil by heavy machinery) will reduce the amount of void space in the soil and thereby reduce the amount of rainfall that infiltrates through the soil to ground water. Natural soil conditions are very important when siting structures designed for storm water infiltration. In addition, identifying such sites must take into consideration potential ground water impacts

that may result whenever infiltration is part of the storm water management program

Existing Municipal Functions and Municipal Lands

The Part 2 application affords municipalities the opportunity to discuss alternatives in the *Proposed Storm Water Management Program*. When considering the wide range of municipal functions, applicants need to establish which agencies will be responsible for implementing each portion of a storm water management program. (This could be outlined in the *Adequate Legal Authority* chapter of the Part 2 application, as discussed in Chapter 3 of this guidance.) Many of these agencies, will have primary missions other than dealing with storm water or water quality. Expansion of the established charter of an agency to include an element of storm water control may require legislative action, moderately expanding the scope of other municipal agencies' missions to include storm water concerns can be much more cost effective than the initiation of entirely new programs.

Applicants should identify existing municipal functions that impact the quality of storm water discharges. These functions may include snow removal activities such as road deicing, vehicle maintenance operations, and herbicide, pesticide, and fertilizer application to public lands. Municipalities can modify these activities to improve storm water quality through oversight of future land development, modifications to flood management structures, changes in materials used or in material handling or application practices, maintenance of roads, and installation of structures such as retention basins

The municipal agency (or agencies) responsible for storm water runoff control should also consider the extent to which municipal lands and activities contribute pollutants to runoff. The same BMPs recommended for private lands may also be incorporated into the development and maintenance of a municipality's own lands and activities. For example, reduced use of pesticides and fertilizers on park land and open spaces usually decreases the contribution of these contaminants to storm water runoff. Implementing BMPs on municipal lands also shows the municipality's commitment to an effective storm water management program BMPs are discussed in greater detail in Section 6.4 of this guidance.

Other Environmental Impacts

Municipalities should consider those activities that can directly or indirectly alter the natural hydrograph of a stream and potentially degrade an otherwise stable aquatic habitat. These factors are particularly important when considering impacts to wetlands, riparian areas, ground water, small rivers, and streams. In addition, the installation of detention or rapid infiltration ponds may have negative impacts on ground water. The installation of culverts or concrete drainage channels and other such structures typically increases the volume and velocity of runoff, which can lead to increased erosion, siltation, and sedimentation in receiving waters. Therefore, installation of these structures can contribute to the degradation of a neighboring habitat.

Public Involvement

Municipal applicants must ensure that they provide adequate public education and ample opportunities for public participation. Public participation should focus on spreading awareness of program objectives and components. Education and public involvement programs must be defined as part of the *Proposed Storm Water Management Program* [§122.26(d)(2)(iv)]. Generally, the public should be involved as early as possible in storm water management initiatives.

Conflict and confusion can be minimized if the program includes a schedule for initial public contact and milestones for public involvement throughout the development and implementation phases. Public education programs are expected to target specific

audiences, including those regulated or affected by the storm water management program (e.g., developers, building contractors, and industrial operators) and those that can assist with program implementation (e.g., volunteers and citizens). For example, one large municipal applicant (Seattle) described an existing public participation program in its Part 1 Application submission. Elements of this program may be instructive to municipalities completing Part 2 of the application because it has generic components that are likely to be applicable to other large (and perhaps medium) municipalities. Excerpts from Seattle's public involvement program are provided in Exhibit 2-3 for reference.

Elements of this municipality's program that are particularly important to consider include of the role of an advisory and outreach group and its relationship to the entire process. Effective public participation programs clearly identify the role of the public

The potential exists for a considerable range in the level of participation the public may actually have in the decision-making process. Generally, the municipal authority is going to make the decisions. However, the authority can choose to use the "participation" process to simply inform the public of decisions, or to allow the views of the public to be registered prior to decision milestones In other cases, although uncommon, the public may have an actual voice or vote in making decisions.

The timing and frequency of meetings and the duration of the groups established for public participation will usually be dictated by the nature of the issues being addressed For example, an ad hoc group established to address a single issue may discover that the issue cannot be effectively addressed without consideration of a broader range of issues that the municipality may also be considering In this instance it may be appropriate for the group to expand its scope, hold regular meetings, and actively participate in the authority's decision making process Therefore, applicants should outline in their Part 2 applications how such coordination will be accomplished

Intergovernmental Coordination

If a number of municipal entities (e.g., multiple cities or a city and a county) are participating in the permit application process as coapplicants, various mechanisms can be used to improve intergovernmental coordination to ensure that the roles and responsibilities of each entity are well defined. Each entity must fulfill its responsibilities to implement applicable program measures. Examples of some of the appropriate coordination techniques and their benefits include:

- **Memoranda of agreement (MOA).** MOAs can define specific municipal roles, responsibilities, and points of coordination that help minimize duplication of effort and ensure accountability;

- **Cross-training of staff.** This allows for the identification of gaps in staffing (e.g., neglected areas of responsibility or insufficient staff levels) as well as providing the benefits of increased versatility and opportunities for learning from others;

- **Interagency advisory committees.** Their objective is to arm decision makers with a comprehensive understanding of the implications of proposed activities or decisions; and

- **Regularly scheduled intermunicipal staff meetings.** These can facilitate an open and thorough exchange of information and solidify new lines of communication

Exhibit 2-3
Excerpts from a Public Involvement Program

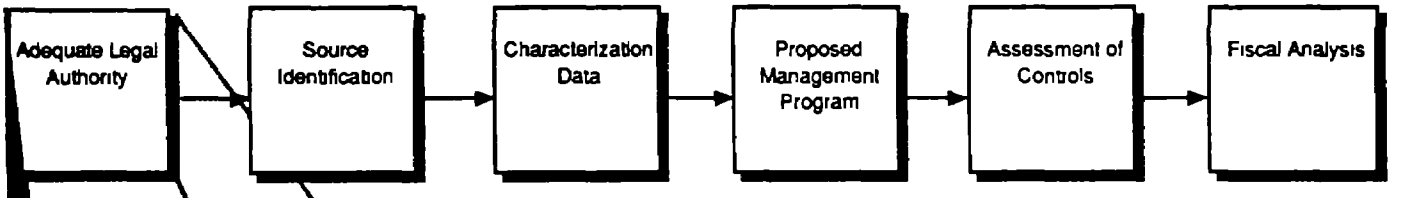
The public involvement program [of the City of Seattle] has been designed to assist in developing an acceptable city-wide plan for addressing drainage and water quality problems. Acceptable is defined as a plan that is both technically sound and sensitive to the needs and interests of the citizens. The involvement program has two major elements: a Citizen Advisory Committee (CAC) and a community outreach effort The initial role of the CAC was to provide guidance to City staff and consultants preparing various sections of a Comprehensive Drainage Plan Until the adoption of the Comprehensive Drainage Plan by the City Council, the CAC provided direction on drainage policy issues, assisted with the public review of the draft plan and environmental impact statement (EIS), and helped coordinate comments sent to the city from the public during the review period. Following council adoption of the plan, the CAC was reconstituted into a Drainage and Wastewater Advisory Committee which serves as an on-going sounding board to the Drainage and Wastewater Utility, the mayor, and the City Council on both sewer and drainage matters.

The community outreach effort was established for two purposes. The first was to ensure adequate public review and support of the Comprehensive Drainage Plan and EIS Comments received during the review were used by the Drainage and Wastewater Utility, the mayor, and the City Council in making decisions about the Drainage Plan and the City's on-going drainage program. The second purpose was to begin educating residents and business people about the importance of their role in solving flooding, landslide, and water quality problems throughout the city. This community outreach/education role remains an on-going effort of the Drainage and Wastewater Utility.

Source City of Seattle, *NPDES Storm Water Permit Application, Part 1*, City of Seattle, November 1991 37

Single municipalities with separate governing functions may face the same challenges as coapplicants when they prepare their Part 2 applications. Many of the same coordination steps may be necessary within a single municipal jurisdiction. The need for *intra*governmental coordination may be most crucial in large municipalities that have functions that impact storm water quality spread throughout the organizational structure of the municipality. For example, a planning department may be in charge of implementing a stream buffer policy, while a public works department may plan, site, and construct storm water BMPs Still other agencies may be responsible for implementing erosion and sediment control requirements, and permitting and inspection functions. Storm water-related responsibilities within governmental organizations may be allocated in this manner due to the relatively recent emergence of storm water quality as an important issue. Nonetheless, effective coordination within the government of a single municipality may be as critical to the success of the storm water management program as is intergovernmental coordination for coapplicants Therefore, applicants should outline in their Part 2 applications how such coordination will be accomplished.

CHAPTER 3
ADEQUATE LEGAL AUTHORITY



Adequate Legal Authority

Part 1

- Identify existing ordinances that control storm water discharges to the MS4
- Determine gaps in legal authority and develop schedule

Part 2

- **Demonstrate that legal authority has been obtained to control industrial discharges, illicit discharges, dumping, and contributions of pollutants from coapplicants.**
- **Show that legal authorities are enforceable.**

3.0 ADEQUATE LEGAL AUTHORITY

3.1 BACKGROUND

A crucial requirement of the NPDES storm water regulation is that a municipality must demonstrate that it has adequate legal authority to control the contribution of pollutants in storm water discharged to its MS4. This guidance manual and the storm water program emphasize development and implementation of storm water management programs as described in Chapter 6. In order to have an effective municipal storm water management program, a municipality must have adequate legal authority to control the contribution of pollutants discharged to the MS4.

Part 1 of the permit application requires applicants to describe their existing legal authority to control the discharge of pollutants from MS4s and evaluate the adequacy of these ordinances. Where existing ordinances were lacking, a proposed schedule to obtain the necessary authority was included with the Part 1 application. In Part 2 of the application, municipal applicants must demonstrate that they now possess adequate legal authority to:

- Control construction site and other industrial discharges to the MS4;
- Prohibit illicit discharges and control spills and dumping;
- Control potential sources of pollutants from discharges to or from coapplicants' MS4s, or MS4s that are interconnected or shared with other entities;
- Require compliance with all regulations and statutes, and
- Carry out inspection, surveillance, and monitoring procedures

Section 3.2 reviews each of these regulatory requirements. Section 3.3 describes specific procedures a municipality may use to demonstrate adequate legal authority.

3.2 SUMMARY OF REGULATORY REQUIREMENTS

3.2.1 Control Construction Site and Other Industrial Discharges to the MS4.

> §122.26(d)(2)(i)(A) [The applicant must demonstrate that it can control] through ordinance, permit, contract, order or similar means, the contribution of pollutants to the municipal storm sewer by storm water discharges associated with industrial activity and the quality of storm water discharged from sites of industrial activity.

The municipality, as a permittee, is responsible for compliance with its permit and must have the authority to implement the conditions in its permit. To comply with its permit, a municipality must have the authority to hold dischargers accountable for their contributions to separate storm sewers.

"Control," in this context, means not only to require disclosure of information, but also to limit, discourage, or terminate a storm water discharge to the MS4. For example, construction sites (of 5 or more acres) and other industrial activities that discharge storm water through MS4s are required to obtain individual NPDES permits or coverage under general NPDES permits from EPA or an authorized NPDES State. These permits require compliance with applicable Federal and State regulations. However, a municipality, to satisfy its permit conditions may need to impose additional requirements on discharges

from permitted industrial facilities, as well as discharges from industrial facilities and construction sites not required to obtain permits. Therefore, a municipality should develop a mechanism to assure that all industrial facilities and constructions sites that discharge to the MS4 know their obligation to comply with the applicable terms of the municipality's storm water ordinances.

3.2.2 Prohibit Illicit Discharges and Control Spills and Dumping

> §122.26(d)(2)(i)(B) [The applicant must demonstrate that it can prohibit] through ordinance, order or similar means, illicit discharges to the municipal separate storm sewer
>
> §122.26(d)(2)(i)(C) [The applicant must demonstrate that it can control] through ordinance, order or similar means the discharge to a municipal separate storm sewer of spills, dumping or disposal of materials other than storm water

To demonstrate that it possesses adequate legal authority to control storm water discharges, a municipality must be able to effectively prohibit illicit discharges and illegal dumping An illicit discharge is "any discharge that is not composed entirely of storm water except discharges pursuant to a NPDES permit . . . and discharges resulting from fire fighting activities" [40 CFR 122.26(b)(2)].

3.2.3 Control Contributions of Coapplicants

> §122 26(d)(2)(i)(D) [The applicant must demonstrate that it can control] through interagency agreements among coapplicants the contribution of pollutants from one portion of the municipal system to another portion of the municipal system

An operator of a MS4 may participate in an application with one or more other operators, or may submit an individual application for the separate storm sewer it operates. As indicated in the box above, the operator of a discharge from a large or medium MS4 may submit, through the use of interjurisdictional agreements, a system-wide permit application. The system-wide application can accommodate existing storm water programs, on a watershed basis, as well as programs which must take into account regional differences in climate, geography, and political institutions. Such an application should cover issues of liability, financial contributions, access to records, enforcement responsibilities, and any other applicable areas of mutual concern.

When two or more municipalities submit a joint application, each coapplicant must demonstrate that it individually possesses adequate legal authority over the entire municipal system it operates or owns. A coapplicant need not fulfill every component of legal authority specified in the regulations, as long as the combined legal authority of all coapplicants satisfies the regulatory criteria for every segment of the MS4 (including authority over all sources that discharge to the MS4).

As coapplicants, for example, a county and a flood control district within that county may together possess adequate legal authority. The flood control district may have legal authority to build, operate, and maintain structures associated with major drainage channels within the county. The county itself may have legal authority to control pollutants in discharges from privately owned lands to the MS4s and legal authority to build, operate, and maintain structures associated with minor drainage channels that tie into major drainage channels. In this situation, the combined legal authority of the coapplicants may be adequate for the system, provided that the only discharge to major drainage channels comes from the county's separate storm sewer system. As another example, a department of transportation or flood control district with no land use authority could be a co-permittee with

a city that does possess land use authority over the entire jurisdiction.

Coapplicants also may use interjurisdictional agreements to show adequate legal authority and to ensure planning, coordination, and the sharing of the resource burden of permit compliance When more than one entity is submitting an application for a MS4 (either as coapplicants or as individual applicants for different parts of a system), the role of each party must be well defined. Each applicant or coapplicant must show the ability to fulfill its responsibilities, including legal authority for the separate storm sewers it owns or operates.

Applicants and coapplicants may use the procedures outlined in Section 3 3 to demonstrate adequate legal authority in their Part 2 permit applications. These procedures are guidelines, however, and are not intended to be the only possible approaches that applicants may follow.

3.2.4 Require Compliance with all Regulations and Statutes

To meet the requirements of §122 26(d)(2)(i)(E), the applicant must show that it has adequate authority to enforce its ordinances.

> **§122 26(d)(2)(i)(E)** [The applicant must demonstrate that it can require] compliance with conditions in ordinances, permits, contracts or orders

One acceptable way to support a declaration of adequate legal authority, including the ability to enforce appropriate ordinances, is for the municipality to provide a certification from the Municipal General Counsel or equivalent The certification should state that the applicant has the legal authority to apply and enforce the requirements of §122 26(d)(2)(i)(A)-(F) in State or local courts The certification would, therefore, cite specific ordinances and the reasons why they are enforceable. The statement should discuss what the municipality can do to ensure full compliance with §122.26(d)(2)(i).

In a Part 2 application, through a statement from the Municipal General Counsel or through some other method, a municipality should identify the administrative and legal procedures available to mandate compliance with appropriate ordinances, and, therefore, with permit conditions. Applications should contain descriptions of how ordinances are implemented and appealed In particular, a municipality should indicate if it can issue administrative orders and injunctions or if it must go through the court system for enforcement actions

3.2.5 Carry Out Inspection, Surveillance, and Monitoring Procedures

In their Part 2 applications, municipalities must propose programs to control the contributions of pollutants from industrial facilities and prohibit illicit discharges For both of these activities, municipalities must have the legal authority to carry out inspection, surveillance, and monitoring procedures necessary to determine compliance.

> **§122.26(d)(2)(i)(F).** [The applicant must demonstrate that it can carry] out all inspection, surveillance and monitoring procedures necessary to determine compliance and noncompliance with permit conditions including the prohibition on illicit discharges to the municipal separate storm sewer.

To meet this requirement, municipalities may wish to consider establishing ordinances that require industrial facilities to perform inspections and report the results to the city. In many municipalities, these facilities may perform similar inspections under a pretreatment program. In their Part 2 applications, municipalities should provide

documentation of their authority to enter, sample, inspect, review, and copy records, etc., as well as demonstrate their authority to require regular reports

3.3 PROCEDURES FOR DEMONSTRATING ADEQUATE LEGAL AUTHORITY

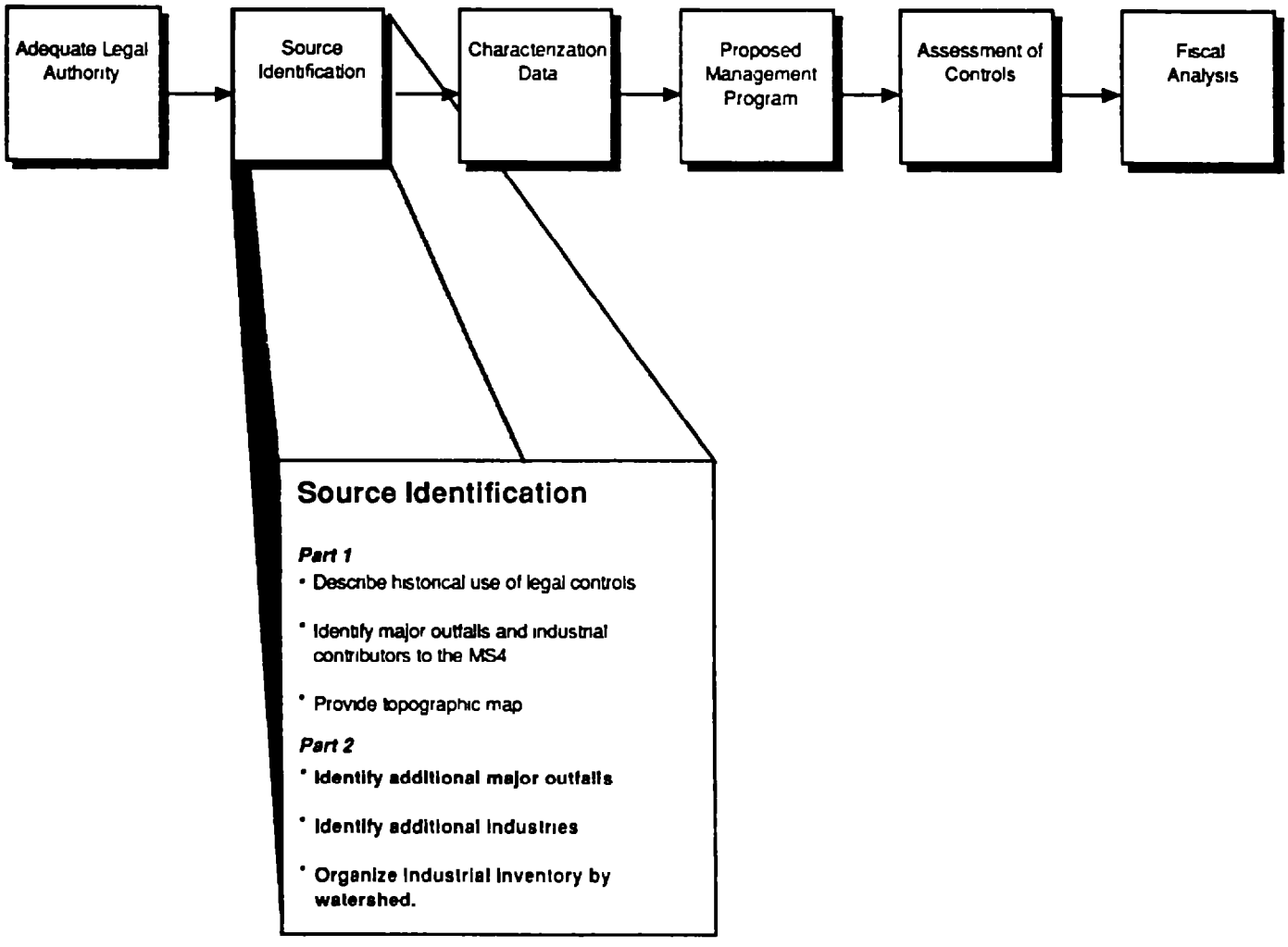
The Part 2 application requires the applicant or coapplicants to cite and describe specific ordinances currently in effect and demonstrate that the jurisdiction for these ordinances covers the entire area served by the MS4. In addition, the applicant may elect to discuss specific changes in ordinances passed since the submission of the Part 1 permit application to illustrate how legal authority has evolved to meet the regulatory requirements in §122.26(d)(2)(i). One method by which an applicant can partially demonstrate that it has adequate legal authority is to develop a matrix that compares, in a side-by-side format, the regulatory requirements in §122.26(d)(2)(i)(A)-(F) and the municipality's legal authority. Once completed, the matrix would indicate whether an adequate legal framework exists to address all key regulatory requirements identified in §122.26(d)(2)(i)(A)-(F). Furthermore, the matrix could also illustrate where the authority to mandate compliance is vested.

In order to support an assertion of adequate legal authority, applicants should include the complete text of the applicable portions of the ordinances or other such provisions in the application. The applicant should also provide a specific explanation of why and how the language of a particular ordinance or other authority meets Federal regulatory requirements. The application should indicate to whom the ordinance applies and how it will operate to control, prevent, or stop discharges that violate permit conditions. For example, the municipality may describe and provide an excerpt from a city ordinance that prohibits non-storm water discharges to the MS4.

Appendix C illustrates one way to detail the existence of ordinances that establish the legal authority required in §122.26(d)(2)(i). A narrative discussion of the historical use of these ordinances to control pollutants in storm water discharges also may be included. The example in Appendix C shows what the applicant may do to satisfy §122.26(d)(2)(i).

Substantial effort should be devoted to obtaining the necessary legal authority before the Part 2 application is submitted. However, some municipalities may find that the two-year application process does not allow enough time to secure adequate legal authority as described in this section. This may be due to the need for State statutory or legislative changes. In this instance, the Part 2 application must include a detailed description of what changes are needed and a schedule of when they will be accomplished. The schedule must include timetables for drafting proposed changes, public comment periods, and final authorizations.

CHAPTER 4
SOURCE IDENTIFICATION



4.0 SOURCE IDENTIFICATION

4.1 BACKGROUND

In Part 1 of the NPDES storm water permit application, applicants are required to identify the location of known major outfalls discharging to waters of the United States from MS4s. Applicants also are required to provide information and data on existing land use activities. The identification of outfalls and land use activities is the first step in the process of:

- Identifying the sources of pollutants in storm water runoff;
- Linking the sources of pollutants in runoff to specific water quality impacts and other impacts that may result in degradation of aquatic resources;
- Identifying those activities or physical factors that have the most significant impact on water quality;
- Defining control measures that yield improvements in storm water quality; and
- Developing methodologies by which engineers, urban planners, and managers can make long term decisions that not only provide for economic growth, but also have discernible environmental benefits through imposed storm water controls.

The source identification requirements in the Part 2 permit application reflect three basic steps. First, municipalities must identify any major outfalls that were not already identified in the Part 1 application. Second, applicants must compile an inventory of industrial activities that may discharge storm water to a MS4. Third and finally, applicants must organize the inventory of industrial activities on a watershed basis.

Organizing the inventory by watershed allows the municipality to focus on activities within discrete areas that may contribute pollutants in storm water discharges to waters of the United States. For example, combining outfall data with the industrial inventory organized by watershed may help the municipality to identify probable areas of illicit connections. This information will also be useful for municipalities when they develop specific strategies [e.g., best management practices (BMPs)] as part of their proposed storm water management programs. The following sections discuss regulatory requirements and procedures for completing the source identification section of the Part 2 permit application. Section 4.2 provides guidance on identifying major outfalls, Section 4.3 provides guidance on compiling an inventory of industrial dischargers, and Section 4.4 provides guidance on organizing the inventory of industrial discharges by watershed.

4.2 MAJOR OUTFALLS

The first portion of the Part 2 Source Identification provision states:

> §122.26(d)(2)(ii) *Source identification.* [The applicant must provide the] location of any major outfall that discharges to waters of the United States that was not reported [in Part 1 of the application]

4.2.1 Definition of a Major Outfall

According to 40 CFR 122 26(b)(5), a major outfall is a MS4 outfall that discharges from a single pipe with an inside diameter of at least 36 inches The term also includes discharges from a single conveyance other than a circular pipe serving a drainage area of more than 50 acres.

For those municipal separate storm sewer systems that receive storm water runoff from lands zoned for industrial activity, major outfalls also include outfalls that discharge from a single pipe with an inside diameter of 12 inches or more, or discharge from other than a circular pipe associated with a drainage area of 2 acres or more. This definition also applies to outfalls of drainage areas that have both industrial and non-industrial activity. For example, if a three acre drainage area is zoned half woodland and half industrial, the discharges from that area would still be considered a major outfall. Because the definition of major outfall includes consideration of drainage area, municipalities may need to consider conveyances such as ditches and swales when identifying major outfalls

4.2.2 Identifying Major Outfalls

The first step in this section of the Part 2 application is the identification of major outfalls not identified in the Part 1 application [§122.26(d)(2)(ii), cited in box above]. When identifying these major outfalls, municipalities should build upon the approach used in the Part 1 application. One way to identify major outfalls is a review of sewer system maps. These maps can provide information on sewer system type (e g , separate storm versus combined sewer), pipe size, and outfall location However, depending upon the age of the sewer system maps, they may not provide complete information about newly developed areas or improvements to older areas Often, interviews with sewer system maintenance personnel can provide information on the most recent changes to the sewer system The municipality should also consider conducting field surveys (e g., visual inspection of the banks of receiving waters) to locate major outfalls

When submitting a Part 2 permit application, municipalities should include a brief description of how additional major outfalls were identified This description is not intended to be a lengthy list of each sewer system employee interviewed, but rather an outline of the methods employed

4.3 INVENTORY OF INDUSTRIAL DISCHARGERS

The second step in this portion of the Part 2 application is assembling an inventory of industrial storm water dischargers

> **§122 26(d)(2)(ii)** *Source Identification*
> Provide an inventory, organized by watershed of the name and address, and a description (such as SIC codes) which best reflects the principal products or services provided by each facility which may discharge, to the municipal separate storm sewer, storm water associated with industrial activity

This section describes how municipalities may develop the inventory of industrial facilities Section 4.4, below, provides guidance on organizing these facilities by watershed.

4.3.1 Facilities that must be Included in the Inventory

As stated above, applicants must provide an inventory of each facility that may discharge to the MS4 storm water associated with industrial activity. Industrial storm water dischargers that must be included in this inventory fall into 11 classes of industrial activities as defined in the November 1990

regulations Six of these classes were defined in a narrative format and five were defined by Standard Industrial Classification (SIC) codes. Specific categories of industries are identified in §122.26(b)(14)(i)-(xi). Exhibit 4-1 provides a list of the SIC codes and industry categories cited in the regulatory definition.

4.3.2 Identifying the Industrial Facilities

As a first step in developing a comprehensive industrial storm water inventory, the applicant must review **facility notifications**. Industrial facilities were required to notify municipalities by May 15, 1991, of their intent to discharge storm water to the municipal storm sewer system [§122.26(a)(vi)(4)]. Each facility should have submitted to the municipality information including facility name, facility location, and facility type (such as SIC code or other industry categorization).

In addition, municipalities should explore other sources of information on industrial facilities to help identify gaps in inventory. One specific source of information a municipality should review is **facility information submitted under other programs**. For example, SIC codes are often required for air pollution permit applications, hazardous materials management permits, pretreatment program applications, building permits, business licenses, or local tax rolls. A municipality may take the list of SIC codes provided in Exhibit 4-1 and compare it with existing information on SIC codes or industrial categories which has been submitted by industrial facilities under other programs.

Under 40 CFR 122.28, facilities that discharge storm water associated with industrial activity must submit an individual permit application, participate in a storm water group permit application, or file a **Notice of Intent (NOI)** to be covered by a general permit. These applications and NOIs are another source of information on industrial dischargers. For existing facilities, applications or NOIs were to be submitted by October 1, 1992; for new facilities, they must be submitted prior to the commencement of industrial activity However, in the Intermodel Surface Transportation Efficiency Act of 1991, Congress provided that permit application requirements be reserved for industrial activities owned or operated by municipalities with a population of less than 100,000, with the exception of airports, power plants, and uncontrolled sanitary landfills If EPA is the permitting authority in a State, applications and NOIs should be submitted to EPA, if a State has NPDES authority, they should be submitted to the State. Section 308 of the CWA provides the legal authority for any individual (including a municipality) to obtain information from the NPDES permitting authority. A municipality may be able to obtain a list of the facilities in its jurisdiction that have applied for coverage under a general or individual permit or that have applied for coverage as a member of a group

Additional sources of information on industrial facilities may include **zoning maps** showing industrial parks, manufacturing and industrial listings in **telephone books, trade association listings, pretreatment industrial waste surveys, the Chamber of Commerce Manufacturing Directory, and Dunn and Bradstreet.**

In the Part 2 application, a municipality should provide a brief description of the sources it reviewed in identifying the industrial dischargers. As part of the proposed storm water management program, which is described in Chapter 6, municipalities should describe a plan for collecting new or updated information on industrial dischargers throughout the life of the permit.

Exhibit 4-1
Industry Categories Cited in the
Definition of Storm Water Associated with Industrial Activity

1. Facilities subject to storm water effluent limitations guidelines, new source performance standards, or toxic pollutant effluent standards under 40 CFR Subchapter N (except facilities with toxic pollutant effluent standards which are exempted under category 11 below.

2 Facilities described by SIC 24 (except 2434), 26 (except 265 and 267), 28 (except 283), 29, 311, 32 (except 323), 33, 3441, 373 *

3. Facilities described by SIC 10 through 14 (mineral industry), including:

   - active or inactive mining operations, except for areas of coal mining operations no longer meeting the definition of a reclamation area under 40 CFR 434 11(1) because the performance bond issued to the facility by the appropriate SMCRA authority has been released, or areas of non-coal mining operations which have been released from applicable State or Federal reclamation requirements after December 17, 1990, and

   - oil and gas exploration, production, processing, or treatment operations, or transmission facilities that discharge storm water contaminated by contact with or that has come into contact with, any overburden, raw material, intermediate products, finished products, by-products, or waste products located on the site of such operations

4 Hazardous waste treatment, storage, or disposal facilities, including those that are operating under interim status or a permit under Subtitle C of RCRA.

5. Landfills, land application sites, and open dumps that receive or have received any industrial wastes (waste that is received from any of the facilities described under this subsection) including those that are subject to regulation under Subtitle D of RCRA.

6. Facilities involved in the recycling of materials (metal scrapyards, battery reclaimers, salvage yards, and automobile junkyards) including but not limited to SIC 5015 and 5093

7. Steam electric power generating facilities, including coal handling sites.

8. Transportation facilities described by SIC 40, 41, 42 (except 4221-25), 43, 44, 45, and 5171, which have vehicle maintenance shops, equipment cleaning operations, or airport deicing operations. Only those portions of the facility that are either involved in vehicle maintenance (including vehicle rehabilitation, mechanical repairs, painting, fueling, and lubrication), equipment cleaning operations, airport deicing operations, or which are otherwise identified under 1 - 7 or 9 - 11 are associated with industrial activity.

(Continued)

Exhibit 4-1 (continued)

9. Treatment works treating domestic sewage or any other sewage sludge or wastewater treatment device or system, used in the storage treatment, recycling, and reclamation of municipal or domestic sewage, including land dedicated to the disposal of sewage sludge that is located within the confines of the facility, with a design flow of 1.0 mgd or more, or required to have an approved pretreatment program under 40 CFR Part 403. Not included are farm lands, domestic gardens, or lands used for sludge management where sludge is beneficially reused and which are not located within the facility, or areas that are in compliance with Section 405 of the CWA.

10 Construction activity including clearing, grading, and excavation activities except operations that result in the disturbance of less than five acres of total land area which are not part of a larger common plan of development or sale **

11 Facilities described by SIC 20, 21, 22, 23, 2434, 25, 265, 267, 27, 283, 285, 30, 31 (except 311), 323, 34 (except 3441), 35, 36, 37 (except 373), 38, 39, 4221-25, (and which are not otherwise included within categories 2 - 10).*

Source 55 FR 48065, November 16, 1990

*Please note the SIC 285 is covered under Category 11 Also note that for the industries identified in Category 11, the term includes only storm water discharges from all areas (except access roads and rail lines) where material handling equipment or activities, raw materials, intermediate products, final products, waste materials, by-products, or industrial machinery are exposed to storm water

**On June 4, 1992, the United States Court of Appeals for the Ninth Circuit found that EPA's rationale for exempting construction sites of less than five acres and certain uncontaminated storm water discharges from Category 11 light industrial facilities from Phase I of the storm water program to be invalid and has remanded these exemptions for further proceedings (see *Natural Resources Defense Council* v *EPA* No 91-70176)

4.4 ORGANIZING THE INDUSTRIAL INVENTORY BY WATERSHED

Once the industrial inventory is complete, the applicant must organize the inventory by watershed, or drainage area. The main objective of this requirement is to associate discrete discharges with specific watersheds, which may help the municipality identify relationships between pollutant sources and receiving water quality problems To help organize the industrial inventory by watershed, municipalities should consider the long-term benefits of using automated database systems to help organize and update information on

- Locations of major outfalls or system modifications;
- Land use designations and composition;
- Dischargers of storm water associated with industrial activity,
- Other NPDES permit holders,
- Location/inventory of structural controls, and
- Locations of illicit connections

This information can help satisfy the requirement that discharges of storm water associated with industrial activity be organized by watershed Using an automated database system or the map submitted in the Part 1 application may be helpful in satisfying this requirement However, the regulations do not require Part 2 applicants to use a particular database or submit certain information, and municipalities may elect to use other methods.

The following procedure is provided as an example of one way to organize industrial dischargers by watershed:

1. Create a transparent overlay of tax maps covering the entire area served by the MS4.

2 Indicate on the maps the location of each industrial activity according to its address with an appropriate symbol or code.

3 Produce an overlay of existing watersheds from a topographical map, for example, United States Geological Survey (USGS) maps, covering the area that the MS4 supports Previously performed hydrological surveys may be helpful in delineating the boundaries of existing watersheds Municipalities may elect to sub-divide existing watersheds into smaller units if this will assist in management planning.

4 Align the tax map and watershed overlay so that industrial activity locations can be transposed to the watershed overlay.

A number of PC-based tools can be used to organize information on facilities and outfalls. For example, computer-aided design (CAD) packages, in conjunction with third-party software packages, are specifically designed to present information on separate transparent layers that can be "turned off and on" when necessary One layer could contain information on watershed topography and another could contain the locations of industrial storm water dischargers. Additional layers might contain information on the layout of the municipal system, locations of structural source controls and outfalls, and land-use patterns (both present and future)

A CAD-based system can be useful, not only in presenting information easily and graphically, but also in its ability to transfer spatial data, such as XYZ coordinates, to commonly available PC-based database applications This spatial data can be merged with other databases containing more generic information including facility name, address, and SIC codes However, one potential drawback to CAD systems is that most of them cannot store "real-world" (e g., latitude-longitude) coordinates and are not generally designed for spatial analyses

Information stored in a CAD format may also be input into a Geographic Information System (GIS) With some conversion, the CAD system coordinates may be transformed into the "real-world" coordinates typically employed by GIS GIS are integrated database management systems designed for the input, storage, retrieval, analysis, output, and display of geographically or spatially indexed data

The key attribute of GIS is the relational database capabilities that make these systems powerful tools for conducting spatial analyses. Using GIS, a municipality could overlay several layers of data and derive new information from this existing information. For example, using GIS, an applicant could overlay a map showing the 100-year flood plain with a map showing locations of industrial facilities The GIS could then calculate the amount of industrial area within the 100-year flood plain and plot this data on a new overlay This type of spatial analysis might be a powerful tool in the design of the municipality's storm water management program.

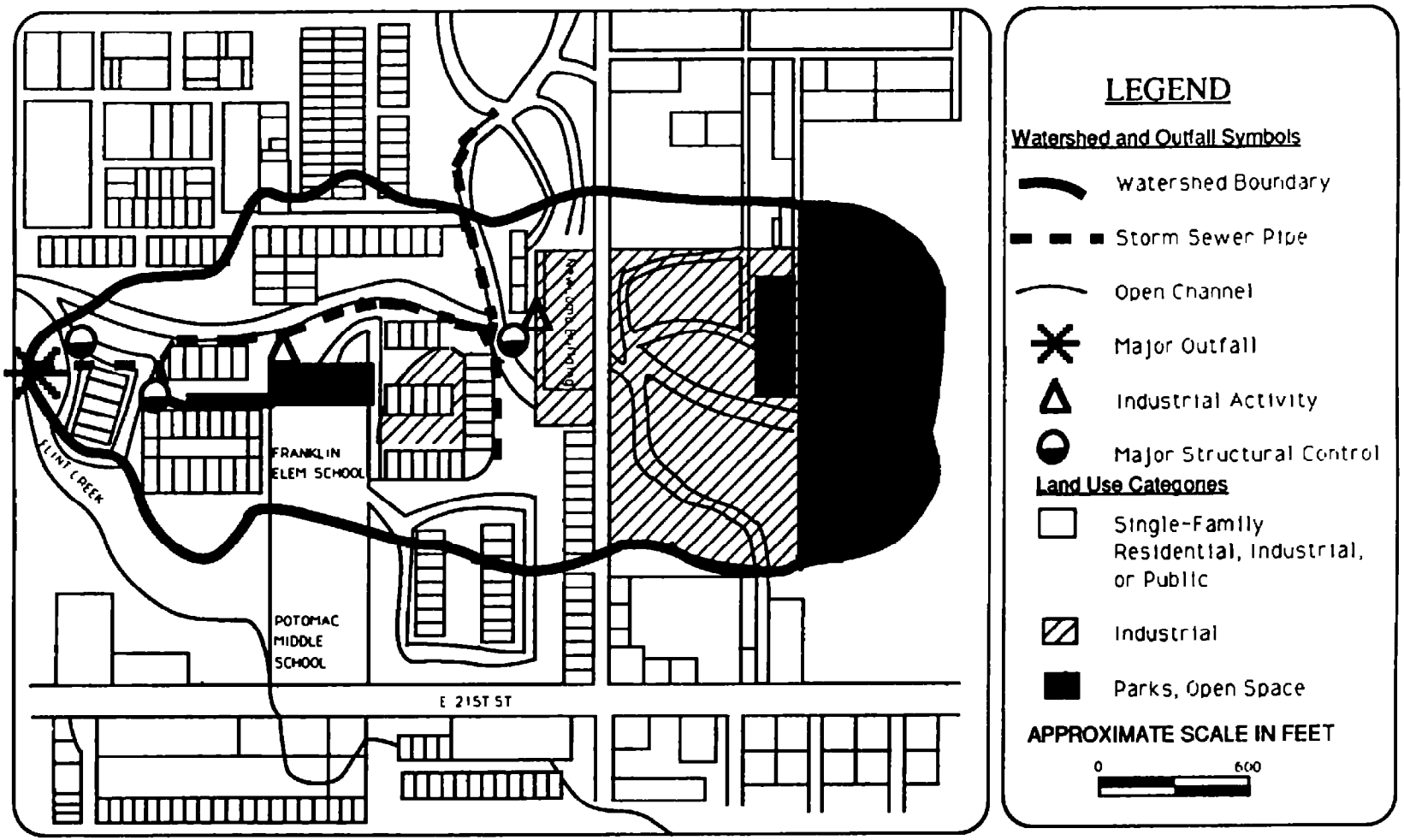
Another benefit of GIS is the ability for common data to be shared efficiently among several agencies. For example, the flood management agency, department of transportation, and storm water control agency could all contribute data to and use analyses from the same GIS. On the other hand, one potential drawback to GIS is their relatively high cost. Often, developing accurate, appropriate base maps is one of the most resource intensive parts of the system.

The techniques presented in this section to organize industrial dischargers by watershed are not the only methods that the applicant can use For example, municipalities may elect to present the information in tabular form. Using a CAD, GIS, or other automated system is entirely up to the municipality There is no requirement that municipalities use these systems in the development of either the Part 1 or Part 2 NPDES permit applications Each applicant will have to examine its existing resources (including computer systems, personnel, and budget) and projected needs before deciding which method will be the most efficient and most useful in the long term

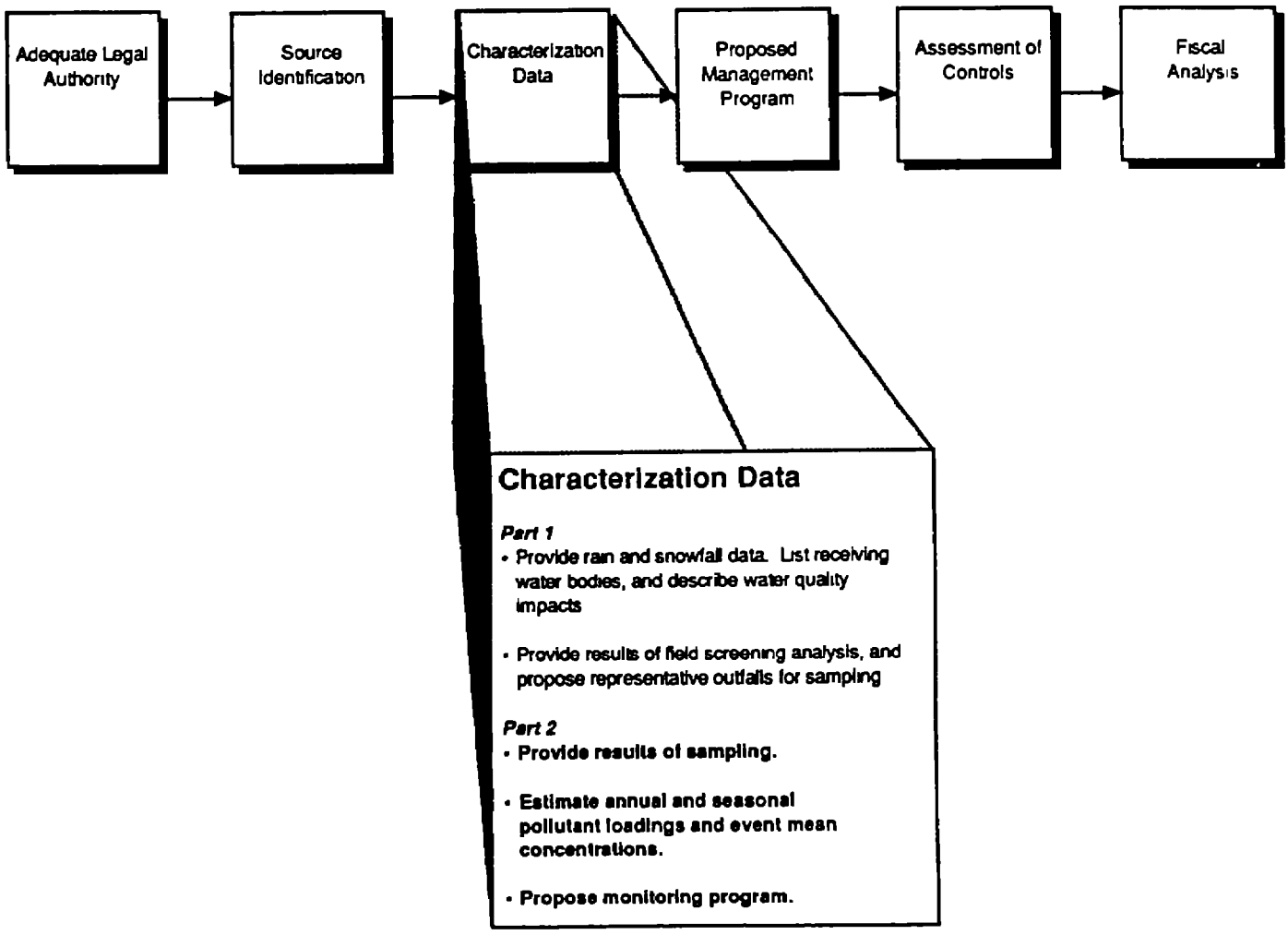
A discussion of maintaining and/or updating the industrial inventory is provided in Section 6.3.3.2 of this guidance.

Exhibit 4-2 illustrates an example of the procedure discussed in Sections 4.3 and 4 4

Exhibit 4-2
Example of a Map Organizing Industry by Watershed



CHAPTER 5
CHARACTERIZATION DATA



5.0 CHARACTERIZATION DATA

5.1 BACKGROUND

5.1.1 Objective of this Section

This section addresses the requirements for reporting the physical and chemical characteristics of municipal storm water runoff as specified by 40 CFR 122 26(d)(2)(iii). These requirements describe the minimum quantitative and descriptive data necessary to begin characterizing storm water discharges.

The applicant is encouraged to provide additional information, if available, which may provide a basis for a more effective storm water management program. The additional information may also help the permitting authority make more informed decisions regarding the specifications of the permit to be issued.

The NPDES permit application regulations require the applicant to identify all major outfalls that are part of the MS4 [§122.26(d)(1)(iii) and 126(d)(2)(ii)]. Part 1 requires the municipality to propose a sampling plan that identifies 5-10 outfalls that would be appropriate for representative data collection under Part 2 of the application [§122.26(d)(1)(iv)(E)]. The next step is to collect and analyze samples from these outfalls (or others designated by the permitting authority) for a variety of pollutant parameters from 3 representative storm events.

5.1.2 Potential Impacts of Storm Water Runoff

The Nationwide Urban Runoff Program (NURP) study showed that discharges from MS4s contribute to the degradation of water quality in the Nation's waters (EPA, 1983). The NURP study also concluded that the effects of urban runoff on receiving water quality are very site specific The effects depend on the types, size, and hydrology of the water body, the designated beneficial use, the pollutants which affect that use, the urban runoff quality characteristics, and the amounts of urban runoff dictated by local rainfall patterns and land use. *The National Water Quality Inventory, 1990 Report to Congress* as required by Section 305(b) of the Clean Water Act, stated that one-third of the impairment in assessed waters is due to storm water runoff (EPA, 1990d)

Quantity Impacts

Urbanization often increases the quantity and reduces the quality of storm water runoff For example, vegetated or forested areas with pervious surfaces are often replaced with impervious surfaces (e g , concrete and asphalt) that prevent or minimize the amount of rainfall available for ground water recharge This increases the volume and velocity of storm water runoff.

Vegetated areas play a crucial role in ground water recharge and in the maintenance of stream baseflow This is especially true during extended dry periods, when ground water is often the only source that preserves stream baseflow In highly urbanized areas, ground water recharge may be so severely reduced that ground water flow to perennial streams during dry periods is not sufficient. Further, the natural hydrology of a watershed is often altered by urbanization, because developing areas often provide drainage appurtenances that rapidly conduct storm water runoff away from these areas Such drainage may also affect the geometry of natural streams, especially where natural streams have been modified through the installation of man-made channels Ultimately, reduced perviousness due to urbanization increases the magnitude and the frequency of localized flooding which can have the long term effect of substantially increasing the width of natural streams through erosion and scouring

Increases in peak discharge velocity and runoff volume can also result in substantial erosion of natural streambanks and the washout of benthic habitats. Since streambeds often consist of unconsolidated silt and sediment, they may be stripped away substantially by excessive discharge velocities Increased discharge velocities can also lead to undercutting and destabilization of streambanks, which may cause erosion that extends beyond the natural boundary of the streambank

Further, silt and sediment can increase the turbidity of the receiving water, thus interfering with the growth of aquatic plants which depend on photosynthesis. Increased turbidity can also interfere with aquatic feeding, eliminate spawning areas for fish, and cause abrasion and clogging of fish gills. Also, because silt and sediment may remain in the watershed, they can blanket benthic habitats and severely reduce streamflow capacity

In the presence of excessive volumes of storm water runoff and discharge velocities, the net impact on receiving waters can be almost indistinguishable from impacts commonly associated with the discharge of toxics (e g , increased mortality, reduced biodiversity, and reduced reproduction)

Deposition and Resuspension of Toxicants

Research is currently on-going to examine the impact of the deposition and resuspension of toxicants as a result of wet weather events Questions about the survivability of benthic habitats when exposed to toxicants in deposited sediments still remain. The impact of resuspended toxicants from the sediments is not well known since toxics are often bound to sediment particles that may reduce the concentrations available for biological uptake and subsequent bioaccumulation The applicant should also be aware that different metal contaminants in sediments can exhibit different solubilities Under varying conditions of pH and temperatures, metals deposited in sediment can become soluble again and be reintroduced into the water column

Excessive Bacterial Levels

The NURP study final report concluded that "coliform bacteria are present at high levels in urban runoff and can be expected to exceed EPA water quality criteria during and immediately after storm events." Thus is of significant concern, particularly in swimming and shellfish areas.

Dissolved Oxygen Depression

The presence of oxygen-consuming pollutants in receiving waters can lead to severe dissolved oxygen depression Factors that can cause dissolved oxygen depression include the resuspension of biodegradable organic material (which can occur in the presence of high flow velocities) or the discharge of organic pollutants in storm water discharges. The NURP study demonstrated that storm water discharges exhibit biochemical oxygen demand (BOD) levels in excess of levels commonly associated with secondary treated effluent from publicly owned treatment works (POTWs). Severe dissolved oxygen depression could contribute to fish kills, which are one of the most readily observable impacts of pollution on receiving waters.

Eutrophication

Eutrophication, or the aging of a water body, can be accelerated by excessive nutrient loadings from storm water. Advanced stages of eutrophication are often associated with substantial variations in dissolved oxygen concentration. Nutrients of concern are nitrogen and phosphorus. Phosphorus is typically the growth-limiting nutrient for plants in fresh water systems. Storm water discharges routinely contain excess concentrations of these nutrients, which can lead to excessive algal growth, commonly referred to as algal blooms. Excessive concentrations of algae can cause odor and taste problems in drinking water and can result in aesthetically unpleasant

environments. In addition, the eventual decomposition of large concentrations of algae can depress dissolved oxygen in the water body to levels where fish kills occur. In nature, the process of eutrophication occurs over a substantial period of time, however, storm water discharges can rapidly accelerate this process.

Exceedance of Chronic Toxicity Criterion

Long-term exposure to toxics in excess of chronic toxicity criteria can cause sublethal effects on aquatic life. Indicators of chronic toxicity include reduced fertility, reproduction, and growth rates and a decline in the diversity of aquatic organisms The NURP study clearly indicated that storm water discharges contain concentrations of trace metals, such as lead, cadmium, zinc, and copper in amounts that exceed the chronic toxicity criteria. Prolonged exposure to chronic concentration levels of toxics can also be lethal to aquatic organisms, primarily from the bioaccumulation of toxics within the cell tissue of the organism over a extended period of time

Thermal Impacts

The temperature of storm water runoff may become significantly elevated via conductive and convective heat transfer with impervious, man-made surfaces In the case of contact with impervious surfaces, the resulting temperature elevation of storm water runoff can be substantial. For example, the surface temperature of parking lots during summer months may exceed 100 degrees Fahrenheit. Consequently, storm water runoff from these parking lots will be elevated in temperature. Many aquatic organisms are extremely sensitive to changes in water temperature. Increased water temperature also reduces dissolved oxygen in streams, rivers, lakes, and wetlands Therefore, significant discharges of storm water at elevated temperatures can, over the long term, lead to the alteration of aquatic populations.

5.1.3 Use of the Characterization Data

The NURP study analyzed storm water discharge from 28 sites representing 12 major river basins of the United States NURP detected 77 EPA priority pollutants present in the storm water discharges sampled, including samples with concentrations that exceeded water quality criteria for certain pollutants. Those pollutants detected in at least 10 percent of the samples studied in NURP are identified in Exhibit 5-1.

The data gathered for storm water discharge characterization can be used to create a baseline measurement of pollutant concentration and loadings The data also can be used to evaluate the effectiveness of best management practices (BMPs) as well as help identify storm water control priorities. In addition, it can be used to help identify the sources of pollutants in storm water runoff, to help establish an effective monitoring program for the life of the permit, and to help predict the impact of storm water runoff on receiving waters that are known to be impaired.

5.1.4 Storm Water Sampling and Analysis Procedures

The regulation requires that the process of collecting quantitative data for storm water characterization follow certain guidelines

> §122.26(d)(2)(iii) *Characterization data* When "quantitative data" for a pollutant are required under paragraph (d)(1)(iii)(A)(3) of this paragraph, the applicant must collect a sample of effluent in accordance with 40 CFR 122.21(g)(7) and analyze it for the pollutant in accordance with analytical methods approved under 40 CFR part 136 When no analytical method is approved the applicant may use any suitable method but must provide a description of the method

Exhibit 5-1. Priority Pollutants Detected in at Least 10% of NURP Samples.

PARAMETERS	FREQUENCY OF DETECTION (%)
Metals and Inorganics	
Antimony	13
Arsenic	52
Beryllium	12
Cadmium	48
Chromium	58
Copper	91
Cyanides	23
Lead	94
Nickel	43
Selenium	11
Zinc	94
Pesticides	
Alpha hexachlorocyclohexane (alpha-BHC)	20
Alpha endosulfan	19
Chlordane	17
Lindane (gamma BHC)	15
Halogenated aliphatics	
Methane, dichloro-	11
Phenols and cresols	
Phenol	14
Phenol, pentachloro-	19
Phenol, 4-nitro	10
Phthalate esters	
Phthalate, bis(2-ethylhexyl)	22
Polycyclic aromatic hydrocarbons	
Chrysene	10
Fluoranthene	16
Phenanthrene	12
Pyrene	15

Source: U.S. Environmental Protection Agency, *Results of the Nationwide Urban Runoff Program*. EPA Planning Division (National Technical Information Service (NTIS) Accession No. PB84-8552). December 1983.

The data collection procedures must follow the guidelines for storm water sampling outlined in §122.21(g)(7), *Effluent Characteristics*. This portion of the NPDES regulation describes the conditions under which a storm water discharge will be sampled, and which collection procedure (grab sample versus flow-weighted composite sample) is required for the water quality parameter being analyzed. These guidelines are discussed in more detail in Sections 5.3.2 and 5.3.4 of this guidance manual. In addition, EPA has available a *Storm Water Sampling Guidance Document* that describes in detail the methods used for storm water discharge sampling (EPA, 1992a).

The methods for the chemical analyses of storm water discharge samples must be conducted in accordance with 40 CFR Part 136, *Guidelines for Establishing Test Procedures for the Analysis of Pollutants*. These guidelines refer the applicant to EPA-approved methods and cite the source of the approved methods (e.g., Standard Methods for the Examination of Water and Wastewater, ASTM methods, etc.). Note that alternative methods (i.e., those not included in Part 136) may be used under certain circumstances (see Section 5.3.4) as described in 40 CFR Part 136, and reiterated in the Characterization Data section of Part 2 of the storm water discharge NPDES permit.

The specific constituent pollutants and water quality parameters that must be analyzed in the storm water samples are presented in Section 5.3.4.

5.2 SUMMARY OF REGULATORY REQUIREMENTS

The following is a summary of the characterization data requirements for the Part 2 application:

- Quantitative data on physical and chemical characteristics of the discharge taken from at least 5 to 10 representative outfalls chosen by the permitting authority (Section 5.3).
- Estimates of both the annual pollutant load and event mean concentration of the cumulative discharges from all municipal outfalls during a storm event (Section 5.4),
- A proposed schedule to provide estimates for each major outfall of the seasonal pollutant load and the event mean concentration for constituents detected in required sampling (Section 5.5); and
- A proposed monitoring program for the life of the permit that meets specific requirements established in the regulations (Section 5.6).

5.3 QUANTITATIVE AND QUALITATIVE DATA REQUIREMENTS

5.3.1 Selection of Representative Sampling Sites

In the Part 1 application, the municipality is required to describe a plan for obtaining characterization data [§122.26(d)(1)(iv)(E)]. The plan should reflect the requirements of §122.26(d)(2)(iii).

Different types and intensities of land use activities influence, in part, the types of pollutants and the pollutant concentrations in municipal storm water runoff. Therefore, Part 1 of the permit application [§122.26(d)(1)(iii)(B)(2)] requires the applicant to describe the land use activity within the area to be covered by the permit. In Part 1, the applicant also must select a subset of all the major outfalls (see Section 4.2.1 for definition of major outfall) identified that represented surface runoff discharge of the various land use activities described. In some cases, a municipality preparing a Part 2 application may want to supplement its sampling program by collecting and analyzing samples from major outfalls that were not identified in the Part 1 application or designated by the permitting authority. This additional sampling may provide the

municipality with data that better characterizes its MS4 discharges

5.3.2 Criteria for Storm Water Discharge Sampling

Land use activities are not the only factors that affect the pollutant composition of storm water runoff Storm water composition also varies according to the nature of the storm event (e.g , duration, volume), and the composition may vary throughout the duration of a single storm event (i.e., the initial discharge, or "first flush," tends to have higher pollutant loads). In order to obtain data that represents an "average" storm event, EPA requires samples from three separate storm events to characterize the surface water runoff; however, the permitting authority may allow exemptions

> §122.26(d)(2)(iii)(A)(1) For each outfall or field screening point designated under this subparagraph, samples shall be collected of storm water discharges from three storm events occurring at least one month apart in accordance with the requirements at §122 21(g)(7) (the Director may allow exemptions to sampling three storm events when climatic conditions create good cause for such exemptions),

The criteria for sampling storm water discharge are detailed in §122.21(g)(7), *Effluent Characterization* EPA's *Storm Water Sampling Guidance Document* addresses these criteria. For the purpose of this discussion, a brief synopsis of these criteria follows:

- For each outfall or field screening point selected, samples must be collected from three separate storm events.
- The three storm events must be at least one month apart
- Each sampled storm event must have a rainfall of at least 0 1 inch in the drainage area
- There must be no storm event in excess of 0 1 inch in the drainage area for at least 72 hours prior to the sampled storm event
- The rainfall event should not vary by plus or minus 50 percent from the average or median per storm volume and duration for the region.

EPA understands that climatic conditions may make it difficult for some municipalities to sample storm events meeting these criteria. For example, storm events may be so infrequent in arid and semi-arid areas that sufficient samples cannot be obtained by the application deadline. In other areas, storms may be so frequent that it may not be possible to wait the required 72 hours between storm events. In such cases, the applicant should confer with the permitting authority in advance. In instances where representative storm events do not occur prior to the application due date, the municipality should submit its application with as much information as possible. It should include an explanation [certified by a principal executive officer or ranking elected official in accordance with §122.22(a)(3)] as to why sampling data were unavailable.

The municipality may need to perform some initial research and calculation to meet the requirements listed above In order to determine what constitutes an average storm event for the area, the applicant should contact the National Weather Service or National Oceanographic and Atmospheric Administration's National Climate Center Weather data is also available commercially and from airports. The applicant may also refer to the information provided in the *Storm Water Sampling Guidance Document.*

5.3.3 Narrative Description of Storm Event

> **§122.26(d)(2)(iii)(A)(2)** A narrative description shall be provided of the date and duration of the storm event(s) sampled, rainfall estimates of the storm event which generated the sampled discharge and the duration between the storm event sampled and the end of the previous measurable (greater than 0.1 inch rainfall) storm event;

Under §122.26(d)(2)(iii)(A)(2), the municipality must provide a narrative description of each storm that produced the discharge to be chemically and physically characterized. Such a narrative description must include:

- The date and duration of the rainfall event that produced the discharge sampled. Measurements describing the peak intensity of the storm, if available, should also be reported,
- The amount of rainfall. Rainfall conditions may vary significantly across large drainage areas, so rainfall characteristics should be spatially averaged over the drainage area, if possible. If more than one rain gauge is used, averages should be reported. Rain gauges operated near the drainage area by the National Weather Service may be used, or the discharger may collect this information,
- The time elapsed since the last rainfall event greater than 0.1 inches. Historical rainfall data from rainfall gauges can be used to provide this information. If a gauge records only daily data, municipal field personnel could be asked to provide information on times during the day a rainfall event began or ended

5.3.4 Chemicals/Water Quality Parameters to be Measured

The storm water discharge samples must be analyzed for a number of pollutant parameters.

> **§122.26(d)(2)(iii)(A)(3)** For samples collected and described under paragraphs (d)(2)(iii)(A)(1) and (A)(2) of this section, quantitative data shall be provided for the organic pollutants listed in Table II, the pollutants listed in Table III (toxic metals, cyanide, and total phenols) of appendix D of 40 CFR part 122, and for the following pollutants:
>
> Total suspended solids (TSS)
> Total dissolved solids (TDS)
> COD
> BOD_5
> Oil and grease
> Fecal coliform
> Fecal streptococcus
> Ph
> Total Kjeldahl nitrogen
> Nitrate plus nitrite
> Dissolved phosphorus
> Total ammonia plus organic nitrogen
> Total phosphorus
>
> *[Note that total kjeldahl nitrogen is actually a substitute for total ammonia plus organic nitrogen.]*

The complete list of chemicals is provided in Exhibits 5-2, 5-3, and 5-4. Exhibits 5-2 and 5-3 are derived from 40 CFR Part 122, Appendix D, Tables II and III, respectively. Exhibit 5-4 comes from the text of the regulation (see box above). The EPA-approved analysis procedure for the pollutants in Exhibits 5-2 and 5-3 can be found in 40 CFR Part 136. If a municipality is seeking approval to use an alternative method of analysis, then a request should be made according to procedures outlined in 40 CFR 136.4.

Exhibit 5-2: Pollutants Listed in Table II in Appendix D of 40 CFR Part 122

Pollutant		Pollutant	
Volatiles		**Acid Compounds**	
Acrolein	1 2 Dichloropropane	2 Chlorophenol	
Acrylonitrile	1,3-Dichloropropylene	2,4-Dichlorophenol	
Benzene	Ethylbenzene	2,4-Dimethylphenol	
Bromoform	Methyl bromide	4,6-Dinitro-o-cresol	
Carbon tetrachloride	Methyl chloride	2,4-Dinitrophenol	
Chlorobenzene	Methylene chloride	2-Nitrophenol	
Chlorodibromomethane	1,1,2,2-Tetrachloroethane	4-Nitrophenol	
Chloroethane	Tetrachloroethylene	p-Chloro-m-cresol	
2 Chloroethylvinyl ether	Toluene	Pentachlorophenol	
Chloroform	1,2-trans-Dichloroethylene	Phenol	
Dichlorobromomethane	1,1,1-Trichloroethane	2,4,6-Trichlorophenol	
1,1 Dichloroethane	1,1,2-Trichloroethane		
1,2 Dichloroethane	Trichloroethylene		
1 1-Dichloroethylene	Vinyl chloride		
Base/Neutral		**Pesticides**	
Acenaphthene	Diethyl phthalate	Aldrin	Endrin
Acenaphthylene	Dimethyl phthalate	Alpha BHC	Endrin aldehyde
Anthracene	Di n-butyl phthalate	Beta BHC	Heptachlor
Benzidine	2 4-Dinitrotoluene	Gamma BHC	Heptachlor epoxide
Benzo(a)anthracene	2,6-dinitrotoluene	Delta-BHC	PCB-1242
Benzo(a)pyrene	Di-n-octyl phthalate	Chlordane	PCB-1254
3,4-Benzofluoranthene	1,2-diphenylhydrazine (as azobenzene)	4 4'-DDT	PCB-1221
Benzo(ghi)perylene		4 4 DDE	PCB-1232
Benzo(k)fluoranthene	Fluoranthene	4,4'-DDD	PCB-1248
Bis(2 chloroethoxy)methane	Fluorene	Dieldrin	PCB-1260
Bis(2-chloroethyl)ether	Hexachlorobenzene	Alpha-endosulfan	PCB 1016
Bis(2-chloroisopropyl)ether	Hexachlorobutadiene	Beta-endosulfan	Toxaphene
Bis(2-ethylhexyl)phthalate	Hexachlorocyclopentadiene	Endosulfan sulfate	
4-bromophenyl phenyl ether	Hexachloroethane		
Butylbenzyl phthalate	Indeno(1,2,3-cd)pyrene		
2-Chloronaphthalene	Isophorone		
4-Chlorophenyl phenyl ether	Naphthalene		
Chrysene	Nitrobenzene		
Dibenzo(a h)anthracene	N-nitrosodimethylamine		
1,2 Dichlorobenzene	N nitrosodi-n-propylamine		
1,3-Dichlorobenzene	N-nitrosodiphenylamine		
1,4-Dichlorobenzene	Phenanthrene		
3,3 -Dichlorobenzidine	Pyrene		
	1,2,4-trichlorobenzene		

Source 40 CFR Part 122 Appendix D

Exhibit 5-3: Pollutants Listed in Table III in Appendix D of 40 CFR Part 122

Pollutant	Pollutant	Pollutant
Antimony, total	Copper, total	Silver, total
Arsenic, total	Lead, total	Thallium, total
Beryllium, total	Mercury, total	Zinc, total
Cadmium, total	Nickel, total	Cyanide, total
Chromium, total	Selenium, total	Phenols, total

Source 40 CFR Part 122, Appendix D

Exhibit 5-4. Conventional Pollutants Listed in Section 122.26(d)(2)(iii)(A)(3)

Pollutant	Pollutant
Total suspended solids (TSS)	pH
Total dissolved solids (TDS)	Total Kjeldahl nitrogen (TKN)*
COD	Nitrate plus nitrite
BOD_5	Dissolved phosphorus
Oil and grease	Total ammonia plus organic nitrogen
Fecal coliform	Total phosphorus
Fecal streptococcus	

* Total ammonia plus organic nitrogen is interchangeable with TKN

Source 40 CFR 122.26(d)(2)(iii)(A)(3)

Section 122.21(g)(7) specifies that certain pollutant parameters will be analyzed on grab samples taken from the outfall, whereas the remainder of the pollutant parameters require that composite samples be taken from the outfall. These types of sampling procedures are differentiated as follows:

Grab samples: discrete, individual samples taken within a short period of time (usually less than 15 minutes). Analysis of grab samples characterizes the quality of a storm water discharge at a given time of the discharge. The following measurements must be made from grab samples:

- pH
- Temperature
- Cyanide
- Total phenols
- Residual chlorine
- Oil and grease
- Fecal coliform
- Fecal streptococcus

Note that measurements of temperature and pH must be taken in the field to avoid time-dependent changes that may occur between sampling time and actual analyses.

Flow-weighted composite samples: single unit volumes composed of a mixture of samples collected proportional to flow throughout the entire runoff event or at least for the first three hours of the storm water event, if it lasts more than three hours. The flow-weighted composite sample must consist of at least three discrete aliquots per hour from the storm water discharge, or a continuous sampler may be used.

All parameters (see Exhibits 5-2, 5-3, 5-4) not listed under the description of grab samples above must be analyzed from flow-

weighted composite samples Details on taking flow-weighted composite samples may be found in the EPA *Storm Water Sampling Guidance Document*.

5.3.5 Additional Quantitative Data

Section 122 26(d)(2)(iii)(A) concludes with a provision that allows the permitting authority to request additional quantitative data if necessary to determine permit conditions.

> §122 26 (d)(2)(iii)(A)(4) Additional limited quantitative data required by the Director for determining permit conditions (the Director may require that quantitative data shall be provided for additional parameters, and may establish sampling conditions such as the location, season of sample collection, form of precipitation (snow melt, rainfall) and other parameters necessary to insure representativeness),

To ensure the storm water discharge system is accurately represented, the permitting authority may require that quantitative data include additional parameters and may establish specific sampling conditions, such as:

- Location where the sample is taken;
- Season of sample collection;
- Form of precipitation (snowmelt, rainfall);
- Evidence of impact to aquatic ecosystems, or
- Other parameters necessary to ensure the system is accurately characterized.

The data generated from the qualitative and quantitative analyses described under §122.26 (d)(2)(iii)(A) will be used to calculate the annual pollutant loads and event mean concentrations for each pollutant as described in subsequent parts of this section Estimates of annual pollutant loads and event mean concentrations would then be used to assist in establishing storm water management priorities and selecting BMPs

5.4 ESTIMATION OF SYSTEM-WIDE EVENT MEAN CONCENTRATIONS AND ANNUAL POLLUTANT LOADS

The applicant must submit estimates of the event mean concentration and annual pollutant load of the cumulative discharges to waters of the United States from all identified municipal outfalls.

> §122.26(d)(2)(iii)(B) Estimates of the annual pollutant load of the cumulative discharges to waters of the United States from all identified municipal outfalls and the event mean concentration of the cumulative discharges to waters of the United States from all identified municipal outfalls during a storm event (as described under §122 21(g)(7)) for BOD_5, COD, TSS, dissolved solids, total nitrogen, total ammonia plus organic nitrogen, total phosphorus, dissolved phosphorus, cadmium, copper, lead, and zinc Estimates shall be accompanied by a description of the procedures for estimating constituent loads and concentrations, including any modelling, data analysis, and calculation methods,

Estimates of annual pollutant loads will be somewhat imprecise; however, municipalities should exercise best professional judgement in deriving these estimates A description of what assumptions were made to derive pollutant loadings must be included.

Under §122.26(d)(2)(iii)(B) (see box above) applicants must provide the following:

- Estimates for the event mean concentration for pollutants listed in Exhibit 5-5 below, which can be used to estimate the annual pollutant load associated with all municipal outfalls identified under §122 26(d)(1)(iii) and (d)(2)(ii);

- A description of the procedures for estimating constituent loads and concentrations, and
- Details on data analysis, models used, and calculation methods

Data sources and procedures that municipal applicants may use to estimate event mean concentrations and annual pollutant loads of the cumulative discharges are discussed below.

The primary purpose for estimating annual pollutant loads and event mean concentrations is to assign priorities for implementing BMPs. Municipalities should consider the magnitude of individual pollutant loadings when assigning priorities to resources to reduce these loadings. The areas receiving the highest priority for implementation of BMPs will be those portions of the MS4 that appear to contribute the largest load of pollutants to the system. Therefore, it is the relative value of these calculations that is of importance within this regulation, not the absolute value.

Over time the accuracy of the available methods to calculate loads and concentrations will improve and the use of these estimates may assume a larger role in determining permit conditions and estimating the success of the comprehensive municipal storm water management program. The emphasis for now, however, is on the application of the most practicable methods to reasonably estimate annual loads and event mean concentrations.

5.4.1 Data Sources

The Part 1 application requires municipalities to submit all existing storm water sampling data, along with all relevant water quality data, sediment data, fish tissue or other biosurvey data taken over the past 10 years. All historical data must accompanied by a narrative description of the watershed served by the outfall from which the data are obtained, a description of the sampling and quality control program, and the monitoring location of the receiving water.

To estimate an annual pollutant load for a given pollutant, a value must be derived for the average concentration, or event mean concentration, of that pollutant. To derive this value, applicants may use either site-specific data, or data from a national or regional study, such as NURP.

Municipalities with adequate historical data may choose to use these data to estimate annual pollutant loads in the Part 2 application. However, many applicants may not have enough site-specific data to develop valid estimates. These applicants may choose to use generic data (e.g., from regional and national studies), such as the data provided in the NURP study. The NURP study's estimated range of detected concentration for specific pollutants is summarized in Exhibit 5-6.

Exhibit 5-5: Pollutants for which Event Mean Concentrations and Annual Pollutant Loads Must be Calculated

Pollutant	Pollutant
BOD_5	Total phosphorus
COD	Dissolved phosphorus
TSS	Cadmium
Dissolved solids	Copper
Total nitrogen	Lead
Total ammonia plus organic nitrogen	Zinc

Source: 40 CFR 122.26(d)(2)(iii)(B) (55 FR 48070, November 16, 1990)

Exhibit 5-6. NURP Study Range of Detected Concentration for Specific Pollutants

Parameter	Concentrations µg/L
Metals and inorganics:	
Antimony	2.6 - 23
Arsenic	1 - 50.5
Beryllium	1 - 49
Cadmium	1 - 14
Chromium	1 - 90
Copper	1 - 100
Cyanides	2 - 300
Lead	4 - 23,000
Nickel	1 - 182
Selenium	0.2 - 0.8
Zinc	10 - 2400
Pesticides:	
Alpha-hexachlorocyclohexane (alpha-BHC)	0.027 - 0.10
Alpha-endosulfan	0.008 - 0.20
Chlordane	n/a
Lindane (gamma-BHC)	0.007 - 0.1
Halogenated aliphatics:	
Methane, dichloro-	5 - 14.5
Phenols and cresols:	
Phenol	1 - 13
Phenol, pentachloro-	1 - 115
Phenol, 4-nitro	1 - 37
Phthalate esters:	
Phthalate, bis(2-ethylhexyl)	4 - 62
Polycyclic aromatic hydrocarbons:	
Chrysene	0.6 - 10
Fluoranthene	0.3 - 2
Phenanthrene	0.3 - 10
Pyrene	0.3 - 16

Source. U.S. Environmental Protection Agency, *Results of the Nationwide Urban Runoff Program*, EPA Planning Division (National Technical Information Service (NTIS) Accession No. PB84-8552) December 1983

The applicant should be aware of limitations associated with data from national and regional studies before deciding on methods to estimate pollutant loadings In some cases, it may be more appropriate to use any available site-specific data rather than data from national or regional studies. For example, the NURP study did not collect pollutant concentration data from industrial areas. In this instance, even limited site specific concentration data from industrial areas may be more meaningful.

EPA encourages applicants to seek data from a variety of sources to better characterize the quality of their storm water discharges. Regardless of the data source, a description of the procedures for estimating constituent loads and concentrations, including any modeling, data analysis, and calculation methods, must be included

There will be a degree of uncertainty associated with estimating pollutant loadings in the Part 2 application The requirement to calculate pollutant loadings and concentrations is intended to be a planning and screening effort to assign program priorities, and not necessarily to determine absolute values.

5.4.2 Event Mean Concentrations

Event mean concentrations (C_i in Equation 1 on page 5-16) are determined from analyses of flow-weighted composite samples collected from each of the designated field screening points. Section 2.2.4 of the *Storm Water Sampling Guidance Document* describes procedures for collecting flow-weighted composite samples (EPA, 1992a). Concentration values must be reported in the applicant's Part 2 Permit Application for each representative storm event sampled. The applicant should report the average of these results as the event mean concentration for each parameter measured Municipalities are encouraged to present data in a tabular format However, the applicant has flexibility to present the data in other ways, provided the data is clearly presented.

As stated previously, applicants must sample storm events for at least three hours, or for the entire storm event if it lasts less than three hours If a storm event lasts more than three hours, the applicant may choose among three approaches for calculating the event mean concentration of the storm First, the applicant may report the event mean concentration for the first three hours of the event (or longer, if the applicant monitored more than three hours). Second, if the applicant has data available on the correlation between flow and concentration which allows it to be more specific about the event mean concentration, an estimation technique may be used to derive the event mean concentration. If the applicant uses such an estimation technique, the methodology must be explained Third and finally, the applicant may monitor the entire storm event and report the actual event mean concentration.

Whichever approach the applicant uses, the same method should be used to derive event mean concentrations in the future. This will assist the applicant in identifying meaningful trends in changes in event mean concentrations over time.

5.4.3 Annual Pollutant Loadings

Municipalities may choose from a variety of acceptable procedures for estimating the annual pollutant loads of the cumulative discharge. This guidance contains an example of calculating the annual pollutant loads using the "simple method," which is adapted from Schueler (1987). The guidance also discusses some dynamic models that applicants may wish to employ.

Regardless of which method applicants choose, they must describe and document the specific technique used. The description should include (but is not limited to) the key equations used to calculate reported values, such as.

- Assumptions for selecting site-specific parameters (e.g., runoff coefficients),

- References to any source documentation (e g , previously completed studies or reference textbooks), and

- Justification for any assumed parameter values

The Simple Method

The following method of computing pollutant loadings is referred to as the "simple method" and is adapted from Schueler (1987). For purposes of satisfying Part 2 application requirements, the simple method provides a quick and reasonable estimate of pollutant loadings with a minimal amount of data required Although the regulations require a system wide (cumulative) annual pollutant load calculation for each of the pollutants listed in Exhibit 5-5 (above), the single pollutant load values provide limited insights into potential problem areas and what BMPs might yield the best results. Consequently, the municipality may want to consider using the simple method to estimate "individual" pollutant loadings from drainage areas The individual pollutant loadings can be aggregated to derive a cumulative annual pollutant loading for the entire MS4 In the procedure below, for example, Step 1 computes the annual loading for each outfall of the MS4 Then in Step 2, the resulting pollutant loadings are summed to derive annual pollutant loads on a per-watershed basis In Step 3, the annual pollutants loads for each watershed are summed to derive a system-wide annual pollutant load.

As stated above, this procedure is only one example of how a municipality could calculate a system-wide annual pollutant load. Estimates of annual pollutant loads for individual outfalls, watersheds, or other discrete areas are not specifically required by the regulations However, municipalities will find such estimates helpful in making relative comparisons among different areas of the MS4 Ultimately, these estimates could assist the municipality with selecting BMPs and assigning priorities to potential problem areas

Step 1: Use the Simple Method to Calculate Annual Pollutant Loads on a Per-Outfall Basis

The first step in this example is to calculate annual pollutant loads for individual outfalls. However, the applicant may choose to begin by calculating annual pollutant loads for each watershed or other discrete area. As stated above, this example uses the simple method, which is given by the following equation:

EQUATION 1:

$$L_i = \left[\frac{(P)(CF)(Rv_i)}{12}\right](C_i)(A_i)(2.72)$$

where: L_i = Annual pollutant load (lb/outfall/yr)
P = Annual precipitation (in/yr)
CF = Correction factor that adjusts for storms where no runoff occurs (a value of 0 9 is typically used)
Rv_i = Weighted-average runoff coefficient for the area served by the outfall (the calculation of runoff coefficients is discussed below)
C_i = Event mean concentration of pollutant (mg/L)
A_i = Catchment area (acres)

The numbers 12 and 2.72 are conversion factors that account for unit conversions.

Each of the parameters in Equation 1 is defined below:

- **Annual pollutant load** is the total amount of a specific pollutant discharged in pounds per time period (in this case, per year) for the particular segment of the MS4 being modeled (in this case for each outfall) Pollutant loads may also be expressed for alternative time periods, or on a system-wide or watershed basis

- **Annual precipitation** is the total inches of rainfall occurring in a single year plus the contribution of snowmelt Estimates of the annual rainfall can be based on the rainfall data provided in Part 1 of the application

- **Correction factor** is an adjustment factor for the number of storm events that do not actually produce any runoff (i.e., the percentage of storm events that have a total accumulation greater than a specific threshold value). This value will vary by region. Without this adjustment factor, the municipality would be assuming that all storm events produce runoff, which may or may not be the case. A typical value for this correction factor is 0.9 (90%). However, this value can vary between climatic regions. Municipalities should review historical rainfall data to estimate the percentage of storm events that produce runoff versus the number of storm events per year.

- **Weighted-average runoff coefficient** is a relative measure of imperviousness or the percentage of rainfall that becomes surface runoff Runoff coefficients are a function of the type of surface, intensity of the rainfall, the degree of soil saturation and storativity (storage capacity) of the soil. To determine runoff coefficients, the municipality may use Equations 2 or 3 (which follow). Alternatively, the municipality may use actual field measurements, relevant hydrologic studies, average values published in civil engineering reference manuals, or default values provided in Exhibit 3-12 of EPA's *NPDES Storm Water Sampling Guidance Document*

- **Event mean concentration of pollutant** is the event mean concentration value for the specific pollutant determined from the analysis of flow-weighted composite samples. Equation 1 requires a value for each pollutant concentration As discussed previously, the applicant may use site-specific concentration data (e g , storm water sampling data) or generic (e g , NURP) data to derive event mean concentrations In other words, the applicant should use best professional judgement to decide which of the following concentration values to use

  – a mean concentration value from the NURP study;

  OR

  – an average of all event mean concentrations from all samples over three representative storm events;

  OR

  – an event mean concentration attributable to a specific land use activity

  The applicant will have to consider the extent of the variability of the data when selecting an appropriate concentration value. NURP or other regional studies used to estimate pollutant concentrations can be compared to existing site-specific data in order to assess the uncertainty associated with generic approaches.

- **Catchment area** is the size of the drainage area for the particular segment of the MS4 being modeled (in this case, the outfall drainage area). Areas that are served by combined sewers or that are not otherwise served by the MS4 should not be included

Weighted-average runoff coefficient. Runoff coefficients can be based on flow measurements or estimated from land use characteristics. In order to determine an average runoff coefficient for an area with a diversity of land

use activities, the following equation should be used to estimate a weighted-average runoff coefficient

EQUATION 2

$$Rv_i = \frac{(\sum A_i R_v)}{\sum A_i}$$

where. Rv_i = Weighted-average runoff coefficient
A_i = Catchment area (acres)
R_v = Catchment runoff coefficient

As an alternative to Equation 2, Equation 3 can be used to estimate weighted-average runoff coefficients from percent imperviousness data (Shelley, 1986)

EQUATION 3

$$Rv_i = 0.05 + 0.009 * I$$

where. Rv_i = Weighted-average runoff coefficient
I = Percent imperviousness

The percent imperviousness can be estimated from land use data Residential land can be assumed to be 24% impervious, commercial land 75% impervious; industrial land 55% impervious; and open space 15% impervious The percent imperviousness of residential land was estimated from the following empirical equation of NURP and USGS data, which relates population density to percent imperviousness

EQUATION 4

$$I = 9 * D^{0.5}$$

where I = Percent imperviousness
D = Population density (persons/acre)

Similar to Equation 1, individual parameters for Equations 2, 3, and 4 can be used on a system-wide basis, or modified to reflect more realistic conditions within smaller or discrete segments (e g , individual watersheds or outfalls).

Step 2. Use the Per-Outfall Annual Pollutant Loads to Calculate Per-Watershed Annual Pollutant Loads

If the simple method is used to compute the annual loading on a per-outfall basis, Equation 5 may be used to estimate annual pollutant loadings on a per watershed basis. The approach of computing pollutant loadings on a watershed basis is used by some counties where larger watersheds are segregated into smaller watersheds or drainage areas on the basis of similar land use designations One county uses this method in conjunction with forecasts of future development within the county to develop preliminary estimates of future pollutant loadings This approach minimizes the possibility of computing an annual pollutant loading that is too conservative.

EQUATION 5

$$L_w = \sum L_i$$

where: L_w = Annual pollutant load for a particular watershed
$\sum L_i$ = Summation of individual annual pollutant loadings from all major outfalls within a specific watershed

Step 3: Use the Watershed-Based Annual Pollutant Loads to Calculate System-Wide Annual Pollutant Loads

To calculate the annual loadings system-wide, use the following equation

EQUATION 6

$$L_n = \sum L_w$$

where L_n = Annual pollutant load for an entire MS4

ΣL_w = Summation of individual annual pollutant loadings from all watersheds within a municipal separate storm sewer system

Dynamic Models

In instances where a municipality has a significant amount of historical data for the drainage areas serviced by storm sewer outfalls, including historical precipitation data and receiving water concentration and flow data, the MS4 may elect to use dynamic models to derive pollutant loads and to analyze the effects of MS4 discharges on receiving waters.

Dynamic models are designed to calculate a complete probability distribution for the output being modeled Therefore, dynamic models take into consideration the inherent variability of data associated with MS4 discharges, such as variations in concentration, flow rate, and runoff volume

One benefit of using a dynamic model is that the calculation of a complete probability distribution allows the modeler to consider a multitude of "what-if" scenarios. For example, when sufficient historical data is available, the modeler could consider the benefits and risks associated with alternative BMP strategies.

Dynamic models have one additional benefit over steady-state models in that dynamic models determine the entire discharge concentration frequency distribution. Consequently, this would enable the modeler to examine the effects of storm water discharges on receiving water quality in terms of the frequency by which water quality standards may be exceeded For purposes of computing pollutant loadings, a number of models are available including EPA's Stormwater Management Model (SWMM) and Hydrologic Simulation Program (HSPF), U S Army Corps of Engineers' Storage, Treatment, Overflow, Runoff Model (STORM), and Illinois State Water Survey's Model QILLUDAS (or Auto-QI).

Regardless of the method employed, the applicant must document how pollutant loadings are derived Applicants must provide estimates of annual pollutant loads and event mean concentrations for each outfall with their Part 2 applications. However, some outfalls will need to be more completely characterized, and conditions will change after the permit is approved. This is one reason why, as described in Section 5.4, data collection will continue throughout the term of the permit. Estimates of the individual contribution of pollutant loadings for each watershed or major outfall will help the applicant select priorities for specific watersheds.

5.5 PROPOSED SCHEDULE FOR SEASONAL LOADS AND REPRESENTATIVE EVENT MEAN CONCENTRATIONS OF MAJOR OUTFALLS

> **§122.26(d)(2)(iii)(C)** A proposed schedule to provide estimates for each major outfall identified in either paragraph (d)(2)(ii) or (d)(1)(iii)(B)(*1*) of this section of the seasonal pollutant load and of the event mean concentration of a representative storm for any constituent detected in any sample required under paragraph (d)(2)(iii)(A) of this section;

Seasonal pollutant loads are important because they are a more accurate representation of loadings that may occur during a short time interval. To further refine the annual pollutant load estimates, Part 2 requires the applicant to propose a schedule to estimate seasonal

pollutant loadings and event mean concentrations for each major outfall

The quality of the data available when the Part 2 application is prepared will affect the accuracy and usefulness of the initial estimates of pollutant loadings and average concentrations These estimates can be improved as more site-specific data are collected during the term of the permit. A long-term site specific monitoring program will capture the variability in data that is essential to estimate more accurate pollutant loadings over time. Therefore, the impacts associated with these loadings can also be estimated with greater certainty. In addition, a site specific record collected over a longer time frame allows the effectiveness of the comprehensive municipal storm water management program to be evaluated

Estimates must be submitted for any contaminant detected in any sample required under the Part 2 sampling effort [§122.26(d)(2)(iii)(B)]. Seasonal pollutant load estimates are required for any pollutants listed in Exhibits 5-2, 5-3, and 5-4 that were detected during the sampling procedure described in Section 5 3.4. Therefore, the analyses required for seasonal pollutant loads will potentially be more comprehensive than the analyses of annual pollutant loads This results from the possibility that additional pollutants will be detected as part of the storm water characterization studies.

In some regions, precipitation patterns vary significantly from season to season, resulting in significantly different pollutant loadings throughout the year. In arid and semi-arid parts of the country, pollutants accumulate during dry spells, resulting in significantly higher pollutant concentrations in storm water discharges after extended dry weather Because of the buildup of accumulated pollutants, pollutant concentrations in discharges from MS4s are typically highest during the "first flush," or initial discharge

In other regions, pollutants that accumulate in snow may lead to high pollutant concentrations in runoff from the spring thaw Therefore, using an annual average pollutant loading might disguise the impact of shock loadings (discharges that occur within a very short time period and which often exceed acute toxicity criteria) of certain pollutants. Numerous factors contribute to the total volume of snowmelt runoff including shortwave and longwave radiation, condensation or vaporization, convected heat transfer by wind, heat content of rain water, and conductive heat transfer from the ground. Therefore, for regions with significant snowfall, pollutant loading estimates need to be adjusted to account for the additional volume of runoff attributable to snowmelt.

Since snowmelt runoff can occur in either the presence or absence of a storm event, the computation of seasonal pollutant loadings becomes significantly more complex The determination of total snowmelt runoff, however, is beyond the scope of this manual. Affected municipalities are encouraged to contact the U.S. Geological Survey or the Army Corps of Engineers for historical data on snowmelt runoff.

The effects of pollutant load can also vary by season Nutrient pollutant loads from storm water discharges can promote algal blooms in receiving waters during the spring and summer, but they may be of little consequence during winter in surface waters with good flushing characteristics. Quantifying seasonal variations in pollutant loads may aid the development of more cost-effective storm water management programs.

Pollutant loads also may vary significantly from one outfall to another. Within a drainage area, the type of land use, the percent of surface that is impervious, and the extent of exposure of storm water to contaminants affect the pollutant load from an outfall. Procedures for estimating seasonal pollutant loadings must be proposed for major outfalls only

Under §122.26(d)(2)(iii)(C) the regulation requires a schedule to provide estimates of:

- The seasonal pollutant load for each identified major outfall.
- The event mean concentration of a representative storm for any constituent detected in any sample required.

The following steps can be taken to develop a proposed schedule for estimating seasonal loadings at major outfalls:

1. Use historical or long-term hydrologic data to define seasons.
2. Describe the procedure to be used to estimate seasonal loads. This could be an adaption of the simple method or another mathematical model used for annual loads (e.g., instead of using a total annual rainfall accumulation, use an average rainfall accumulation associated with a specific season). If the simple method is used, the municipality could still use Equation 1. However, the amount of rainfall (P) would no longer be an annual value. Instead, it would be the amount of rainfall associated with a particular season defined by the municipality. In addition, the applicant may have to adjust the average runoff coefficient to reflect seasonal changes (e.g., frozen ground can behave like an impervious surface and substantially increase the amount of runoff). Lastly, substantial differences in the frequency and duration of seasonal storm events may increase or decrease the correction factor CF (e.g., during a dry season, the number of storms that actually produce runoff may be substantially lower than during a wet weather season).
3. Identify data elements that need to be refined. In cases where there is substantial seasonal variation, revised runoff coefficient values may be necessary. For example, during rainy seasons, ground surfaces are more saturated than during the dry season. As a result, the same amount of rainfall in the wet season will lead to a greater volume of storm water runoff than in the dry season.
4. Proposed procedures for collecting the appropriate data or otherwise improving estimates.
5. Provide an approximate time frame for data collection and submission of seasonal load estimates.

Proposed procedures for estimating seasonal pollutant loadings and event mean concentrations should explain when and how data used for the estimates will be obtained. The data can be based on site-specific information, or they can be obtained from municipal systems with similar characteristics (such as Regional NURP data).

5.6 COLLECTION OF REPRESENTATIVE DATA FOR PROPOSED MONITORING PROGRAM FOR THE TERM OF THE PERMIT

Under §122.26(d)(2)(iii)(D), applicants are given the opportunity to propose monitoring programs to be carried out during the term of the permit.

> **§122.26(d)(2)(iii)(D)** A proposed monitoring program for representative data collection for the term of the permit that describes the location of outfalls or field screening points to be sampled (or the location of instream stations), why the location is representative, the frequency of sampling, parameters to be sampled, and a description of sampling equipment.

Applicants should consider their specific needs and identify priorities for the proposed

monitoring program After receiving the Part 2 application, the permitting authority will review proposed monitoring programs and make appropriate adjustments when establishing permit conditions

The applicant must propose a monitoring program for representative data collection for the term of the permit that describes:

- The location of outfalls or field screening points to be sampled (or the location of instream stations);
- Why the location is representative;
- The frequency of sampling;
- Parameters to be sampled, and
- A description of sampling equipment.

Municipalities must submit sampling data over the life of a permit so that changes in storm water quality can be assessed Like initial sampling data, the data from an ongoing monitoring program can be used by the municipality to allocate resources to achieve reduction in pollutants The monitoring data will also serve as an environmental indicator of the success of the storm water management program. Many municipalities may require an extended period of time (possibly the entire permit term) and substantial data to definitively evaluate the effectiveness of a storm water management program. Therefore, a plan for data collection must be proposed by the municipality for the five-year term of the permit. During the permit term, the results of the monitoring program will be submitted in the municipality's annual report [§122.42(c)(4), discussed in Section 7.3 of this guidance].

5.6.1 Goals of a Monitoring Program

The first and most important step in developing a proposed monitoring program is to define the program's objectives as clearly as possible Development of monitoring program goals should be closely coordinated with development of the proposed storm water management program Applicants are required to propose monitoring programs as part of their proposed management programs to reduce pollutants from industrial site runoff. The monitoring plan is part of *Characterization Data* [§122 26(d)(2)(iii)]. The storm water management program is discussed in Section 6.

A comprehensive monitoring program should be designed to support specific goals, including:

- Characterizing discharges;
- Evaluating the source of specific pollutants,
- Evaluating the performance of specific source controls; and
- Identifying the full range of chemical, physical, and biological water quality impacts.

5.6.1.1 Characterizing Discharges

Monitoring pollutants in discharges from MS4s serves several purposes. Quantitative data on specific pollutants in storm water runoff can support estimates of annual and seasonal pollutant loadings and modelling efforts to identify the magnitude of water quality impacts. Over the long term, monitoring data may suggest that new outfalls should be selected for sampling As municipalities gain experience in storm water sampling, they likely will target BMPs that achieve the greatest improvements in storm water quality.

5.6.1.2 Evaluating the Source(s) of Specific Pollutants

Some sources of storm water (e.g., industrial sources that must be covered by NPDES permits, highways with heavy traffic flows, and large parking lots) are expected to generate significantly higher concentrations of pollutants than typical urban runoff. Monitoring efforts to quantify sources of

priority pollutants can provide support for resource allocations to address pollutant sources posing the greatest environmental risk How proposed monitoring efforts will be structured to identify and quantify pollutant sources should be discussed in proposed storm water management programs.

The monitoring program may also include procedures to conduct dry-weather monitoring over the term of the permit to help detect illicit discharges and improper dumping. This can include recording visual observations and odors observed in dry weather flows.

5.6.1.3 Evaluating the Performance of Specific Controls

Pollutant removal efficiencies are fairly well known for certain structural BMPs. However, sampling may still be necessary to ensure that the BMP is meeting original design expectations The expected pollutant removal efficiency for a structural control must take into account site-specific conditions For example, an infiltration basin has a certain expected pollutant removal efficiency, but actual field efficiency is affected by subsurface soil conditions and the extent and frequency of maintenance

The efficiency of a particular structural control will be affected by many factors, such as detention time. However, efforts to determine the efficiency of structural controls must include consideration of pollutant concentrations and flow volumes into and out of the control. The efficiency of nonstructural source controls can be characterized by comparing discharges at a given location before and after the control measures are implemented. Over time, sufficient monitoring data may be gathered to draw substantive conclusions about the effectiveness of certain BMPs. Alternatively, discharges from a sampling site with source controls can be compared with discharges from a similar site that lacks source controls Efforts to monitor the effectiveness of controls should be closely coordinated with the assessment of control efficiencies discussed in Chapter 7

5.6.1.4 Identifying the Full Range of Chemical, Physical, and Biological Water Quality Impacts

Characterizing the effect of storm water discharges on water quality is complicated by a number of factors. EPA recommends an integrated approach to assessing water quality impacts associated with discharges from MS4s Monitoring procedures that help assess water quality impacts include:

- Discharge and receiving water monitoring to support water quality models and to identify hydraulic impacts of increased peak flows and to identify parameters of concern, and
- In-stream monitoring of water chemistry;
- Bioassessments and biosurveys; and
- Sediment sampling

Discharge and Receiving Water Monitoring to Support Water Quality Models

As discussed above, when there is sufficient historical data available from monitoring, these data may be used as inputs to models that predict or validate the effects of pollutant loadings from MS4s on receiving water quality characteristics. In addition to monitoring data, data on receiving water quality characteristics are also necessary to calibrate a particular model.

Once the model has been calibrated to reflect site-specific conditions, future monitoring data could be used to validate long term reductions in pollutant loadings, the effectiveness of nonstructural BMPs, and/or pollutant removal efficiencies of existing structural controls

The information gathered from this approach may also help define those BMPs that which appear to be the most effective. For example, in developing areas, monitoring data could eventually support future planning efforts that would seek to minimize the impact of future development on local receiving waters.

In-stream Monitoring

Using models to estimate pollutant concentrations in receiving waters can be inaccurate In-stream monitoring can directly measure pollutant concentrations. General designs for in-stream monitoring are:

- **Monitoring above and below a set location** This method is generally more useful for evaluating control effectiveness than documenting the severity of a diffuse source of pollutants

- **Monitoring at different times** Monitoring at different times and seasons can provide valuable information on seasonal variations in pollutant concentrations Dry weather in-stream monitoring can be compared with in-stream monitoring during storm events

- **Paired watersheds.** Evaluating similar water bodies can document management program improvements by controlling for meteorologic and hydrologic variability. This approach can also be used to compare receiving waters to background conditions associated with undeveloped watersheds

Detailed guidance on applying these approaches is provided in the draft *Nonpoint Source Monitoring and Evaluation Guide*, February 26, 1988, Nonpoint Source Branch, U S EPA

Bioassessments and Biosurveys

A biological assessment, or "bioassessment," is an evaluation of the biological condition of a water body using biological surveys and other direct measurements of resident biota in surface waters. A biological survey or "biosurvey," consists of collecting, processing, and analyzing representative portions of a resident aquatic community to determine the community structure and function Biosurveys and bioassessments can be used directly to evaluate the overall biological integrity (structure and/or functional characteristics) of an aquatic community Deviations from the biological integrity can be measured directly using biosurveys only when the impacted community is compared against a predetermined reference condition. Without the proper reference conditions, biosurveys may underestimate the extent of impairment.

Biosurveys are useful in that they can assess or detect the aggregate effect of impacts upon an aquatic community where discharges are multiple, complex, and variable, and where point, nonpoint, and storm water discharges are all affecting the biological condition of the receiving water Because of this, biosurveys cannot measure the impacts of one particular discharge or effluent being discharged to receiving waters. Currently, biosurveys cannot be used as a predictive water quality assessment tools.

Biosurveys provide a useful monitor of both aggregate ecological impact and historical trends in the condition of an aquatic ecosystem They can also detect impacts that other assessment methods may miss. More importantly, biosurveys can detect impacts caused by habitat degradation such as channelization, sedimentation, and historical contamination that disrupt the interactive balance of the components of the aquatic community

Sediment Sampling

Pollutants, both organic and inorganic, associated with storm water discharges may become physically or chemically bound with sediment particles Depending upon the size distribution of the sediment particles, a portion of the contaminated sediment particles will settle out of the water column Consequently, the potential exists for a buildup of contaminated sediment over time. The effects of heavily contaminated sediments on both benthic habitat and water quality have been documented to the extent that EPA is developing sediment quality criteria (SQC) that will allow assessments of the toxicological effects of contaminated sediments on varying types of receiving waters.

The amount of sediment material found in storm water discharges suggests that applying sediment quality criteria could be a useful component of a monitoring program. For example, sediment quality criteria could be a valuable preventative tool to ensure that point source discharges of storm water do not cause or contribute to the contamination of sediments

In addition, a MS4 could make comparisons of field measurements to sediment quality criteria as a means of providing an early warning of a potential problem. Consequently, an early warning could provide an opportunity to take corrective action to prevent further contamination For long term planning, consideration could also be given to the feasibility of establishing target levels or goals that would ensure that point sources discharges of storm water do not contribute to sediment contamination

5.6.2 Monitoring Procedures

Monitoring procedures will depend on the objectives of the monitoring effort To a large extent, the type of receiving water will be an important factor in developing monitoring procedures and techniques. For example, grab samples may be appropriate for monitoring discharges from a retention pond, while composite samples may be appropriate for monitoring flows into the pond The following information, at a minimum, should be included for each sampling site.

- The criteria for storm selection,
- Whether grab, composite, continuous, or other sampling techniques are to be used,
- The criteria on when to begin and end sample collection;
- The basis for selecting the time interval between sequentially collected samples,
- How seasonal factors affect the selection of monitoring frequencies,
- The method of estimating rates or volumes of flow passing the sampling point, and
- The analytical methods used for analyzing pollutant parameters and their detection limits

Location of Monitoring Sites and Description of Drainage Basins

The selection of monitoring sites should depend on the goals of the monitoring program. Applicants should identify the location of each proposed monitoring site and the boundary of its drainage basin. They should describe the estimated size and land use characteristics of the drainage basin for each sampling location The applicant also should explain why the sampling sites are representative or will otherwise provide information to support a monitoring program goal. Other monitoring sites can be selected to evaluate unique conditions in the drainage area that have significant or unusual potential for generating pollutants in storm water discharges

Samples should be analyzed in accordance with the analytical methods approved under 40 CFR Part 136

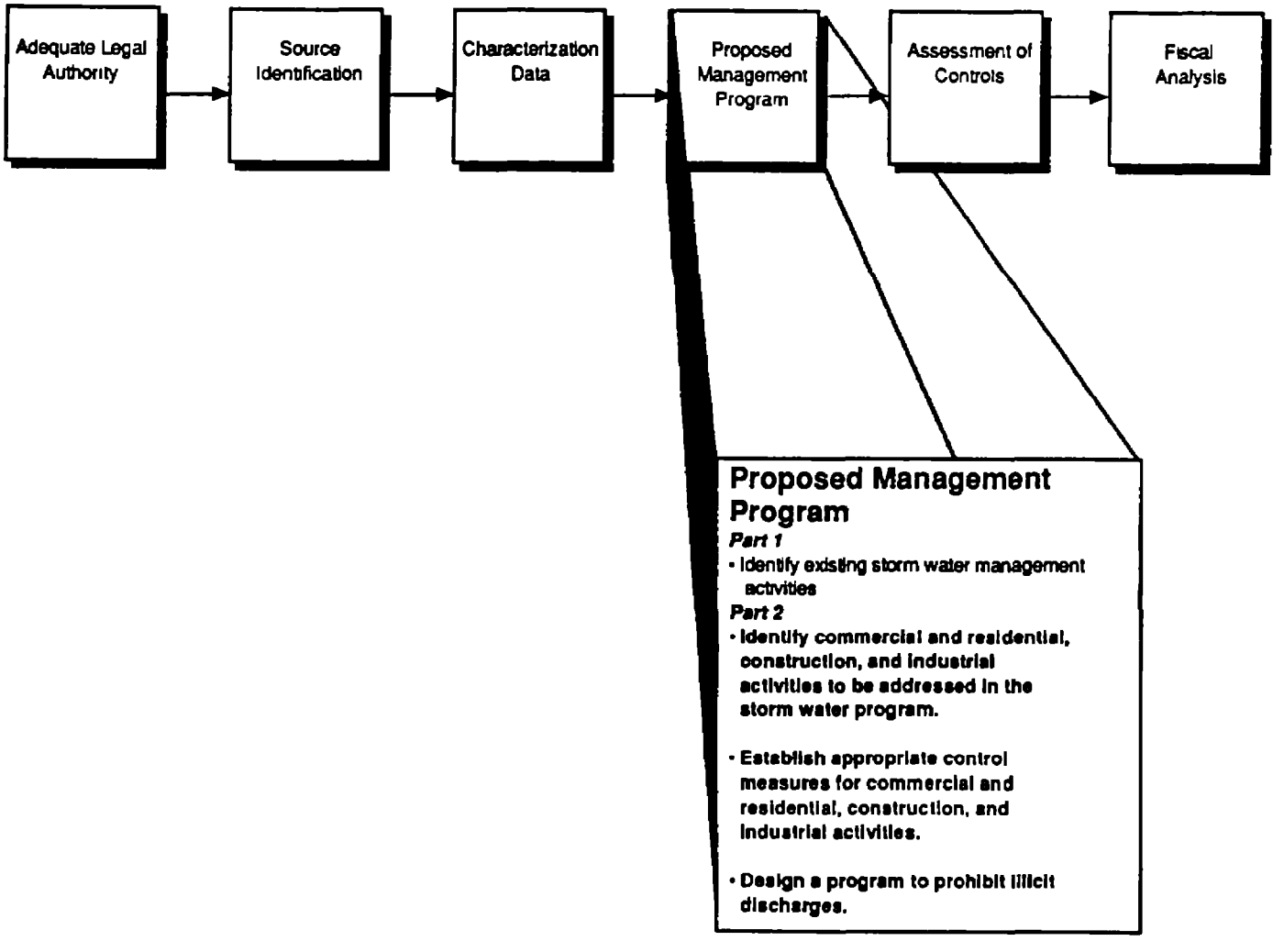
Parameters to be Analyzed

The applicant must list all parameters to be analyzed, which should depend on the objective of the sampling effort. For example, it may only be necessary to monitor several indicator parameters (such as TSS, settleable solids, nutrient, and a metal) to characterize the pollutant removal efficiency of a wet pond.

Sampling Equipment

The applicant must describe the equipment to be used in the proposed sampling program. Only the primary pieces of equipment need be identified. Descriptions can be made by reference to equipment supplied by a vendor or manufacturer if distinctive enough to be readily identified.

CHAPTER 6
PROPOSED MANAGEMENT PROGRAM



6.0 PROPOSED MANAGEMENT PROGRAM

6.1 BACKGROUND

Under the Part 2 application requirements, municipalities must propose site-specific storm water management programs. This is the most important aspect of the permit application. The Part 2 application requirements provide each MS4 with the flexibility to design a program that best suits its site-specific factors and priorities.

The regulations require the applicant to provide a description of the range of control measures considered for implementation during the term of the permit. Applicants must meet all the requirements of the Part 2 application regulation. However, flexibility in developing permit conditions is encouraged by allowing municipalities to emphasize the controls that best apply to their MS4. For example, a municipality that expects significant new development may focus more on requirements for new development and construction, while a municipality that does not expect significant new development may focus more on a program to prohibit illicit discharges or control industrial contributions. In any case, a satisfactory proposed management program will address· management practices; control techniques and systems; design and engineering methods, and other measures to ensure the reduction of pollutants to the "maximum extent practicable (MEP)."

If the municipality proposes a thorough and complete program, the permitting authority is likely to incorporate all or part of the proposed management program into the NPDES storm water permit written for that municipality Therefore, the proposed programs provide municipalities with the opportunity to have substantial input into their NPDES permit conditions.

This section of the guidance manual describes the minimum information requirements for proposed storm water management programs. Examples of how the program elements should be addressed are provided. These examples illustrate minimum information requirements for the program elements, and occasions when municipalities may opt to go beyond minimum requirements in order to meet the MEP standard

6.2 SUMMARY OF REGULATORY REQUIREMENTS

The municipality must develop and submit a proposed management program that covers the duration of the permit The program must integrate the information and actions described in the Part 1 application and portions of the Part 2 application (see Chapters 3, 4, and 5 of this guidance). The regulatory requirements for the proposed management program are in 40 CFR 122.26(d)(2)(iv)

At a minimum, the proposed management program must include:

- A comprehensive planning process that involves both public participation and intergovernmental coordination;
- A description of management practices, control techniques, and system design and engineering methods to reduce the discharge of pollutants to the MEP; and
- A description of staff and equipment available to set up and assess the storm water management program.

Additional provisions under §122 26(d)(2)(iv)(A) require applicants to include:

- Programs to control storm water runoff from commercial and residential areas, construction sites, and industrial

facilities (including waste handling sites), (Section 6 3),

- Identification of structural control measures to be included in these proposed programs, such as detention controls, infiltration controls, and filtration controls that the municipality plans to apply to the activities addressed in its storm water management program (Section 6.4); and

- Programs to detect and remove illicit discharges, and to control and prevent improper disposal into the MS4 of materials such as used oil or seepage from municipal sanitary sewers (Section 6.5).

6.3 PROGRAMS TO CONTROL STORM WATER RUNOFF FROM COMMERCIAL AND RESIDENTIAL AREAS, CONSTRUCTION SITES, AND INDUSTRIAL FACILITIES

A proposed management program must identify the activities or areas that require controls to reduce pollutants in storm water runoff Specifically, a proposed management program must address storm water runoff from **commercial and residential areas** (Section 6.3.1), **construction sites** (Section 6.3.2), and **industrial facilities** (Section 6.3 3). Also, areas where illicit connections or illegal discharges may occur must be identified (Section 6.5).

In addition to the requirements of the proposed storm water management program, other provisions of the Part 1 and Part 2 applications require information that will help enable the municipality to focus on identifying activities and areas that may need control measures Examples of these provisions include

- Identification of sources [Part 1, §122 2(d)(1)(iii)(B)(3)-(4), and Part 2, §122 26(d)(2)(ii)];

- Identification of water bodies that may be adversely affected by storm water runoff [Part 1, §122 26(d)(1)(iv)(C)],

- Organization of sources by watershed [Part 2, §122 26(d)(2)(ii)],

- Description of land use activities [Part 1, §122 26(d)(1)(iii)(B)(2)];

- Results of field screening analysis [Part 1, §122 26(d)(1)(iv)(D)];

- Results of the sampling program [Part 2, §122 26(d)(2)(iii)(A)(3)],

- Estimates of annual pollutant loads and event mean concentrations, and schedules to submit seasonal pollutant loads and event mean concentrations [Part 2, §122.26(d)(2)(iii)(B) and (C)], and

- Findings from an on-going monitoring program [Part 2, §122 26(d)(2)(iii)(D)].

6.3.1 Commercial and Residential Activities

Under §122.26(d)(2)(iv)(A), applicants must propose structural and source control measures to reduce pollutants from commercial and residential areas.

> **§122.26(d)(2)(iv)(A)** [The proposed management program must include a] description of structural and source control measures to reduce pollutants from runoff from commercial and residential areas that are discharged from the municipal storm sewer system that are to be implemented during the life of the permit, accompanied with an estimate of the expected reduction of pollutant loads and a proposed schedule for implementing such controls

To ensure that proposed control measures are effective, the applicant should study how storm water runoff from pollutant sources affects the existing municipal system, how the proposed

control measures will enhance the existing system, and what impact the proposed measures will have on receiving waters The control measures should recognize and emphasize the interaction between pollutant sources and the physical attributes of the municipal system and receiving waters.

Specific commercial and residential activities that must be addressed include maintenance activities and a maintenance schedule for structural controls to reduce pollutants in storm water runoff. This provision is discussed in Section 6.4.2. Other activities to be addressed include:

- Post-construction controls to reduce pollutants in discharges to MS4s resulting from new development and significant redevelopment (Section 6 3 1 1),
- Practices for maintaining and operating public streets, roads, and highways that will reduce the impact on receiving waters from storm water runoff discharges (Section 6 3 1 2);
- Procedures to assure that the impacts on receiving waters from flood management projects are assessed, and that existing structural control devices have been evaluated to determine if retrofit controls are feasible (Section 6 3.1.3);
- A program to monitor pollutants in runoff from operating or closed municipal landfills that identifies priorities and procedures for inspections and establishing and implementing control measures (Section 6.3 1.4); and
- A program to reduce to the maximum extent practicable, pollutants in storm water runoff associated with the application of pesticides, herbicides, and fertilizer (Section 6 3.1.5).

To reduce pollutants in storm water runoff from commercial and residential activities, a proposed management program might include the use of infiltration devices, detention and retention basins, vegetated swales, water quality inlets (which may include oil and water or oil/grit separators), screens, channel stabilization/riparian habitat enhancement efforts, wetland restoration and preservation projects, as well as various source control strategies and other nonstructural control measures

6.3.1.1 New Development and Significant Redevelopment

Summary of Regulatory Requirement

New development or redevelopment often increases impervious land surfaces, which usually leads to increased pollutant levels in storm water runoff Chemical and thermal changes in storm water runoff are commonly associated with new development and can adversely affect the quality of receiving waters In addition, urbanization results in an increase in the volume of storm water discharges.

The Nationwide Urban Runoff Program (NURP) study (EPA, 1983) and more recent investigations indicate that controlling the contribution of pollutants in storm water discharges at the onset of land development is the most cost-effective approach to storm water quality management Mitigating problems caused by pollutants after they have entered a MS4 is often more expensive and less efficient than preventing or reducing the discharge of pollutants at the source Therefore, a satisfactory proposed management program will propose structural and nonstructural measures to reduce pollutants in storm water discharges from areas of new development and redevelopment Examples of such measures are discussed below

§122.26(d)(2)(iv)(A)(2) [The applicant must include a] description of planning procedures including a comprehensive master plan to develop, implement and enforce controls to reduce the discharge of pollutants from municipal separate storm sewers which receive discharges from areas of new development and significant redevelopment Such plan shall address controls to reduce pollutants in discharges from municipal separate storm sewers after construction is completed

Provisions under §122.26(d)(2)(iv)(A)(2) focus on the reduction of pollutants in storm water runoff **after** construction in areas where new development or redevelopment is completed Controls that are required **during** construction are discussed in Section 6.3 2 of this guidance

Post-Construction Controls

Proposed storm water management programs should include planning procedures for both during and after construction to implement control measures to ensure that pollution is reduced to the maximum extent practicable in areas of new development and redevelopment. Design criteria and performance standards may be used to assist in meeting this objective

Further, storm water management program goals should be reviewed during planning processes that guide development to appropriate locations and steer intensive land uses away from sensitive environmental areas A municipality may, for example, include provisions in the planning process that ensure that all new development in targeted areas or zones provides for a certain percentage of undisturbed area to assist in preserving post-development runoff quality and velocity as similar as possible to pre-development conditions In its Part 2 application, a municipality should describe how it plans to implement the proposed standards (e g , through an ordinance requiring approval of storm water management programs, a review and approval process, and adequate enforcement)

The proposed storm water management program should identify and include **planning procedures** and **control measures** that will be used in the municipality.

Planning Procedures

Comprehensive planning procedures typically involve incorporation of land use goals and objectives into a plan document or a plan map. These plans are often called Master Plans, Comprehensive Land Use Plans, or Comprehensive Zoning Plans

Comprehensive or master plans are often non-binding. They provide support and direction to local officials that have the authority to make land use decisions

While applicants do not need to submit a complete comprehensive or master plan with the Part 2 application, they should detail the planning process employed by the municipality. They must thoroughly describe how the municipality's comprehensive plan is compatible with the storm water regulations The description should clearly

- Identify management objectives for streams, wetlands, and other receiving waters;
- Identify areas where urban development is likely to occur and areas that are sensitive to the effects of urbanization. Consideration should be given to receiving waters, topography, soil types, ground water uses and potential impacts, and other relevant factors;
- Describe standards such as design criteria and performance standards for storm water controls for new developments, such as buffer zones,

open space preservation, erosion and sediment controls, etc.;

- Describe other measures to minimize the effects of new development on storm water quality (these may include local code and ordinance requirements); and

- Identify or discuss the site development review process for the evaluation and approval of storm drainage or storm water management programs. Requirements in drainage or storm water management programs can be coordinated with review of other related plans such as those for site grading or landscaping.

There will be great variation among municipalities in their sophistication of land use planning. If the municipality has recently updated its land use plan, it may detail storm water quality issues. In other instances, there may be no policy to include storm water quality considerations in land use decisions. In such cases, the applicant must describe how consideration of those activities that affect storm water quality are to be incorporated into the municipality's comprehensive or master plan and its approval process for construction projects.

Control Measures

Most traditional storm water control measures focus on efficient collection and conveyance of storm water runoff to an offsite location. This approach can increase downstream property damage due to increased storm water runoff quantity and flow velocity. Corrective action often involves expensive public works projects, such as enlarging and reinforcing channels or constructing swales to provide an adequate outfall from affected or damaged areas. The traditional approach has typically involved downstream channel stabilization projects. However, these projects may also result in increased storm water runoff quantity and flow velocity.

Some recent approaches to storm water management include preserving the natural features of a watershed by maintaining vegetative cover and establishing buffer zones and open space or green areas. The benefit of employing this approach is the protection afforded to riparian areas and wetlands, as well as the preservation of a stable watershed. One additional benefit from this approach includes maintaining ground water recharge through infiltration. These approaches to storm water management minimize the impact of erosion, flooding, and other damage to natural drainage features such as streams, wetlands, and lakes. Preservation of natural habitat can be achieved through effective storm water quality control measures. More recent approaches use storm water to:

- Recharge ground water sources with runoff from impervious areas;

- Preserve baseflows of surface water bodies;

- Augment water supplies used for street cleaning and other municipal functions, such as watering public lawns,

- Increase recreational opportunities including swimming, fishing, and boating; and

- Sometimes, augment drinking water supplies if it is treated and in compliance with all applicable drinking water standards.

The municipality should consider storm water controls and structural concerns in planning, zoning, and site or subdivision plan approval. An example of effective structural control is described in Exhibit 6-1. Non-structural control measures are highly recommended for new development. They can be included during the planning, site-selection, and development stages. Examples of non-structural controls include street sweeping, buffer strip preservation, and public education.

Exhibit 6-1
Storm Water Programs in Delaware and Florida

Delaware requirements for on-site measures include water quality ponds with permanent pools Ponds must be designed to release the equivalent volume of runoff from the first 1/2 inch of runoff from the site over a 24-hour period and have a storage volume designed to accommodate at least 1/2 inch of runoff from the site Water quality ponds without permanent pools may also be used in Delaware's program. These pools are to be designed to release the first inch of runoff from the site over a 24-hour period.

Developers are instructed to consider infiltration practices only after ponds are eliminated for engineering or hardship reasons. Infiltration structures must be designed to accept at least the first inch of runoff from all streets, roadways, and parking lots Other practices may be acceptable if they meet the equivalent removal efficiency of 80 percent for suspended solids. More stringent requirements may be established on a case-by-case basis.

The 80 percent removal efficiency for suspended solids that Delaware requires takes into account pollutant settling. The 24-hour detention period allows for substantial settling where most of the pollutant removal occurs. In addition, the requirement that the first inch of runoff be released over a period of no less than 24 hours reduces downstream erosion

Source Schueler, 1987

For significant redevelopment, municipalities can incorporate both structural and nonstructural storm water controls However, there are generally far more constraints and limitations on the control opportunities available at redevelopment sites One of the primary constraints is the availability of sufficient open area to accommodate structural controls such as detention ponds. In instances where redevelopment is occurring in densely urbanized areas, storm water runoff volumes may be so large that sufficient storage capacity can not be provided without further compounding problems associated with siting and retrofitting existing storm water conveyance systems In such cases, the municipality should consider nonstructural control measures such as traffic flow control, the use of porous construction materials for roads and parking lots, revisions to street sweeping or deicing policies, or public education programs

6.3.1.2 Public Streets, Roads, and Highways

Summary of Regulatory Requirement

Public streets, roads, and highways can be significant sources of pollutants in discharges from MS4s Therefore, proposed management programs must include a description of practices for operation and maintenance of public streets, roads, and highways, and procedures for reducing the impact of runoff from these areas on receiving waters.

§122.26(d)(2)(iv)(A)(3) [The application must include a] description of practices for operating and maintaining public streets, roads and highways and procedures for reducing the impact on receiving waters of discharges from municipal storm sewer systems, including pollutants discharged as a result of deicing activities

Road maintenance practices, especially snow management and road repair, and traffic are significant sources of pollutants in storm water discharges Measures to reduce the pollutants in storm water runoff from these sources should be addressed in the proposed management program

Snow Management

Deicing salts are the main source of pollutants in runoff of urban snowmelt. Municipalities can reduce these pollutants by calibrating equipment, educating equipment operators, using alternative deicing materials, and properly storing deicing materials. As alternatives to deicing salts, the Federal Highway Administration is considering many materials that may be less polluting. However, most of these deicers contain sodium or chloride ions that are harmful to roadside trees, shrubs, and soils. One deicer, calcium magnesium acetate (CMA) may be the best option for environmentally sensitive areas (Chollar, 1990) In salt storage facilities, salt piles should be completely covered, storage and handling areas should have impervious surfaces, and contaminated runoff should be contained

Road Repair

Road maintenance and repair activities may contribute pollutants through erosion caused by the elimination of stabilizing vegetation from roadside shoulders and ditches. Maintenance crews can decrease the potential for erosion by disturbing only the area under repair. Graded areas should also be limited in size so that repairs can be completed the same day and graded areas stabilized by the end of the workday. Other measures to reduce pollutants in storm water include scheduling potential pollutant-causing repair work during dry seasons, when possible

Municipal equipment yards and maintenance shops that support road maintenance activities can also be significant sources of pollutants Therefore, municipalities should consider instituting procedures that address spill prevention, material management practices, and good housekeeping

Traffic

Oil and grease and metals from traffic are the pollutants of most concern with respect to aquatic toxicity and their ability to "wash off" roadways and enter a MS4

In almost all instances, the pollutant concentrations in initial storm water discharge from heavily travelled streets is significant When the initial runoff reaches the velocity needed to entrain particulates, highly soluble pollutants that have accumulated between storms are transported to the storm sewer system. Therefore, shortly after a storm event begins, the pollutant loading in the initial flow to a MS4 is often the greatest

Pollutants from traffic can be minimized by using nonstructural controls (e.g, traffic reduction and improved traffic management), structural controls (e g, traditional and innovative BMPs), and changing maintenance activities. Traditional structural controls to reduce pollutants in road runoff include vegetated swales, infiltration devices and detention/retention basins Highways often afford opportunities for using structural controls such as detention basins on entrance or exit ramps and upstream or downstream of culvert crossings (Steward, 1992). Smaller roads may also have low-cost structural control opportunities available at culvert crossings such as vegetated swales Many structural controls can also be placed on public or private land that is outside the right-of-way, but still may be proximate enough to capture road runoff. Any time controls are placed at culvert crossings, potential wetland impacts and instream treatment issues need to be considered

Maintenance activities that can reduce pollutants in storm water discharges include catch basin cleaning, litter control, and targeted street sweeping For municipalities that have

developed transportation plans under the Clean Air Act, applicants should describe how they will review the plan, and amend it where appropriate, to address water quality concerns Potential locations for installing new structural controls to reduce pollutants from road and highway runoff should be identified by applicants.

6.3.1.3 Flood Management Projects

Summary of Regulatory Requirement

The traditional focus of storm water management in many communities has been water quantity (i e., flood) control. The proposed management program must demonstrate that flood management projects take into account the effects on the water quality of receiving water bodies, and the program must discuss whether existing structural flood control devices can be retrofitted to control water quality.

> §122.26(d)(2)(iv)(A)(4) [The application must include a] description of procedures to assure that flood management projects assess the impacts on the water quality of receiving water bodies and that existing structural flood control devices have been evaluated to determine if retrofitting the device to provide additional pollutant removal from storm water is feasible

Opportunities for pollutant reduction should be considered when determining specific controls to be proposed as the MEP standard in the storm water management program.

Control Measures

Storm water management devices and structures that focus solely on water quantity are usually not designed to remove pollutants, and may sometimes harm aquatic habitat and aesthetic values. For example, channels that are completely lined with concrete typically do not provide for aquatic habitat and tend to increase potentially erosive velocities and elevate ambient water temperatures, resulting in downstream channel enlargement and increased pollutant loadings However, this condition can be mitigated through alternative stabilization methods.

Channel management measures that can enhance streams and their ecological values include corridor preservation, biological bank treatment, and, where necessary, geomorphic restoration (Ferguson, 1991). The municipality may also install structural devices to dampen the hydraulic energy of the flow and minimize downstream erosion. As another example, willow saplings could be planted between rip-rap, timbers, and other stabilization structures that are anchored into terraces on the side of the streambank.

Flood-control projects can be built or subsequently modified to address water quantity and water quality concerns. Sometimes existing flood control structures can be retrofitted to provide water quality benefits as well as water quantity control (EPA, 1989b). Basin retrofits are a common example. For such a retrofit, dry flood control or detention basins can be converted to wet basins by modifying outlet orifices Additional storage can be obtained by raising the elevation of the basin embankment.

Dry retention basins, or extended dry or wet retention basins can be used to improve water quality. Dry retention basins are not as efficient or as effective in improving water quality as extended dry or wet retention basins, but dry retention basins are generally less costly to design and maintain. The decision to use dry retention or extended dry or wet retention basins should consider all these factors.

Optimally, such measures should be considered in the planning process (discussed previously). However, they can also be implemented later in the land development

process (e g , site review or public facilities requirements stage)

If a flood control authority is responsible for a portion of the MS4, the applicant should take the lead in coordinating efforts to incorporate pollutant reduction considerations in flood control projects EPA recommends the use of Memoranda of Agreement and Memoranda of Understanding to clarify roles and responsibilities between two or more political entities.

6.3.1.4 Municipal Waste Facilities

Applicants must describe programs that identify measures to monitor and reduce pollutants in storm water discharges from facilities that handle municipal waste, including sewage sludge.

> §122.26(d)(2)(iv)(A)(5) [The application must include a] description of a program to monitor pollutants in runoff from operating or closed municipal landfills or other treatment, storage or disposal facilities for municipal waste which shall identify priorities and procedures for inspections and establishing and implementing control measures for such discharges

The first step is to identify facilities that handle municipal waste and summarize their operations The types of facilities that should be included are

- Active or closed municipal waste landfills,
- Publicly owned treatment works, including water and wastewater treatment plants,
- Incinerators,
- Municipal solid waste transfer facilities
- Land application sites,
- Uncontrolled sanitary landfills,
- Maintenance and storage yards for waste transportation fleets and equipment,
- Sites for disposing or treating sludge from municipal treatment works; and
- Other treatment, storage, or disposal facilities for municipal waste.

Applicants may combine this part of the proposed management program with the program established under §122 26(d)(2)(iv)(C), which sets standards for monitoring and controlling pollutants from similar types of solid waste facilities (e.g , those with hazardous wastes, or subject to the requirements of SARA Title III—Section 313 of the Emergency Protection and Community Right-to-Know Act). Monitoring should include all the parameters listed in §122 26(d)(2)(iv)(C) and any additional parameters listed in an effluent guideline. Procedures to evaluate, inspect, monitor, and establish control measures for municipal waste sites over the term of the NPDES permit should be described For example, after one year of monitoring each waste handling facility category listed above, the municipality may have collected enough data to decide which facilities or types of facilities should receive a higher priority for pollutant reduction More attention could then be focused on the high-priority sites

6.3.1.5 Pesticides, Herbicides, and Fertilizers

The proposed management program must include a description of procedures to reduce the contribution of pollutants associated with pesticides, herbicides, and fertilizers discharged to the MS4.

§122 26(d)(2)(iv)(A)(6) [The application must include a] description of a program to reduce to the maximum extent practicable, pollutants in discharges from municipal separate storm sewers associated with the application of pesticides, herbicides and fertilizer which will include, as appropriate, controls such as educational activities, permits, certifications and other measures for commercial applicators and distributors, and controls for application in public right-of-ways and at municipal facilities

The proposed program should include educational measures for the public and commercial applicators, and should include integrated pest management measures that rely on non-chemical solutions to pest control. The program should also describe how educational materials will be developed and distributed Applicants are encouraged to consider providing information for the collection and proper disposal of unused pesticides, herbicides, and fertilizers, or to establish their own program An effective and safe program would include

- Development of an inventory of products that may be accepted under the program, and collection of the Material Safety Data Sheets (MSDSs) for these products,
- Identification of transportation, storage, and disposal requirements,
- A shelf-life program to dispose of expired products,
- Applicator training or certification (the pretreatment program may be helpful as a source of industry-specific information or as a model approach for obtaining and tracking information on chemical applicators and distributors), and
- Safety training

Any certification/training program for the collection and disposal of pesticides, herbicides, and fertilizers must be in compliance with Federal, State, and local laws such as the Resource Conservation and Recovery Act, the Federal Insecticide, Fungicide, and Rodenticide Act, the Department of Transportation's hazardous materials regulations, and State and local ordinances.

In addition, applicants must include a discussion of controls for the application of pesticides, herbicides, and fertilizers in public-rights-of-way and at municipal facilities Planting low-maintenance vegetation, such as perennial ground covers, reduces pesticide and herbicide use. Native vegetation is often preferable because there is less need to apply fertilizers and herbicides, and to perform other forms of maintenance, such as mowing (Horner, 1988).

If herbicides are used, a herbicide-use plan must be proposed as part of the storm water management program The plan might include

- A list of selected herbicides and their specific uses,
- Information about the formulations of various products, including how to recognize the chemical constituents from the label, and directions and precautions for applicators that explain if products should be diluted, mixed, or only used alone,
- Application methods and estimated quantities to be used,
- Equipment use and maintenance,
- Training in safe use, storage, and disposal of pesticides (safety requirements for individual products are listed on the products' MSDSs),
- Inspection and monitoring procedures, and

- Recordkeeping and public notice procedures

6.3.2 Construction Sites

As specified in §122 26(d)(2)(iv)(D), applicants must describe proposed regulatory programs to reduce pollutants in storm water runoff from construction sites to the MS4.

> **§122.26(d)(2)(iv)(D)** [The application must include a] description of a program to implement and maintain structural and nonstructural best management practices to reduce pollutants in storm water runoff from construction sites to the municipal storm sewer system

This part of the proposed management program must address

- Implementation of BMPs,
- Procedures for reviewing site plans to ensure that they are consistent with local sediment and erosion control plans,
- Inspection of construction sites; and
- Enforcement measures and educational activities for construction site developers and operators

EPA encourages municipalities to (1) coordinate requirements to reduce pollutants in construction site runoff with management programs to reduce pollutants from new development, and (2) maintain, to the degree possible, pre-construction hydrologic conditions (Section 6 3 1 1) Applicants are encouraged to describe these two proposed management program components together Implementation of this program component will rely on the establishment and maintenance of both structural and nonstructural BMPs This requirement extends to all construction activity within the municipality

All construction sites, regardless of size, must be addressed by the municipality To begin to identify these sites, the applicant should obtain lists of construction site operators that are covered by general or individual storm water NPDES permits from the NPDES permitting authority However, construction sites not covered by a storm water discharge permit also need to be addressed by the municipality. The best way to identify these construction sites and implement an effective BMP program to reduce pollutants in their runoff is through the site planning process (see Section 6.3.2.1).

The BMPs envisioned for construction site runoff are generally well established technologies and practices They rely predominantly on erosion and sediment controls and other measures applicable to construction sites (e.g , control of solid wastes, and prohibitions on discharging concrete truck washing runoff into storm drains) The technologies proposed should be referenced, and a description of when and how the controls will be used should be included Municipality-specific technical guidance for construction site operators, such as handbooks and inspection checklists, are examples of suitable reference sources If an applicant chooses to develop such handbooks and checklists, they should be referenced and described in the application

The major requirements of this program component include

- Site planning that considers the potential impacts on water quality,
- Nonstructural and structural best management practices,
- Procedures that consider physical site characteristics when identifying priorities for inspection and enforcement, and
- Educational and training measures for construction site operators

Each of these requirements, and the reasons that they are important elements of a proposed storm water management program, is described in more detail below

6.3.2.1 Site Planning

Sediment runoff rates from construction sites are typically 10 to 20 times greater than those of agricultural lands, and 1,000 to 2,000 times those of forest lands. Over a short period, construction sites can contribute more sediment to streams than had been deposited over several decades Runoff from construction sites can also include other pollutants such as phosphorus and nitrogen from fertilizer, pesticides, petroleum derivatives, construction chemicals, and solid wastes

To address these problems, the proposed management program should describe procedures for site planning that consider potential water quality impacts

> §122 26(d)(2)(iv)(D)(1) [The program for construction sites must include a] description of procedures for site planning which incorporate consideration of potential water quality impacts

The objective is for the municipality and the developer to address storm water discharges from construction activity early in the project design process so that potential water quality impacts can be eliminated or minimized and consequences to the aquatic environment assessed Nonstructural approaches to minimize the generation of runoff from the construction site will also need to be considered These measures may include phasing development to coincide with seasonal dry periods, minimizing areas that are cleared and graded to only the portion of the site that is necessary for construction, exposing areas for the briefest period possible, and stabilizing and reseeding disturbed areas rapidly after construction activity is completed

It is often easier and more effective to incorporate storm water quality controls during the site plan review process or earlier The process typically culminates with the developer of the construction site submitting detailed engineering plans to the municipality for review and approval

Upon completion of the site plan review stage, the developer and the municipality have invested considerable time and money into the project If storm water quality issues are considered only after significant detailed engineering has gone into the project, municipal site reviewers may only address minor drainage issues. In recent years, however, many municipalities have developed separate teams of site inspectors to implement erosion and sediment control measures in the field. In these municipalities, site inspectors should be part of the site review team (if they are not already) in order to incorporate their expertise on the appropriate erosion and sediment controls for the given circumstances

The above discussion reinforces the importance of site planning, as described in the section on site planning for new development (Section 6.3 1) In general, the sooner planners consider storm water quality issues, the better the opportunity for efficient and effective pollutant reduction In some cases storm water issues should be considered in the conceptual stage of planning (e g , as a planning or zoning function)

Some municipalities include a final step in the planning process that requires a developer to provide a far greater level of design detail than earlier conceptual design approvals This step may be required as a condition of the final approval for certain zoning categories Municipalities with such a step in the development process can consider potential storm water quality issues in detail at this stage Municipalities that do not currently require such detailed plans should consider adopting this procedure as part of their storm water management program

6.3.2.2 Nonstructural and Structural BMPs for Construction Activities

This component of the proposed management program should describe requirements for nonstructural and structural BMPs that operators of construction activities that discharge to MS4s must meet

> **§122.26(d)(2)(iv)(D)(2)** [The program for construction sites must include a] description of requirements for nonstructural and structural best management practices

As indicated above, applicants must propose site review and approval procedures that address sediment and erosion controls, storm water management, and other appropriate measures Approvals should be clearly tied to commitments to implement structural and nonstructural BMPs during the construction process Appropriate structural and nonstructural control requirements will vary by project Project type, size, and duration, as well as soil composition, site slope, and proximity to sensitive receiving waters will determine the appropriate structural and nonstructural BMPs Municipalities should acquire the authority to require operators to install and maintain applicable erosion and sediment control plans Exhibit 6-2 summarizes common construction-site BMPs

A description of the local erosion and sediment control law or ordinance is needed to satisfy this program requirement The description should include information that links the enforcement of the law or ordinance to the legal authority of the applicant, as discussed in Section 3 of this manual

While many municipalities have erosion and sediment control ordinances in place, their effectiveness is often limited because they are not adequately implemented and enforced Examples include silt fencing that is not maintained or excavated soils that are placed directly on top of the silt fencing Therefore, construction sites covered under NPDES permit regulations must indicate whether they are in compliance with State and local sediment and erosion control plans Site inspections are expected to be the primary enforcement mechanism by which erosion and sediment controls are maintained

To ensure that developers are in compliance with erosion and sediment control plans, applicants may wish to consider expanding the use of performance bonds. This approach might depart from a traditional site bonding approach For example, the size of bonds could be based on the amount of earth disturbed, the slope of the site, changes in grades, soil type, proximity to surface waters, sensitivity of surrounding area, and other relevant factors. In addition, the bond could clearly specify the storm water quality controls that must be included in the development. Appropriate maintenance and site cleanup could be tied to the bond-release process

6.3.2 3 Site Inspections and Enforcement of Controls For Construction Sites

Storm water BMPs associated with construction activities are highly susceptible to damage due to the intensity of activities commonly associated with construction Consequently, inspections are crucial to the effective operation of storm water BMPs Therefore, the proposed management program should describe construction site inspection and enforcement procedures The procedures should be flexible so that they can be tailored to specific construction activities and physical characteristics of the construction site

> **§122 26(d)(2)(iv)(D)(3)** [The program for construction sites must include a] description of procedures for identifying priorities for inspecting sites and enforcing control measures which consider the nature of the construction activity, topography, and the characteristics of soils and receiving water quality

Exhibit 6-2
Construction Site Controls and Their Applicability

Control Type	Slope Protection	Waterway Protection	Surface Drainage	Enclosed Drainage	Large Flat Areas	Borrow Areas	Adjacent Properties
Non-structural (cover)							
temporary seeding	●		●		●	●	●
mulching & matting	●				●	●	
plastic covering	●					●	
retain natural vegetation	●	●	●	●	●	●	●
buffer zones	●	●	●	●	●	●	●
seeding & planting	●				●	●	
sodding	●		●		●	●	●
topsoiling					●	●	
Structural-erosion control							
gravel entry/truck wash			●		●		
road stabilization			●				
dust control							
pipe slope drains					●	●	●
subsurface drains	●						
surface roughening	●				●		
gradient terraces	●					●	
bioengineered slopes	●					●	
level spreader			●				
interceptor dikes/swales	●					●	●
check dams			●				●
outlet protection		●	●				
riprap	●	●	●				
vegetative streambank stabilization		●					
bioengineered streambank stabilization		●					
structural streambank stabilization		●					
Structural-sediment retention							
filter fence		●		●			●
gravel filter berm	●	●			●		●
storm drain inlet protection	●			●			●
sediment trap or sump		●	●		●	●	●
sediment pond or basin		●	●	●	●	●	●

Source: Modified from WDOE *Public Review Draft - Stormwater Management Manual for the Puget* [illegible] Washington State Department of Ecology, Publication #90-73 June 1991

Effective inspection and enforcement requires adequate staff, systematic inspection procedures, penalties to deter infractions, and intervention by the municipal authority to correct violations. Enforcement mechanisms, such as the ability to require additional storm water controls, administrative penalties (e.g., stop work orders) and injunctive relief (via citizen suits) also must be described. In addition, the applicant should describe who has the authority to require compliance.

Proposed procedures for inspecting construction sites may include minimum frequencies and an inspector's checklist. For example, the State of Delaware requires a minimum of one inspection every two weeks for sites over 50,000 square feet.

The proposed program should also specify the minimum number of inspectors that will be employed during the permit term and how they will be trained. For example, some erosion and sediment control programs require that certified private inspectors be used. In such case, procedures for inspector training and certification must also be described.

In formulating procedures to identify priorities for inspecting sites and enforcing control measures, applicants are encouraged to begin early in the process (i.e., at the site planning stage, as discussed previously) and continue throughout all ground disturbing activities. Once the nature of the construction activity has been established or perhaps modified during the site plan review process, the physical site constraints can be evaluated so that effective controls can be implemented.

For example, if the controls specified in the site plan prove to be ineffective, or if changes occur that were not anticipated during the planning process, site inspection and enforcement mechanisms can be required to mitigate the potential for pollutants to enter a downstream MS4. In this instance, a perimeter barrier, such as a temporary diversion dike, could be used to divert the concentrated runoff to a pipe slope drain terminating with a level spreader. The spreader would dissipate the erosive velocity of the runoff and release it into an undisturbed area beyond the limits of the clearing and grading at the toe of the slope.

The proximity and sensitivity of the receiving water to which the construction site discharges is an important consideration. For construction sites that discharge to receiving waters that do not support their designated use or other waters of special concern, additional construction site controls are probably warranted and should be strongly considered. These receiving waters are identified in the Part 1 municipal NPDES storm water permit application [§122.26(d)(1)(i)(C)].

6.3.2.4 Educational Measures for Construction Site Operators

Construction site operators often need training and education about the sources, control, and impacts of pollutants in runoff from construction sites (see Virginia, 1988). Therefore, applicants must describe examples of informational materials and activities to be used in education programs.

> §122.26(d)(2)(iv)(D)(4). [The program for construction sites must include a] description of appropriate educational and training measures for construction site operators.

Implementation and enforcement of erosion and sediment controls have historically been major problems even with many programs that may be otherwise exemplary. Therefore, technical information on how to incorporate storm water management with erosion and sediment control and other BMP training courses are recommended for municipal employees and construction site operators. Training on the available alternatives will help operators recognize and correct problems promptly. *Tools for such training* include videos, workshops, seminars, and demonstrations or field trips

An acceptable program must include a training program, which should be supplemented by a certification program for all construction site operators (contractors and developers) plan reviewers, and inspectors that work on sites that discharge to a MS4. For example, one NPDES State has a certification program based on adequate training and minimum-competency level testing of all private individuals involved in the preparation and implementation of erosion and sediment control plans.

6.3.3 Program to Control Pollutants in Storm Water Discharges from Waste Handling Sites and from Industrial Facilities

> §122.26(d)(2)(iv)(C) [The application must include a] description of a program to monitor and control pollutants in storm water discharges to municipal systems from municipal landfills, hazardous waste treatment, disposal and recovery facilities, industrial facilities that are subject to Section 313 of Title III of the Superfund Amendments and Reauthorization Act of 1986 (SARA), and industrial facilities that the municipal permit applicant determines are contributing a substantial pollutant loading to the municipal storm sewer system.

The storm water regulations envision that NPDES permitting authorities and municipal operators will cooperate to develop programs to monitor and control pollutants in storm water discharges to municipal systems from various sites that handle waste and certain industrial facilities.

Operators responsible for storm water discharges associated with industrial activity must obtain NPDES permits from EPA or an authorized NPDES State. These industrial storm water permits will establish requirements such as controls, practices, and monitoring for storm water discharges from the industrial facilities to the MS4. The industrial storm water permits will also provide a basis for enforcement actions directly against the industrial owner or operator.

NPDES permits for MS4s will establish responsibilities for municipal system operators to control pollutants from industrial storm water discharged through their system. Proposed storm water management programs must address the reduction of pollutants in storm water discharges from municipal landfills; hazardous waste treatment, storage and disposal facilities; facilities subject to SARA Title III; and other priority industrial facilities, as determined by the applicant. Municipalities should consider the information gathered for the Part 1 application and other parts of the Part 2 application (particularly the Source Identification and Characterization Data components) when prioritizing storm water discharges from these sites. In addition, Appendix B contains a list of pollutants commonly associated with various industries.

In the Part 2 application, the Source Identification component (see Section 4 of this guidance manual) requires the applicant to provide an inventory of pollutant sources, organized by watershed. This inventory identifies and describes the products and services of each industrial facility that may discharge storm water to the MS4. The *Source Identification* component suggests applicants use standard industrial classification (SIC) codes for this description. EPA strongly recommends this information be used to identify priority waste handling sites and industrial facilities. A similar technique could be developed for sites that do not meet the regulatory definition of "storm water discharge associated with industrial activity" (i.e. not included in the *Source Identification* and *Discharge Characterization* components), but are identified as a high priority under the proposed management program. Applicants can obtain information on how SIC codes are used to describe the industrial facilities located within their jurisdictions from their NPDES permitting authority.

Characterization data should also be evaluated. Applicants should analyze quantitative data from representative outfalls to establish a monitoring and control program.

An integral part of this requirement is the adequacy of the applicant's legal authority. If a municipality believes that a discharge of storm water associated with industrial activity violates the industrial facility's NPDES permit limits, but the municipality does not have authority over the discharge, the municipality should contact the NPDES permitting authority for appropriate action. Examples of possible actions by the NPDES permitting authority are:

- For a facility that already has a NPDES individual permit, the permit may be reopened and further controls imposed,
- For a facility covered by a NPDES general permit, an individual site-specific permit application may be required, or
- For a facility not covered by a NPDES storm water permit, a permit may be required

The municipality is ultimately responsible for discharges from their MS4. Consequently, the proposed storm water management program should describe how the municipality will help EPA and authorized NPDES States

- Identify priority industries discharging to their systems,
- Review and evaluate storm water pollution prevention plans and other procedures that industrial facilities must develop under general or individual permits;
- Establish and implement BMPs to reduce pollutants from these industrial facilities (or require industry to implement them), and
- Inspect and monitor industrial facilities to verify that the industries discharging storm water to the municipal systems are in compliance with their NPDES storm water permit, if required

6.3.3.1 Identifying Priorities

Proposed management programs must clearly identify priority industrial facilities.

> **§122.26(d)(2)(iv)(C)(1).** [The applicant must] identify priorities and procedures for inspections and establishing and implementing control measures for such discharges

This section discusses how applicants might identify priority facilities Section 6.3.3.2 discusses how municipalities might develop procedures for inspections and implementation of control measures

At a minimum, priority facilities include:

- Operating and closed municipal landfills;
- Hazardous waste treatment, disposal or recovery facilities, and
- Facilities subject to SARA Title III

Municipalities must identify these and other priority industrial facilities and describe the criteria used to identify them For example, information from the Toxics Release Inventory is one source a municipality could use to identify industrial facilities subject to SARA Title III. Other sources may include CWA Section 205 or 208 use-attainability studies, other studies that indicate a site-specific beneficial use impairment immediately downstream of a storm water outfall, or records of industrial pretreatment programs or other permit programs that identify facilities that may be the source of a use impairment or

a major contribution of pollutants The program should also describe procedures for modifying the inventory of priority industries based on additional evaluation that occurs throughout the permit term

Applicants may initially focus their implementation efforts on known pollution sources The municipality may have previously identified these sources, or they may be identified through existing information compiled during the permit application process However, the initial management program implementation strategy should be based on information gathered while completing the *Adequate Legal Authority, Source Identification,* and *Discharge Characterization* sections of the permit application (See Chapters 3, 4, and 5, respectively)

During the term of the permit, as additional information becomes available, the municipality should target and set priorities for other program elements that emerge. For example, if the municipality has incomplete characterization data about waste handling sites identified in this program component because the inventory of dischargers to the MS4 has not been completed, the municipality could propose to direct monitoring programs to those areas Upon acquiring sufficient characterization data, the priority of the sites discharging to these portions of the MS4 can be either determined or modified

As noted above, when identifying priority sites, applicants must consider all the facilities listed in §122 26(d)(2)(iv)(C)(*1*). When municipalities develop criteria for identifying additional priority industrial facilities, they are advised to consider, at a minimum:

- The type of industrial activity (SIC codes can help characterize the type of industrial activity),
- The use and management of chemicals or raw products at the facility and the likelihood that storm water discharge from the site will be contaminated; and
- The size and location of the facility in relation to sensitive watersheds

6.3.3.2 Developing Procedures

This program component should describe the specific steps that the municipality will take if it identifies a waste handling site or priority industrial facility when preparing the Part 2 application or during the permit term [§122.26(d)(2)(iv)(C)(*1*), printed in the box above]. The proposed management program must include procedures for inspecting priority industrial sites. The results of inspection may be used as a basis for requiring storm water management controls and enhanced pollution prevention measures. It should also establish an inspection schedule for each priority facility at the time it is identified

Applicants may want to consider establishing prior notification procedures The applicant will need to evaluate the legal authority it has over priority facilities to determine if prior notification is required This is another example of how EPA expects the different components of the application process to be linked In this instance, the Adequate Legal Authority section is tied directly to the prior notification procedure of the inspection and evaluation component of the proposed management plan

Applicants also should consider developing inspection documents such as standard forms or checklists for recording observations Forms and checklists can be used to identify high risk areas of priority facilities and to make comparisons among sites When characterization data or baseline estimates are factored into the evaluation process, the effectiveness of pollution prevention activities at a particular site could be quantified and compared to similar sites Other procedures that applicants should describe to effectively incorporate inspections as well as establish and implement control measures for these types of discharges can be derived from monitoring data

Applicants also should describe a procedure for conducting follow-up inspections, where necessary, as part of this program component For example, follow-up inspections might be needed to verify the installation of a specific control or implementation of a practice specified in a negotiated agreement between the municipality and the industrial site A system-wide approach to establishing priorities for inspection procedures is recommended. The system-wide approach should begin with the evaluation of existing information, followed by the identification and evaluation of new information during the permit term. Therefore, applicants should link these procedures with information from the *Source Identification* and *Discharge Characterization* components

6.3.3.3 Establishing and Implementing Controls

A municipality must consider if it should place more stringent controls on discharges associated with industrial activity than are required in an industrial facility's existing NPDES storm water permit [§122 26(d)(2)(iv)(C)(1) printed in box above] Usually, the municipality will not need to impose controls beyond those required in the industrial facility's NPDES storm water permit (for more information on appropriate controls, refer to *Storm Water Management for Industrial Activities, Developing Pollution Prevention Plans and Best Management Practices*, EPA 832-R-92-006, September, 1992)

However, nothing in the Federal regulations would prohibit the municipality from requiring additional controls beyond the permit requirements for industrial activities For this reason, EPA recommends that municipal applicants incorporate a provision in the proposed storm water management program that allows the municipality to require priority industrial facilities to implement the controls necessary for the municipality to meet its permit responsibilities

Finally, the applicant should suggest procedures for requiring pollutant control measures in runoff from priority industrial facilities Applicants should provide information to the industrial facilities that discharge to the MS4s and industry-specific guidance on appropriate control measures that industries discharging to their systems should follow (WDOE, 1991).

Priority industrial facilities should focus on controlling activities such as the use, storage, and handling of toxic chemicals Standard methods for implementing control measures at different types of facilities should be described To facilitate this, municipalities should obtain copies of the pollution prevention plans developed by industrial permittees Control measures that the municipality may suggest include preventing exposure of pollutant sources to precipitation, on-site pretreatment, and oil/water separators Applicants should provide a schedule for setting up this program component at priority industrial facilities. The schedule should include educational services for industrial site operators and technical BMP guidance, training courses, videos, workshops, and seminars for plan reviewers, inspectors, contractors, and developers

6.3.3.4 Inspection and Monitoring

The proposed management program should describe the inspection procedures that will be followed Storm water inspections can be coupled with inspections for other purposes (e.g., pretreatment programs, fire and safety) Proposed management programs should address minimum frequency for routine inspections. For example, how often, how much of the site, and how long an inspection may take are appropriate to explain in this proposed management program component Applicants should also describe procedures for conducting inspections and provide an inspector's checklist

In addition these inspection procedures should identify the minimum number of inspectors that will be employed and describe

the programs to train them For example, if the number of inspectors is expected to increase over the term of the permit, it should be noted in the proposed management program. Also, if storm water inspections are combined with other program inspections, means of cross-training inspectors and coordinating schedules should be outlined

Municipalities are urged to evaluate pollution prevention plans and discharge monitoring data collected by the industrial facility to ensure that the facility is in compliance with its NPDES storm water permit Site inspections should include (1) an evaluation of the pollution prevention plan and any other pertinent documents, and (2) an on-site visual inspection of the facility to evaluate the potential for discharges of contaminated storm water from the site and to assess the effectiveness of the pollution prevention plan. A municipality could begin the inspection process with information from the facility's notification to the municipality, which should have been submitted by May 15, 1991. Industrial facilities must also submit an individual NPDES permit application, participate in a group storm water permit application, or file a Notice of Intent (NOI) to be covered by a general permit to the NPDES permitting authority Section 308 of the CWA provides the legal authority for any individual (including a municipality) to obtain information from the NPDES permitting authority

The proposed management program also must include a description of a monitoring program for storm water discharges associated with industrial facilities [§122.26(d)(2)(iv)(C)(2)]

The monitoring program should describe the framework and rationale for selecting monitoring sites Sites that may be appropriate for monitoring include locations with several upstream industrial facilities, industrial facilities that are representative of a significant number of similar facilities, and priority industrial sites with significant potential for high levels of pollutants in their storm water discharges The description of the proposed

§122.26(d)(2)(iv)(C)(2) [The application must describe] a monitoring program for storm water discharges associated with the industrial facilities identified in paragraph (d)(2)(iv)(C) of this section, to be implemented during the term of the permit, including the submission of qualitative data on the following constituents any pollutants limited in effluent guidelines subcategories, where applicable; any pollutant listed in an existing NPDES permit for a facility; oil and grease, COD, pH, BOD_5, TSS, total phosphorus, total Kjeldahl nitrogen, nitrate plus nitrite nitrogen, and any information on discharges required under 40 CFR 122.21(g)(7)(iii) and (iv).

monitoring program should address how the monitoring data will be used and what the frequency of the monitoring will be.

Identifying who will actually conduct the monitoring (e g , industry or municipality) is appropriate to include in the program description. Linking this element of the monitoring program to the Adequate Legal Authority section of the permit application is vital The legal authority to require monitoring should prescribe the specific monitoring protocols required elsewhere in the regulation [§122 26(d)(2)(i)(F)]. Applicants should describe proposed procedures for monitoring industrial facilities, including methods for determining parameters to be sampled throughout the term of the permit **At a minimum, parameters that must be considered for monitoring include**

- Any pollutant limited in effluent limitations guidelines for the subcategory of industry;
- Any pollutant that is controlled in a NPDES permit for the process discharge from an industrial site,
- Oil and grease, COD, pH, BOD_5, TSS, total phosphorus, total Kjeldahl nitrogen, nitrate plus nitrite nitrogen; and

- Certain pollutant(s) known or suspected to be in the discharge, based on §122.21(g)(7)(iii) and (iv) (Section 5.3).

If a municipality believes (based on the results of monitoring and inspections) that an industrial facility is not meeting its NPDES permit requirements, the municipality should petition the NPDES authority to either require the facility to change its pollution prevention plan or institute an enforcement action. Municipalities may also file citizen suits under CWA Section 505 to enforce the conditions of the NPDES permit.

6.4 STRUCTURAL CONTROLS

6.4.1 Description of Structural Controls

Applicants are required to identify the location of major structural controls for storm water (retention basins, detention basins, major infiltration devices, etc.) in Part 1 of the application [§122.26(d)(1)(iii)(B)(5)]. In Part 2, applicants must describe additional controls that they plan to implement [§122.26(d)(2)(iv)]. The controls must address the activities described in Section 6.3. In addition, the applicant must describe maintenance procedures [§122.26(d)(2)(iv)(A)(1), discussed in Section 6.4.2]. Later, when the municipality submits its annual report, it will have to report on its progress in implementing these controls [§122.42(c)(1), discussed in Section 7.3 of this guidance].

The matrix in Exhibit 6-3 provides information on commonly used structural and source control BMPs. Structural practices to control urban storm water runoff rely on three basic mechanisms: **detention, infiltration,** and filtration. More detailed technical information about source controls (particularly in the selection of structural BMPs) is available in the technical BMP manuals (MWCOG, 1991, Schueler, 1987, WDOE 1991; and EPA 1990c). The following summary of structural and source control BMPs draws extensively from those manuals.

Applicants should note that CWA Section 404 permits may be required for some structural controls, including any control projects that involve the discharge of dredged or fill material into waters of the United States, including wetlands. States may also require permits that address water quality and quantity. To the extent possible, municipalities should avoid locating structural controls in natural wetlands. Before considering siting of controls in a natural wetland, the municipality should demonstrate that it is not possible or practicable to construct them in sites that do not contain natural wetlands, and that the use of other nonstructural or source controls are not practicable or as effective. In addition, impacts to wetlands should be minimized by identifying those wetlands that are severely degraded or that depend on runoff as the primary water source. Moreover, natural wetlands should only be used in conjunction with other practices, so that the wetland serves a "final polishing" function (usually targeting reduction of primary nutrients and sediments). Finally, practices should be used that settle solids, regulate flow, and remove contaminants prior to discharging storm water into a wetland.

Another concern for siting controls is the possible adverse effect that infiltration and detention controls may have on ground water. This issue is addressed in more detail in Section 7.2.3.

Exhibit 6-3
Structural Controls Matrix

CONTROL AND MAINTENANCE REQUIREMENTS	ADVANTAGES	DISADVANTAGES
Extended Detention Dry Basin • Periodic mowing • Regular debris removal • Sediment removal annually	• Provides peak flow control • Possible to provide good particulates removal • Can serve large development • Requires less capital cost and land area when compared to wet basin • Does not usually release warmed or oxygen-depleted water downstream • Protects against downstream channel erosion • Can create valuable wetland and meadow habitat when properly landscaped	• Low removal rates for soluble pollutants • Generally not feasible for drainage areas less than 10 acres • If not adequately maintained, can become a nuisance; (becomes unsightly, breeds mosquitos, and creates undesirable odors) • Periodic mowing and maintenance can be detrimental to nesting birds or other animals inhabiting the area
Vegetative Filter Strip • Inspection • Fertilizer use if necessary to maintain stable vegetation	• Low maintenance requirements • Can be used as part of the runoff conveyance system to provide pretreatment • Can reduce particulate pollutant levels in areas where runoff velocity is low to moderate • Enhances urban wildlife habitat diversity • Economical	• May concentrate water, significantly reducing effectiveness • Soluble pollutant removal highly variable • Limited feasibility in highly urbanized areas where runoff velocities are high and flow is concentrated • Requires periodic repair, regrading, and sediment removal to prevent channelization • Maintenance can be detrimental to nesting birds or other animals inhabiting the area • Fertilizer use can lead to higher nutrient loadings in storm water runoff
Grassed Swale • Periodic mowing • Fertilizer use if necessary to maintain stable vegetation	• Requires minimal land area • Can be used as part of the runoff conveyance system to provide pretreatment • Can provide sufficient runoff control to replace curb and gutter in single-family residential subdivisions and on highway medians • Economical and aesthetically pleasing	• Low pollutant removal rates • Leaching from culverts and fertilized lawns may actually increase the presence of trace metals and nutrients • Fertilizer use can lead to higher nutrient loadings in storm water runoff

Exhibit 6-3 (continued)
Structural Controls Matrix

CONTROL AND MAINTENANCE REQUIREMENTS	ADVANTAGES	DISADVANTAGES
Porous Pavement • Routine removal of fine particles from surface • May need weight limit of traffic imposed for protection	• Provides ground water recharge • Provides water quality control without additional consumption of land • Can provide peak flow control • High removal rates for sediment, nutrients, organic matter, and trace metals • When operating properly can replicate pre-development hydrologic conditions • Eliminates the need for storm water drainage, conveyance, and treatment systems off-site	• Requires regular maintenance • Possible risks of ground water contamination • Only feasible where soil is permeable, of sufficient depth to bedrock and water table, and gentle slopes are present • Not suitable for areas with high traffic volume or heavy vehicles • Need extensive feasibility tests, inspections, and very high level of construction workmanship • High failure rate due to clogging • Not suitable to serve large offsite pervious areas • Limited use in snowy climates where sanding and salting operations occur
Concrete Grid Pavement • Periodic mowing, if planted	• Provides peak flow control • Provides ground water recharge • Provides water quality control without additional consumption of land	• Requires regular maintenance • Not suitable for area with high traffic volume • Possible risk of contaminating ground water • Only feasible where soil is permeable, of sufficient depth to bedrock and water table, and gentle slopes are present
Filtration Basin • Periodic vacuuming and power washing	• Ability to accommodate moderately large-sized development (3-80 acres) • Flexibility to provide or not provide ground water recharge • Can provide peak volume control	• Requires pretreatment of storm water through sedimentation to prevent filter media from premature clogging

Exhibit 6-3 (continued)
Structural Controls Matrix

CONTROL AND MAINTENANCE REQUIREMENTS	ADVANTAGES	DISADVANTAGES
Wet Retention Basin • Periodic dredging, preferably from forebay area, if properly designed • Mowing of impoundment to prevent successional growth	• Provides peak flow control • Can serve large developments; most effective for large, intensively developed sites • Enhances species diversity, aesthetics, and provides recreational benefits • Little ground water discharge • Permanent pool in wet ponds helps prevent scour and resuspension of sediments • Provides moderate to high removal of both particulate and soluble pollutants	• Generally not feasible for drainage area less than 10 acres • Potential for safety and liability issues if not properly built and maintained • If not adequately maintained, can become a nuisance; (becomes unsightly, breeds mosquitos, and creates undesirable odors) • Requires considerable space, which limits use in densely urbanized areas with expensive land and property values • Not suitable for hydrologic soil groups "A" and "B" (SCS classification) • Potential for thermal discharge and oxygen depletion, which may severely impact downstream aquatic life
Extended Detention Wet Basin • Periodic dredging of sediment forebay	• Provides peak flow control • Can serve large developments; most effective for large, intensively developed sites • Enhances species diversity, aesthetics, and provides recreational benefits • Permanent pool in wet ponds helps prevent scour and resuspension of sediments • Provides better nutrient removal than traditional wet basin	• Not feasible for drainage area less than 10 acres • Potential for safety and liability issues if not properly built and maintained • If not adequately maintained, can become a nuisance; (becomes unsightly, breeds mosquitoes, and creates undesirable odors) • Requires considerable space, which limits use in densely urbanized areas with expensive land and property values • Not suitable for hydrologic soil groups "A" and "B" (SCS classification) • Potential for thermal discharge and oxygen depletion, which may severely impact downstream aquatic life

Sources Modified from MWCOG, 1991, Schueler 1987, and WDOE, 1991

6.4.1.1 Detention Controls

Detention controls temporarily store storm water runoff to control peak runoff rates and provide a reduction in pollutant concentrations by the gravitational settling of suspended solids and associated contaminants Except for incidental losses due to evaporation or percolation, essentially all the detained water is subsequently discharged to a surface water conveyance (e.g., a stream or MS4). The most common examples of detention practices are extended detention basins and wet (retention) basins

Variations on these basic detention controls include constructed storm water wetlands and multiple pond systems These types of controls also rely on detaining flows (leading to sedimentation) as the primary means of pollutant removal Recent investigations suggest that wetlands vegetation within a detention control can also reduce nutrient loads and certain other pollutants by incorporating them into plant tissue

If properly designed, detention controls can protect downstream channels by reducing the frequency of bankfull flood events and associated erosion Reduction in velocity and sediment load is also important for minimizing the adverse impacts of discharges to MS4s Detention facilities also can provide terrestrial and aquatic wildlife habitat if they are landscaped and planted appropriately

When considering detention controls, the municipality should consider the potential negative effects of downstream warming that may be caused by the shallowness of the water in the control The municipality should also consider negative impacts of detention controls, such as reduced baseflow; bacterial contamination due to waterfowl, and potential impacts to wildlife from concentrated contaminants, waterfowl diseases, and maintenance practices Safety and liability issues and nuisance factors, such as mosquitoes and odor, also should be considered. Setting detention controls in sensitive floodplains or in existing wetlands should generally be avoided. The flooding effect of impounding and detaining water is a particular concern if the upstream watershed drains more than 250 acres, because the volume of runoff and required detention times can cause inundation of upstream channels to occur.

Detention controls incorporating multiple pond systems and/or constructed storm water wetlands also treat runoff through the processes of absorption, filtration, biological uptake, volatilization, precipitation, and microbial decomposition. Recent investigations by the Metropolitan Washington Council of Governments suggest that multiple pond systems, in particular, have shown potential to provide higher and more consistent levels of treatment than traditional detention controls The redundancy afforded by the multiple pond system generally increases the reliability of the control. However, the potential concerns and drawbacks affecting retention basins also apply to these systems Many of these systems are currently being designed to include vegetative buffers and deep water areas to enhance wildlife habitat and to improve the appearance of the facility If a municipality selects one of these more innovative designs, it should recognize that periodic maintenance is necessary The effectiveness of these controls, like most controls, depends on proper operation, maintenance, and monitoring of the entire system

Wet (Retention) Basins

Wet (retention) basins are designed to maintain a permanent pool of water and temporarily store storm water runoff until it is released at a controlled rate Unlike extended detention ponds, wet basins cannot detain runoff for long times, because most of their storage capacity is needed for holding the permanent pool Enhanced designs include a forebay to trap incoming sediment where it can be easily removed A fringe wetland also can be established around the perimeter of the basin Similar to detention controls, locating

retention basins in sensitive floodplains or existing wetlands should be avoided if possible

Extended Detention Basins

Extended detention basins temporarily detain a portion of storm water runoff for 24 to 48 hours after a storm, gradually releasing the stored water through a fixed opening to allow urban pollutants to settle out. The basins normally return to a "dry" condition between storm events and do not have any permanent standing water. These basins are typically composed of two stages: an upper stage, which remains dry except during larger storms, and a lower stage, which is designed for typical storms. Pollutant removal from extended detention basins can be enhanced if they are equipped with plunge pools near the inlet, a micropool at the outlet, and an adjustable reverse-sloped pipe as the extended detention control device.

Water Quality Inlets

Water quality inlets (also referred to as catch basins) are small underground systems that, like retention basins, rely on settling to remove pollutants before discharging water to the MS4. Several designs of water quality inlets exist. In their simplest form, catch basins are single-chambered storm water inlets with the bottom lowered to provide 2 to 4 feet of additional space between the outlet pipe and the bottom of the structure for collection of trash and sediment. Some water quality inlets include a second chamber with a sand filter to provide additional removal by filtration. The first chamber provides effective removal of coarse particles and helps prevent premature clogging of the filter media.

Water quality inlets may include an oil/grit separator. There are 3 basic types of oil/grit separators the spill control (SC), the coalescing plate interceptor (CPI), and a design credited to the American Petroleum Institute (API). Most of the oil/grit separators that are promoted for use in reducing hydrocarbon loads in storm water are a modification of the API design, although there are appropriate applications for all three separator designs. Oil/grit separators based on the API design consist of three chambers. The first chamber removes coarse material and debris. The second chamber provides separation of oil, grease, and gasoline from the storm water runoff; and the third chamber provides a safety relief should a blockage occur.

Recent experiences have shown that, because of their volume limitations, oil/grit separators have limited pollutant removal effectiveness. They are perhaps the best example of a structural control that is only effective with frequent maintenance. Proper disposal of the standing water, trapped sediments, and floating hydrocarbons are problems in the few locations that have been studied.

Constructed Storm Water Wetlands

Constructed storm water wetlands are a hybrid, drawing on elements of detention and retention basins. Constructed storm water wetlands are shallow pools and are often designed to simulate the pollutant removal functions of natural wetlands. Enhanced designs may include a sediment forebay, carefully contoured topography, and multiple species of wetland plants. Constructed storm water wetlands, while a promising technology for pollutant removal from storm water, may not replicate all the ecological functions of natural wetlands.

6.4.1.2 Infiltration Controls

Infiltration controls rely chiefly on absorption to treat storm water discharges. In the ideal case, storm water percolates through a porous medium and into native soils where filtration and biological action remove pollutants. Typical controls of this type include infiltration trenches, infiltration basins, filtration basins, porous pavement, and concrete or block pavers. Systems that rely on soil absorption work best in deep, highly permeable soils that

are at least four feet away from the seasonal ground-water table.

The Soil Conservation Service (SCS) classifies soils into four major soil groups A-D. The soil groups are as follows:

Group A: Sand, loamy sand
Group B: Sandy loam, loam
Group C: Silt loam, sandy clay loam
Group D: Clay loam, silty clay loam, sandy clay, silty clay, and clay

Soils in Group A provide the highest infiltration rate while soils in Group D provide the lowest Suitable soils for infiltration-type controls typically fall in soil groups A and B. Other types of soils may be suitable, provided the clay content does not exceed 30 percent (clay has very low hydraulic conductivity). The clay content of soil may be determined from the SCS soil textural triangle, which can be found in many civil engineering references texts

If suitable soils are available, the widespread use of infiltration in a watershed can be useful in helping to maintain, restore, or replicate pre-development hydrology. Specific benefits of infiltration often include increased dry-weather baseflow in streams and a reduction in the frequency of bankfull floods. However, infiltration systems are not recommended unless soil conditions warrant. Also, infiltration should not be used where ground water requires protection. For example, the use of infiltration-type controls may not be appropriate in areas that recharge sole source aquifers.

Infiltration Basins

Infiltration basins are areas that intercept incoming storm water runoff and temporarily store it until it gradually infiltrates into the soil surrounding the basin. Infiltration basins should be designed to control drainage areas ranging from about 5 to 50 acres. They also should drain within 48 to 72 hours to maintain aerobic conditions favoring bacteria that aid in pollutant removal, and to ensure that the basin is ready to receive the next storm The runoff entering the basin is usually pretreated to remove coarse sediment that may clog the surface soil pores on the basin floor. Concentrated runoff may flow through a sediment trap or by sheet flow (vegetative filter strip).

Infiltration Trenches

Infiltration trenches are shallow (e.g., 2 to 10 feet deep) excavated ditches or vaults that have been backfilled with a coarse stone aggregate. The aggregate forms an underground reservoir that has approximately 40 percent void space. Storm water runoff diverted into the trench gradually infiltrates from the bottom of the trench into the subsoil and eventually into the ground water. Variations in the design of infiltration trenches include dry wells and percolation pits that are designed to control small volumes of runoff, such as the runoff from a rooftop A more complex variation is the enhanced infiltration trench, which is equipped with filter fabric or a more extensive pretreatment system to remove sediment and oil. Depending on the quality of the runoff, pretreatment may be necessary to lower the failure rate of the trench. Infiltration trenches are generally best suited for drainage areas of less than 10 acres They are particularly applicable for use on residential lots, small commercial areas, down slope from parking lots, and under drainage swales.

Grassed Swales

A grassed swale is an infiltration method that is usually used as a form of pretreatment before discharging runoff to another storm water control device (e.g., a detention basin) However, the grassed swale itself is a control that can remove significant amounts of pollutants through sediment entrainment A grassed swale is a shallow, vegetated, man-made ditch with the bottom elevation above the water table to allow runoff to infiltrate into the ground water. The vegetation helps to

prevent erosion, filters sediment, and allows for some uptake of nutrients.

Porous Pavement

Porous pavement, which is basically traditional asphalt aggregate without the fine particles, is an alternative to conventional pavement. Proper design and application of this control can reduce or eliminate the need for curbs and gutters, storm drains and sewers, and offside controls. Instead, runoff is diverted through a porous asphalt layer into an underground stone reservoir. The stored runoff gradually exfiltrates out of the stone reservoir into the subsoil. Soil considerations are important when evaluating the appropriateness of this control. Generally, grades should be gentle, and subsoil should be at least 3 feet thick (to bedrock) and moderately permeable (capable of infiltrating about one half inch per hour). Because porous pavement tends to clog with fine sediments and because it loses its effectiveness under heavy loads, its application should generally be limited to low-traffic areas (e g., overflow parking areas) and areas that are not exposed to large bearing loads caused by heavy vehicles

Concrete Grid Pavement

Concrete grid pavement has concrete blocks with regularly interdispersed void areas that are filled with pervious materials, such as gravel, sand, or grass The blocks are typically placed on a sand or gravel base. They are usually designed to provide a load-bearing surface adequate for supporting vehicles, while allowing infiltration of surface water into the underlying soil

6.4.1.3 Filtration Controls

Filtration controls treat storm water flows by using vegetation or sand to filter and settle pollutants Generally, these controls are most effective before the flows become concentrated (e g sheet flow) In certain instances, infiltration and treatment in the subsoil also may occur through the processes of absorption and adsorption After passing through the filtration media, the treated water is usually directed to a stream or MS4, although it may be evaporated or percolated into the ground Filtration controls include filter strips, grass swales, and sand filters Sand filters are particularly useful for ground water protection Applicants must consider the influence of climate when they select vegetative systems

Vegetative Filter Strips

Vegetative filter strips (also called bio-filters) are vegetated sections of land designed to accept runoff as overland sheet flow from upstream development They may adopt any natural vegetated form, from grassy meadow to small forest. The dense vegetative cover facilitates sediment reduction and pollutant removal Filter strips cannot treat high-velocity flows Therefore, these strips generally have been recommended for use in agriculture and low-density development and other situations where runoff does not tend to be concentrated Unlike grassed swales, filter strips are effective only for overland sheet flow, as opposed to concentrated flow Grading and level spreaders can be used to reduce the energy of concentrated flows and distribute the runoff evenly across the filter strip Vegetative filter strips are often used as pretreatment for other structural practices, such as infiltration trenches Leaving a buffer of natural vegetation along an urban stream valley is an example of a vegetative filter strip and also an example of a nonstructural control

Filtration Basins

Filtration basins are usually small impoundments lined with filter media, such as sand or gravel Storm water drains through the filter media and perforated pipes into the subsoil For optimal pollutant removal, recommended detention times range from 24 to 48 hours with a maximum drainage area of about 50 acres Grassed swales or other structural controls can be used to filter coarse sediments and thereby minimize clogging of the filter medium

6.4.2 Maintenance Activities

After summarizing the location of major structural storm water controls, applicants must submit a description of maintenance activities and a maintenance schedule for structural controls to reduce pollutants.

> §122.26(d)(2)(iv)(A)(1) [The application must include a] description of maintenance activities and a maintenance schedule for structural controls to reduce pollutants (including floatables) in discharges from municipal separate storm sewers.

Typical maintenance requirements include:

- Inspection of basins and ponds after every major storm for the first few months after construction and annually thereafter,
- Mowing of grass filter strips and swales at the frequency necessary to prevent woody growth and promote dense vegetation,
- Regular removal of litter and debris from dry ponds, forebays, and water quality inlets,
- Periodic stabilization and revegetation of eroded areas,
- Periodic removal and replacement of filter media from infiltration trenches and filtration ponds,
- Deep tilling of infiltration basins to maintain infiltrative capability, and
- Frequent vacuuming or jet hosing of porous pavement or concrete grid pavements

Lack of maintenance often limits the effectiveness of storm water structural controls such as detention/retention basins and infiltration devices. Maintenance programs should address measures for catch basins and drainage channels in addition to major structural controls

The proposed program should provide for maintenance logs and identify specific maintenance activities for each class of control, such as removing sediment from retention ponds every five years, cleaning catch basins annually, and removing litter from channels twice a year. If maintenance activities are scheduled infrequently, inspections must be scheduled to ensure that the control is operating adequately. In cases where scheduled maintenance is not appropriate, maintenance should be based on inspections of the control structure or frequency of storm events If maintenance depends on the results of inspections or if it occurs infrequently, the applicant must provide an inspection schedule. The applicant should also identify the municipal department(s) responsible for the maintenance program.

Municipalities should use caution in adopting controls that do not have sufficient history of use for their performance characteristics and maintenance requirements to be adequately evaluated A good example is the oil/grit separator used on small commercial or retail sites Some municipalities have required the use of these technologies, but due to poor performance, municipalities have often rescinded the requirement. In these cases, it is not clear whether the control technology was ineffective or the maintenance program was flawed

Because maintenance is critical to successful program implementation, it must be considered throughout the term of the permit Applicants may wish to develop a matrix that identifies maintenance tasks on a timeline indicating criteria for inspection, repair, and replacement PERT charts, GANT charts, or other critical path analyses (available for personal computers) can help organize a maintenance program and schedule. For a summarized

listing of appropriate maintenance activities and schedules refer to the matrix in Exhibit 6-3.

6.4.3 Considerations for Planning and Siting Controls

The storm water management program should describe the criteria used to identify that a particular structural control is warranted and the circumstances under which it will be required. The possibilities for new control sites should be evaluated for their storm water quality control potential. Guidelines and performance standards that identify specific structural controls for new development should be proposed in the procedures for new development. From this evaluation, priorities based on the feasibility of implementing a particular control at a given location can be determined.

6.4.3.1 Use of Municipal Lands

Applicants should discuss existing major structural controls and sites that have the potential for new structural controls which could be installed on municipal lands and other major rights-of-way (e g , major roads and highways) Note that existing controls are identified in Part 1 applications [(§122 26(d)(1)(iii)(B)(5)] The location of publicly owned parks, recreational areas, and other open areas are also identified [§122 26(d)(1)(iii)(6)]

To determine what storm water quality controls are necessary for public lands and facilities, current activities and functions that may affect the quality of storm water discharges should be identified Such activities and functions include parks, trails, and other recreational land uses, road maintenance and snow management, and storage and repair yards/shops for municipal vehicles An inventory of public land uses may be necessary to help make determinations of what controls are needed An effective inventory should involve coordination among all of the local departments and agencies that have authority over the use of public lands and facilities

Opportunities for controlling storm water quality problems that are identified through the inventory process can be evaluated on a site-specific basis and included in the proposed management program

There are several benefits to the establishment of structural controls on municipal lands:

- Municipal lands often provide greater retrofit opportunities because they typically do not require additional property purchases;
- Municipal lands ensure opportunities to provide future maintenance and security in preservation of the retrofit control,
- Applicants may be able to adapt existing municipal functions (such as industrial pretreatment program implementation, fire-safety inspections, and flood-control activities) to address storm water quality concerns (Expanding their mission to address storm water concerns may be more cost-effective than initiating entirely new programs.),
- Applicants may be able to adapt functions of development on municipal lands (such as planning, zoning, and construction oversight functions), and
- After considering controls on municipal lands, the applicant will be in a better position to address the private land under its jurisdiction

As a precaution, however, applicants need to consider potential conflicts arising over the multiple use of public lands Criteria other than land ownership (e g , locating controls downstream of developed areas) also should be considered when deciding where to locate storm water runoff controls

6.4.3.2 Use of Private Lands

A municipality also may incorporate storm water quality controls into its land use plan to indicate controls that may be necessary for new development Some of the best opportunities to prevent pollution and to implement effective storm water quality controls occur during development Local governments typically play a strong role in overseeing new development and have, or can adapt, administrative infrastructure to address storm water quality concerns.

The storm water management process should begin with land use planning and zoning and continue through the development and redevelopment processes. Municipalities generally can obtain commitments from land developers more easily prior to relinquishing jurisdictional leverage over the parcel where the potential control is to be located Leverage can be achieved through plan approval or zoning changes The negotiation process for the dedication, condemnation, or other acquisition of land and the process for getting the land developer to construct or otherwise implement controls will vary dramatically among municipalities, particularly among those in different States

Source and structural controls are most cost-effective when development is planned with storm water quality controls in mind However, it is probably more appropriate for the municipality to propose a flexible plan that specifies a variety of program objectives through the development process rather than identifying a certain priority and rigid schedule Other benefits of early and flexible planning include ecological diversity, wetlands preservation, and the creation of controls that also function as amenities Comprehensive land use plans zoning ordinances, and subdivision ordinances are important mechanisms to implement these controls early in the development process Consideration of storm water quality during pre-development is one of the most effective ways to implement controls This is because the maximum flexibility (and opportunity) to incorporate BMPs exists prior to final land use decisions and construction activities (see Section 6.3 1 1)

6.4.3.3 Siting Considerations

Imperviousness

The degree of imperviousness affects the concentration of pollutants in storm water, which in turn affects the type of structural controls that may be necessary. As the imperviousness of an area increases, the runoff volume and the pollutant loading increase. Studies show that runoff from industrial areas, which generally have a high degree of imperviousness, can have a wider variety and greater concentration of pollutants than runoff from other land uses Recent studies also indicate that the degree of imperviousness can be inferred from the level of degradation in urban receiving streams (For example, see Schueler 1991 and Klien 1979) Population projections will not indicate the degree to which industrial land use will increase unless planning and zoning information is also considered

Soil Conditions

Controls designed to infiltrate storm water will be affected by site specific soil conditions For example, clay content of the soil and the antecedent moisture content (degree of soil saturation at the time of a given storm event) will strongly influence the effectiveness, and therefore the applicability, of infiltration controls for a given location

6.5 PROGRAM AND SCHEDULE TO DETECT AND REMOVE ILLICIT DISCHARGES AND IMPROPER DISPOSAL

NPDES permits for discharges from MS4s require effective detection and removal from the MS4 of illicit or improper discharges and disposal

§122 26(d)(2)(iv)(B) [The application must include a] description of a program, including a schedule, to detect and remove (or require the discharger to the municipal separate storm sewer to obtain a separate NPDES permit for) illicit discharges and improper disposal into the storm sewer

The NURP study concluded that the quality of urban runoff can be adversely impacted by illicit connections and illegal dumping. Often, large amounts of wastes, particularly used oils, are improperly disposed of in storm sewers. Elimination of these sources of pollutants would result in a dramatic improvement in the quality of storm water discharges from MS4s. Procedures to eliminate such discharges should be an important part of the proposed management program.

The regulatory requirement cited above is intended to directly implement the mandate of Section 402(p)(3)(B)(ii) of the CWA, which requires permits for MS4s to effectively prohibit non-storm water discharges into storm sewers In certain instances, the most appropriate action will be for the municipality to ensure that illicit discharges become covered by a NPDES permit However, in most cases, elimination of illicit discharges or improper dumping is the appropriate focus of this program component The quality of storm water runoff from inner-city core areas, particularly in older parts of the country, would benefit most from this component

The applicant should propose a schedule for implementing this program component throughout the initial permit term This schedule should reflect the priorities identified by the municipality during the application process and be based on the problems particular to the specific MS4

6.5.1 Prohibiting Illicit Discharges

The proposed management program must include a description of inspection procedures, orders, ordinances, and other legal authorities necessary to prevent illicit discharges to the MS4.

§122.26(d)(2)(iv)(B)(1) [The application must include a] description of a program, including inspections, to implement and enforce an ordinance, orders or similar means to prevent illicit discharges to the municipal separate storm sewer system; this program description shall address all types of illicit discharges, however the following category of non-storm water discharges or flows shall be addressed where such discharges are identified by the municipality as sources of pollutants to waters of the United States . . . . *[these sources are listed in the guidance].*

This proposed management program component also should describe how the prohibition on illicit discharges will be implemented and enforced. The description should include a schedule and allocation of staff and resources A direct linkage should exist between this program component and the adequate legal authority requirements for the ordinances and orders to effectively implement the prohibition of illicit discharges

While this program component is required to prohibit all types of illicit discharges, the following categories of non-storm water discharges need only be prohibited by the MS4 when they are identified by the MS4 as sources of pollutants to waters of the United States:

- Water line flushing
- Landscape irrigation
- Diverted stream flows
- Rising ground waters
- Uncontaminated ground water infiltration [as defined at 40 CFR 35.2005(20)] to separate storm sewers
- Uncontaminated pumped ground water
- Discharges from potable water sources
- Foundation drains
- Air conditioning condensation
- Irrigation water

- Springs
- Water from crawl space pumps
- Footing drains
- Lawn watering
- Individual residential car washing
- Flows from riparian habitats and wetlands
- Dechlorinated swimming pool discharges
- Street wash water

While EPA does not consider these flows to be innocuous, they are only regulated by the storm water program to the extent that they may be identified as significant sources of pollutants to waters of the United States under certain circumstances. If an applicant knows, for example, that landscape irrigation water from a particular site flows through and picks up pesticides or excess nutrients from fertilizer applications, there may be a reasonable potential for a storm water discharge to result in a water quality impact. In such an event, the applicant should contact the NPDES permitting authority to request that the authority order the discharger to the MS4 to obtain a separate NPDES permit (or in this case, the discharge could be controlled through the storm water management program of the MS4)

The applicant should consider the specific land use, age, and stage of development in this program component. For example, one study in an established metropolitan area found that 60 percent of automobile-related businesses had improper storm drain connections. While some of the problems discovered in this study were the result of improper plumbing or illegal connections to storm drains, the majority of the connections were approved by the municipality when they were built

For problem identification and problem-solving, a municipality may elect to implement a follow-up study that traces identified pollution incidents to their source (e.g , up the system) A variety of pollutant-tracing techniques and field screening can be used to identify illicit discharges

6.5.2 Field Screening

Part 1 of the application requires applicants to submit the results of field screening studies to evaluate the possible occurrence of illicit connections and improper dumping [§122 26(d)(1)(iv)(D)]. Dry weather flows that were encountered during the initial field screening were sampled and analyzed. The analysis was intended to provide information about illicit connections and improper dumping.

In Part 2, applicants are required to propose procedures for continued field screening during the term of the permit.

> §122 26(d)(2)(iv)(B)(2) [The application must include a] description of procedures to conduct on-going field screening activities during the life of the permit, including areas or locations that will be evaluated by such field screens

Applicants can propose to use procedures similar to those used for field screening required in Part 1 of the application or they can propose alternative procedures and techniques. The Part 1 field screening requirements are found in §122.26(d)(1)(iv)(D) and are explained in the Part 1 guidance manual

The Part 2 proposed field screening program component should describe areas of the system where the continuation of the field screening program will be conducted and the rationale for selecting these areas For example, the rationale for continuing field screening at a given location might be that a wide variation in results was obtained during the initial screens In addition, the applicant should propose field screening for a portion of any recently-identified major outfalls that were not known to the applicant when it prepared its Part 1 application, provided sampling of these outfalls is safe and practicable

The potential for illicit discharges and improper disposal is generally higher for areas of older development, areas with many automobile-related industries, and areas with significant numbers of heavy industrial facilities Therefore, in most cases applicants should include these areas in the proposed field screening program.

The description of the field screening component should provide a detailed summary of the departmental responsibility for field activities, frequency of inspections, procedures and equipment to be used, and the procedures for documenting field activities, both in the field and in the office. Generally, the Part 2 field screening program should reflect a continuously narrowing process to trace illicit and improper sources.

6.5.3 Investigation of Potential Illicit Discharges

In order to submit a comprehensive proposed management program, applicants are required to describe procedures for investigating portions of the municipal system where field screening or other information indicates a reasonable potential for illicit discharges

> §122 26(d)(2)(iv)(B)(3). [The application must include a] description of procedures to be followed to investigate portions of the separate storm sewer system that, based on the results of the field screen, or other appropriate information, indicate a reasonable potential of containing illicit discharges or other sources of non-storm water (such procedures may include sampling procedures for constituents such as fecal coliform, fecal streptococcus, surfactants (MBAS), residual chlorine, fluorides and potassium, testing with fluorometric dyes, or conducting in storm sewer inspections where safety and other considerations allow Such description shall include the location of storm sewers that have been identified for such evaluation)

Applicants should propose criteria to identify portions of the system where follow-up investigations are appropriate For example, calculating a frequency distribution of dry weather flows at each screening site could aid in establishing criteria to identify where follow-up investigations are appropriate.

Procedures to investigate priority locations for illicit connections include inspection of the storm sewer system, use of remote-control cameras, on-site inspections and dye-testing at priority or suspect facilities, and additional discharge monitoring to pinpoint pollutant sources. In some cases, these investigations may be coordinated with pretreatment program inspections. Such approaches are summarized in Exhibit 6-4. Coordinating inspections can be a very effective use of resources. For example, portions of the sanitary sewer system that need evaluation to detect illicit discharge may already be undergoing inspection by operators of the municipal treatment plant.

A checklist should be developed for inspectors to use to detect illicit connections. The checklist should be structured to ensure a comprehensive evaluation of the problem and stipulate the use of the easiest and least expensive detection methods first

Regardless of the format in which information is compiled (e.g., table, list, text description), EPA suggests that the applicant prepare a map identifying the location of suspected problem areas. The map should be provided as part of the Part 2 application.

The proposed program component description should describe a step-by-step process to investigate, identify, and prohibit illicit discharges If field screening leads to positive tests of fecal coliform, fecal streptococcus, surfactants, residual chlorine, fluorides, or potassium, a municipality should reconsider whether any of the non-storm water discharges described in Section 6.5.1 are the source (see previous section)

Exhibit 6-4
Sample Illicit Discharge Investigation Procedures Options

Results of Initial Field Screen	Procedures for Detailed Analysis	Comments
Plumbing unidentifiable	Cameras	Effective for identifying deterioration
Uncertain use of facility	On-site inspections	May be combined with other inspections
Several facilities or complex plumbing	Dye-testing	Simple and accurate if system not interconnected
Unusual pollutants	Monitoring	Particularly useful for fingerprinting

6.5.4 Spill Response and Prevention

The proposed management program must describe procedures that the municipality will implement during the term of the permit to prevent contain, and respond to spills that may discharge into the MS4

> §122 26(d)(2)(iv)(B)(4) [The application must include a] description of procedures to prevent, contain, and respond to spills that may discharge into the municipal separate storm sewer

The municipality and the property owners (and/or operators) of sites where spills may occur need to implement procedures to prevent, contain and respond to spills One way to implement these procedures is to modify the land use planning process and ordinance enforcement Such modifications would require notification and emergency preparedness procedures for any land use activity that could lead to leaks and spills Another method is to coordinate with on-going programs in other regulated areas where detection of spills is important, such as pretreatment and hazardous materials management. The goal of a spill-prevention program is to reduce the frequency and extent of spills of hazardous materials, oils, and other materials which can cause water quality impairment. Spill-containment programs may establish minimum chemical storage and handling requirements, require users to submit prevention and control plans, and ensure site inspections The content of the descriptions that should be submitted with the Part 2 application for each of these program elements is discussed in more detail below

Spill-response programs are intended to reduce risk to the public and the environment Although these programs tend to focus on issues of public health and safety, such as exposure to toxic materials, fires, or explosions, spill-response teams should attempt to prevent or minimize contamination of surface water, ground water, and soil. Spill-response programs often require a coordinated response from a number of municipal departments (e.g., fire, police, health, and public works). Municipalities should describe how response procedures within these programs attempt to mitigate potential pollutant discharges to surface waters

For example, some industrial pretreatment programs specifically require that leaks or spills be routed to the storm sewer rather than the sanitary sewer generally to protect worker health and safety and to protect biological treatment capabilities This issue serves to reinforce the need for coordination between the various municipal programs that are related in some way to storm water.

The proposed program should identify the municipal departments responsible for implementing the program, and also should address employee training, reporting procedures, containment of spills, storage and disposal activities, documentation, and follow-up procedures Generally, the proposed program for spill response and prevention should focus on good housekeeping and materials management practices, which are discussed in more detail below.

One of the initial elements in the development of a successful spill response and prevention program is to assess the potential of various sources at a particular property to contribute pollutants to the storm water discharges from the site This assessment should inventory the land use, types of materials handled, and the location and types of materials management activities. Factors to consider when evaluating the pollution potential of runoff from various portions of a site include those that are likely to lead to the identification of specific structural or nonstructural controls to address problems

Other factors to consider are the toxicity and quantity of any chemicals used, produced, stored, or discharged from the site, the history of any NPDES permit violations from a site, history of significant leaks or spills of toxic or hazardous pollutants, and the designated uses of the receiving waters

This program element should also include a description of storm water management controls that are appropriate for the site that would control or allow for the mitigation of any leak or spill and a proposal to implement such controls. The priorities developed in the implementation proposal should reflect the nature of identified sources of pollutants at the site

The description of spill response and prevention activities should include the steps a municipality will take to prevent, and when necessary, adequately respond to spills discharged to its MS4. The MS4 might identify special training requirements for municipal employees in order to respond to spills of hazardous chemicals from a particular facility into the storm sewer system.

Sources with the greatest potential for spills to occur (or cause the most severe damage) should be identified in the proposed storm water management program If appropriate, specific materials handling procedures and storage requirements should be identified for these sources Requirements for these sources could be modeled after the Spill Prevention, Control, and Countermeasure (SPCC) Plans that are required for certain facilities under Section 311 of the CWA

Under the SPCC program, for example, personnel are trained and given responsibility for inspecting the facility for leaks and spills. These inspections include equipment and materials handling areas, which need to be investigated for evidence of, or the potential for, pollutants entering the drainage system Procedures to ensure the availability of appropriate personnel and equipment for cleaning up spills must be identified A system to ensure that appropriate corrective action has occurred in response to inadequacies identified during the inspection is also established under the program.

Not all of the SPCC program elements may be necessary for municipal applicants However, EPA recommends that the proposed storm water management program describe how the records of inspections will be maintained and made available for investigations of causal factors and program effectiveness Incidents of leaks, spills, and

improper dumping, along with other information describing the quality and quantity of storm water discharges should be included in the records Inspections and maintenance activities, such as containment berm integrity testing or the cleaning of oil/water separators should be documented and recorded in a maintenance log

6.5.5 Public Awareness and Reporting Program

Applicants must propose a management program component that promotes, publicizes, and facilitates public reporting of illicit discharges or water quality impacts associated with discharges from MS4s

> §122 26(d)(2)(iv)(B)(5) [The application must include a] description of a program to promote, publicize, and facilitate public reporting of the presence of illicit discharges or water quality impacts associated with discharges from municipal separate storm sewers

Timely reporting by the public of improper disposal and illicit discharges are critical components of programs to control such sources.

To enhance public awareness, programs may include setting up a public information hotline number, educating school students, establishing community and volunteer "watchdog" groups (e g , "Adopt-a-Stream Program"), using inserts into utility bills; and newspaper, television and radio announcements to inform the public about what to look for and how to report incidents The public awareness efforts should clarify to the public that they are the ultimate beneficiaries of a successful storm water management program

6.5.6 Proper Management of Used Oil and Toxics

EPA estimates that annually, 267 million gallons of used oil, including 135 million gallons of used oil from do-it-yourself automobile oil changes, are disposed of improperly An additional 70 million gallons of used oil, most coming from service stations and repair shops, are used for road oiling (55 FR 48056, November 16, 1990). If private individuals find the proper disposal of used oil or toxic materials difficult, incidents of improper disposal increase For example, when a large fraction of service stations do not accept do-it-yourself used oil, improper disposal into the municipal storm sewer rises. Therefore, applicants are required to propose a program component that will facilitate the proper disposal of used oil and toxics from households by establishing municipally operated collection sites, or ensuring that privately-operated collection sites are available.

> §122.26(d)(2)(iv)(B)(6) [The application must include a] description of educational activities, public information activities, and other appropriate activities to facilitate the proper management and disposal of used oil and toxic materials

The proposed program should describe outreach plans to handlers of used oil and to the public, and operating plans for oil and household waste collection programs.

Examples of effective public outreach for these types of programs include dedicated municipal phone numbers (e.g , a used oil/toxic materials hotline), pamphlets, and requirements that oil retailers post the location of the nearest used oil collection facility. Programs can also inform the public about alternatives to toxic materials. Catch basin/storm sewer inlet stenciling programs can also be proposed as part of the program to increase public awareness of the connection between storm sewers and local water resources

6.5.7 Infiltration of Seepage

In order to effectively complete this portion of a proposed management plan, the applicant must describe controls to limit infiltration of seepage from municipal sanitary sewers to MS4s, if necessary.

> §122.26(d)(2)(iv)(B)(7). [The application must include a] description of controls to limit infiltration of seepage from municipal sanitary sewers to municipal separate storm sewer systems where necessary.

Raw sewage can seep from sanitary sewage collection systems through leaks and cracks in aging pipes, poorly constructed manholes and joints, and main breaks. Sewage from a leaky sanitary system can flow to storm sewers or contaminate ground water supplies. Interaction between sanitary sewers and separate storm sewers may occur at manholes and where sanitary sewer laterals and storm sewer trenches cross. Separate storm sewers and sanitary sewers may share the same trench, which is generally filled with very porous material such as gravel.

One indication of seepage from a sewage collection system may be infiltration of water. Often, the rate of exfiltration (seepage) from sanitary collection systems is significantly greater than the rate of infiltration into the system. An EPA study on sewer exfiltration found significant ratios of the rate of exfiltration of sewage to the rate of infiltration of ground water or storm water into sanitary sewers. Field and laboratory results found this ratio to vary between 1.5 to 1 and 14 to 1.

In some cases, preventive maintenance surveys or on-going infiltration and inflow (I&I) programs to determine where water is entering a sanitary sewer system may be modified to locate the source and fate of exfiltration from the system

Identifying infiltration of seepage into a MS4 is a good example of the need for various municipal functions to be effectively coordinated. Proposed storm water management programs might discuss how personnel responsible for inspections of the sanitary sewer system could inspect for sources of exfiltration during I&I inspections, and pass any findings to personnel responsible for maintaining the MS4. If seepage is believed to be a problem, a coordinated effort with the maintenance department of the municipal sanitary sewer system is recommended.

The proposed storm water management program also should include provisions to address the discovery of previously unknown problems. There should be procedures to enact a coordinated program between the operators of the storm sewer and sanitary sewer (which in many cases will be within the same municipal agency or department).

EPA recommends that the proposed storm water management program describe controls that will be used to address seepage from malfunctioning septic systems in areas not served by a sewage treatment works. Malfunctioning septic systems may lead to more significant surface runoff pollution problems than ground water problems. A malfunctioning septic system is less likely to cause ground water contamination where an impervious bacterial mat in the soil retards the downward movement of wastewater. (Poorly located septic systems that are operating properly are the greatest threat to ground water).

Surface malfunctions of septic systems are caused by clogged or impermeable soils, or when stopped up or collapsed pipes force untreated wastewater to the surface. Surface malfunctions can vary in degree from occasional damp patches on the surface to constant pooling or runoff of wastewater to a storm sewer. An improper remedy for a surface malfunction is to install a pipe or trench over soil absorption systems to route untreated overflow away from the septic

system. This results in direct discharges to drainage ditches, empty lots, or surface waters

Proper controls range from prescribing maximum intervals between tank pump-out to the installation of sand filters. Discharge from sand filters to surface waters may require a separate NPDES permit, because such discharge is not storm water.

Additional information about the most appropriate controls for use in correcting malfunctioning septic systems is probably best obtained from local or regional sources. Organizations such as extension services, soil and water conservation districts, and planning agencies may be good sources of information about methods that have been successful (and also those that have failed).

By obtaining this type of information, the applicant can determine what control techniques have been successful in correcting malfunctioning septic systems in similar types of soils The value of this approach is that the applicant will know that a certain control technique has been used to correct a malfunctioning septic system in the same types of soils that occur in the municipality. Where only part of the MS4 drainage area is served by septic systems, proposed programs should address setting and maintenance of septic systems, including draft requirements and implementation procedures.

6.6 SIGNATORY AND CERTIFICATION REQUIREMENTS

Under the Federal NPDES regulations [§122 22(a)], all NPDES permit applications (including municipal storm water permit applications) must be signed by an authorized person, as defined in the regulations Permit applications submitted by a municipality, State, Federal, or other public agency must be signed by either a principal executive officer or ranking elected official [§122 22(a)(3)] To fulfill the signatory requirements, the person signing the municipal application must provide his or her name (printed or typed), title, and date signed In addition, the applicant should provide the name, address, and telephone number of the person signing the application or another point of contact that can answer questions about the application.

In addition, §122.22(d) states that any person signing a permit application must make the following certification:

"I certify under penalty of law that this document and all attachments were prepared under my direction or supervision in accordance with a system designed to assure that qualified personnel properly gather and evaluate the information submitted. Based on my inquiry of the person or persons who manage the system, or those persons directly responsible for gathering the information, the information submitted is, to the best of my knowledge and belief, true, accurate, and complete. I am aware that there are significant penalties for submitting false information, including the possibility of fine and imprisonment for knowing violations."

6.7 IMPLEMENTATION OF THE STORM WATER PROGRAM

EPA anticipates that municipal storm water management programs will mature over time to reflect advances in technology, additional data collection, changing conditions, program development, stage of implementation, and improvements in water quality. Therefore, applicants may emphasize different program components to reflect implementation priorities. The proposed management program should clearly identify each of the program components and include a schedule for implementation. Each component of the Part 2 application should be classified as: full implementation, phased implementation, pilot study, or feasibility analysis In annual reports on the progress of storm water management programs, municipalities must report on the status of implementing program provisions [§122 42(c)(1), or Section 7.3 of the guidance].

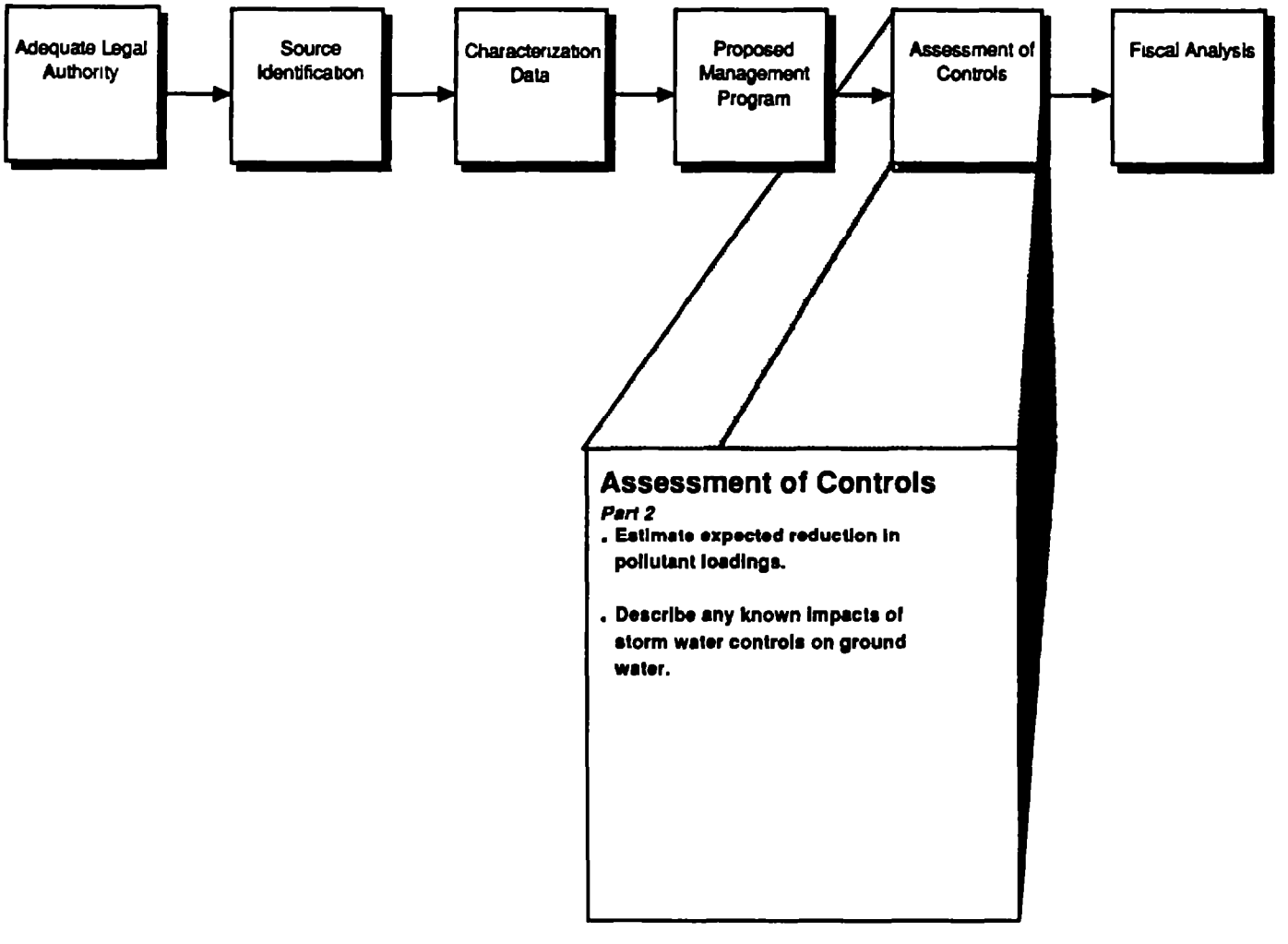
- **Full Implementation.** Fully implemented components should be proposed when the municipality is

prepared to begin or continue full implementation after its permit is issued and it expects to continue the component throughout the life of the permit. Full implementation of a program component is generally the preferred way of demonstrating the required level of control.

- **Phased Implementation.** Phased implementation should be proposed when the level of effort to implement the component will vary during the term of the permit. Phased implementation may be appropriate when additional data must be collected or technical guidance, training materials, or appropriate ordinances must be developed prior to full implementation. A schedule that includes milestones should be part of the description.

- **Pilot Studies.** Although the municipality must implement and comply with *each* provision of the municipal storm water permit, the municipality may choose to carry out pilot studies that involve limited experimental implementation of a program component. In some cases, pilot studies may be authorized by the permit. Used to evaluate the effectiveness of a program component, pilot studies may be appropriate when a technology is unproven or when data must be collected to develop operating standards or procedures. A schedule including milestones should be included in the description of a pilot study. This schedule should provide options for phased implementation of the program component, showing alternatives based on various possible results of the pilot study.

CHAPTER 7
ASSESSMENT OF CONTROLS



7.0 ASSESSMENT OF CONTROLS

7.1 BACKGROUND

Part 2 applications require that municipalities estimate the effectiveness of their proposed storm water quality management programs The regulations require an initial estimate or assessment because the performance of appropriate management controls is highly dependent on site-specific factors Program effectiveness can be estimated through both direct measurements (such as reductions in annual pollutant loads) and indirect measurements (such as measurements that demonstrate increased public awareness of storm water quality issues) At a minimum, applicants must submit estimated reductions in pollutant loads expected to result from implemented controls and describe known impacts of storm water controls on ground water

> 122 26(d)(2)(v) *Assessment of controls* [The application must include] estimated reductions in loadings of pollutants for discharges of municipal storm sewer constituents from municipal storm sewer systems expected as the result of the municipal storm water quality management program The assessment shall also identify known impacts of storm water controls on ground water

Reductions in pollutant loads due to the implementation and maintenance of structural controls provide direct measurements of the effectiveness of the storm water management program In addition, EPA encourages applicants to go beyond the minimum requirement and assess the effectiveness of their storm water management program through other direct measurements as well as indirect measurements As discussed below, indirect measurements provide surrogate estimates of qualitative factors, such as increased public awareness of storm water quality issues

Estimates of the effectiveness of the storm water management program will assist the municipality and the permit writer in:

- Determining whether the most cost-effective best management practices (BMPs) are included in the storm water management program;
- Ensuring that the storm water management program includes adequate public participation programs and intergovernmental coordination,
- Establishing on-going monitoring inspection and surveillance programs that help refine estimates of program effectiveness, and
- Developing a strategy to evaluate progress toward achieving water quality goals

7.2 ASSESSMENT OF STORM WATER MANAGEMENT PROGRAM

For some components of a proposed storm water management program, such as structural controls (e g , vegetative streambank stabilization, sediment pond or basin, etc.), the effect on pollution in storm water runoff is observable, and pollutant removal efficiencies can be estimated directly For other components, pollutant reductions may be difficult to quantify Applicants may need to use indirect estimates. For example, a program component may address source controls such as changing the behavior of citizens in the community, or improving the municipal control of industrial or commercial runoff For

components of the proposed management program where pollutant removal efficiency cannot be reasonably estimated, applicants are strongly encouraged to identify some indirect measurement that can be used to evaluate the success of the practice

7.2.1 Direct Measurements of Program Effectiveness

As discussed above, 40 CFR 122.26(d)(2)(v) requires that applicants submit estimates of expected pollutant load reductions with their Part 2 applications To supplement these estimates, applicants could provide estimates of other direct measurements of program effectiveness including

- Removal efficiencies of BMPs that control storm water quality,
- Reductions in the volume of storm water discharged,
- Reductions in event mean concentrations or
- Reductions in seasonal pollutant loadings

Such direct estimates do not have to be verified with quantitative data, but can be based on accepted engineering design practices However the applicant should describe its procedures for estimating the effectiveness of the control Applicants should present estimates of pollutant load reductions or other measurements separately for each component of the proposed management program Applicants should provide estimated reductions on a watershed basis and system-wide basis

Reductions in pollutant loadings can be estimated by first estimating the pollutant loading (based on concentrations and flows) that would result without the control measure The value should then be multiplied by the efficiency of the control expressed in terms of a fraction or percentage. Estimated control efficiencies can be obtained from published sources, such as Schueler (1987) (see bibliography in Appendix A) Note that for most control measures, the pollutant removal efficiency differs for different classes of pollutants

After the municipality's storm water management program is implemented, the municipality can work to refine its initial assessment of the program. For example, the permit will require applicants to submit estimates of event mean concentrations and estimates of annual pollutant loadings for each outfall in the system [§122.26(d)(2)(iii)(C), discussed in Section 5.5 of this guidance]. These estimates can be compared with the applicant's initial estimates

In addition, the estimated removal efficiencies can be refined through the monitoring program required by §122.26(d)(2)(iii)(D) (discussed in Section 5.6 of this guidance) To refine these estimates, the monitoring program should include measurements at the inflow and outflow points of the control Throughout the permit term, the municipality must submit refinements to its assessment or additional direct measurements of program effectiveness in its annual report (Section 7 3)

The applicant should use direct measurements of program effectiveness as it begins to assess its long-term progress in improving water quality through storm water management practices Direct measurements of program effectiveness may not provide meaningful conclusions on trends in water quality improvements for a couple of permit terms. However, applicants are encouraged to use direct measurements of program effectiveness, such as annual pollutant loads, event mean concentrations, and seasonal pollutant loadings, to begin to estimate long-term trends Several statistical methods that rely on linear regression have been developed

to model these measurements to determine if trends exist

7.2.2 Indirect Measurements of Program Effectiveness

When pollutant reductions cannot be estimated through direct measurement, appropriate indirect measurements may be used. These may include the estimated level of increased enforcement activity, increased public awareness, or reduction in number of illegal dumping incidents For example, a field screening program to identify illicit connections and improper dumping in Fort Worth, Texas, used reductions in observations of indicator pollutants as a measure of the success of the program (Fort Worth, 1988).

Other possible indirect measurements include.

- Gallons of used oil recycled,
- Amount of household hazardous waste collected,
- Number of educational brochures on storm water quality distributed;
- Number of public hearings on storm water and attendance at these hearings,
- Circulation of an annual report or periodic newsletters on progress in meeting storm water quality goals,
- Number of reports of illicit discharges or illegal dumping
- Number of spill clean-ups,
- Number of sewer inlets stenciled,
- Acres of open space,
- Number of construction and erosion and sediment control plans submitted and approved

Many of these indirect measurements will help to indicate whether the storm water management program includes adequate public participation and intergovernmental coordination.

7.2.3 Impacts of Storm Water Controls on Ground Water

Structural BMPs may have an impact on other media. Therefore, the Part 2 application requires that applicants discuss known impacts of storm water controls on ground water. Impacts should be identified separately for each component of the proposed management program. These controls may increase the quantity of ground water (such as infiltration leading to recharge), but degrade the quality of the ground water For example, in arid parts of the Southwest, imported water is often used for irrigation. This increases the quantity of ground water, but, because of high levels of nutrients and total suspended and dissolved solids in the irrigation water, also results in impacts on ground water quality

In addition, the applicant should evaluate whether structural controls for storm water impact other media, such as wetlands

7.3. ANNUAL REPORTS ON THE EFFECTIVENESS OF THE STORM WATER MANAGEMENT PROGRAM

Under §122.42(c), applicants must provide annual reports on the progress of their storm water management programs. These reports, which are due on the anniversaries of permit issuance, must include

- The status of implementing the components of the storm water management program that are required by the permit,
- Proposed changes to the storm water management programs that are established as permit conditions,

- Revisions, if necessary, to the assessment of controls and the fiscal analysis reported in the permit application;

- Summary of data, including monitoring data, that are accumulated throughout the reporting year,

- Projected annual expenditures and budget for the year following each annual report;

- A summary describing the number and nature of enforcement actions, inspections, and public education programs; and

- Identification of water quality improvements or degradation

Applicants should refer to the specific regulatory language in §122 42(c) for a more complete discussion of annual reporting requirements

Although the Part 2 application requirements do not specifically address annual reporting requirements, applicants should consider their strategy for preparing annual reports when they complete their Part 2 applications. A municipality may develop a strategy to assess the progress of its storm water management program throughout the term of the permit in addition to providing a baseline assessment of its program. To develop the strategy, applicants should

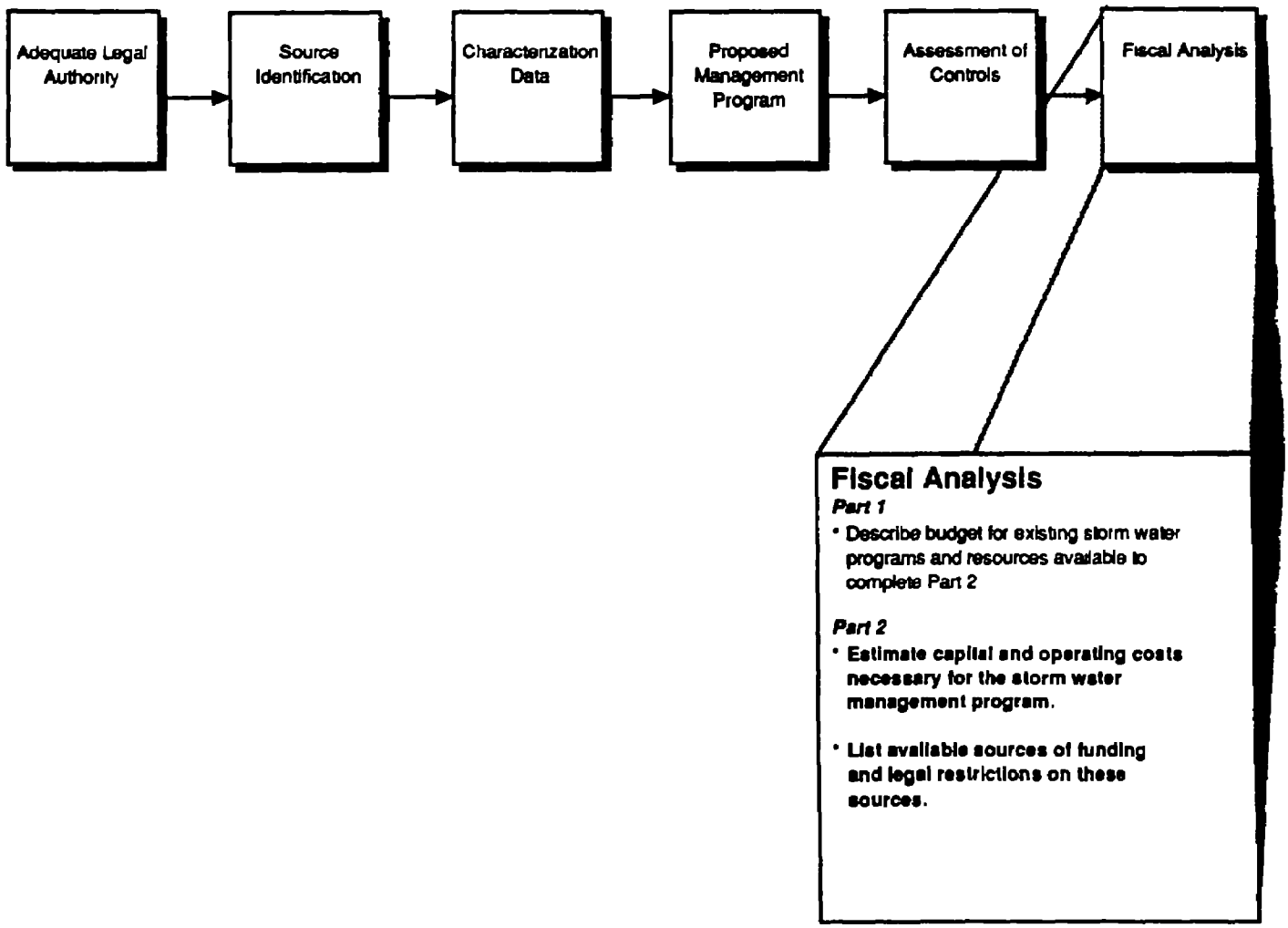
- Identify the direct or indirect measurements that will be used to track the long-term progress of the applicant's program towards achieving improvements in storm water quality (the results of this assessment would appear in the municipality's annual report);

- Discuss the role of monitoring data in substantiating or refining their assessment of the progress of their program towards established objectives and goals; and

- Discuss how future additions or revisions to the assessment measurements or strategy will be implemented by the municipality (e.g , what roles and responsibilities will participating municipal agencies and/or organizations have in this area)

It is anticipated that many municipalities will use the same criteria or measurements that were used in the baseline assessment to develop their long-term assessment strategy. This is an acceptable approach provided that the municipality delineates how their program provides for a longer term assessment of the progress of their storm water management program The municipality is encouraged to consider in advance the information requirements for annual reporting that are identified above when developing their long-term assessment strategy

CHAPTER 8
FISCAL ANALYSIS



8.0 FISCAL ANALYSIS

8.1 BACKGROUND

NPDES permits for discharges from MS4s will require municipal permittees to implement management programs, conduct long term storm water monitoring, and provide other information Because these activities will result in expense to the municipality, a fiscal analysis is required in the Part 2 application

Applicants must provide yearly cost estimates for these programs. Applicants also must provide a schedule indicating when funds will be available Examining the levels of proposed spending and funding allows the permitting authority to gauge the ability of the applicant to implement the program and predict its effectiveness The fiscal analysis also will help the permit writer determine whether the applicant has met the statutory requirement of reducing the discharge of pollutant to the MS4 to the maximum extent practicable Finally, the estimates help the applicant evaluate the feasibility and cost-effectiveness of its program A municipality must update its fiscal analysis each year for the annual report on the progress in implementing their storm water management program [40 CFR 122 42(c)(3) and (5), discussed in Section 7.3 of this guidance]

8.2 PROCEDURE FOR CONDUCTING A FISCAL ANALYSIS

Under §122 26(d)(2)(vi), each applicant must demonstrate sufficient financial resources to implement the conditions of the permit

Adequate resources may be demonstrated by performing a fiscal analysis of the estimated capital and operation and maintenance expenditures required to complete the activities required by the regulations This fiscal analysis must be performed for each fiscal year to be covered by the permit (5 years, in most cases). The analysis must describe the source of the funds used to meet the necessary expenditures, including any legal restrictions on the appropriated funds

> **§122.26(d)(2)(vi)** [The application must include] for each fiscal year to be covered by the permit, a fiscal analysis of the necessary capital and operation and maintenance expenditures necessary to accomplish the activities of the programs under paragraphs (d)(2)(iii) and (iv) of this section. Such analysis shall include a description of the source of funds that are proposed to meet the necessary expenditures, including legal restrictions on the use of such funds

The following procedure is an example of a method of conducting the necessary fiscal analysis

Step 1. Identify the major tasks for each component covered by this application requirement, including

- Elements of the proposed management program,
- Estimates of seasonal loads and event mean concentrations for each major outfall covered by the permit, and
- Proposed monitoring program.

Step 2. Develop a schedule outlining when each of the tasks identified in Step 1 will be undertaken Some tasks may be performed just once, others may be on-going For example, the schedule should include, among other things·

- The installation of any new control measures identified in the proposed

management program [§122 26(d)(2)(iv), discussed in Section 6 4],

- A maintenance schedule for structural best management practices (BMPs) [§122 26(d)(2)(iv)(A)(*1*), discussed in Section 6 4.3];

- Development of seasonal pollutant loadings and event mean concentrations of a representative storm [§122.26(d)(2)(iii)(C), discussed in Section 5.5];

- Monitoring program for representative data collection for the term of the permit [§122.26(d)(2)(iii)(D), discussed in Section 5 6],

- Monitoring program for industrial facilities [§122 26(d)(2)(iv)(C)(2), discussed in Section 6.3.3];

- On-going field screening program for illicit discharges [§122.26(d)(2)(iv)(B), discussed in Section 6 5],

- Development of certification programs for construction workers or pesticide applicators, if appropriate [§122 26(d)(2)(iv), discussed in Sections 6.3.1 and 6 3 2], and

- Implementation schedules for other components of the storm water application that have not been fully implemented at the time of application, such as additional legal authority or comprehensive development plans

Step 3. Estimate the capital expenses necessary to accomplish the tasks identified in Step 1 and determine a schedule for purchase Applicants may elect to define categories of capital expenditures such as "monitoring equipment," "miscellaneous monitoring supplies," "personal protective equipment," etc

Step 4. Estimate other non-capital costs to implement the tasks identified in Step 1. Use the schedule developed in Step 2 to spread costs over the term of the permit Costs should be presented as a total annual cost for each proposed program component In addition, estimates of the total annual costs and annual per capita costs should be provided. Per capita costs can be compared with the per capita costs of other programs, such as sewage treatment programs.

These costs may include items such as :

- Newspaper ads announcing new programs or recycling centers;

- Holding public meetings or hearings, and

- Labor for department personnel to speak to citizens groups

Step 5. Identify funding to be applied. Applicants must describe the sources of funding and any legal restrictions on that funding Sources may include general revenues, storm water utilities, plan review fees, permit fees, industrial/commercial user fees, special assessment district funds, and revenue bonds Some funding sources, such as general revenues based on property taxes, are generally unrestricted, but can be allocated by local officials annually In a few cases, a local property tax may be dedicated to finance a storm water management program For example, one county finances its storm water management program through a dedicated property tax of $0.135 per $100 assessed valuation Other municipalities add special assessments to property tax bills.

A storm water utility is another source of funding dedicated to financing storm water management activities The storm water utility offers the advantage of a stable and predictable source of funds. Other advantages of storm water utilities over general revenues are that utility charges can be more equitably based on

the user's contribution to local storm water problems, and a utility provides a mechanism to incorporate incentives for on-site storm water management

In many cases, municipalities will evaluate sources of funds that are not currently available, such as a new storm water utility. In these cases, applicants must include a schedule of when funds will be available. For example, it usually takes a municipality 18 to 24 months of planning before local elected officials authorize a storm water utility, and another 6 to 12 months to implement the utility (Lindsey, 1988) Key milestones for planning and implementing the funding mechanism must be identified in the schedule The following components have been found to be important in establishing storm water utilities

- Determining the most appropriate administrative structure for implementing a storm water management program,
- Adopting a storm water utility ordinance,
- Estimating revenue needs and planning for cost recovery,
- Establishing a utility rate structure and billing system,
- Establishing a system for developer contributions, and
- Implementing a public information program

Step 6. Compare the funding sources with the funding needs. As a last step in this process, the municipality must ensure that adequate funding is available to cover the cost of implementing the storm water management program If adequate funding is not available, the municipality must consider alternate sources of funding such as a storm water utility

APPENDIX A:
BIBLIOGRAPHY

BIBLIOGRAPHY*

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* For additional sources of information, applicants may wish to consult the documents listed in the bibliography of Urban Drainage & Flood Control District, *Urban Storm Drainage Criteria Manual, Vol III*, Urban Drainage and Flood Control District, Denver CO September 1, 1992

APPENDIX B:
PART 2 APPLICATION REQUIREMENTS

certify, pursuant to 5 U S.C 605(b) that these amendments do not, have a significant impact on a substantial number of small entities.

List of Subjects in 40 CFR Parts 122, 123, and 124

Administrative practice and procedure Environmental protection, Reporting and recordkeeping requirements, Water pollution control

Authority: Clean Water Act, 33 U S C 1251 *et seq*

Dated October 31, 1990

William K. Reilly,

Administrator

For the reasons stated in the preamble, parts 122, 123, and 124 of title 40 of the Code of Federal Regulations are amended as follows

PART 122—EPA ADMINISTERED PERMIT PROGRAMS; THE NATIONAL POLLUTANT DISCHARGE ELIMINATION SYSTEM

Subpart B—Permit Application and Special NPDES Program Requirements

1 The authority citation for part 122 continues to read as follows

Authority Clean Water Act 33 U S C 1251 *et seq*

2 Section 122 1 is amended by revising paragraph (b)(2)(iv) to read as follows

§ 122.1 Purpose and scope

* * * * *

(b) * * *

(2) * * *

(iv) Discharges of storm water as set forth in § 122 26, and

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3 Section 122 21 is amended by revising paragraph (c)(1) by removing the last sentence of paragraph (f)(7), by removing paragraph (f)(9) by adding two sentences at the end of paragraph (g)(3) by revising paragraph (g)(7) introductory text by removing and reserving paragraph (g)(10) and by revising the introductory text of paragraph (k) to read as follows

§ 122 21 Application for a permit (applicable to State programs, see § 123 25)

* * * * *

(c) *Time to apply* (1) Any person proposing a new discharge, shall submit an application at least 180 days before the date on which the discharge is to commence unless permission for a later date has been granted by the Director Facilities proposing a new discharge of storm water associated with industrial activity shall submit an application 180 days before that facility commences industrial activity which may result in a discharge of storm water associated with that industrial activity Facilities described under § 122.26(b)(14)(x) shall submit applications at least 90 days before the date on which construction is to commence. Different submittal dates may be required under the terms of applicable general permits Persons proposing a new discharge are encouraged to submit their applications well in advance of the 90 or 180 day requirements to avoid delay. See also paragraph (k) of this section and § 122.26 (c)(1)(i)(G) and (c)(1)(ii).

* * * * *

(g) * * *

(3) * * * The average flow of point sources composed of storm water may be estimated. The basis for the rainfall event and the method of estimation must be indicated.

* * * * *

(7) *Effluent characteristics.* Information on the discharge of pollutants specified in this paragraph (except information on storm water discharges which is to be provided as specified in § 122 26) When "quantitative data" for a pollutant are required, the applicant must collect a sample of effluent and analyze it for the pollutant in accordance with analytical methods approved under 40 CFR part 136 When no analytical method is approved the applicant may use any suitable method but must provide a description of the method When an applicant has two or more outfalls with substantially identical effluents the Director may allow the applicant to test only one outfall and report that the quantitative data also apply to the substantially identical outfalls The requirements in paragraphs (g)(7) (iii) and (iv) of this section that an applicant must provide quantitative data for certain pollutants known or believed to be present do not apply to pollutants present in a discharge solely as the result of their presence in intake water, however, an applicant must report such pollutants as present Grab samples must be used for pH, temperature, cyanide total phenols, residual chlorine, oil and grease, fecal coliform and fecal streptococcus For all other pollutants 24-hour composite samples must be used However, a minimum of one grab sample may be taken for effluents from holding ponds or other impoundments with a retention period greater than 24 hours In addition, for discharges other than storm water discharges, the Director may waive composite sampling for any outfall for which the applicant demonstrates that the use of an automatic sampler is infeasible and that the minimum of four (4) grab samples will be a representative sample of the effluent being discharged For storm water discharges, all samples shall be collected from the discharge resulting from a storm event that is greater than 0 1 inch and at least 72 hours from the previously measurable (greater than 0.1 inch rainfall) storm event Where feasible, the variance in the duration of the event and the total rainfall of the event should not exceed 50 percent from the average or median rainfall event in that area For all applicants, a flow-weighted composite shall be taken for either the entire discharge or for the first three hours of the discharge. The flow-weighted composite sample for a storm water discharge may be taken with a continuous sampler or as a combination of a minimum of three sample aliquots taken in each hour of discharge for the entire discharge or for the first three hours of the discharge, with each aliquot being separated by a minimum period of fifteen minutes (applicants submitting permit applications for storm water discharges under § 122 26(d) may collect flow weighted composite samples using different protocols with respect to the time duration between the collection of sample aliquots, subject to the approval of the Director) However, a minimum of one grab sample may be taken for storm water discharges from holding ponds or other impoundments with a retention period greater than 24 hours For a flow-weighted composite sample, only one analysis of the composite of aliquots is required For storm water discharge samples taken from discharges associated with industrial activities, quantitative data must be reported for the grab sample taken during the first thirty minutes (or as soon thereafter as practicable) of the discharge for all pollutants specified in § 122 26(c)(1) For all storm water permit applicants taking flow-weighted composites quantitative data must be reported for all pollutants specified in § 122 26 except pH temperature, cyanide total phenols residual chlorine, oil and grease fecal coliform, and fecal streptococcus, The Director may allow or establish appropriate site-specific sampling procedures or requirements including sampling locations, the season in which the sampling takes place the minimum duration between the previous measurable storm event and the storm event sampled the minimum or maximum level of precipitation required for an appropriate storm event, the form of precipitation sampled (snow melt or rain fall) protocols for collecting samples under 40 CFR part 136 and additional time for submitting data on a

case-by-case basis. An applicant is expected to "know or have reason to believe" that a pollutant is present in an effluent based on an evaluation of the expected use, production, or storage of the pollutant, or on any previous analyses for the pollutant. (For example, any pesticide manufactured by a facility may be expected to be present in contaminated storm water runoff from the facility.)

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(k) *Application requirements for new sources and new discharges.* New manufacturing, commercial, mining and silvicultural dischargers applying for NPDES permits (except for new discharges of facilities subject to the requirements of paragraph (h) of this section or new discharges of storm water associated with industrial activity which are subject to the requirements of § 122.26(c)(1) and this section (except as provided by § 122.26(c)(1)(ii)) shall provide the following information to the Director, using the application forms provided by the Director:

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4 Section 122.22(b) introductory text is revised to read as follows

§ 122.22 Signatories to permit applications and reports (applicable to State programs, see § 123.25).

• • • • •

(b) All reports required by permits, and other information requested by the Director shall be signed by a person described in paragraph (a) of this section, or by a duly authorized representative of that person. A person is a duly authorized representative only if

• • • • •

5 Section 122.26 is revised to read as follows

§ 122.26 Storm water discharges (applicable to State NPDES programs, see § 123.25)

(a) *Permit requirement.* (1) Prior to October 1, 1992, discharges composed entirely of storm water shall not be required to obtain a NPDES permit except:

(i) A discharge with respect to which a permit has been issued prior to February 4, 1987;

(ii) A discharge associated with industrial activity (see § 122.26(a)(4));

(iii) A discharge from a large municipal separate storm sewer system;

(iv) A discharge from a medium municipal separate storm sewer system;

(v) A discharge which the Director, or in States with approved NPDES programs either the Director or the EPA Regional Administrator, determines to contribute to a violation of a water quality standard or is a significant contributor of pollutants to waters of the United States. This designation may include a discharge from any conveyance or system of conveyances used for collecting and conveying storm water runoff or a system of discharges from municipal separate storm sewers, except for those discharges from conveyances which do not require a permit under paragraph (a)(2) of this section or agricultural storm water runoff which is exempted from the definition of point source at § 122.2. The Director may designate discharges from municipal separate storm sewers on a system-wide or jurisdiction-wide basis. In making this determination the Director may consider the following factors:

(A) The location of the discharge with respect to waters of the United States as defined at 40 CFR 122.2;

(B) The size of the discharge;

(C) The quantity and nature of the pollutants discharged to waters of the United States; and

(D) Other relevant factors.

(2) The Director may not require a permit for discharges of storm water runoff from mining operations or oil and gas exploration, production, processing or treatment operations or transmission facilities, composed entirely of flows which are from conveyances or systems of conveyances (including but not limited to pipes, conduits, ditches, and channels) used for collecting and conveying precipitation runoff and which are not contaminated by contact with or that has not come into contact with, any overburden, raw material, intermediate products, finished product, byproduct or waste products located on the site of such operations.

(3) *Large and medium municipal separate storm sewer systems.* (i) Permits must be obtained for all discharges from large and medium municipal separate storm sewer systems.

(ii) The Director may either issue one system-wide permit covering all discharges from municipal separate storm sewers within a large or medium municipal storm sewer system or issue distinct permits for appropriate categories of discharges within a large or medium municipal separate storm sewer system including, but not limited to all discharges owned or operated by the same municipality, located within the same jurisdiction; all discharges within a system that discharge to the same watershed, discharges within a system that are similar in nature, or for individual discharges from municipal separate storm sewers within the system.

(iii) The operator of a discharge from a municipal separate storm sewer which is part of a large or medium municipal separate storm sewer system must either:

(A) Participate in a permit application (to be a permittee or a co-permittee) with one or more other operators of discharges from the large or medium municipal storm sewer system which covers all, or a portion of all, discharges from the municipal separate storm sewer system;

(B) Submit a distinct permit application which only covers discharges from the municipal separate storm sewers for which the operator is responsible; or

(C) A regional authority may be responsible for submitting a permit application under the following guidelines:

(*1*) The regional authority together with co-applicants shall have authority over a storm water management program that is in existence, or shall be in existence at the time part 1 of the application is due;

(*2*) The permit applicant or co-applicants shall establish their ability to make a timely submission of part 1 and part 2 of the municipal application;

(*3*) Each of the operators of municipal separate storm sewers within the systems described in paragraphs (b)(4)(i), (ii), and (iii) or (b)(7)(i), (ii), and (iii) of this section, that are under the purview of the designated regional authority, shall comply with the application requirements of paragraph (d) of this section.

(iv) One permit application may be submitted for all or a portion of all municipal separate storm sewers within adjacent or interconnected large or medium municipal separate storm sewer systems. The Director may issue one system-wide permit covering all or a portion of all municipal separate storm sewers in adjacent or interconnected large or medium municipal separate storm sewer systems.

(v) Permits for all or a portion of all discharges from large or medium municipal separate storm sewer systems that are issued on a system-wide, jurisdiction-wide, watershed or other basis may specify different conditions relating to different discharges covered by the permit, including different management programs for different drainage areas which contribute storm water to the system.

(vi) Co-permittees need only comply with permit conditions relating to discharges from the municipal separate storm sewers for which they are operators.

(4) *Discharges through large and medium municipal separate storm sewer systems* In addition to meeting the requirements of paragraph (c) of this section, an operator of a storm water discharge associated with industrial activity which discharges through a large or medium municipal separate storm sewer system shall submit, to the operator of the municipal separate storm sewer system receiving the discharge no later than May 15, 1991, or 180 days prior to commencing such discharge: the name of the facility; a contact person and phone number; the location of the discharge; a description, including Standard Industrial Classification, which best reflects the principal products or services provided by each facility; and any existing NPDES permit number.

(5) *Other municipal separate storm sewers.* The Director may issue permits for municipal separate storm sewers that are designated under paragraph (a)(1)(v) of this section on a system-wide basis, jurisdiction-wide basis, watershed basis or other appropriate basis, or may issue permits for individual discharges

(6) *Non-municipal separate storm sewers* For storm water discharges associated with industrial activity from point sources which discharge through a non-municipal or non-publicly owned separate storm sewer system, the Director, in his discretion, may issue a single NPDES permit, with each discharger a co-permittee to a permit issued to the operator of the portion of the system that discharges into waters of the United States; or, individual permits to each discharger of storm water associated with industrial activity through the non-municipal conveyance system

(i) All storm water discharges associated with industrial activity that discharge through a storm water discharge system that is not a municipal separate storm sewer must be covered by an individual permit, or a permit issued to the operator of the portion of the system that discharges to waters of the United States, with each discharger to the non-municipal conveyance a co-permittee to that permit

(ii) Where there is more than one operator of a single system of such conveyances, all operators of storm water discharges associated with industrial activity must submit applications

(iii) Any permit covering more than one operator shall identify the effluent limitations or other permit conditions, if any, that apply to each operator

(7) *Combined sewer systems* Conveyances that discharge storm water runoff combined with municipal sewage are point sources that must obtain NPDES permits in accordance with the procedures of § 122.21 and are not subject to the provisions of this section.

(8) Whether a discharge from a municipal separate storm sewer is or is not subject to regulation under this section shall have no bearing on whether the owner or operator of the discharge is eligible for funding under title II, title III or title VI of the Clean Water Act. *See* 40 CFR part 35, subpart I, appendix A(b)H.2.j.

(b) *Definitions.* (1) *Co-permittee* means a permittee to a NPDES permit that is only responsible for permit conditions relating to the discharge for which it is operator.

(2) *Illicit discharge* means any discharge to a municipal separate storm sewer that is not composed entirely of storm water except discharges pursuant to a NPDES permit (other than the NPDES permit for discharges from the municipal separate storm sewer) and discharges resulting from fire fighting activities.

(3) *Incorporated place* means the District of Columbia, or a city, town, township, or village that is incorporated under the laws of the State in which it is located.

(4) *Large municipal separate storm sewer system* means all municipal separate storm sewers that are either:

(i) Located in an incorporated place with a population of 250,000 or more as determined by the latest Decennial Census by the Bureau of Census (appendix F); or

(ii) Located in the counties listed in appendix H, except municipal separate storm sewers that are located in the incorporated places, townships or towns within such counties; or

(iii) Owned or operated by a municipality other than those described in paragraph (b)(4) (i) or (ii) of this section and that are designated by the Director as part of the large or medium municipal separate storm sewer system due to the interrelationship between the discharges of the designated storm sewer and the discharges from municipal separate storm sewers described under paragraph (b)(4) (i) or (ii) of this section. In making this determination the Director may consider the following factors:

(A) Physical interconnections between the municipal separate storm sewers,

(B) The location of discharges from the designated municipal separate storm sewer relative to discharges from municipal separate storm sewers described in paragraph (b)(4)(i) of th section;

(C) The quantity and nature of pollutants discharged to waters of the United States;

(D) The nature of the receiving waters, and

(E) Other relevant factors, or

(iv) The Director may, upon petition, designate as a large municipal separate storm sewer system, municipal separate storm sewers located within the boundaries of a region defined by a storm water management regional authority based on a jurisdictional, watershed, or other appropriate basis that includes one or more of the systems described in paragraph (b)(4) (i), (ii), (iii) of this section.

(5) *Major municipal separate storm sewer outfall* (or "major outfall") means a municipal separate storm sewer outfall that discharges from a single pipe with an inside diameter of 36 inches or more or its equivalent (discharge from a single conveyance other than circular pipe which is associated with a drainage area of more than 50 acres), or for municipal separate storm sewers that receive storm water from lands zoned for industrial activity (based on comprehensive zoning plans or the equivalent), an outfall that discharge from a single pipe with an inside diameter of 12 inches or more or from its equivalent (discharge from other than a circular pipe associated with a drainage area of 2 acres or more)

(6) *Major outfall* means a major municipal separate storm sewer outfall.

(7) *Medium municipal separate storm sewer system* means all municipal separate storm sewers that are either:

(i) Located in an incorporated place with a population of 100,000 or more but less than 250,000, as determined by the latest Decennial Census by the Bureau of Census (appendix G), or

(ii) Located in the counties listed in appendix I, except municipal separate storm sewers that are located in the incorporated places, townships or towns within such counties, or

(iii) Owned or operated by a municipality other than those described in paragraph (b)(4) (i) or (ii) of this section and that are designated by the Director as part of the large or medium municipal separate storm sewer system due to the interrelationship between the discharges of the designated storm sewer and the discharges from municipal separate storm sewers described under paragraph (b)(4) (i) or (ii) of this section. In making this determination the Director may cons the following factors

(A) Physical interconnections between the municipal separate storm sewers;

(B) The location of discharges from the designated municipal separate storm sewer relative to discharges from municipal separate storm sewers described in paragraph (b)(7)(i) of this section;

(C) The quantity and nature of pollutants discharged to waters of the United States;

(D) The nature of the receiving waters, or

(E) Other relevant factors; or

(iv) The Director may, upon petition, designate as a medium municipal separate storm sewer system, municipal separate storm sewers located within the boundaries of a region defined by a storm water management regional authority based on a jurisdictional, watershed, or other appropriate basis that includes one or more of the systems described in paragraphs (b)(7) (i), (ii), (iii) of this section.

(8) *Municipal separate storm sewer* means a conveyance or system of conveyances (including roads with drainage systems, municipal streets, catch basins, curbs, gutters, ditches, man-made channels, or storm drains)

(i) Owned or operated by a State, city, town, borough, county, parish, district, association, or other public body (created by or pursuant to State law) having jurisdiction over disposal of sewage, industrial wastes, storm water, or other wastes, including special districts under State law such as a sewer district, flood control district or drainage district, or similar entity, or an Indian tribe or an authorized Indian tribal organization, or a designated and approved management agency under section 208 of the CWA that discharges to waters of the United States,

(ii) Designed or used for collecting or conveying storm water;

(iii) Which is not a combined sewer; and

(iv) Which is not part of a Publicly Owned Treatment Works (POTW) as defined at 40 CFR 122 2

(9) *Outfall* means a *point source* as defined by 40 CFR 122 2 at the point where a municipal separate storm sewer discharges to waters of the United States and does not include open conveyances connecting two municipal separate storm sewers or pipes, tunnels or other conveyances which connect segments of the same stream or other waters of the United States and are used to convey waters of the United States.

(10) *Overburden* means any material of any nature, consolidated or unconsolidated, that overlies a mineral deposit, excluding topsoil or similar naturally-occurring surface materials that are not disturbed by mining operations.

(11) *Runoff coefficient* means the fraction of total rainfall that will appear at a conveyance as runoff.

(12) *Significant materials* includes, but is not limited to: raw materials; fuels, materials such as solvents, detergents, and plastic pellets; finished materials such as metallic products; raw materials used in food processing or production; hazardous substances designated under section 101(14) of CERCLA; any chemical the facility is required to report pursuant to section 313 of title III of SARA; fertilizers; pesticides; and waste products such as ashes, slag and sludge that have the potential to be released with storm water discharges.

(13) *Storm water* means storm water runoff, snow melt runoff, and surface runoff and drainage.

(14) *Storm water discharge associated with industrial activity* means the discharge from any conveyance which is used for collecting and conveying storm water and which is directly related to manufacturing, processing or raw materials storage areas at an industrial plant. The term does not include discharges from facilities or activities excluded from the NPDES program under 40 CFR part 122. For the categories of industries identified in paragraphs (b)(14) (i) through (x) of this section, the term includes, but is not limited to, storm water discharges from industrial plant yards; immediate access roads and rail lines used or traveled by carriers of raw materials, manufactured products, waste material, or by-products used or created by the facility; material handling sites; refuse sites; sites used for the application or disposal of process waste waters (as defined at 40 CFR part 401); sites used for the storage and maintenance of material handling equipment, sites used for residual treatment, storage, or disposal; shipping and receiving areas; manufacturing buildings; storage areas (including tank farms) for raw materials, and intermediate and finished products; and areas where industrial activity has taken place in the past and significant materials remain and are exposed to storm water For the categories of industries identified in paragraph (b)(14)(xi) of this section, the term includes only storm water discharges from all the areas (except access roads and rail lines) that are listed in the previous sentence where material handling equipment or activities, raw materials, intermediate products, final products, waste materials, by-products, or industrial machinery are exposed to storm water. For the purposes of this paragraph, material handling activities include the storage, loading and unloading, transportation, or conveyance of any raw material, intermediate product, finished product, by-product or waste product. The term excludes areas located on plant lands separate from the plant's industrial activities, such as office buildings and accompanying parking lots as long as the drainage from the excluded areas is not mixed with storm water drained from the above described areas. Industrial facilities (including industrial facilities that are Federally, State, or municipally owned or operated that meet the description of the facilities listed in this paragraph (b)(14)(i)–(xi) of this section) include those facilities designated under the provisions of paragraph (a)(1)(v) of this section. The following categories of facilities are considered to be engaging in "industrial activity" for purposes of this subsection.

(i) Facilities subject to storm water effluent limitations guidelines, new source performance standards, or toxic pollutant effluent standards under 40 CFR subchapter N (except facilities with toxic pollutant effluent standards which are exempted under category (xi) in paragraph (b)(14) of this section);

(ii) Facilities classified as Standard Industrial Classifications 24 (except 2434), 26 (except 265 and 267), 28 (except 283), 29, 311, 32 (except 323), 33, 3441, 373;

(iii) Facilities classified as Standard Industrial Classifications 10 through 14 (mineral industry) including active or inactive mining operations (except for areas of coal mining operations no longer meeting the definition of a reclamation area under 40 CFR 434 11(1) because the performance bond issued to the facility by the appropriate SMCRA authority has been released, or except for areas of non-coal mining operations which have been released from applicable State or Federal reclamation requirements after December 17, 1990) and oil and gas exploration, production, processing, or treatment operations, or transmission facilities that discharge storm water contaminated by contact with or that has come into contact with, any overburden, raw material, intermediate products, finished products, byproducts or waste products located on the site of such operations. (inactive mining operations are mining sites that are not being actively mined but which have an identifiable owner/operator; inactive mining sites do not include sites where mining claims are being maintained prior to disturbances associated with the extraction, beneficiation, or processing of mined

materials, nor sites where minimal activities are undertaken for the sole purpose of maintaining a mining claim);

(iv) Hazardous waste treatment, storage, or disposal facilities, including those that are operating under interim status or a permit under subtitle C of RCRA;

(v) Landfills, land application sites, and open dumps that receive or have received any industrial wastes (waste that is received from any of the facilities described under this subsection) including those that are subject to regulation under subtitle D of RCRA.

(vi) Facilities involved in the recycling of materials, including metal scrapyards, battery reclaimers, salvage yards, and automobile junkyards, including but limited to those classified as Standard Industrial Classification 5015 and 5093.

(vii) Steam electric power generating facilities, including coal handling sites.

(viii) Transportation facilities classified as Standard Industrial Classifications 40, 41, 42 (except 4221-25), 43, 44, 45, and 5171 which have vehicle maintenance shops, equipment cleaning operations, or airport deicing operations. Only those portions of the facility that are either involved in vehicle maintenance (including vehicle rehabilitation, mechanical repairs, painting, fueling, and lubrication), equipment cleaning operations, airport deicing operations, or which are otherwise identified under paragraphs (b)(14) (i)-(vii) or (ix)-(xi) of this section are associated with industrial activity;

(ix) Treatment works treating domestic sewage or any other sewage sludge or wastewater treatment device or system, used in the storage treatment, recycling and reclamation of municipal or domestic sewage including land dedicated to the disposal of sewage sludge that are located within the confines of the facility with a design flow of 1.0 mgd or more or required to have an approved pretreatment program under 40 CFR part 403. Not included are farm lands, domestic gardens or lands used for sludge management where sludge is beneficially reused and which are not physically located in the confines of the facility, or areas that are in compliance with section 405 of the CWA.

(x) Construction activity including clearing, grading and excavation activities except operations that result in the disturbance of less than five acres of total land area which are not part of a larger common plan of development or sale.

(xi) Facilities under Standard Industrial Classifications 20, 21, 22, 23, 2434, 25, 265, 267, 27, 283, 285, 30, 31 (except 311), 323, 34 (except 3441), 35, 36, 37 (except 373), 38, 39, 4221-25, (and which are not otherwise included within categories (ii)-(x)).

(c) *Application requirements for storm water discharges associated with industrial activity*—(1) *Individual application.* Dischargers of storm water associated with industrial activity are required to apply for an individual permit, apply for a permit through a group application, or seek coverage under a promulgated storm water general permit. Facilities that are required to obtain an individual permit, or any discharge of storm water which the Director is evaluating for designation (*see* 40 CFR 124.52(c)) under paragraph (a)(1)(v) of this section and is not a municipal separate storm sewer, and which is not part of a group application described under paragraph (c)(2) of this section, shall submit an NPDES application in accordance with the requirements of § 122.21 as modified and supplemented by the provisions of the remainder of this paragraph. Applicants for discharges composed entirely of storm water shall submit Form 1 and Form 2F. Applicants for discharges composed of storm water and non-storm water shall submit Form 1, Form 2C, and Form 2F. Applicants for new sources or new discharges (as defined in § 122.2 of this part) composed of storm water and non-storm water shall submit Form 1, Form 2D, and Form 2F.

(i) Except as provided in § 122.26(c)(1)(ii)-(iv), the operator of a storm water discharge associated with industrial activity subject to this section shall provide:

(A) A site map showing topography (or indicating the outline of drainage areas served by the outfall(s) covered in the application if a topographic map is unavailable) of the facility including each of its drainage and discharge structures, the drainage area of each storm water outfall, paved areas and buildings within the drainage area of each storm water outfall, each past or present area used for outdoor storage or disposal of significant materials, each existing structural control measure to reduce pollutants in storm water runoff, materials loading and access areas, areas where pesticides, herbicides, soil conditioners and fertilizers are applied, each of its hazardous waste treatment, storage or disposal facilities (including each area not required to have a RCRA permit which is used for accumulating hazardous waste under 40 CFR 262.34), each well where fluids from the facility are injected underground; springs, and other surface water bodies which receive storm water discharges from the facility;

(B) An estimate of the area of impervious surfaces (including paved areas and building roofs) and the total area drained by each outfall (within a mile radius of the facility) and a narrative description of the following: Significant materials that in the three years prior to the submittal of this application have been treated, stored or disposed in a manner to allow exposure to storm water; method of treatment, storage or disposal of such materials; materials management practices employed, in the three years prior to the submittal of this application, to minimize contact by these materials with storm water runoff; materials loading and access areas; the location, manner and frequency in which pesticides, herbicides, soil conditioners and fertilizers are applied; the location and a description of existing structural and non-structural control measures to reduce pollutants in storm water runoff and a description of the treatment the storm water receives, including the ultimate disposal of any solid or fluid wastes other than by discharge;

(C) A certification that all outfalls that should contain storm water discharges associated with industrial activity have been tested or evaluated for the presence of non-storm water discharges which are not covered by a NPDES permit; tests for such non-storm water discharges may include smoke tests, fluorometric dye tests, analysis of accurate schematics, as well as other appropriate tests. The certification shall include a description of the method used, the date of any testing, and the on site drainage points that were directly observed during a test;

(D) Existing information regarding significant leaks or spills of toxic or hazardous pollutants at the facility that have taken place within the three years prior to the submittal of this application;

(E) Quantitative data based on samples collected during storm events and collected in accordance with § 122.21 of this part from all outfalls containing a storm water discharge associated with industrial activity for the following parameters:

(*1*) Any pollutant limited in an effluent guideline to which the facility is subject;

(*2*) Any pollutant listed in the facility's NPDES permit for its process wastewater (if the facility is operating under an existing NPDES permit);

(*3*) Oil and grease, pH, BOD5, COD, TSS, total phosphorus, total Kjeldahl nitrogen, and nitrate plus nitrite nitrogen;

(*4*) Any information on the discharge required under paragraph § 122.21(g)(7)(iii) and (iv) of this part;

(5) Flow measurements or estimates of the flow rate and the total amount of discharge for the storm event(s) sampled, and the method of flow measurement or estimation; and

(6) The date and duration (in hours) of the storm event(s) sampled, rainfall measurements or estimates of the storm event (in inches) which generated the sampled runoff and the duration between the storm event sampled and the end of the previous measurable (greater than 0.1 inch rainfall) storm event (in hours);

(F) Operators of a discharge which is composed entirely of storm water are exempt from the requirements of § 122.21 (g)(2), (g)(3), (g)(4), (g)(5), (g)(7)(i), (g)(7)(ii), and (g)(7)(v); and

(G) Operators of new sources or new discharges (as defined in § 122.2 of this part) which are composed in part or entirely of storm water must include estimates for the pollutants or parameters listed in paragraph (c)(1)(i)(E) of this section instead of actual sampling data, along with the source of each estimate. Operators of new sources or new discharges composed in part or entirely of storm water must provide quantitative data for the parameters listed in paragraph (c)(1)(i)(E) of this section within two years after commencement of discharge, unless such data has already been reported under the monitoring requirements of the NPDES permit for the discharge. Operators of a new source or new discharge which is composed entirely of storm water are exempt from the requirements of § 122.21 (k)(3)(ii), (k)(3)(iii), and (k)(5).

(ii) The operator of an existing or new storm water discharge that is associated with industrial activity solely under paragraph (b)(14)(x) of this section, is exempt from the requirements of § 122.21(g) and paragraph (c)(1)(i) of this section. Such operator shall provide a narrative description of

(A) The location (including a map) and the nature of the construction activity;

(B) The total area of the site and the area of the site that is expected to undergo excavation during the life of the permit;

(C) Proposed measures, including best management practices, to control pollutants in storm water discharges during construction, including a brief description of applicable State and local erosion and sediment control requirements;

(D) Proposed measures to control pollutants in storm water discharges that will occur after construction operations have been completed, including a brief description of applicable State or local erosion and sediment control requirements;

(E) An estimate of the runoff coefficient of the site and the increase in impervious area after the construction addressed in the permit application is completed, the nature of fill material and existing data describing the soil or the quality of the discharge, and

(F) The name of the receiving water.

(iii) The operator of an existing or new discharge composed entirely of storm water from an oil or gas exploration, production, processing, or treatment operation, or transmission facility is not required to submit a permit application in accordance with paragraph (c)(1)(i) of this section, unless the facility:

(A) Has had a discharge of storm water resulting in the discharge of a reportable quantity for which notification is or was required pursuant to 40 CFR 117.21 or 40 CFR 302.6 at anytime since November 16, 1987, or

(B) Has had a discharge of storm water resulting in the discharge of a reportable quantity for which notification is or was required pursuant to 40 CFR 110.6 at any time since November 16, 1987; or

(C) Contributes to a violation of a water quality standard.

(iv) The operator of an existing or new discharge composed entirely of storm water from a mining operation is not required to submit a permit application unless the discharge has come into contact with, any overburden, raw material, intermediate products, finished product, byproduct or waste products located on the site of such operations.

(v) Applicants shall provide such other information the Director may reasonably require under § 122.21(g)(13) of this part to determine whether to issue a permit and may require any facility subject to paragraph (c)(1)(ii) of this section to comply with paragraph (c)(1)(i) of this section.

(2) *Group application for discharges associated with industrial activity.* In lieu of individual applications or notice of intent to be covered by a general permit for storm water discharges associated with industrial activity, a group application may be filed by an entity representing a group of applicants (except facilities that have existing individual NPDES permits for storm water) that are part of the same subcategory (*see* 40 CFR subchapter N, part 405 to 471) or, where such grouping is inapplicable, are sufficiently similar as to be appropriate for general permit coverage under § 122.28 of this part. The part 1 application shall be submitted to the Office of Water Enforcement and Permits, U.S. EPA, 401 M Street, SW., Washington, DC 20460 (EN-336) for approval. Once a part 1 application is approved, group applicants are to submit Part 2 of the group application to the Office of Water Enforcement and Permits. A group application shall consist of:

(i) *Part 1.* Part 1 of a group application shall:

(A) Identify the participants in the group application by name and location. Facilities participating in the group application shall be listed in nine subdivisions, based on the facility location relative to the nine precipitation zones indicated in appendix E to this part.

(B) Include a narrative description summarizing the industrial activities of participants of the group application and explaining why the participants, as a whole, are sufficiently similar to be a covered by a general permit.

(C) Include a list of significant materials stored exposed to precipitation by participants in the group application and materials management practices employed to diminish contact by these materials with precipitation and storm water runoff.

(D) Identify ten percent of the dischargers participating in the group application (with a minimum of 10 dischargers, and either a minimum of two dischargers from each precipitation zone indicated in appendix E of this part in which ten or more members of the group are located, or one discharger from each precipitation zone indicated in appendix E of this part in which nine or fewer members of the group are located) from which quantitative data will be submitted in part 2. If more than 1,000 facilities are identified in a group application, no more than 100 dischargers must submit quantitative data in Part 2. Groups of between four and ten dischargers may be formed. However, in groups of between four and ten, at least half the facilities must submit quantitative data and at least one facility in each precipitation zone in which members of the group are located must submit data. A description of why the facilities selected to perform sampling and analysis are representative of the group as a whole in terms of the information provided in paragraph (c)(1)(i)(B) and (i)(C) of this section, shall accompany this section. Different factors impacting the nature of the storm water discharges such as processes used and material management shall be represented to the extent feasible in a manner roughly equivalent to their proportion in the group.

(ii) *Part 2.* Part 2 of a group application shall contain quantitative

data (NPDES Form 2F), as modified by paragraph (c)(1) of this section, so that when part 1 and part 2 of the group application are taken together, a complete NPDES application (Form 1, Form 2C, and Form 2F) can be evaluated for each discharger identified in paragraph (c)(2)(i)(D) of this section.

(d) *Application requirements for large and medium municipal separate storm sewer discharges.* The operator of a discharge from a large or medium municipal separate storm sewer or a municipal separate storm sewer that is designated by the Director under paragraph (a)(1)(v) of this section, may submit a jurisdiction-wide or system-wide permit application. Where more than one public entity owns or operates a municipal separate storm sewer within a geographic area (including adjacent or interconnected municipal separate storm sewer systems), such operators may be a coapplicant to the same application. Permit applications for discharges from large and medium municipal storm sewers or municipal storm sewers designated under paragraph (a)(1)(v) of this section shall include:

(1) *Part 1.* Part 1 of the application shall consist of:

(i) *General information.* The applicants' name, address, telephone number of contact person, ownership status and status as a State or local government entity.

(ii) *Legal authority.* A description of existing legal authority to control discharges to the municipal separate storm sewer system. When existing legal authority is not sufficient to meet the criteria provided in paragraph (d)(2)(i) of this section, the description shall list additional authorities as will be necessary to meet the criteria and shall include a schedule and commitment to seek such additional authority that will be needed to meet the criteria.

(iii) *Source identification.* (A) A description of the historic use of ordinances, guidance or other controls which limited the discharge of non-storm water discharges to any Publicly Owned Treatment Works serving the same area as the municipal separate storm sewer system.

(B) A USGS 7.5 minute topographic map (or equivalent topographic map with a scale between 1:10,000 and 1:24,000 if cost effective) extending one mile beyond the service boundaries of the municipal storm sewer system covered by the permit application. The following information shall be provided:

(*1*) The location of known municipal storm sewer system outfalls discharging to waters of the United States;

(*2*) A description of the land use activities (e.g. divisions indicating undeveloped, residential, commercial, agricultural and industrial uses) accompanied with estimates of population densities and projected growth for a ten year period within the drainage area served by the separate storm sewer. For each land use type, an estimate of an average runoff coefficient shall be provided;

(*3*) The location and a description of the activities of the facility of each currently operating or closed municipal landfill or other treatment, storage or disposal facility for municipal waste;

(*4*) The location and the permit number of any known discharge to the municipal storm sewer that has been issued a NPDES permit;

(*5*) The location of major structural controls for storm water discharge (retention basins, detention basins, major infiltration devices, etc.); and

(*6*) The identification of publicly owned parks, recreational areas, and other open lands.

(iv) *Discharge characterization.* (A) Monthly mean rain and snow fall estimates (or summary of weather bureau data) and the monthly average number of storm events.

(B) Existing quantitative data describing the volume and quality of discharges from the municipal storm sewer, including a description of the outfalls sampled, sampling procedures and analytical methods used.

(C) A list of water bodies that receive discharges from the municipal separate storm sewer system, including downstream segments, lakes and estuaries, where pollutants from the system discharges may accumulate and cause water degradation and a brief description of known water quality impacts. At a minimum, the description of impacts shall include a description of whether the water bodies receiving such discharges have been:

(*1*) Assessed and reported in section 305(b) reports submitted by the State, the basis for the assessment (evaluated or monitored), a summary of designated use support and attainment of Clean Water Act (CWA) goals (fishable and swimmable waters), and causes of nonsupport of designated uses;

(*2*) Listed under section 304(l)(1)(A)(i), section 304(l)(1)(A)(ii), or section 304(l)(1)(B) of the CWA that is not expected to meet water quality standards or water quality goals;

(*3*) Listed in State Nonpoint Source Assessments required by section 319(a) of the CWA that, without additional action to control nonpoint sources of pollution, cannot reasonably be expected to attain or maintain water quality standards due to storm sewer construction, highway maintenance and runoff from municipal landfills and municipal sludge adding significant pollution (or contributing to a violation of water quality standards);

(*4*) Identified and classified according to eutrophic condition of publicly owned lakes listed in State reports required under section 314(a) of the CWA (include the following: A description of those publicly owned lakes for which uses are known to be impaired; a description of procedures, processes and methods to control the discharge of pollutants from municipal separate storm sewers into such lakes; and a description of methods and procedures to restore the quality of such lakes);

(*5*) Areas of concern of the Great Lakes identified by the International Joint Commission;

(*6*) Designated estuaries under the National Estuary Program under section 320 of the CWA;

(*7*) Recognized by the applicant as highly valued or sensitive waters;

(*8*) Defined by the State or U.S. Fish and Wildlife Service's National Wetlands Inventory as wetlands; and

(*9*) Found to have pollutants in bottom sediments, fish tissue or biosurvey data.

(D) *Field screening.* Results of a fie screening analysis for illicit connect and illegal dumping for either selected field screening points or major outfalls covered in the permit application. At a minimum, a screening analysis shall include a narrative description, for either each field screening point or major outfall, of visual observations made during dry weather periods. If any flow is observed, two grab samples shall be collected during a 24 hour period with a minimum period of four hours between samples. For all such samples, a narrative description of the color, odor, turbidity, the presence of an oil sheen or surface scum as well as any other relevant observations regarding the potential presence of non-storm water discharges or illegal dumping shall be provided. In addition, a narrative description of the results of a field analysis using suitable methods to estimate pH, total chlorine, total copper, total phenol, and detergents (or surfactants) shall be provided along with a description of the flow rate. Where the field analysis does not involve analytical methods approved under 40 CFR part 136, the applicant shall provide a description of the method used including the name of the manufacturer of the test method along with the range and accuracy of the test. Field screening points shall be either major outfalls or other outfall points (or

any other point of access such as manholes) randomly located throughout the storm sewer system by placing a grid over a drainage system map and identifying those cells of the grid which contain a segment of the storm sewer system or major outfall. The field screening points shall be established using the following guidelines and criteria:

(*1*) A grid system consisting of perpendicular north-south and east-west lines spaced ¼ mile apart shall be overlayed on a map of the municipal storm sewer system, creating a series of cells;

(*2*) All cells that contain a segment of the storm sewer system shall be identified; one field screening point shall be selected in each cell; major outfalls may be used as field screening points;

(*3*) Field screening points should be located downstream of any sources of suspected illegal or illicit activity;

(*4*) Field screening points shall be located to the degree practicable at the farthest manhole or other accessible location downstream in the system, within each cell, however, safety of personnel and accessibility of the location should be considered in making this determination;

(*5*) Hydrological conditions; total drainage area of the site; population density of the site, traffic density; age of the structures or buildings in the area; history of the area; and land use types;

(*6*) For medium municipal separate storm sewer systems, no more than 250 cells need to have identified field screening points, in large municipal separate storm sewer systems, no more than 500 cells need to have identified field screening points, cells established by the grid that contain no storm sewer segments will be eliminated from consideration, if fewer than 250 cells in medium municipal sewers are created, and fewer than 500 in large systems are created by the overlay on the municipal sewer map then all those cells which contain a segment of the sewer system shall be subject to field screening (unless access to the separate storm sewer system is impossible), and

(*7*) Large or medium municipal separate storm sewer systems which are unable to utilize the procedures described in paragraphs (d)(1)(iv)(D) (*1*) through (*6*) of this section, because a sufficiently detailed map of the separate storm sewer systems is unavailable, shall field screen no more than 500 or 250 major outfalls respectively (or all major outfalls in the system, if less), in such circumstances, the applicant shall establish a grid system consisting of north-south and east-west lines spaced ¼ mile apart as an overlay to the boundaries of the municipal storm sewer system, thereby creating a series of cells; the applicant will then select major outfalls in as many cells as possible until at least 500 major outfalls (large municipalities) or 250 major outfalls (medium municipalities) are selected; a field screening analysis shall be undertaken at these major outfalls.

(E) *Characterization plan.* Information and a proposed program to meet the requirements of paragraph (d)(2)(iii) of this section. Such description shall include: the location of outfalls or field screening points appropriate for representative data collection under paragraph (d)(2)(iii)(A) of this section, a description of why the outfall or field screening point is representative, the seasons during which sampling is intended, a description of the sampling equipment. The proposed location of outfalls or field screening points for such sampling should reflect water quality concerns (*see* paragraph (d)(1)(iv)(C) of this section) to the extent practicable.

(v) *Management programs.* (A) A description of the existing management programs to control pollutants from the municipal separate storm sewer system. The description shall provide information on existing structural and source controls, including operation and maintenance measures for structural controls, that are currently being implemented. Such controls may include, but are not limited to. Procedures to control pollution resulting from construction activities; floodplain management controls; wetland protection measures; best management practices for new subdivisions, and emergency spill response programs. The description may address controls established under State law as well as local requirements.

(B) A description of the existing program to identify illicit connections to the municipal storm sewer system. The description should include inspection procedures and methods for detecting and preventing illicit discharges, and describe areas where this program has been implemented.

(vi) *Fiscal resources.* (A) A description of the financial resources currently available to the municipality to complete part 2 of the permit application. A description of the municipality's budget for existing storm water programs, including an overview of the municipality's financial resources and budget, including overall indebtedness and assets, and sources of funds for storm water programs.

(2) *Part 2.* Part 2 of the application shall consist of.

(i) *Adequate legal authority.* A demonstration that the applicant can operate pursuant to legal authority established by statute, ordinance or series of contracts which authorizes or enables the applicant at a minimum to.

(A) Control through ordinance, permit, contract, order or similar means, the contribution of pollutants to the municipal storm sewer by storm water discharges associated with industrial activity and the quality of storm water discharged from sites of industrial activity;

(B) Prohibit through ordinance, order or similar means, illicit discharges to the municipal separate storm sewer;

(C) Control through ordinance, order or similar means the discharge to a municipal separate storm sewer of spills, dumping or disposal of materials other than storm water;

(D) Control through interagency agreements among coapplicants the contribution of pollutants from one portion of the municipal system to another portion of the municipal system;

(E) Require compliance with conditions in ordinances, permits, contracts or orders; and

(F) Carry out all inspection, surveillance and monitoring procedures necessary to determine compliance and noncompliance with permit conditions including the prohibition on illicit discharges to the municipal separate storm sewer.

(ii) *Source identification* The location of any major outfall that discharges to waters of the United States that was not reported under paragraph (d)(1)(iii)(B)(*1*) of this section Provide an inventory, organized by watershed of the name and address, and a description (such as SIC codes) which best reflects the principal products or services provided by each facility which may discharge, to the municipal separate storm sewer, storm water associated with industrial activity;

(iii) *Characterization data* When "quantitative data" for a pollutant are required under paragraph (d)(a)(ui)(A)(*3*) of this paragraph, the applicant must collect a sample of effluent in accordance with 40 CFR 122.21(g)(7) and analyze it for the pollutant in accordance with analytical methods approved under 40 CFR part 136. When no analytical method is approved the applicant may use any suitable method but must provide a description of the method. The applicant must provide information characterizing the quality and quantity of discharges covered in the permit application, including:

(A) Quantitative data from representative outfalls designated by the Director (based on information received

in part 1 of the application, the Director shall designate between five and ten outfalls or field screening points as representative of the commercial, residential and industrial land use activities of the drainage area contributing to the system or, where there are less than five outfalls covered in the application, the Director shall designate all outfalls) developed as follows

(*1*) For each outfall or field screening point designated under this subparagraph, samples shall be collected of storm water discharges from three storm events occurring at least one month apart in accordance with the requirements at § 122.21(g)(7) (the Director may allow exemptions to sampling three storm events when climatic conditions create good cause for such exemptions).

(*2*) A narrative description shall be provided of the date and duration of the storm event(s) sampled, rainfall estimates of the storm event which generated the sampled discharge and the duration between the storm event sampled and the end of the previous measurable (greater than 0 1 inch rainfall) storm event.

(*3*) For samples collected and described under paragraphs (d)(2)(iii)(A)(*1*) and (A)(*2*) of this section, quantitative data shall be provided for the organic pollutants listed in Table II, the pollutants listed in Table III (toxic metals, cyanide and total phenols) of appendix D of 40 CFR part 122, and for the following pollutants

Total suspended solids (TSS)
Total dissolved solids (TDS)
COD
BOD_5
Oil and grease
Fecal coliform
Fecal streptococcus
pH
Total Kjeldahl nitrogen
Nitrate plus nitrite
Dissolved phosphorus
Total ammonia plus organic nitrogen
Total phosphorus

(*4*) Additional limited quantitative data required by the Director for determining permit conditions (the Director may require that quantitative data shall be provided for additional parameters, and may establish sampling conditions such as the location, season of sample collection, form of precipitation (snow melt, rainfall) and other parameters necessary to insure representativeness)

(B) Estimates of the annual pollutant load of the cumulative discharges to waters of the United States from all identified municipal outfalls and the event mean concentration of the cumulative discharges to waters of the United States from all identified municipal outfalls during a storm event (as described under § 122.21(c)(7)) for BOD_5, COD, TSS, dissolved solids, total nitrogen, total ammonia plus organic nitrogen, total phosphorus, dissolved phosphorus, cadmium, copper, lead, and zinc. Estimates shall be accompanied by a description of the procedures for estimating constituent loads and concentrations, including any modelling, data analysis, and calculation methods;

(C) A proposed schedule to provide estimates for each major outfall identified in either paragraph (d)(2)(ii) or (d)(1)(iii)(B)(*1*) of this section of the seasonal pollutant load and of the event mean concentration of a representative storm for any constituent detected in any sample required under paragraph (d)(2)(iii)(A) of this section; and

(D) A proposed monitoring program for representative data collection for the term of the permit that describes the location of outfalls or field screening points to be sampled (or the location of instream stations), why the location is representative, the frequency of sampling, parameters to be sampled, and a description of sampling equipment

(iv) *Proposed management program* A proposed management program covers the duration of the permit It shall include a comprehensive planning process which involves public participation and where necessary intergovernmental coordination, to reduce the discharge of pollutants to the maximum extent practicable using management practices, control techniques and system, design and engineering methods, and such other provisions which are appropriate. The program shall also include a description of staff and equipment available to implement the program Separate proposed programs may be submitted by each coapplicant. Proposed programs may impose controls on a systemwide basis, a watershed basis, a jurisdiction basis, or on individual outfalls. Proposed programs will be considered by the Director when developing permit conditions to reduce pollutants in discharges to the maximum extent practicable Proposed management programs shall describe priorities for implementing controls Such programs shall be based on.

(A) A description of structural and source control measures to reduce pollutants from runoff from commercial and residential areas that are discharged from the municipal storm sewer system that are to be implemented during the life of the permit accompanied with an estimate of the expected reduction of pollutant loads and a proposed schedule for implementing such controls At a minimum, the description shall include.

(*1*) A description of maintenance activities and a maintenance schedule for structural controls to reduce pollutants (including floatables) in discharges from municipal separate storm sewers;

(*2*) A description of planning procedures including a comprehensive master plan to develop, implement and enforce controls to reduce the discharge of pollutants from municipal separate storm sewers which receive discharges from areas of new development and significant redevelopment. Such plan shall address controls to reduce pollutants in discharges from municipal separate storm sewers after construction is completed. (Controls to reduce pollutants in discharges from municipal separate storm sewers containing construction site runoff are addressed in paragraph (d)(2)(iv)(D) of this section.

(*3*) A description of practices for operating and maintaining public streets, roads and highways and procedures for reducing the impact on receiving waters of discharges from municipal storm sewer systems, including pollutants discharged as a result of deicing activities.

(*4*) A description of procedures to assure that flood management projects assess the impacts on the water quality of receiving water bodies and that existing structural flood control devices have been evaluated to determine if retrofitting the device to provide additional pollutant removal from storm water is feasible.

(*5*) A description of a program to monitor pollutants in runoff from operating or closed municipal landfills or other treatment, storage or disposal facilities for municipal waste, which shall identify priorities and procedures for inspections and establishing and implementing control measures for such discharges (this program can be coordinated with the program developed under paragraph (d)(2)(iv)(C) of this section), and

(*6*) A description of a program to reduce to the maximum extent practicable, pollutants in discharges from municipal separate storm sewers associated with the application of pesticides, herbicides and fertilizer which will include, as appropriate, controls such as educational activities, permits, certifications and other measures for commercial applicators and distributors, and controls for application in public right-of-ways and at municipal facilities

(B) A description of a program, including a schedule, to detect and remove (or require the discharger to the municipal separate storm sewer to obtain a separate NPDES permit for) illicit discharges and improper disposal into the storm sewer. The proposed program shall include:

(*1*) A description of a program, including inspections, to implement and enforce an ordinance, orders or similar means to prevent illicit discharges to the municipal separate storm sewer system; this program description shall address all types of illicit discharges, however the following category of non-storm water discharges or flows shall be addressed where such discharges are identified by the municipality as sources of pollutants to waters of the United States: water line flushing, landscape irrigation, diverted stream flows, rising ground waters, uncontaminated ground water infiltration (as defined at 40 CFR 35.2005(20)) to separate storm sewers, uncontaminated pumped ground water, discharges from potable water sources, foundation drains, air conditioning condensation, irrigation water, springs, water from crawl space pumps, footing drains, lawn watering, individual residential car washing, flows from riparian habitats and wetlands, dechlorinated swimming pool discharges, and street wash water (program descriptions shall address discharges or flows from fire fighting only where such discharges or flows are identified as significant sources of pollutants to waters of the United States).

(*2*) A description of procedures to conduct on-going field screening activities during the life of the permit, including areas or locations that will be evaluated by such field screens.

(*3*) A description of procedures to be followed to investigate portions of the separate storm sewer system that, based on the results of the field screen, or other appropriate information, indicate a reasonable potential of containing illicit discharges or other sources of non-storm water (such procedures may include sampling procedures for constituents such as fecal coliform, fecal streptococcus, surfactants (MBAS), residual chlorine, fluorides and potassium, testing with fluorometric dyes, or conducting in storm sewer inspections where safety and other considerations allow. Such description shall include the location of storm sewers that have been identified for such evaluation).

(*4*) A description of procedures to prevent, contain, and respond to spills that may discharge into the municipal separate storm sewer;

(*5*) A description of a program to promote, publicize, and facilitate public reporting of the presence of illicit discharges or water quality impacts associated with discharges from municipal separate storm sewers;

(*6*) A description of educational activities, public information activities, and other appropriate activities to facilitate the proper management and disposal of used oil and toxic materials; and

(*7*) A description of controls to limit infiltration of seepage from municipal sanitary sewers to municipal separate storm sewer systems where necessary;

(C) A description of a program to monitor and control pollutants in storm water discharges to municipal systems from municipal landfills, hazardous waste treatment, disposal and recovery facilities, industrial facilities that are subject to section 313 of title III of the Superfund Amendments and Reauthorization Act of 1986 (SARA), and industrial facilities that the municipal permit applicant determines are contributing a substantial pollutant loading to the municipal storm sewer system. The program shall:

(*1*) Identify priorities and procedures for inspections and establishing and implementing control measures for such discharges;

(*2*) Describe a monitoring program for storm water discharges associated with the industrial facilities identified in paragraph (d)(2)(iv)(C) of this section, to be implemented during the term of the permit, including the submission of quantitative data on the following constituents: any pollutants limited in effluent guidelines subcategories, where applicable; any pollutant listed in an existing NPDES permit for a facility; oil and grease, COD, pH, BOD_5, TSS, total phosphorus, total Kjeldahl nitrogen, nitrate plus nitrite nitrogen, and any information on discharges required under 40 CFR 122.21(g)(7) (iii) and (iv).

(D) A description of a program to implement and maintain structural and non-structural best management practices to reduce pollutants in storm water runoff from construction sites to the municipal storm sewer system, which shall include:

(*1*) A description of procedures for site planning which incorporate consideration of potential water quality impacts;

(*2*) A description of requirements for nonstructural and structural best management practices;

(*3*) A description of procedures for identifying priorities for inspecting sites and enforcing control measures which consider the nature of the construction activity, topography, and the characteristics of soils and receiving water quality; and

(*4*) A description of appropriate educational and training measures for construction site operators.

(v) *Assessment of controls.* Estimated reductions in loadings of pollutants from discharges of municipal storm sewer constituents from municipal storm sewer systems expected as the result of the municipal storm water quality management program. The assessment shall also identify known impacts of storm water controls on ground water.

(vi) *Fiscal analysis.* For each fiscal year to be covered by the permit, a fiscal analysis of the necessary capital and operation and maintenance expenditures necessary to accomplish the activities of the programs under paragraphs (d)(2) (iii) and (iv) of this section. Such analysis shall include a description of the source of funds that are proposed to meet the necessary expenditures, including legal restrictions on the use of such funds.

(vii) Where more than one legal entity submits an application, the application shall contain a description of the roles and responsibilities of each legal entity and procedures to ensure effective coordination.

(viii) Where requirements under paragraph (d)(1)(iv)(E), (d)(2)(ii), (d)(2)(iii)(B) and (d)(2)(iv) of this section are not practicable or are not applicable, the Director may exclude any operator of a discharge from a municipal separate storm sewer which is designated under paragraph (a)(1)(v), (b)(4)(ii) or (b)(7)(ii) of this section from such requirements. The Director shall not exclude the operator of a discharge from a municipal separate storm sewer identified in appendix F, G, H or I of part 122, from any of the permit application requirements under this paragraph except where authorized under this section.

(e) *Application deadlines.* Any operator of a point source required to obtain a permit under paragraph (a)(1) of this section that does not have an effective NPDES permit covering its storm water outfalls shall submit an application in accordance with the following deadlines:

(1) For any storm water discharge associated with industrial activity identified in paragraph (b)(14) (i)–(xi) of this section, that is not part of a group application as described in paragraph (c)(2) of this section or which is not covered under a promulgated storm water general permit, a permit application made pursuant to paragraph (c) of this section shall be submitted to the Director by November 18, 1991.

(2) For any group application submitted in accordance with paragraph (c)(2) of this section:

(i) Part 1 of the application shall be submitted to the Director, Office of Water Enforcement and Permits by March 18, 1991;

(ii) Based on information in the part 1 application, the Director will approve or deny the members in the group application within 60 days after receiving part 1 of the group application.

(iii) Part 2 of the application shall be submitted to the Director, Office of Water Enforcement and Permits no later than 12 months after the date of approval of the part 1 application.

(iv) Facilities that are rejected as members of a group by the permitting authority shall have 12 months to file an individual permit application from the date they receive notification of their rejection

(v) A facility listed under paragraph (b)(14) (i)–(xi) of this section may add on to a group application submitted in accordance with paragraph (e)(2)(i) of this section at the discretion of the Office of Water Enforcement and Permits, and only upon a showing of good cause by the facility and the group applicant, the request for the addition of the facility shall be made no later than February 18,1992; the addition of the facility shall not cause the percentage of the facilities that are required to submit quantitative data to be less than 10%, unless there are over 100 facilities in the group that are submitting quantitative data. approval to become part of group application must be obtained from the group or the trade association representing the individual facilities.

(3) For any discharge from a large municipal separate storm sewer system:

(i) Part 1 of the application shall be submitted to the Director by November 18, 1991.

(ii) Based on information received in the part 1 application the Director will approve or deny a sampling plan under paragraph (d)(1)(iv)(E) of this section within 90 days after receiving the part 1 application.

(iii) Part 2 of the application shall be submitted to the Director by November 16, 1992.

(4) For any discharge from a medium municipal separate storm sewer system.

(i) Part 1 of the application shall be submitted to the Director by May 18, 1992

(ii) Based on information received in the part 1 application the Director will approve or deny a sampling plan under paragraph (d)(1)(iv)(E) of this section within 90 days after receiving the part 1 application

(iii) Part 2 of the application shall be submitted to the Director by May 17, 1993.

(5) A permit application shall be submitted to the Director within 60 days of notice, unless permission for a later date is granted by the Director (*see* 40 CFR 124.52(c)), for:

(i) A storm water discharge which the Director, or in States with approved NPDES programs, either the Director or the EPA Regional Administrator, determines that the discharge contributes to a violation of a water quality standard or is a significant contributor of pollutants to waters of the United States (see paragraph (a)(1)(v) of this section);

(ii) A storm water discharge subject to paragraph (c)(1)(v) of this section.

(6) Facilities with existing NPDES permits for storm water discharges associated with industrial activity shall maintain existing permits. New applications shall be submitted in accordance with the requirements of 40 CFR 122.21 and 40 CFR 122.26(c) 180 days before the expiration of such permits. Facilities with expired permits or permits due to expire before May 18, 1992, shall submit applications in accordance with the deadline set forth under paragraph (c)(1) of this section.

(f) *Petitions.* (1) Any operator of a municipal separate storm sewer system may petition the Director to require a separate NPDES permit (or a permit issued under an approved NPDES State program) for any discharge into the municipal separate storm sewer system.

(2) Any person may petition the Director to require a NPDES permit for a discharge which is composed entirely of storm water which contributes to a violation of a water quality standard or is a significant contributor of pollutants to waters of the United States.

(3) The owner or operator of a municipal separate storm sewer system may petition the Director to reduce the Census estimates of the population served by such separate system to account for storm water discharged to combined sewers as defined by 40 CFR 35 2005(b)(11) that is treated in a publicly owned treatment works. In municipalities in which combined sewers are operated, the Census estimates of population may be reduced proportional to the fraction, based on estimated lengths, of the length of combined sewers over the sum of the length of combined sewers and municipal separate storm sewers where an applicant has submitted the NPDES permit number associated with each discharge point and a map indicating areas served by combined sewers and the location of any combined sewer overflow discharge point.

(4) Any person may petition the Director for the designation of a large or medium municipal separate storm sewer system as defined by paragraphs (b)(4)(iv) or (b)(7)(iv) of this section.

(5) The Director shall make a final determination on any petition received under this section within 90 days after receiving the petition.

6. Section 122.28(b)(2)(i) is revised to read as follows:

§ 122.28 General permits (applicable to State NPDES programs, see § 123.25).

* * * * *

(b) * * *

(2) *Requiring an individual permit.* (i) The Director may require any discharger authorized by a general permit to apply for and obtain an individual NPDES permit. Any interested person may petition the Director to take action under this paragraph. Cases where an individual NPDES permit may be required include the following:

(A) The discharger or "treatment works treating domestic sewage" is not in compliance with the conditions of the general NPDES permit.

(B) A change has occurred in the availability of demonstrated techno or practices for the control or abate of pollutants applicable to the point source or treatment works treating domestic sewage.

(C) Effluent limitation guidelines are promulgated for point sources covered by the general NPDES permit.

(D) A Water Quality Management plan containing requirements applicable to such point sources is approved.

(E) Circumstances have changed since the time of the request to be covered so that the discharger is no longer appropriately controlled under the general permit, or either a temporary or permanent reduction or elimination of the authorized discharge is necessary;

(F) Standards for sewage sludge use or disposal have been promulgated for the sludge use and disposal practice covered by the general NPDES permit, or

(G) The discharge(s) is a significant contributor of pollutants. In making this determination, the Director may consider the following factors:

(*1*) The location of the discharge with respect to waters of the United States,

(*2*) The size of the discharge;

(*3*) The quantity and nature of the pollutants discharged to waters of the United States, and

(*4*) Other relevant factors.

* * * * *

7. Section 122.42 is amended by adding paragraph (c) to read as follows.

§ 122.42 Additional conditions applicable to specified categories of NPDES permits (applicable to State NPDES programs, see § 123.25).

. . . . .

(c) *Municipal separate storm sewer systems.* The operator of a large or medium municipal separate storm sewer system or a municipal separate storm sewer that has been designated by the Director under § 122.26(a)(1)(v) of this part must submit an annual report by the anniversary of the date of the issuance of the permit for such system. The report shall include:

(1) The status of implementing the components of the storm water management program that are established as permit conditions.

(2) Proposed changes to the storm water management programs that are established as permit condition. Such proposed changes shall be consistent with § 122.26(d)(2)(iii) of this part; and

(3) Revisions, if necessary, to the assessment of controls and the fiscal analysis reported in the permit application under § 122.26(d)(2)(iv) and (d)(2)(v) of this part.

(4) A summary of data, including monitoring data, that is accumulated throughout the reporting year;

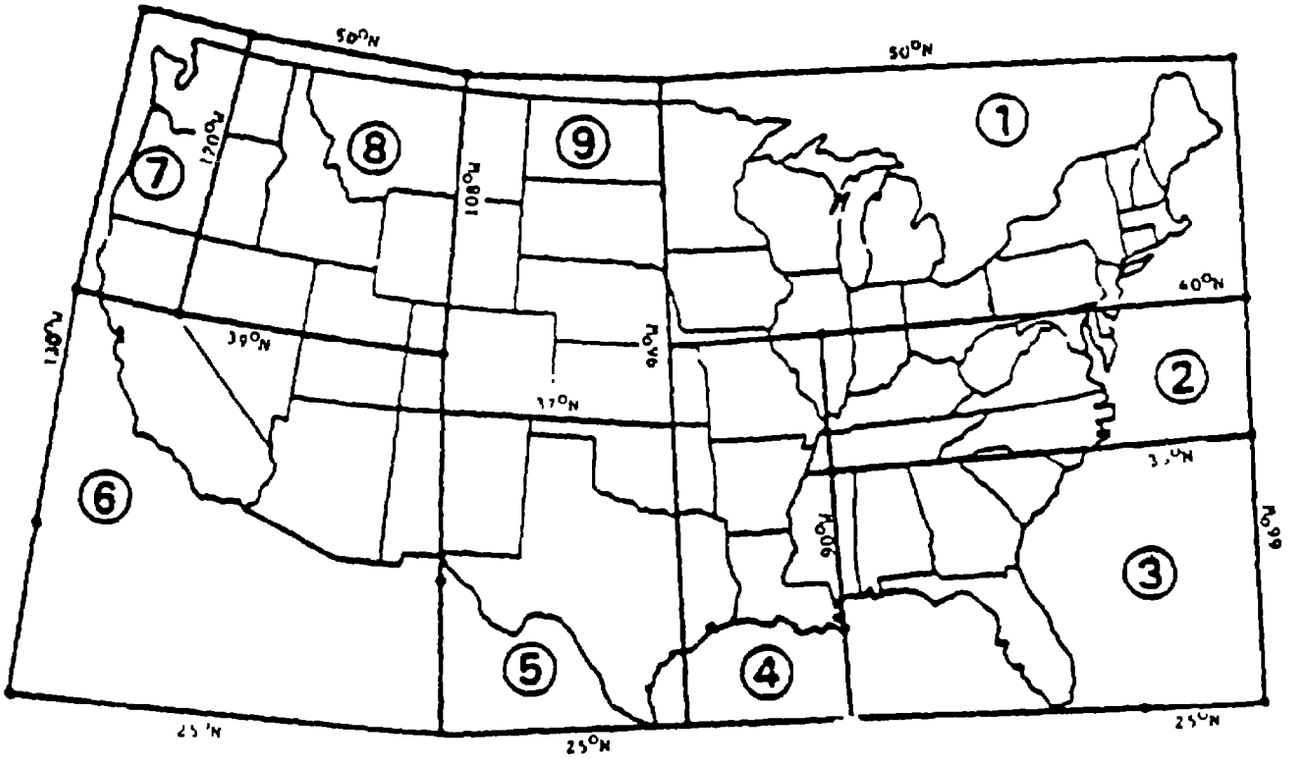
(5) Annual expenditures and budget for year following each annual report;

(6) A summary describing the number and nature of enforcement actions, inspections, and public education programs;

(7) Identification of water quality improvements or degradation;

7a. Part 122 is amended by adding appendices E through I as follows:

Appendix E to Part 122—Rainfall Zones of the United States



Not Shown: Alaska (Zone 7), Hawaii (Zone 7), Northern Mariana Islands (Zone 7), Guam (Zone 7), American Samoa (Zone 7), Trust Territory of the Pacific Islands (Zone 7), Puerto Rico (Zone 3), Virgin Islands (Zone 3).

Source: Methodology for Analysis of Detention Basins for Control of Urban Runoff Quality, prepared for U.S. Environmental Protection Agency, Office of Water, Nonpoint Source Division, Washington, DC, 1986.

Appendix F to Part 122—Incorporated Places With Populations Greater Than 250,000 According to Latest Decennial Census by Bureau of Census.

State	Incorporated place
Alabama	Birmingham.
Arizona	Phoenix.
	Tucson.
California	Long Beach.
	Los Angeles.
	Oakland.
	Sacramento.
	San Diego.
	San Francisco.
	San Jose.
Colorado	Denver.
District of Columbia	
Florida	Jacksonville.
	Miami.
	Tampa.
Georgia	Atlanta.
Illinois	Chicago.
Indiana	Indianapolis.
Kansas	Wichita.
Kentucky	Louisville.
Louisiana	New Orleans.
Maryland	Baltimore.
Massachusetts	Boston.
Michigan	Detroit.
Minnesota	Minneapolis.
	St. Paul.

State	Incorporated place
Missouri	Kansas City
	St. Louis.
Nebraska	Omaha.
New Jersey	Newark.
New Mexico	Albuquerque
New York	Buffalo.
	Bronx Borough.
	Brooklyn Borough.
	Manhattan Borough.
	Queens Borough.
	Staten Island Borough.
North Carolina	Charlotte
Ohio	Cincinnati
	Cleveland.
	Columbus.
	Toledo
Oklahoma	Oklahoma City
	Tulsa.
Oregon	Portland.
Pennsylvania	Philadelphia
	Pittsburgh
Tennessee	Memphis.
	Nashville/Davidson.
Texas	Austin.
	Dallas
	El Paso
	Fort Worth
	Houston.
	San Antonio
Virginia	Norfolk.
	Virginia Beach
Washington	Seattle.
Wisconsin	Milwaukee

Appendix G to Part 122—Incorporated Places With Populations Greater Than 100,000 and Less Than 250,000 According to Latest Decennial Census by Bureau of Census

State	Incorporated place
Alabama	Huntsville
	Mobile
	Montgomery
Alaska	Anchorage
Arizona	Mesa
	Tempe
Arkansas	Little Rock
California	Anaheim
	Bakersfield
	Berkeley
	Concord
	Fremont
	Fresno
	Fullerton
	Garden Grove
	Glendale
	Huntington Beach
	Modesto
	Oxnard
	Pasadena
	Riverside
	San Bernadino
	Santa Ana
	Stockton
	Sunnyvale
	Torrance
Colorado	Aurora
	Colorado Springs
	Lakewood
	Pueblo
Connecticut	Bridgeport
	Hartford
	New Haven
	Stamford
	Waterbury
Florida	Fort Lauderdale
	Hialeah
	Hollywood
	Orlando
	St. Petersburg
Georgia	Columbus
	Macon
	Savannah
Idaho	Boise City
Illinois	Peoria
	Rockford
Indiana	Evansville
	Fort Wayne
	Gary
	South Bend
Iowa	Cedar Rapids
	Davenport
	Des Moines
Kansas	Kansas City
	Topeka
Kentucky	Lexington-Fayette
Louisiana	Baton Rouge
	Shreveport
Massachusetts	Springfield
	Worcester
Michigan	Ann Arbor
	Flint
	Grand Rapids
	Lansing
	Livonia
	Sterling Heights
	Warren
Mississippi	Jackson
Missouri	Independence
	Springfield
Nebraska	Lincoln
Nevada	Las Vegas
	Reno
New Jersey	Elizabeth
	Jersey City
	Paterson
New York	Albany
	Rochester
	Syracuse
	Yonkers
North Carolina	Durham
	Greensboro
	Raleigh
	Winston-Salem
Ohio	Akron
	Dayton
	Youngstown
Oregon	Eugene
Pennsylvania	Allentown
	Erie
Rhode Island	Providence
South Carolina	Columbia
Tennessee	Chattanooga
	Knoxville
Texas	Amarillo
	Arlington
	Beaumont
	Corpus Christi
	Garland
	Irving
	Lubbock
	Pasadena
	Waco
Utah	Salt Lake City
Virginia	Alexandria
	Chesapeake
	Hampton
	Newport News
	Portsmouth
	Richmond
	Roanoke
Washington	Spokane
	Tacoma
Wisconsin	Madison

Appendix H to Part 122—Counties with Unincorporated Urbanized Areas With a *Population of 250,000 or More* According to the Latest Decennial Census by the Bureau of Census

State	County	Unincorporated urbanized population
California	Los Angeles	812,884
	Sacramento	449,856
	San Diego	304,758
Delaware	New Castle	257,184
Florida	Dade	781,949
Georgia	DeKalb	386,379
Hawaii	Honolulu	688,178
Maryland	Anne Arundel	271,458
	Baltimore	801,308
	Montgomery	447,993
	Prince George's	450,188
Texas	Harris	409,801
Utah	Salt Lake	304,832
Virginia	Fairfax	527,178
Washington	King	336,800

Appendix I to Part 122—Counties With Unincorporated Urbanized Areas Greater Than 100,000, But Less Than 250,000 According to the Latest Decennial Census by the Bureau of Census

State	County	Unincorporated urbanized population
Alabama	Jefferson	102,917
Arizona	Pima	111,479
California	Alameda	187,474
	Contra Costa	158,452
	Kern	117,231
	Orange	210,893
	Riverside	115,719
	San Bernardino	148,844
Florida	Broward	150,370
	Escambia	147,892
	Hillsborough	238,292
	Orange	245,325
	Palm Beach	167,089
	Pinellas	194,389
	Polk	104,150
	Sarasota	110,009
Georgia	Clayton	100,742
	Cobb	204,121
	Richmond	118,529
Kentucky	Jefferson	224,958
Louisiana	Jefferson	140,836
North Carolina	Cumberland	142,727
Nevada	Clark	201,775
Oregon	Multnomah	141,100
	Washington	109,348
South Carolina	Greenville	135,396
	Richland	124,684
Virginia	Arlington	152,599
	Henrico	161,204
	Chesterfield	108,348
Washington	Snohomish	103,490
	Pierce	186,113

PART 123—STATE PROGRAM REQUIREMENTS

8 The authority citation for part 123 continues to read as follows

Authority: Clean Water Act, 33 U.S.C. 1251 *et seq.*

9. Section 123.25 is amended by revising paragraph (a)(9) to read as follows:

§ 123.25 Requirements for permitting.

(a) * * *

(9) § 122.26—(Storm water discharges);

* * * * * *

PART 124—PROCEDURES FOR DECISIONMAKING

10. The authority citation for part 124 continues to read as follows:

Authority: Resource Conservation and Recovery Act, 42 U.S.C. 6901 *et seq.*; Safe Drinking Water Act, 42 U.S.C. 300f *et seq.*, Clean Water Act, 33 U.S.C. 1251 *et seq.*; and Clean Air Act, 42 U.S.C. 1857 *et seq.*

11. Section 124.52 is revised to read as follows.

§ 124.52 Permits required on a case-by-case basis.

(a) Various sections of part 122, subpart B allow the Director to determine, on a case-by-case basis, that certain concentrated animal feeding operations (§ 122.23), concentrated aquatic animal production facilities (§ 122.24), storm water discharges (§ 122.26), and certain other facilities covered by general permits (§ 122.28) that do not generally require an individual permit may be required to obtain an individual permit because of their contributions to water pollution.

(b) Whenever the Regional Administrator decides that an individual permit is required under this section, except as provided in paragraph (c) of this section, the Regional Administrator shall notify the discharger in writing of that decision and the reasons for it, and shall send an application form with the notice. The discharger must apply for a permit under § 122.21 within 60 days of notice, unless permission for a later date is granted by the Regional Administrator. The question whether the designation was proper will remain open for consideration during the public comment period under § 124.11 or § 124.118 and in any subsequent hearing.

(c) Prior to a case-by-case determination that an individual permit is required for a storm water discharge under this section (*see* 40 CFR 122.26 (a)(1)(v) and (c)(1)(v)), the Regional Administrator may require the discharger to submit a permit application or other information regarding the discharge under section 308 of the CWA. In requiring such information, the Regional Administrator shall notify the discharger in writing and shall send an application form with the notice. The discharger must apply for a permit under § 122.26 within 60 days of notice, unless permission for a later date is granted by the Regional Administrator. The question whether the initial designation was proper will remain open for consideration during the public comment period under § 124.11 or § 124.118 and in any subsequent hearing.

Note: The following form will not appear in the Code of Federal Regulations

BILLING CODE 6560-50-M

APPENDIX C:
ADEQUATE LEGAL AUTHORITY

contrivance for the elimination or destruction of human waste, within those portions of the watershed of the city contiguous to the intake of the city's water supply, as hereinafter described, or by placing any foul or putrescible substance, whether solid or liquid, and whether the same be buried or not, within the limits of the portion of the watershed so described.

Sec. 49-6. Application for permit.

(a) Any person who desires to use or develop any vegetated wetland and on and after January 1, 1983, any nonvegetated wetland, within this city, other than for those activities specified in section 49-3 above, shall first file an application for a permit with the wetlands board.

Sec. 49-22. Application for permit.

(a) Any person who desires to use or alter any coastal primary sand dune within this city, other than for those activities specified in section 49-20 above, shall first file an application for a permit with the wetlands board.

1.6 Authority to Meet Part 2 Permit Requirements

The NPDES stormwater permit application regulations require an assessment of whether existing legal authority is sufficient to meet the criteria for Part 2 of the permit application provided in *40 CFR 122.26(d)(2)(i)* as follows:

40 CFR 122.26(d)(2)(i)

A demonstration that the applicant can operate pursuant to legal authority established by statute, ordinance or series of contracts which authorizes or enables the applicant at a minimum to:

(A) Control through ordinance, permit, contract, order or similar means, the contribution of pollutants to the municipal storm sewer system by storm water discharges associated with industrial activity and the quality of storm water discharged from sites of industrial activity;

(B) Prohibit through ordinance, order or similar means, illicit discharges to the municipal separate storm sewer;

(C) Control through ordinance, order or similar means the discharge to a municipal separate storm sewer of spills, dumping or disposal of materials other than storm water;

(D) Control through interagency agreements among coapplicants the contribution of pollutants from one portion of the municipal system to another portion of the municipal system;

(E) Require compliance with conditions in ordinances, permits, contracts or order; and

(F) Carry out all inspection, surveillance and monitoring procedures necessary to determine compliance and noncompliance with permit conditions including the prohibition on illicit discharges to the municipal separate storm sewer.

The City Code sections identified above are referenced in an assessment of the individual Part 2 legal authority criteria.

(A) Control through ordinance, permit, contract, order or similar means, the contribution of pollutants to the municipal storm sewer system by storm water discharges associated with industrial activity and the quality of storm water discharged from sites of industrial activity. Section 39.1-19 of the City Code prohibits the discharge of sanitary sewer flow to the storm sewer system. Section 39.2-5 of the City Code prohibits the discharge of any sewage from a private sewage disposal facility on any public or private property in the City. Section 41.1-4 of the City Code prohibits pollutants to be discharged to the storm sewer system including the discharge of industrial process water, wash water, or other unpermitted industrial discharges in Section 41.1-4(c). Section 41.1-5 of the City Code provides the City with authority to order the correction of drainage problems on any site in the City. Sections 9-10, 30-69, 41-16, and 41-17 of the City Code prohibit pollution of waters of the City and littering. Sections 42-20.1 and 42-20.2 of the City Code prohibit the obstruction of drains or drainage areas. Sections 42-24, 42-25, and 42-46 of the City Code establish regulations for protecting the City from spills or deposits of liquid wastes. Section 46-28 of the City Code prohibits pollution of the City's water supply.

For development or redevelopment of industrial sites, the City's Zoning Ordinance establishes lot size, yard size, and maximum lot coverage requirements for industrial activity. Chapter 15 of the City Code establishes erosion and sedimentation control regulations If development or redevelopment of industrial sites occurs within a Chesapeake Bay Preservation Area, Section 494 of the City's Zoning Ordinance and Chapter 32.2 of the City Code establish stringent criteria for stormwater management, protection of water quality, and use of Best Management Practices. Chapter 49 of the City Code protects development within wetlands or coastal primary sand dunes by requiring a permit application with the wetlands board.

Enforcement provisions and penalties for violations of the referenced sections of City Code are also provided in specific chapters. Chapter 27 of the City Code provides additional authority for the abatement of nuisances.

(B) Prohibit through ordinance, order or similar means, illicit discharges to the municipal separate storm sewer. Section 39.1-19 of the City Code prohibits the discharge of sanitary sewer flow to the storm sewer system. Section 39.2-5 of the City Code prohibits the discharge of any sewage from a private sewage disposal facility on any public or private property in the City. Section 41.1-4 of the City Code prohibits pollutants to be discharged to the storm sewer system. Section 41.1-5 of the City Code provides the City with authority to order the correction of drainage problems on any site in the City. Sections 9-10, 30-69, 41-16, and 41-17 of the City Code prohibit pollution of waters of the City and littering. Sections 42-20.1 and 42-20.2 of the City Code prohibit the obstruction of drains or drainage areas. Sections 42-24, 42-25, and 42-46 of the City Code establish regulations for protecting the City from spills or deposits of liquid wastes. Section 46-28 of the City Code prohibits pollution of the City's water supply.

Enforcement provisions and penalties for violations of the referenced sections of City Code are also provided in specific chapters. Chapter 27 of the City Code provides additional authority for the abatement of nuisances.

(C) Control through ordinance, order or similar means the discharge to a municipal separate storm sewer of spills, dumping or disposal of materials other than storm water. Section 39.1-19 of the City Code prohibits the discharge of sanitary sewer flow to the storm sewer system. Section 39.2-5 of the City Code prohibits the discharge of any sewage from a private sewage disposal facility on any public or private property in the City. Section 41.1-4 of the City Code prohibits pollutants to be discharged to the storm sewer system. Sections 9-10, 30-69, 41-16, and 41-17 of the City Code prohibit pollution of waters of the City and littering. Sections 42-24, 42-25, and 42-46 of the City Code establish regulations for protecting the City from spills or deposits of liquid wastes. Section 46-28 of the City Code prohibits pollution of the City's water supply.

Enforcement provisions and penalties for violations of the referenced sections of City Code are also provided in specific chapters. Chapter 27 of the City Code provides additional authority for the abatement of nuisances.

(D) Control through interagency agreements among coapplicants the contribution of pollutants from one portion of the municipal system to another portion of the municipal system. The City of Norfolk owns the entire separate storm water system and is an individual NPDES permit applicant.

The City of Norfolk relies on its In-Town Reservoir System as a vital part of the water supply system. To protect water quality within the In-Town Reservoir System, the City of Norfolk will seek an intermunicipal agreement with the City of Virginia Beach to control nonpoint source pollution for the areas of the In-Town Reservoir System bordering and located within the jurisdiction of the City of Virginia Beach. After approval of Part 1 of the application by the EPA, the City of Norfolk will meet with the City of Virginia Beach to discuss the development of an agreement before submittal of Part 2 of the application on November 16, 1992.

(E) Require compliance with conditions in ordinances, permits, contracts or order. Enforcement provisions and penalties for violations of the referenced sections of City Code are provided in specific chapters. Chapter 27 of the City Codes provides additional authority for the abatement of nuisances.

(F) Carry out all inspection, surveillance and monitoring procedures necessary to determine compliance and noncompliance with permit conditions including the prohibition on illicit discharges to the municipal separate storm sewer. Chapter 41.1, entitled "Storm Water Management", provides authority for the City's Director of Public Works to establish procedures and enforce regulations pertaining to the storm water system in Section 41.1-3. Authority to prohibit and inspect for illicit connections to the storm sewer system is provided to the Department of City Planning and Codes Administration in Section 39.1-19. Authority to enforce violations of private sewage disposal regulations is provided to the Department of Health in Section 39.2-1 of the City Code. For development and redevelopment, the Department of City Planning and Codes Administration has authority over erosion and sediment control plans, the site review process, and stormwater management regulations required for activity within the Chesapeake Bay Preservation Area. Additional authority for enforcement of erosion and sediment control regulations and stormwater management is being established for the Department of Public Works in an ordinance currently under review by the state. Authority to enforce regulations and permits of the City's Tree Ordinance is provided in Section 30-23 of the City Code

1.7 Legal Authority Overview

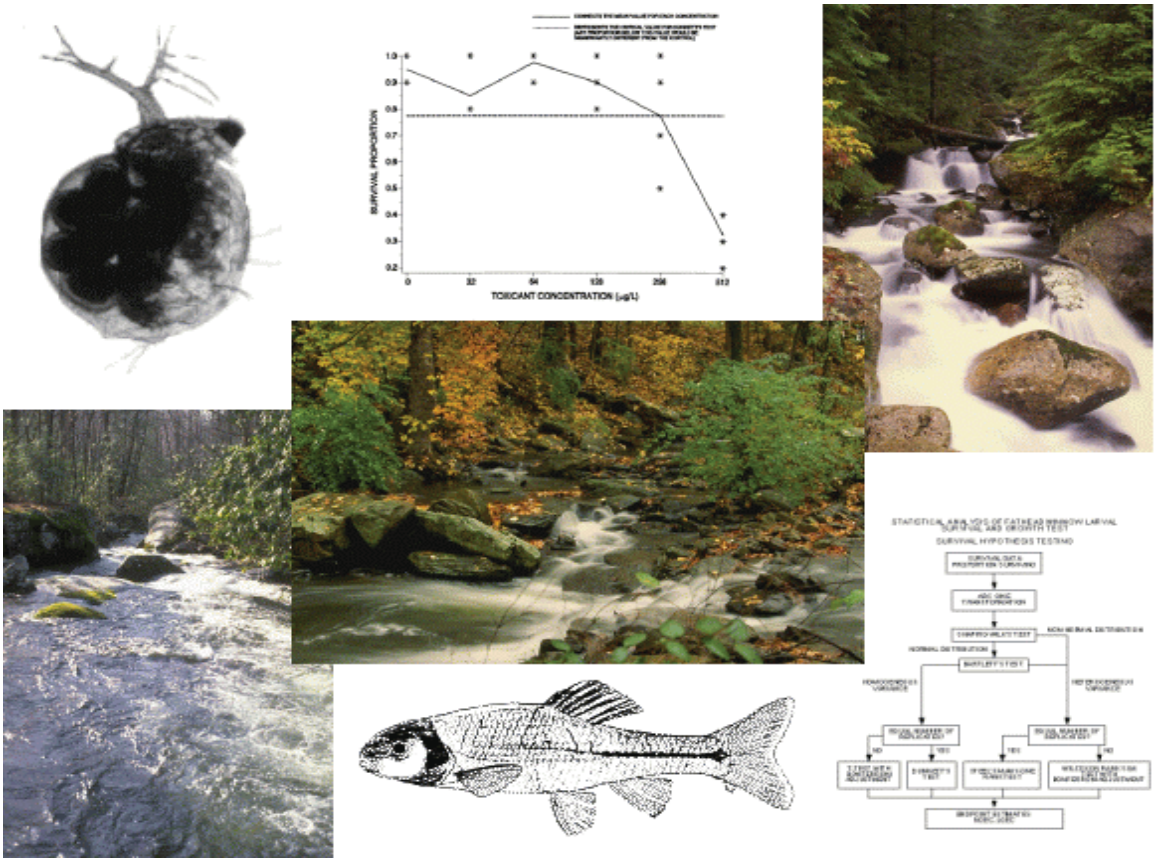
Overall, the City of Norfolk has the existing legal authority, or is in the process of modifying existing City Code with ordinances, to control discharges to the municipal storm sewer system and meet the legal authority requirements of *40 CFR 122 26(d)(2)(i)*.

EPA
United States
Environmental Protection
Agency

Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms

Fourth Edition

October 2002



U.S. Environmental Protection Agency
Office of Water (4303T)
1200 Pennsylvania Avenue, NW
Washington, DC 20460

EPA-821-R-02-013

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SECTION 12

SECTION 12 (Continued)

SECTION 13

SECTION 13 (Continued)

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SECTION 1

INTRODUCTION

1.1 This manual describes chronic toxicity tests for use in the National Pollutant Discharge Elimination System (NPDES) Permits Program to identify effluents and receiving waters containing toxic materials in chronically toxic concentrations. The methods included in this manual are referenced in Table IA, 40 CFR Part 136 regulations and, therefore, constitute approved methods for chronic toxicity tests. They are also suitable for determining the toxicity of specific compounds contained in discharges. The tests may be conducted in a central laboratory or on-site, by the regulatory agency or the permittee.

1.2 The data are used for NPDES permits development and to determine compliance with permit toxicity limits. Data can also be used to predict potential acute and chronic toxicity in the receiving water, based on the LC50, NOEC, IC50 or IC25 (see Section 9, Chronic Toxicity Endpoints and Data Analysis) and appropriate dilution, application, and persistence factors. The tests are performed as a part of self-monitoring permit requirements, compliance biomonitoring inspections, toxics sampling inspections, and special investigations. Data from chronic toxicity tests performed as part of permit requirements are evaluated during compliance evaluation inspections and performance audit inspections.

1.3 Modifications of these tests are also used in toxicity reduction evaluations and toxicity identification evaluations to identify the toxic components of an effluent, to aid in the development and implementation of toxicity reduction plans, and to compare and control the effectiveness of various treatment technologies for a given type of industry, irrespective of the receiving water (USEPA, 1988c; USEPA, 1989b; USEPA 1989c; USEPA, 1989d; USEPA, 1989e; USEPA, 1991a; USEPA, 1991b; and USEPA, 1992).

1.4 This methods manual serves as a companion to the acute toxicity test methods for freshwater and marine organisms (USEPA, 2002a), the short-term chronic toxicity test methods for marine and estuarine organisms (USEPA, 2002b), and the manual for evaluation of laboratories performing aquatic toxicity tests (USEPA, 1991c). In 2002, EPA revised previous editions of each of the three methods manuals (USEPA, 1993a; USEPA, 1994a; USEPA, 1994b).

1.5 Guidance for the implementation of toxicity tests in the NPDES program is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991a).

1.6 These freshwater short-term toxicity tests are similar to those developed for marine and estuarine organisms to evaluate the toxicity of effluents discharged to marine and estuarine waters under the NPDES permit program. Methods are presented in this manual for three species of freshwater organisms from three phylogenetic groups. The methods are all static renewal type seven-day tests except the green alga, *Selenastrum capricornutum*, test which lasts four days.

1.7 The three species for which test methods are provided are the fathead minnow, *Pimephales promelas*; the daphnid, *Ceriodaphnia dubia*; and the green alga, *Selenastrum capricornutum*.

1.7.1 Two of the methods incorporate the chronic endpoint of growth in addition to lethality and one incorporates reproduction. The fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test incorporates teratogenic effects in addition to lethality. The green alga, *Selenastrum capricornutum*, growth test has the advantage of a relatively short exposure period (96 h).

1.8 The validity of the freshwater chronic methods in predicting adverse ecological impacts of toxic discharges was demonstrated in field studies (USEPA, 1984; USEPA, 1985b; USEPA, 1985c; USEPA, 1985d; USEPA, 1986a; USEPA, 1986b; USEPA, 1986c; USEPA, 1986d; Birge et al., 1989; and Eagleson et al., 1990).

1.9 The use of any test species or test conditions other than those described in the methods summary tables in this manual shall be subject to application and approval of alternate test procedures under 40 CFR 136.4 and 40 CFR 136.5.

1.10 These methods are restricted to use by, or under the supervision of, analysts experienced in the use or conduct of aquatic toxicity tests and the interpretation of data from aquatic toxicity testing. Each analyst must demonstrate the ability to generate acceptable test results with these methods using the procedures described in this methods manual.

1.11 This manual was prepared in the established EMSL-Cincinnati format (USEPA, 1983).

SECTION 2

SHORT-TERM METHODS FOR ESTIMATING CHRONIC TOXICITY

2.1 INTRODUCTION

2.1.1 The objective of aquatic toxicity tests with effluents or pure compounds is to estimate the "safe" or "no effect" concentration of these substances, which is defined as the concentration which will permit normal propagation of fish and other aquatic life in the receiving waters. The endpoints that have been considered in tests to determine the adverse effects of toxicants include death and survival, decreased reproduction and growth, locomotor activity, gill ventilation rate, heart rate, blood chemistry, histopathology, enzyme activity, olfactory function, and terata. Since it is not feasible to detect and/or measure all of these (and other possible) effects of toxic substances on a routine basis, observations in toxicity tests generally have been limited to only a few effects, such as mortality, growth, and reproduction.

2.1.2 Acute lethality is an obvious and easily observed effect which accounts for its wide use in the early period of evaluation of the toxicity of pure compounds and complex effluents. The results of these tests were usually expressed as the concentration lethal to 50% of the test organisms (LC50) over relatively short exposure periods (one-to-four days).

2.1.3 As exposure periods of acute tests were lengthened, the LC50 and lethal threshold concentration were observed to decline for many compounds. By lengthening the tests to include one or more complete life cycles and observing the more subtle effects of the toxicants, such as a reduction in growth and reproduction, more accurate, direct, estimates of the threshold or safe concentration of the toxicant could be obtained. However, laboratory life-cycle tests may not accurately estimate the "safe" concentration of toxicants because they are conducted with a limited number of species under highly controlled, steady-state conditions, and the results do not include the effects of the stresses to which the organisms would ordinarily be exposed in the natural environment.

2.1.4 An early published account of a full life-cycle, fish toxicity test was that of Mount and Stephan (1967). In this study, fathead minnows, *Pimephales promelas*, were exposed to a graded series of pesticide concentrations throughout their life cycle, and the effects of the toxicant on survival, growth, and reproduction were measured and evaluated. This work was soon followed by full life-cycle tests using other toxicants and fish species.

2.1.5 McKim (1977) evaluated the data from 56 full life-cycle tests, 32 of which used the fathead minnow, *Pimephales promelas*, and concluded that the embryo-larval and early juvenile life-stages were the most sensitive stages. He proposed the use of partial life-cycle toxicity tests with the early life-stages (ELS) of fish to establish water quality criteria.

2.1.6 Macek and Sleight (1977) found that exposure of critical life-stages of fish to toxicants provides estimates of chronically safe concentrations remarkably similar to those derived from full life-cycle toxicity tests. They reported that "for a great majority of toxicants, the concentration which will not be acutely toxic to the most sensitive life stages is the chronically safe concentration for fish, and that the most sensitive life stages are the embryos and fry". Critical life-stage exposure was considered to be exposure of the embryos during most, preferably all, of the embryogenic (incubation) period, and exposure of the fry for 30 days post-hatch for warm water fish with embryogenic periods ranging from one-to-fourteen days, and for 60 days post-hatch for fish with longer embryogenic periods. They concluded that in the majority of cases, the maximum acceptable toxicant concentration (MATC) could be estimated from the results of exposure of the embryos during incubation, and the larvae for 30 days post-hatch.

2.1.7 Because of the high cost of full life-cycle fish toxicity tests and the emerging consensus that the ELS test data usually would be adequate for estimating chronically safe concentrations, there was a rapid shift by aquatic toxicologists to 30 - 90-day ELS toxicity tests for estimating chronically safe concentrations in the late 1970s. In

1980, USEPA adopted the policy that ELS test data could be used in establishing water quality criteria if data from full life-cycle tests were not available (USEPA, 1980a).

2.1.8 Published reports of the results of ELS tests indicate that the relative sensitivity of growth and survival as endpoints may be species dependent, toxicant dependent, or both. Ward and Parrish (1980) examined the literature on ELS tests that used embryos and juveniles of the sheepshead minnow, *Cyprinodon variegatus*, and found that growth was not a statistically sensitive indicator of toxicity in 16 of 18 tests. They suggested that the ELS tests be shortened to 14 days posthatch and that growth be eliminated as an indicator of toxic effects.

2.1.9 In a review of the literature on 173 fish full life-cycle and ELS tests performed to determine the chronically safe concentrations of a wide variety of toxicants, such as metals, pesticides, organics, inorganics, detergents, and complex effluents, Woltering (1984) found that at the lowest effect concentration, significant reductions were observed in fry survival in 57%, fry growth in 36%, and egg hatchability in 19% of the tests. He also found that fry survival and growth were very often equally sensitive, and concluded that the growth response could be deleted from routine application of the ELS tests. The net result would be a significant reduction in the duration and cost of screening tests with no appreciable impact on estimating MATCs for chemical hazard assessments. Benoit et al. (1982), however, found larval growth to be the most significant measure of effect, and survival to be equally or less sensitive than growth in early life-stage tests with four organic chemicals.

2.1.10 Efforts to further reduce the length of partial life-cycle toxicity tests for fish without compromising their predictive value have resulted in the development of an eight-day, embryo-larval survival and teratogenicity test for fish and other aquatic vertebrates (USEPA, 1981; Birge et al., 1985), and a seven-day larval survival and growth test (Norberg and Mount, 1985).

2.1.11 The similarity of estimates of chronically safe concentrations of toxicants derived from short-term, embryo-larval survival and teratogenicity tests to those derived from full life-cycle tests has been demonstrated by Birge et al. (1981), Birge and Cassidy (1983), and Birge et al. (1985).

2.1.12 Use of a seven-day, fathead minnow, *Pimephales promelas*, larval survival and growth test was first proposed by Norberg and Mount at the 1983 annual meeting of the Society for Environmental Toxicology and Chemistry (Norberg and Mount, 1983). This test was subsequently used by Mount and associates in field demonstrations at Lima, OH (USEPA, 1984), and at many other locations. Growth was frequently found to be more sensitive than survival in determining the effects of complex effluents.

2.1.13 Norberg and Mount (1985) performed three single toxicant fathead minnow larval growth tests with zinc, copper, and DURSBAN®, using dilution water from Lake Superior. The results were comparable to, and had confidence intervals that overlapped with, chronic values reported in the literature for both ELS and full life-cycle tests.

2.1.14 Mount and Norberg (1984) developed a seven-day cladoceran partial life-cycle test and experimented with a number of diets for use in culturing and testing the daphnid, *Ceriodaphnia reticulata* (Norberg and Mount, 1985). As different laboratories began to use this cladoceran test, it was discovered that apparently more than one species was involved in the tests conducted by the same laboratory. Berner (1986) studied the problem and determined that perhaps as many as three variant forms were involved and it was decided to recommend the use of the more common *Ceriodaphnia dubia* rather than the originally reported *Ceriodaphnia reticulata*. The method was adopted for use in the first edition of the freshwater short-term chronic methods (USEPA, 1985e).

2.1.15 The green alga, *Selenastrum capricornutum*, bottle test was developed, after extensive design, evaluation, and application, for the National Eutrophication Research Program (USEPA, 1971). The test was later modified for use in the assessment of receiving waters and the effects of wastes originating from industrial, municipal, and agricultural point and non-point sources (USEPA, 1978a).

2.1.16 The use of short-term toxicity tests including subchronic and chronic tests in the NPDES Program is especially attractive because they provide a more direct estimate of the safe concentrations of effluents in receiving waters than was provided by acute toxicity tests, at an only slightly increased level of effort, compared to the fish full life-cycle chronic and 28-day ELS tests and the 21-day daphnid, *Daphnia magna*, life-cycle test.

2.2 TYPES OF TESTS

2.2.1 The selection of the test type will depend on the NPDES permit requirements, the objectives of the test, the available resources, the requirements of the test organisms, and effluent characteristics such as fluctuations in effluent toxicity.

2.2.2 Effluent chronic toxicity is generally measured using a multi-concentration, or definitive test, consisting of a control and a minimum of five effluent concentrations. The tests are designed to provide dose-response information, expressed as the percent effluent concentration that affects the hatchability, gross morphological abnormalities, survival, growth, and/or reproduction within the prescribed period of time (four to seven days). The results of the tests are expressed in terms of the highest concentration that has no statistically significant observed effect on those responses when compared to the controls or the estimated concentration that causes a specified percent reduction in responses versus the controls.

2.2.3 Use of pass/fail tests consisting of a single effluent concentration (e.g., the receiving water concentration or RWC) and a control **is not recommended**. If the NPDES permit has a whole effluent toxicity limit for acute toxicity at the RWC, it is prudent to use that permit limit as the midpoint of a series of five effluent concentrations. This will ensure that there is sufficient information on the dose-response relationship. For example, the effluent concentrations utilized in a test may be: (1) 100% effluent, (2) (RWC + 100)/2, (3) RWC, (4) RWC/2, and (5) RWC/4. More specifically, if the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

2.2.4 Receiving (ambient) water toxicity tests commonly employ two treatments, a control and the undiluted receiving water, but may also consist of a series of receiving water dilutions.

2.2.5 A negative result from a chronic toxicity test does not preclude the presence of toxicity. Also, because of the potential temporal variability in the toxicity of effluents, a negative test result with a particular sample does not preclude the possibility that samples collected at some other time might exhibit chronic toxicity.

2.2.6 The frequency with which chronic toxicity tests are conducted under a given NPDES permit is determined by the regulatory agency on the basis of factors such as the variability and degree of toxicity of the waste, production schedules, and process changes.

2.2.7 Tests recommended for use in this methods manual may be static non-renewal or static renewal. Individual methods specify which static type of test is to be conducted.

2.3 STATIC TESTS

2.3.1 Static non-renewal tests - The test organisms are exposed to the same test solution for the duration of the test.

2.3.2 Static-renewal tests - The test organisms are exposed to a fresh solution of the same concentration of sample every 24 h or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers.

2.4 ADVANTAGES AND DISADVANTAGES OF TOXICITY TEST TYPES

2.4.1 STATIC NON-RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Simple and inexpensive.
2. Very cost effective in determining compliance with permit conditions.
3. Limited resources (space, manpower, equipment) required; would permit staff to perform many more tests in the same amount of time.
4. Smaller volume of effluent required than for static renewal or flow-through tests.

Disadvantages:

1. Dissolved oxygen (DO) depletion may result from high chemical oxygen demand (COD), biological oxygen demand (BOD), or metabolic wastes.
2. Possible loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Generally less sensitive than static renewal, because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.4.2 STATIC RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Reduced possibility of DO depletion from high COD and/or BOD, or ill effects from metabolic wastes from organisms in the test solutions.
2. Reduced possibility of loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Test organisms that rapidly deplete energy reserves are fed when the test solutions are renewed, and are maintained in a healthier state.

Disadvantages:

1. Require greater volume of effluent than non-renewal tests.
2. Generally less chance of temporal variations in waste properties.

SECTION 3

HEALTH AND SAFETY

3.1 GENERAL PRECAUTIONS

3.1.1 Each laboratory should develop and maintain an effective health and safety program, requiring an ongoing commitment by the laboratory management. This program should include (1) a safety officer with the responsibility and authority to develop and maintain a safety program, (2) the preparation of a formal, written, health and safety plan, which is provided to each of the laboratory staff, (3) an ongoing training program on laboratory safety, and (4) regularly scheduled, documented, safety inspections.

3.1.2 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

3.1.3 Prior to sample collection and laboratory work, personnel will determine that all necessary safety equipment and materials have been obtained and are in good condition.

3.1.4 Guidelines for the handling and disposal of hazardous materials must be strictly followed.

3.2 SAFETY EQUIPMENT

3.2.1 PERSONAL SAFETY GEAR

3.2.1.1 Personnel should use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes. Plastic netting on glass beakers, flasks, and other glassware minimizes breakage and subsequent shattering of the glass.

3.2.2 LABORATORY SAFETY EQUIPMENT

3.2.2.1 Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, chemical spill clean up kits, and eye fountains.

3.2.2.2 Mobile laboratories should be equipped with a telephone or other means to enable personnel to summon help in case of emergency.

3.3 GENERAL LABORATORY AND FIELD OPERATIONS

3.3.1 Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see safety manuals listed in Section 3, Health and Safety, Subsection 3.5). It is recommended that personnel collecting samples and performing toxicity tests not work alone.

3.3.2 Because the chemical composition of effluents is usually only poorly known, they should be considered as potential health hazards, and exposure to them should be minimized. Fume and canopy hoods over the toxicity test areas must be used whenever possible.

3.3.3 It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

3.3.4 All containers are to be adequately labeled to indicate their contents.

3.3.5 Staff should be familiar with safety guidelines on Material Safety Data Sheets for reagents and other chemicals purchased from suppliers. Incompatible materials should not be stored together. Good housekeeping contributes to safety and reliable results.

3.3.6 Strong acids and volatile organic solvents employed in glassware cleaning must be used in a fume hood or under an exhaust canopy over the work area.

3.3.7 Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.8 Mobile laboratories should be properly grounded to protect against electrical shock.

3.4 DISEASE PREVENTION

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, polio, and hepatitis B.

3.5 SAFETY MANUALS

3.5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general safety manuals, including USEPA (1986e) and Walters and Jameson (1984).

3.6 WASTE DISPOSAL

3.6.1 Wastes generated during toxicity testing must be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, state, and Federal rules and regulations. It is extremely important that these rules and regulations be known, understood, and complied with by all persons responsible for, or otherwise involved in performing the toxicity testing activities. Local fire officials should be notified of any potentially hazardous conditions.

SECTION 4

QUALITY ASSURANCE

4.1 INTRODUCTION

4.1.1 Development and maintenance of a toxicity test laboratory quality assurance (QA) program (USEPA, 1991a) requires an ongoing commitment by laboratory management. Each toxicity test laboratory should (1) appoint a quality assurance officer with the responsibility and authority to develop and maintain a QA program; (2) prepare a quality assurance plan with stated data quality objectives (DQOs); (3) prepare a written description of laboratory standard operating procedures (SOPs) for culturing, toxicity testing, instrument calibration, sample chain-of-custody procedures, laboratory sample tracking system, glassware cleaning, etc.; and (4) provide an adequate, qualified technical staff for culturing and testing the organisms, and suitable space and equipment to assure reliable data.

4.1.2 QA practices for toxicity testing laboratories must address all activities that affect the quality of the final effluent toxicity test data, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation.

4.1.3 Quality control practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance and general guidance on good laboratory practices and laboratory evaluation related to toxicity testing, see FDA, (1978); USEPA, (1979d), USEPA (1980b), USEPA (1980c), and USEPA (1991c); DeWoskin (1984); and Taylor (1987).

4.1.4 Guidance for the evaluation of laboratories performing toxicity tests and laboratory evaluation criteria may be found in USEPA (1991c).

4.2 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.2.1 Separate test organism culturing and toxicity testing areas should be provided to avoid possible loss of cultures due to cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation areas into organism culturing or testing areas, and from testing and sample preparation areas into culture rooms.

4.2.2 Laboratory and toxicity test temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 5, Facilities, Equipment and Supplies; and specific toxicity test method).

4.3 TEST ORGANISMS

4.3.1 The test organisms used in the procedures described in this manual are the fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia*, and the green alga, *Selenastrum capricornutum*. The fish and invertebrates should appear healthy, behave normally, feed well, and have low mortality in the cultures, during holding, and in test controls. Test organisms should be positively identified to species (see Section 6, Test Organisms).

4.4 LABORATORY WATER USED FOR CULTURING AND TEST DILUTION WATER

4.4.1 The quality of water used for test organism culturing and for dilution water used in toxicity tests is extremely important. Water for these two uses should come from the same source. The dilution water used in effluent toxicity tests will depend in part on the objectives of the study and logistical constraints, as discussed in detail in Section 7, Dilution Water. For tests performed to meet NPDES objectives, synthetic, moderately hard water should be used.

The dilution water used for internal quality assurance tests with organisms, food, and reference toxicants should be the water routinely used with success in the laboratory. Types of water are discussed in Section 5, Facilities, Equipment and Supplies. Water used for culturing and test dilution should be analyzed for toxic metals and organics at least annually or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. The concentration of the metals Al, As, Cr, Co, Cu, Fe, Pb, Ni, and Zn, expressed as total metal, should not exceed 1 mg/L each, and Cd, Hg, and Ag, expressed as total metal, should not exceed 100 ng/L each. Total organochlorine pesticides plus PCBs should be less than 50 ng/L (APHA, 1992). Pesticide concentrations should not exceed USEPA's Ambient Water Quality chronic criteria values where available.

4.5 EFFLUENT AND RECEIVING WATER SAMPLING AND HANDLING

4.5.1 Sample holding times and temperatures of effluent samples collected for on-site and off-site testing must conform to conditions described in Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

4.6 TEST CONDITIONS

4.6.1 Water temperature should be maintained within the limits specified for each test. The temperature of test solutions must be measured by placing the thermometer or probe directly into the test solutions, or by placing the thermometer in equivalent volumes of water in surrogate vessels positioned at appropriate locations among the test vessels. Temperature should be recorded continuously in at least one test vessel for the duration of each test. Test solution temperatures should be maintained within the limits specified for each test. DO concentration and pH should be checked at the beginning of each test and daily throughout the test period.

4.7 QUALITY OF TEST ORGANISMS

4.7.1 The health of test organisms is primarily assessed by the performance (survival, growth, and/or reproduction) of organisms in control treatments of individual tests. The health and sensitivity of test organisms is also assessed by reference toxicant testing. In addition to documenting the sensitivity and health of test organisms, reference toxicant testing is used to initially demonstrate acceptable laboratory performance (Subsection 4.15) and to document ongoing laboratory performance (Subsection 4.16).

4.7.2 Regardless of the source of test organisms (in-house cultures or purchased from external suppliers), the testing laboratory must perform at least one acceptable reference toxicant test per month for each toxicity test method conducted in that month (Subsection 4.16). If a test method is conducted only monthly, or less frequently, a reference toxicant test must be performed concurrently with each effluent toxicity test.

4.7.3 When acute or short-term chronic toxicity tests are performed with effluents or receiving waters using test organisms obtained from outside the test laboratory, concurrent toxicity tests of the same type must be performed with a reference toxicant, unless the test organism supplier provides control chart data from at least the last five monthly short-term chronic toxicity tests using the same reference toxicant and control conditions (see Section 6, Test Organisms).

4.7.4 The supplier should certify the species identification of the test organisms, and provide the taxonomic reference (citation and page) or name(s) of the taxonomic expert(s) consulted.

4.7.5 If routine reference toxicant tests fail to meet test acceptability criteria, then the reference toxicant test must be immediately repeated.

4.8 FOOD QUALITY

4.8.1 The nutritional quality of the food used in culturing and testing fish and invertebrates is an important factor in the quality of the toxicity test data. This is especially true for the unsaturated fatty acid content of brine shrimp nauplii, *Artemia*. Problems with the nutritional suitability of the food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. *Artemia* cysts, and other foods must be obtained as described in Section 5, Facilities, Equipment, and Supplies.

4.8.2 Problems with the nutritional suitability of food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. If a batch of food is suspected to be defective, the performance of organisms fed with the new food can be compared with the performance of organisms fed with a food of known quality in side-by-side tests. If the food is used for culturing, its suitability should be determined using a short-term chronic test which will determine the affect of food quality on growth or reproduction of each of the relevant test species in culture, using four replicates with each food source. Where applicable, foods used only in chronic toxicity tests can be compared with a food of known quality in side-by-side, multi-concentration chronic tests, using the reference toxicant regularly employed in the laboratory QA program.

4.8.3 New batches of food used in culturing and testing should be analyzed for toxic organics and metals or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. If the concentration of total organochlorine pesticides exceeds 0.15 mg/g wet weight, or the concentration of total organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight, or toxic metals (Al, As, Cr, Cd, Cu, Pb, Ni, Zn, expressed as total metal) exceed 20 µg/g wet weight, the food should not be used (for analytical methods see AOAC, 1990 and USDA, 1989). For foods (e.g., such as YCT) which are used to culture and test organisms, the quality of the food should meet the requirements for the laboratory water used for culturing and test dilution water as described in Section 4.4 above.

4.9 ACCEPTABILITY OF SHORT-TERM CHRONIC TOXICITY TESTS

4.9.1 For the tests to be acceptable, control survival in fathead minnow, *Pimephales promelas*, and the daphnid, *Ceriodaphnia dubia*, tests must be 80% or greater. At the end of the test, the average dry weight of surviving seven-day-old fathead minnows in control chambers must equal or exceed 0.25 mg. In *Ceriodaphnia dubia* controls, 60% or more of the surviving females must have produced their third brood in 7 ± 1 days, and the number of young per surviving female must be 15 or greater. In algal toxicity tests, the mean cell density in the controls after 96 h must equal or exceed 1 x 10^6 cells/mL and not vary more than 20% among replicates. If these criteria are not met, the test must be repeated.

4.9.2 An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test condition summaries). The acceptability of the test would depend on the experience and professional judgment of the laboratory investigator and the reviewing staff of the regulatory authority. Any deviation from test specifications must be noted when reporting data from the test.

4.10 ANALYTICAL METHODS

4.10.1 Routine chemical and physical analyses for culture and dilution water, food, and test solutions must include established quality assurance practices outlined in USEPA methods manuals (USEPA, 1979a and USEPA, 1979b).

4.10.2 Reagent containers should be dated and catalogued when received from the supplier, and the shelf life should not be exceeded. Also, working solutions should be dated when prepared, and the recommended shelf life should be observed.

4.11 CALIBRATION AND STANDARDIZATION

4.11.1 Instruments used for routine measurements of chemical and physical parameters such as pH, DO, temperature, and conductivity, must be calibrated and standardized according to instrument manufacturer's procedures as indicated in the general section on quality assurance (see USEPA Methods 150.1, 360.1, 170.1, and 120.1 in USEPA, 1979b). Calibration data are recorded in a permanent log book.

4.11.2 Wet chemical methods used to measure hardness, alkalinity and total residual chlorine must be standardized prior to use each day according to the procedures for those specific USEPA methods (see USEPA Methods 130.2 and 310.1 in USEPA, 1979b).

4.12 REPLICATION AND TEST SENSITIVITY

4.12.1 The sensitivity of the tests will depend in part on the number of replicates per concentration, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data.

4.13 VARIABILITY IN TOXICITY TEST RESULTS

4.13.1 Factors which can affect test success and precision include (1) the experience and skill of the laboratory analyst; (2) test organism age, condition, and sensitivity; (3) dilution water quality; (4) temperature control; and (5) the quality and quantity of food provided. The results will depend upon the species used and the strain or source of the test organisms, and test conditions, such as temperature, DO, food, and water quality. The repeatability or precision of toxicity tests is also a function of the number of test organisms used at each toxicant concentration. Jensen (1972) discussed the relationship between sample size (number of fish) and the standard error of the test, and considered 20 fish per concentration as optimum for Probit Analysis.

4.14 TEST PRECISION

4.14.1 The ability of the laboratory personnel to obtain consistent, precise results must be demonstrated with reference toxicants before they attempt to measure effluent toxicity. The single-laboratory precision of each type of test to be used in a laboratory should be determined by performing at least five tests with a reference toxicant.

4.14.2 Test precision can be estimated by using the same strain of organisms under the same test conditions and employing a known toxicant, such as a reference toxicant.

4.14.3 Interlaboratory precision data from a 1991 study of chronic toxicity tests with two species using the reference toxicants potassium chloride and copper sulfate are shown in Table 1. Table 2 shows interlaboratory precision data from a study of three chronic toxicity test methods using effluent, receiving water, and reference toxicant sample types (USEPA, 2001a; USEPA, 2001b). The effluent sample was a municipal wastewater spiked with KCl, the receiving waster sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Additional precision data for each of the tests described in this manual are presented in the sections describing the individual test methods.

TABLE 1. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 1991: SUMMARY OF RESPONSES USING A REFERENCE TOXICANT[1]

Organism	Endpoint	No. Labs	% Effluent[2]	SD	CV(%)
Pimephales promelas	Survival, NOEC	146	NA	NA	NA
	Growth, IC25	124	4.67	1.87	40.0
	Growth, IC50	117	6.36	2.04	32.1
	Growth, NOEC	142	NA	NA	NA
Ceriodaphnia dubia	Survival, NOEC	162	NA	NA	NA
	Reproduction, IC25	155	2.69	1.96	72.9
	Reproduction, IC50	150	3.99	2.35	58.9
	Reproduction, NOEC	156	NA	NA	NA

[1] From a national study of interlaboratory precision of toxicity test data performed in 1991 by the Environmental Monitoring Systems Laboratory- Cincinnati, U.S. Environmental Protection Agency, Cincinnati, OH 45268. Participants included Federal, state, and private laboratories engaged in NPDES permit compliance monitoring.

[2] Expressed as % effluent; in reality it was a reference toxicant (KCl) but was not known by the persons conducting the tests.

TABLE 2. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 2000: PRECISION OF RESPONSES USING EFFLUENT, RECEIVING WATER, AND REFERENCE TOXICANT SAMPLE TYPES[1].

Organism	Endpoint	Number of Tests[2]	CV (%)[3]
Pimephales promelas	Growth, IC25	73	20.9
Ceriodaphnia dubia	Reproduction, IC25	34	35.0
Selenastrum capricornutum (with EDTA)	Growth, IC25	21	34.3
	Growth, IC50	22	32.2
Selenastrum capricornutum (without EDTA)	Growth, IC25	21	58.5
	Growth, IC50	22	58.5

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 Represents the number of valid tests (i.e., those that met test acceptability criteria) that were used in the analysis of precision. Invalid tests were not used.

3 CVs based on total interlaboratory variability (including both within-laboratory and between-laboratory components of variability) and averaged across sample types. IC25s or IC50s were pooled for all laboratories to calculate the CV for each sample type. The resulting CVs were then averaged across sample types.

4.14.4 Additional information on toxicity test precision is provided in the Technical Support Document for Water Quality-based Control (see pp. 2-4, and 11-15 in USEPA, 1991a).

4.14.5 In cases where the test data are used in Probit Analysis or other point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis), precision can be described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated endpoints from the replicated tests. In cases where the test data are used in the Linear Interpolation Method, precision can be estimated by empirical confidence intervals derived by using the ICPIN Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). However, in cases where the results are reported in terms of the No-Observed-Effect Concentration (NOEC) and Lowest-Observed-Effect Concentration (LOEC) (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis) precision can only be described by listing the NOEC-LOEC interval for each test. It is not possible to express precision in terms of a commonly used statistic. However, when all tests of the same toxicant yield the same NOEC-LOEC interval, maximum precision has been attained. The "true" no effect concentration could fall anywhere within the interval, NOEC ± (NOEC minus LOEC).

4.14.6 It should be noted here that the dilution factor selected for a test determines the width of the NOEC-LOEC interval and the inherent maximum precision of the test. As the absolute value of the dilution factor decreases, the width of the NOEC-LOEC interval increases, and the inherent maximum precision of the test decreases. When a dilution factor of 0.3 is used, the NOEC could be considered to have a relative variability as high as ± 300%. With a dilution factor of 0.5, the NOEC could be considered to have a relative variability of ± 100%. As a result of the variability of different dilution factors, **USEPA recommends the use of the dilution factor of 0.5 or greater**. Other factors which can affect test precision include: test organism age, condition, and sensitivity; temperature

control; and feeding.

4.15 DEMONSTRATING ACCEPTABLE LABORATORY PERFORMANCE

4.15.1 It is a laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicants before it performs toxicity tests with effluents for permit compliance purposes. To meet this requirement, the intralaboratory precision, expressed as percent coefficient of variation (CV%), of each type of test to be used in the laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (i.e., the same test duration, type of dilution water, age of test organisms, feeding, etc.), and the same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations.

4.16 DOCUMENTING ONGOING LABORATORY PERFORMANCE

4.16.1 Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month with a reference toxicant for each toxicity test method conducted in the laboratory during that month. For a given test method, successive tests must be performed with the same reference toxicant, at the same concentrations, in the same dilution water, using the same data analysis methods. Precision may vary with the test species, reference toxicant, and type of test. Each laboratory's reference toxicity data will reflect conditions unique to that facility, including dilution water, culturing, and other variables; however, each laboratory's reference toxicity results should reflect good repeatability.

4.16.2 A control chart should be prepared for each combination of reference toxicant, test species, test conditions, and endpoints. Toxicity endpoints from five or six tests are adequate for establishing the control charts. Successive toxicity endpoints (NOECs, IC25s, LC50s, etc.) should be plotted and examined to determine if the results (X_1) are within prescribed limits (Figure 1). The chart should plot logarithm of concentration on the vertical axis against the date of the test or test number on the horizontal axis. The types of control charts illustrated (see USEPA, 1979a) are used to evaluate the cumulative trend of results from a series of samples, thus reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. For endpoints that are point estimates (LC50s and IC25s), the cumulative mean ($\bar{X}$) and upper and lower control limits ($\pm$ 2S) are re-calculated with each successive test result. Endpoints from hypothesis tests (NOEC, NOAEC) from each test are plotted directly on the control chart. The control limits would consist of one concentration interval above and below the concentration representing the central tendency. After two years of data collection, or a minimum of 20 data points, the control chart should be maintained using only the 20 most recent data points.

4.16.3 Laboratories should compare the calculated CV (i.e., standard deviation / mean) of the IC25 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-2 in USEPA, 2000b). If the calculated CV exceeds the 75th percentile of CVs reported nationally, the laboratory should use the 75th and 90th percentiles to calculate warning and control limits, respectively, and the laboratory should investigate options for reducing variability. Note: Because NOECs can only be a fixed number of discrete values, the mean, standard deviation, and CV cannot be interpreted and applied in the same way that these descriptive statistics are interpreted and applied for continuous variables such as the IC25 or LC50.

4.16.4 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified. In the case of endpoints that are point estimates (LC50s and IC25s), at the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone. If more than one out of 20 reference toxicant tests fall outside the control limits, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Control limits for the NOECs will also be exceeded occasionally, regardless of how well a laboratory performs. In those instances when the laboratory can document the cause for the outlier (e.g., operator error, culture health or test system failure), the outlier should be excluded from the future calculations of the control limits. If two or more consecutive tests do not fall within the control limits, the results

must be explained and the reference toxicant test must be immediately repeated. Actions taken to correct the problem must be reported.

4.16.5 If the toxicity value from a given test with a reference toxicant falls well outside the expected range for the other test organisms when using the standard dilution water and other test conditions, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Performance should improve with experience, and the control limits for endpoints that are point estimates should gradually narrow. However, control limits of ± 2S will be exceeded 5% of the time by chance alone, regardless of how well a laboratory performs. Highly proficient laboratories which develop very narrow control limits may be unfairly penalized if a test result which falls just outside the control limits is rejected *de facto*. For this reason, the width of the control limits should be considered in determining whether or not a reference toxicant test result falls "well" outside the expected range. The width of the control limits may be evaluated by comparing the calculated CV (i.e., standard deviation / mean) of the IC25 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-2 in USEPA, 2000b). In determining whether or not a reference toxicant test result falls "well" outside the expected range, the result also may be compared with upper and lower bounds for ±3S, as any result outside these control limits would be expected to occur by chance only 1 out of 100 tests (Environment Canada, 1990). When a result from a reference toxicant test is outside the 99% confidence intervals, the laboratory must conduct an immediate investigation to assess the possible causes for the outlier.

4.16.6 Reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. Reference toxicant testing is used for evaluating the health and sensitivity of organisms over time and for documenting initial and ongoing laboratory performance. While reference toxicant test results should not be used as a *de facto* criterion for test rejection, effluent and receiving water test results should be reviewed and interpreted in the light of reference toxicant test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increased test organism sensitivity or toward decreased test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test.

4.17 REFERENCE TOXICANTS

4.17.1 Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$), are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests. EMSL-Cincinnati hopes to release USEPA-certified solutions of cadmium and copper for use as reference toxicants through cooperative research and development agreements with commercial suppliers, and will continue to develop additional reference toxicants for future release. Standard reference materials can be obtained from commercial supply houses, or can be prepared inhouse using reagent grade chemicals. The regulatory agency should be consulted before reference toxicant(s) are selected and used.

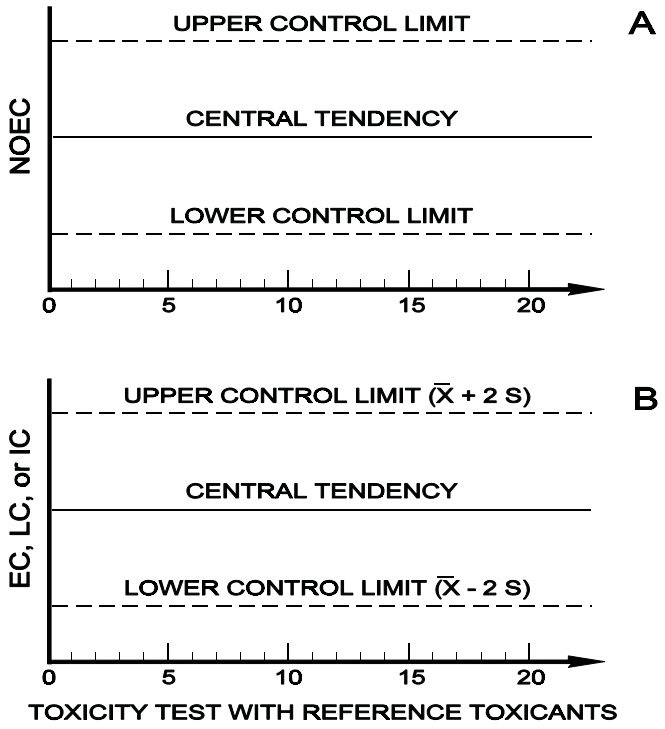

Figure 1. Control charts. (A) hypothesis testing results; (B) point estimates (LC, EC, or IC).

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

$$S = \sqrt{\frac{\sum_{i=1}^{n} X_i^2 - \frac{(\sum_{i=1}^{n} X_i)^2}{n}}{n-1}}$$

Where: X_i = Successive toxicity values from toxicity tests.

n = Number of tests.

$\bar{X}$ = Mean toxicity value.

S = Standard deviation.

4.18 RECORD KEEPING

4.18.1 Proper record keeping is important. A complete file should be maintained for each individual toxicity test or group of tests on closely related samples. This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the toxicity test(s); chemical analysis data on the sample(s); detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions employed; and results of reference toxicant tests. Laboratory data should be recorded on a real-time basis to prevent the loss of information or inadvertent introduction of errors into the record. Original data sheets should be signed and dated by the laboratory personnel performing the tests.

4.18.2 The regulatory authority should retain records pertaining to discharge permits. Permittees are required to retain records pertaining to permit applications and compliance for a minimum of 3 years [40 CFR 122.41(j)(2)].

SECTION 5

FACILITIES, EQUIPMENT, AND SUPPLIES

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities must include equipment for rearing and/or holding organisms. Culturing facilities for test organisms may be desirable in fixed laboratories which perform large numbers of tests. Temperature control can be achieved using circulating water baths, heat exchangers, or environmental chambers. Water used for rearing, holding, acclimating, and testing organisms may be ground water, receiving water, dechlorinated tap water, or reconstituted synthetic water. Dechlorination can be accomplished by carbon filtration, or the use of sodium thiosulfate. Use of 3.6 mg (anhydrous) sodium thiosulfate/L will reduce l.0 mg chlorine/L. After dechlorination, total residual chlorine should be non-detectable. Air used for aeration must be free of oil and toxic vapors. Oil-free air pumps should be used where possible. Particulates can be removed from the air using BALSTON® Grade BX or equivalent filters, and oil and other organic vapors can be removed using activated carbon filters (BALSTON®, C-1 filter, or equivalent).

5.1.2 The facilities must be well ventilated and free from fumes. Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories and/or sample holding areas is not circulated to test organism culture rooms or toxicity test rooms, or that air from toxicity test rooms does not contaminate culture areas. Sample preparation, culturing, and toxicity test areas should be separated to avoid cross contamination of cultures or toxicity test solutions with toxic fumes. Air pressure differentials between such rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely- fitting doors. Organisms should be shielded from external disturbances.

5.1.3 Materials used for exposure chambers, tubing, etc., that come in contact with the effluent and dilution water should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON®) should be used whenever possible to minimize sorption and leaching of toxic substances. These materials may be reused following decontamination. Containers made of plastics, such as polyethylene, polypropylene, polyvinyl chloride, TYGON®, etc., may be used as test chambers or to ship, store and transfer effluents and receiving waters, but they should not be reused unless absolutely necessary, because they could carry over adsorbed toxicants from one test to another, if reused. However, these containers may be repeatedly reused for storing uncontaminated waters, such as deionized or laboratory-prepared dilution waters and receiving waters. Glass or disposable polystyrene containers can be used for test chambers. The use of large (≥ 20 L) glass carboys is discouraged for safety reasons.

5.1.4 New plastic products of a type not previously used should be tested for toxicity before initial use by exposing the test organisms in the test system where the material is used. Equipment (pumps, valves, etc.) which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below (see Section 5, Facilities, Equipment and Supplies, Subsection 5.3.2). Fiberglass and stainless steel, in addition to the previously mentioned materials, can be used for holding, acclimating, and dilution water storage tanks, and in the water delivery system, but once contaminated with pollutants the fiberglass should not be reused. All material should be flushed or rinsed thoroughly with the test media before using in the test.

5.1.5 Copper, galvanized material, rubber, brass, and lead must not come in contact with culturing, holding, acclimation, or dilution water, or with effluent samples and test solutions. Some materials, such as several types of neoprene rubber (commonly used for stoppers), may be toxic and should be tested before use.

5.1.6 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

5.2 TEST CHAMBERS

5.2.1 Test chamber size and shape are varied according to size of the test organism. Requirements are specified in each toxicity test method.

5.3 CLEANING TEST CHAMBERS AND LABORATORY APPARATUS

5.3.1 New plasticware used for sample collection or organism exposure vessels does not require thorough cleaning before use. It is sufficient to rinse new sample containers once with dilution water before use. New glassware must be soaked overnight in 10% acid (see below) and rinsed well in deionized water and dilution water.

5.3.2 All non-disposable sample containers, test vessels, tanks, and other equipment that have come in contact with effluent must be washed after use to remove contaminants as described below.

1. Soak 15 min in tap water and scrub with detergent, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh, dilute (10%, V:V) hydrochloric or nitric acid to remove scale, metals and bases. To prepare a 10% solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with deionized water.

5.3.3 Special requirements for cleaning glassware used in the green alga, *Selenastrum capricornutum*, toxicity tests (Method 1003.0, Section 14). Prepare all graduated cylinders, test flasks, bottles, volumetric flasks, centrifuge tubes and vials used in algal assays as follows:

1. Wash with non-phosphate detergent solution, preferably heated to ≥ 50°C. Brush the inside of flasks with a stiff-bristle brush to loosen any attached material. The use of a commercial laboratory glassware washer or heavy-duty kitchen dishwasher (under-counter type) is highly recommended.
2. Rinse with tap water.
3. Test flasks should be thoroughly rinsed with acetone and a 10% solution (by volume) of reagent grade hydrochloric acid (HCl). It may be advantageous to soak the flasks in 10% HCl for several days. Fill vials and centrifuge tubes with the 10% HCl solution and allow to stand a few minutes; fill all larger containers to about one-tenth capacity with HCl solution and swirl so that the entire surface is bathed.
4. Rinse twice with MILLIPORE® MILLI-Q® OR QPAK™$_2$, or equivalent, water.
5. New test flasks, and all flasks which through use may become contaminated with toxic organic substances, must be rinsed with pesticide-grade acetone or heat-treated before use. To thermally degrade organics, place glassware in a high temperature oven at 400°C for 30 min. After cooling, go to 7. If acetone is used, go to 6.
6. Rinse thoroughly with MILLIPORE® MILLI-Q® or QPAK™$_2$, or equivalent water, and dry in an 105°C oven. All glassware should be autoclaved before use and between uses.
7. Cover the mouth of each chamber with aluminum foil or other closure, as appropriate, before storing.

5.3.4 The use of sterile, disposable pipets will eliminate the need for pipet washing and minimize the possibility of contaminating the cultures with toxic substances.

5.3.5 All test chambers and equipment must be thoroughly rinsed with the dilution water immediately prior to use in each test.

5.4 APPARATUS AND EQUIPMENT FOR CULTURING AND TOXICITY TESTS

5.4.1 Apparatus and equipment requirements for culturing and testing are specified in each toxicity test method. Also, see USEPA, 2002a.

5.4.2 WATER PURIFICATION SYSTEM

5.4.2.1 A good quality, laboratory grade deionized water, providing a resistance of 18 megaohm-cm, must be available in the laboratory and in sufficient quantity for laboratory needs. Deionized water may be obtained from MILLIPORE® Milli-Q®, MILLIPORE® QPAK™$_2$ or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade deionizer with preconditioned water from a Culligan®, Continental®, or equivalent mixed-bed water treatment system.

5.5 REAGENTS AND CONSUMABLE MATERIALS

5.5.1 SOURCES OF FOOD FOR CULTURE AND TOXICITY TESTS

1. Brine shrimp, *Artemia* sp., cysts -- Many commercial sources of brine shrimp cysts are available.
2. Frozen adult brine shrimp, *Artemia* -- Available from most pet supply shops or other commercial sources.
3. Flake fish food -- TETRAMIN® and BIORIL® are available from most pet shops.
4. Trout chow -- Available from commercial sources.
5. Cereal leaves, CEROPHYLL® or equivalent -- Available from commercial sources.
6. Yeast -- Packaged dry yeast, such as Fleischmann's, or equivalent, can be purchased at the local grocery store or commercial sources.
7. Alfalfa Rabbit Pellets -- Available from feed stores as Purina rabbit chow.
8. Algae - Available from commercial sources.

5.5.1.1 All food should be tested for nutritional suitability and chemically analyzed for organochlorine pesticides, PCBs, and toxic metals (see Section 4, Quality Assurance).

5.5.2 Reagents and consumable materials are specified in each toxicity test method section. Also, see Section 4, Quality Assurance.

5.6 TEST ORGANISMS

5.6.1 Test organisms should be obtained from inhouse cultures or from commercial suppliers (see specific test method; Section 4, Quality Assurance; and Section 6, Test Organisms).

5.7 SUPPLIES

5.7.1 See test methods (see Sections 11-14) for specific supplies.

SECTION 6

TEST ORGANISMS

6.1 TEST SPECIES

6.1.1 The species used in characterizing the chronic toxicity of effluents and/or receiving waters will depend on the requirements of the regulatory authority and the objectives of the test. It is essential that good quality test organisms be readily available throughout the year from inhouse or commercial sources to meet NPDES monitoring requirements. The organisms used in the toxicity tests must be identified to species. If there is any doubt as to the identity of the test organism, representative specimens should be sent to a taxonomic expert to confirm the identification.

6.1.2 Toxicity test conditions and culture methods for the species listed in Subsection 6.1.3 are provided in this manual also, see USEPA, 2002a.

6.1.3 The organisms used in the short-term chronic toxicity tests described in this manual are the fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia* (Berner, 1986), and the green alga, *Selenastrum capricornutum*.

6.1.4 Some states have developed culturing and testing methods for indigenous species that may be as sensitive, or more sensitive, than the species recommended in Subsection 6.1.3. However, USEPA allows the use of indigenous species only where state regulations require their use or prohibit importation of the recommended species in Subsection 6.1.3. Where state regulations prohibit importation of non-native fishes or the use of recommended test species, permission must be requested from the appropriate state agency prior to their use.

6.1.5 Where states have developed culturing and testing methods for indigenous species other than those recommended in this manual, data comparing the sensitivity of the substitute species and the one or more recommended species must be obtained in side-by-side toxicity tests with reference toxicants and/or effluents, to ensure that the species selected are at least as sensitive as the recommended species. These data must be submitted to the permitting authority (State or Region) if required. USEPA acknowledges that reference toxicants prepared from pure chemicals may not always be representative of effluents. However, because of the observed and/or potential variability in the quality and toxicity of effluents, it is not possible to specify a representative effluent.

6.1.6 Guidance for the selection of test organisms where the salinity of the effluent and/or receiving water requires special consideration is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991a).

1. Where the salinity of the receiving water is < 1‰, freshwater organisms are used regardless of the salinity of the effluent.
2. Where the salinity of the receiving water is ≥ 1‰, the choice of organisms depends on state water quality standards and/or permit requirements.

6.2 SOURCES OF TEST ORGANISMS

6.2.1 The test organisms recommended in this manual can be cultured in the laboratory using culturing and handling methods for each organism described in the respective test method sections. The fathead minnow, *Pimephales promelas,* culture method is given in Section 11 and not repeated in Section 12. Also, see USEPA (2002a).

6.2.2 Inhouse cultures should be established wherever it is cost effective. If inhouse cultures cannot be maintained or it is not cost effective, test organisms or starter cultures should be purchased from experienced commercial suppliers (see USEPA, 2002a).

6.2.3 Starter cultures of the green algae, *Selenastrum capricornutum, S. minutum,* and *Chlamydomonas reinhardti* are available from commercial suppliers.

6.2.4 Because the daphnid, *Ceriodaphnia dubia*, must be cultured individually in the laboratory for at least seven days before the test begins, it will be necessary to obtain a starter culture from a commercial source at least three weeks before the test is to begin if they are not being cultured inhouse.

6.2.5 If, because of their source, there is any uncertainty concerning the identity of the organisms, it is advisable to have them examined by a taxonomic specialist to confirm their identification. For detailed guidance on identification, see the individual test methods.

6.2.6 FERAL (NATURAL OCCURRING, WILD CAUGHT) ORGANISMS

6.2.6.1 The use of test organisms taken from the receiving water has strong appeal, and would seem to be a logical approach. However, it is generally impractical and not recommended for the following reasons:

1. Sensitive organisms may not be present in the receiving water because of previous exposure to the effluent or other pollutants.
2. It is often difficult to collect organisms of the required age and quality from the receiving water.
3. Most states require collecting permits, which may be difficult to obtain. Therefore, it is usually more cost effective to culture the organisms in the laboratory or obtain them from private, state, or Federal sources. The fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia*, and the green alga, *Selenastrum capricornutum*, are easily cultured in the laboratory or readily available commercially.
4. The required QA/QC records, such as the single laboratory precision data, would not be available.
5. Since it is mandatory that the identity of the test organism be known to species level, it would be necessary to examine each organism caught in the wild to confirm its identity. This would usually be impractical or, at the least, very stressful to the organisms.
6. Test organisms obtained from the wild must be observed in the laboratory for a minimum of one week prior to use, to assure that they are free of signs of parasitic or bacterial infections and other adverse effects. Fish captured by electroshocking must not be used in toxicity testing.

6.2.6.2 Guidelines for collecting natural occurring organisms are provided in USEPA (1973), USEPA (1990) and USEPA (1993b).

6.2.7 Regardless of their source, test organisms should be carefully observed to ensure that they are free of signs of stress and disease, and in good physical condition. Some species of test organisms can be obtained from commercial stock certified as "disease-free".

6.3 **LIFE STAGE**

6.3.1 Young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as larval fish, is required for all tests. In a given test, all organisms should be approximately the same age and should be taken from the same source. Since age may affect the results of the tests, it would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility.

6.4 LABORATORY CULTURING

6.4.1 Instructions for culturing and/or holding the recommended test organisms are included in the respective test methods (also, see USEPA, 2002a).

6.5 HOLDING AND HANDLING TEST ORGANISMS

6.5.1 Test organisms should not be subjected to changes of more than 3°C in water temperature in any 12 h period or 2 units of pH in any 24-h period.

6.5.2 Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that are dropped or touch a dry surface or are injured during handling must be discarded. Dipnets are best for handling larger organisms. These nets are commercially available or can be made from small-mesh nylon netting, silk batting cloth, plankton netting, or similar material. Wide-bore, smooth glass tubes (4 to 8 mm ID) with rubber bulbs or pipettors (such as PROPIPETTE®) should be used for transferring smaller organisms such as larval fish.

6.5.3 Holding tanks for fish are supplied with good quality water (see Section 5, Facilities, Equipment, and Supplies) with flow-through rate of at least two tank volumes per day. Otherwise use a recirculation system where water flows through an activated carbon or undergravel filter to remove dissolved metabolites. Culture water can also be piped through high intensity ultraviolet light sources for disinfection, and to photodegrade dissolved organics.

6.5.4 Crowding must be avoided because it will stress the organisms and lower the DO concentrations to unacceptable levels. The solution of oxygen depends on temperature and altitude. The DO must be maintained at a minimum of 4.0 mg/L. Aerate gently if necessary.

6.5.5 The organisms should be observed carefully each day for signs of disease, stress, physical damage, or mortality. Dead and abnormal organisms should be removed as soon as observed. It is not uncommon for some fish mortality (5-10%) to occur during the first 48 h in a holding tank because of individuals that refuse to feed on artificial food and die of starvation. Organisms in the holding tanks should generally be fed as in the cultures (see culturing methods in the respective methods).

6.5.6 Fish should be fed as much as they will eat at least once a day with live brine shrimp nauplii, *Artemia,* or frozen adult brine shrimp, or dry food (frozen food should be completely thawed before use). Adult brine shrimp can be supplemented with commercially prepared food such as TETRAMIN® or BIORIL® flake food, or equivalent. Excess food and fecal material should be removed from the bottom of the tanks at least twice a week by siphoning.

6.5.7 A daily record of feeding, behavioral observations, and mortality should be maintained.

6.6 TRANSPORTATION TO THE TEST SITE

6.6.1 Organisms are transported from the base or supply laboratory to a remote test site in culture water or standard dilution water in plastic bags or large-mouth screw-cap (500 mL) plastic bottles in styrofoam coolers. Adequate DO is maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags or by use of an airstone supplied by a portable pump. The DO concentration must not fall below 4.0 mg/L.

6.6.2 Upon arrival at the test site, the organisms are transferred to receiving water if receiving water is to be used as the test dilution water. All but a small volume of the holding water (approximately 5%) is removed by siphoning and replaced slowly over a 10 to 15 minute period with dilution water. If receiving water is to be used as the dilution water, caution must be exercised in exposing the test organisms to it, because of the possibility that it might be toxic. For this reason, it is recommended that only approximately 10% of the test organisms be exposed initially

to the dilution water. If this group does not show excessive mortality or obvious signs of stress in a few hours, the remainder of the test organisms may be transferred to the dilution water.

6.6.3 A group of organisms must not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed, or if mortality appears to exceed 10% preceding the test. If the organisms fail to meet these criteria, the entire group must be discarded and a new group obtained. The mortality may be due to the presence of toxicity, if the receiving water is used as dilution water, rather than a diseased condition of the test organisms. If the acclimation process is repeated with a new group of test organisms and excessive mortality occurs, it is recommended that an alternative source of dilution water be used.

6.7 **TEST ORGANISM DISPOSAL**

6.7.1 When the toxicity test(s) is concluded, all test organisms (including controls) should be humanely destroyed and disposed of in an appropriate manner.

SECTION 7

DILUTION WATER

7.1 TYPES OF DILUTION WATER

7.1.1 The type of dilution water used in effluent toxicity tests will depend largely on the objectives of the study.

7.1.1.1 If the objective of the test is to estimate the absolute chronic toxicity of the effluent, a synthetic (standard) dilution water is used. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.2 If the objective of the test is to estimate the chronic toxicity of the effluent in uncontaminated receiving water, the test may be conducted using dilution water consisting of a single grab sample of receiving water (if non-toxic), collected either upstream and outside the influence of the outfall, or with other uncontaminated natural water (ground or surface water) or standard dilution water having approximately the same characteristics (hardness, alkalinity, and conductivity) as the receiving water. Seasonal variations in the quality of receiving waters may affect effluent toxicity. Therefore, the pH, alkalinity, hardness, and conductivity of receiving water samples should be determined before each use. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.3 If the objective of the test is to determine the additive or mitigating effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected immediately upstream or outside the influence of the outfall. A second set of controls, using culture water, should be included in the test.

7.1.2 An acceptable dilution water is one which is appropriate for the objectives of the test; supports adequate performance of the test organisms with respect to survival, growth, reproduction, or other responses that may be measured in the test (i.e., consistently meets test acceptability criteria for control responses); is consistent in quality; and does not contain contaminants that could produce toxicity. Receiving waters, synthetic waters, or synthetic waters adjusted to approximate receiving water characteristics may be used for dilution provided that the water meets the above listed qualifications for an acceptable dilution water. USEPA (2000a) provides additional guidance on selecting appropriate dilution waters.

7.1.3 When dual controls (one control using culture water and one control using dilution water) are used (see Subsections 7.1.1.1 - 7.1.1.3 above), the dilution water control should be used to determine test acceptability. It is also the dilution water control that should be compared to effluent treatments in the calculation and reporting of test results. The culture water control should be used to evaluate the appropriateness of the dilution water source. Significant differences between organism responses in culture water and dilution water controls could indicate toxicity in the dilution water and may suggest an alternative dilution water source. USEPA (2000a) provides additional guidance on dual controls.

7.2 STANDARD, SYNTHETIC DILUTION WATER

7.2.1 Standard, synthetic dilution water is prepared with deionized water and reagent grade chemicals or mineral water (Tables 3 and 4). The source water for the deionizer can be ground water or tap water.

7.2.2 DEIONIZED WATER USED TO PREPARE STANDARD, SYNTHETIC, DILUTION WATER

7.2.2.1 Deionized water is obtained from a MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$ or equivalent system. It is advisable to provide a preconditioned (deionized) feed water by using a Culligan®, Continental®, or

equivalent system in front of the MILLIPORE® System to extend the life of the MILLIPORE® cartridges (see Section 5, Facilities, Equipment, and Supplies).

7.2.2.2 The recommended order of the cartridges in a four-cartridge deionizer (i.e., MILLI-Q® System or equivalent) is (1) ion exchange, (2) ion exchange, (3) carbon, and (4) organic cleanup (such as ORGANEX-Q®, or equivalent) followed by a final bacteria filter. The QPAK™ 2 water system is a sealed system which does not allow for the rearranging of the cartridges. However, the final cartridge is an ORGANEX-Q® filter, followed by a final bacteria filter. Commercial laboratories using this system have not experienced any difficulty in using the water for culturing or testing. Reference to the MILLI-Q® systems throughout the remainder of the manual includes all MILLIPORE® or equivalent systems.

7.2.3 STANDARD, SYNTHETIC FRESHWATER

7.2.3.1 To prepare 20 L of synthetic, moderately hard, reconstituted water, use the reagent grade chemicals in Table 3 as follows:

1. Place 19 L of MILLI-Q®, or equivalent, water in a properly cleaned plastic carboy.
2. Add 1.20 g of $MgSO_4$, 1.92 g $NaHCO_3$, and 0.080g KCl to the carboy.
3. Aerate overnight.
4. Add 1.20 g of $CaSO_4 \cdot 2H_20$ to 1 L of MILLI-Q® or equivalent deionized water in a separate flask. Stir on magnetic stirrer until calcium sulfate is dissolved, add to the 19 L above, and mix well.
5. For *Ceriodaphnia dubia* culturing and testing, add sufficient sodium selenate (Na_2SeO_4) to provide 2 mg selenium per liter of final dilution water.
6. Aerate the combined solution vigorously for an additional 24 h to dissolve the added chemicals and stabilize the medium.
7. The measured pH, hardness, etc., should be as listed in Table 3.

TABLE 3. PREPARATION OF SYNTHETIC FRESHWATER USING REAGENT GRADE CHEMICALS[1]

Water Type	Reagent Added (mg/L)[2]				Approximate Final Water Quality		
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	pH[3]	Hardness[4]	Alkalinity[4]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	57-64
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

1 Taken in part from Marking and Dawson (1973).
2 Add reagent grade chemicals to deionized water.
3 Approximate equilibrium pH after 24 h of aeration.
4 Expressed as mg $CaCO_3$/L.

7.2.3.2 If large volumes of synthetic reconstituted water will be needed, it may be advisable to mix 1 L portions of concentrated stock solutions of $NaHCO_3$, $MgSO_4$, and KCl for use in preparation of the reconstituted waters.

7.2.3.3 To prepare 20 L of standard, synthetic, moderately hard, reconstituted water, using mineral water such as PERRIER® Water, or equivalent (Table 4), follow the instructions below.

1. Place 16 L of MILLI-Q® or equivalent water in a properly cleaned plastic carboy.
2. Add 4 L of PERRIER® Water, or equivalent.
3. Aerate vigorously for 24 h to stabilize the medium.
4. The measured pH, hardness and alkalinity of the aerated water will be as indicated in Table 4.
5. This synthetic water is referred to as diluted mineral water (DMW) in the toxicity test methods.

TABLE 4. PREPARATION OF SYNTHETIC FRESHWATER USING MINERAL WATER[1]

Water Type	Volume of Mineral Water Added (mL/L)[2]	Proportion of Mineral Water (%)	Approximate Final Water Quality pH[3]	Hardness[4]	Alka-linity[4]
Very soft	50	2.5	7.2-8.1	10-13	10-13
Soft	100	10.0	7.9-8.3	40-48	30-35
Moderately Hard	200	20.0	7.9-8.3	80-100	57-64
Hard	400	40.0	7.9-8.3	160-180	110-120
Very hard[5]	---	---	---	---	---

[1] From Mount et al. (1987), and data provided by Philip Lewis, EMSL-Cincinnati, OH.
[2] Add mineral water to Milli-Q® water, or equivalent, to prepare Diluted Mineral Water (DMW).
[3] Approximate equilibrium pH after 24 h of aeration.
[4] Expressed as mg $CaCO_3$/L.
[5] Dilutions of PERRIER® Water form a precipitate when concentrations equivalent to "very hard water" are aerated.

7.3 USE OF RECEIVING WATER AS DILUTION WATER

7.3.1 If the objectives of the test require the use of uncontaminated receiving water as dilution water, and the receiving water is uncontaminated, it may be possible to collect a sample of the receiving water upstream of, or close to, but outside of the zone influenced by the effluent. However, if the receiving water is contaminated, it may be necessary to collect the sample in an area "remote" from the discharge site, matching as closely as possible the physical and chemical characteristics of the receiving water near the outfall.

7.3.2 The sample should be collected immediately prior to the test, but never more than 96 h before the test begins. Except where it is used within 24 h, or in the case where large volumes are required for flow through tests, the sample should be chilled to 0-6°C during or immediately following collection, and maintained at that temperature prior to use in the test.

7.3.3 Receiving water containing debris or indigenous organisms that may be confused with or attack the test organisms should be filtered through a sieve having 60 mm mesh openings prior to use.

7.3.4 Where toxicity-free dilution water is required in a test, the water is considered acceptable if test organisms show the required survival, growth, and reproduction in the controls during the test.

7.3.5 The regulatory authority may require that the hardness of the dilution water be comparable to the receiving water at the discharge site. This requirement can be satisfied by collecting an uncontaminated receiving water with a suitable hardness, or adjusting the hardness of an otherwise suitable receiving water by addition of reagents as indicated in Table 3.

7.4 USE OF TAP WATER AS DILUTION WATER

7.4.1 The use of tap water as dilution water is discouraged unless it is dechlorinated and passed through a deionizer and carbon filter. Tap water can be dechlorinated by deionization, carbon filtration, or the use of sodium thiosulfate. Use of 3.6 mg/L (anhydrous) sodium thiosulfate will reduce 1.0 mg chlorine/L (APHA, 1992). Following dechlorination, total residual chlorine should not exceed 0.01 mg/L. Because of the possible toxicity of thiosulfate to test organisms, a control lacking thiosulfate should be included in toxicity tests utilizing thiosulfate-dechlorinated water.

7.4.2 To be adequate for general laboratory use following dechlorination, the tap water is passed through a deionizer and carbon filter to remove toxic metals and organics, and to control hardness and alkalinity.

7.5 DILUTION WATER HOLDING

7.5.1 A given batch of dilution water should not be used for more than 14 days following preparation because of the possible build-up of bacterial, fungal, or algal slime growth and the problems associated with it. The container should be kept covered and the contents should be protected from light.

SECTION 8

EFFLUENT AND RECEIVING WATER SAMPLING, SAMPLE HANDLING, AND SAMPLE PREPARATION FOR TOXICITY TESTS

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point should be the same as that specified in the NPDES discharge permit (USEPA, 1988a). Conditions for exception would be: (l) better access to a sampling point between the final treatment and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to municipal waste treatment plants or separate wastewater streams in industrial facilities prior to their being combined with other wastewater streams or non-contact cooling water, additional sampling points may be chosen.

8.1.2 The decision on whether to collect grab or composite samples is based on the objectives of the test and an understanding of the short and long-term operations and schedules of the discharger. If the effluent quality varies considerably with time, which can occur where holding times are short, grab samples may seem preferable because of the ease of collection and the potential of observing peaks (spikes) in toxicity. However, the sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitively large number of separate samples and tests. Collection of a 24-h composite sample, however, may dilute toxicity spikes, and average the quality of the effluent over the sampling period. Sampling recommendations are provided below (also see USEPA, 2002a).

8.1.3 Aeration during collection and transfer of effluents should be minimized to reduce the loss of volatile chemicals.

8.1.4 Details of date, time, location, duration, and procedures used for effluent sample and dilution water collection should be recorded.

8.2 EFFLUENT SAMPLE TYPES

8.2.1 The advantages and disadvantages of effluent grab and composite samples are listed below:

8.2.1.1 GRAB SAMPLES

Advantages:

1. Easy to collect; require a minimum of equipment and on-site time.
2. Provide a measure of instantaneous toxicity. Toxicity spikes are not masked by dilution.

Disadvantages:

1. Samples are collected over a very short period of time and on a relatively infrequent basis. The chances of detecting a spike in toxicity would depend on the frequency of sampling and the probability of missing a spike is high.

8.2.1.2 COMPOSITE SAMPLES

Advantages:

1. A single effluent sample is collected over a 24-h period.

2. The sample is collected over a much longer period of time than a single grab sample and contains all toxicity spikes.

Disadvantages:

1. Sampling equipment is more sophisticated and expensive, and must be placed on-site for at least 24 h.
2. Toxicity spikes may not be detected because they are masked by dilution with less toxic wastes.

8.3 EFFLUENT SAMPLING RECOMMENDATIONS

8.3.1 When tests are conducted on-site, test solutions can be renewed daily with freshly collected samples, except for the green alga, *Selenastrum capricornutum*, test which is not renewed.

8.3.2 When tests are conducted off-site, a minimum of three samples are collected. If these samples are collected on Test Days 1, 3, and 5, the first sample would be used for test initiation, and for test solution renewal on Day 2. The second sample would be used for test solution renewal on Days 3 and 4. The third sample would be used for test solution renewal on Days 5, 6, and 7.

8.3.3 Sufficient sample volume must be collected to perform the required toxicity and chemical tests. A 4-L (1-gal) CUBITAINER® will provide sufficient sample volume for most tests.

8.3.4 THE FOLLOWING EFFLUENT SAMPLING METHODS ARE RECOMMENDED:

8.3.4.1 Continuous Discharges

8.3.4.1.1 If the facility discharge is continuous, a single 24-h composite sample is to be taken.

8.3.4.2 Intermittent discharges

8.3.4.2.1 If the facility discharge is intermittent, a composite sample is to be collected for the duration of the discharge but not more than 24 hours.

8.4 RECEIVING WATER SAMPLING

8.4.1 Logistical problems and difficulty in securing sampling equipment generally preclude the collection of composite receiving water samples for toxicity tests. Therefore, based on the requirements of the test, a single grab sample or daily grab sample of receiving water is collected for use in the test.

8.4.2 The sampling point is determined by the objectives of the test. In rivers, samples should be collected from mid-stream and at mid-depth, if accessible. In lakes the samples are collected at mid-depth.

8.4.3 To determine the extent of the zone of toxicity in the receiving water downstream from the outfall, receiving water samples are collected at several distances downstream from the discharge. The time required for the effluent-receiving-water mixture to travel to sampling points downstream from the outfall, and the rate and degree of mixing, may be difficult to ascertain. Therefore, it may not be possible to correlate downstream toxicity with effluent toxicity at the discharge point unless a dye study is performed. The toxicity of receiving water samples from five stations downstream from the discharge point can be evaluated using the same number of test vessels and test organisms as used in one effluent toxicity test with five effluent dilutions.

8.5 EFFLUENT AND RECEIVING WATER SAMPLE HANDLING, PRESERVATION, AND SHIPPING

8.5.1 Unless the samples are used in an on-site toxicity test the day of collection (or hand delivered to the testing laboratory for use on the day of collection), they should be chilled and maintained at 0-6°C until used to inhibit microbial degradation, chemical transformations, and loss of highly volatile toxic substances.

8.5.2 Composite samples should be chilled as they are collected. Grab samples should be chilled immediately following collection.

8.5.3 If the effluent has been chlorinated, total residual chlorine must be measured immediately following sample collection.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time (holding time) from sample collection to first use of each grab or composite sample must not exceed 36 h. EPA believes that 36 h is adequate time to deliver the samples to the laboratories performing the test in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the USEPA Regional Administrator under 40 CFR 136.3(e) should include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond more than 36 h. However, in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, each grab or composite sample may also be used to prepare test solutions for renewal at 24 h, 48 h, and/or 72 h after first use, if stored at 0-6°C, with minimum head space, as described in Subsection 8.5. If shipping problems (e.g., unsuccessful Saturday delivery) are encountered with renewal samples after a test has been initiated, the permitting authority may allow the continued use of the most recently used sample for test renewal. Guidance for determining the persistence of the sample is provided in Subsection 8.7.

8.5.5 To minimize the loss of toxicity due to volatilization of toxic constituents, all sample containers should be "completely" filled, leaving no air space between the contents and the lid.

8.5.6 SAMPLES USED IN ON-SITE TESTS

8.5.6.1 Samples collected for on-site tests should be used within 24 h.

8.5.7 SAMPLES SHIPPED TO OFF-SITE FACILITIES

8.5.7.1 Samples collected for off-site toxicity testing are to be chilled to 0-6°C during or immediately after collection, and shipped iced to the performing laboratory. Sufficient ice should be placed with the sample in the shipping container to ensure that ice will still be present when the sample arrives at the laboratory and is unpacked. Insulating material should not be placed between the ice and the sample in the shipping container unless required to prevent breakage of glass sample containers.

8.5.7.2 Samples may be shipped in one or more 4-L (l-gal) CUBITAINERS® or new plastic "milk" jugs. All sample containers should be rinsed with source water before being filled with sample. After use with receiving water or effluents, CUBITAINERS® and plastic jugs are punctured to prevent reuse.

8.5.7.3 Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. Saturday and Sunday shipping and receiving schedules of private carriers vary with the carrier.

8.6 SAMPLE RECEIVING

8.6.1 Upon arrival at the laboratory, samples are logged in and the temperature is measured and recorded. If the samples are not immediately prepared for testing, they are stored at 0-6°C until used.

8.6.2 Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory, and the sample holding time should not exceed 36 h unless a variance has been granted by the NPDES permitting authority.

8.7 PERSISTENCE OF EFFLUENT TOXICITY DURING SAMPLE SHIPMENT AND HOLDING

8.7.1 The persistence of the toxicity of an effluent prior to its use in a toxicity test is of interest in assessing the validity of toxicity test data, and in determining the possible effects of allowing an extension of the holding time. Where a variance in holding time (> 36 h, but ≤ 72 h) is requested by a permittee, (see Subsection 8.5.4 above), information on the effects of the extension in holding time on the toxicity of samples must be obtained by comparing the results of multi-concentration chronic toxicity tests performed on effluent samples held 36 h with toxicity test results using the same samples after they were held for the requested, longer period. The portion of the sample set aside for the second test should be held under the same conditions as during shipment and holding.

8.8 PREPARATION OF EFFLUENT AND RECEIVING WATER SAMPLES FOR TOXICITY TESTS

8.8.1 When aliquots are removed from the sample container, the head space above the remaining sample should be held to a minimum. Air which enters a container upon removal of sample should be expelled by compressing the container before reclosing, if possible (i.e., where a CUBITAINER® is used), or by using an appropriate discharge valve (spigot).

8.8.2 With the daphnid, *Ceriodaphnia dubia*, and fathead minnow, *Pimephales promelas*, tests, effluents and receiving waters should be filtered through a 60-µm plankton net to remove indigenous organisms that may attack or be confused with the test organisms (see the daphnid, *Ceriodaphnia dubia*, test method for details). Receiving waters used in green alga, *Selenastrum capricornutum*, toxicity tests must be filtered through a 0.45-µm pore diameter filter before use. It may be necessary to first coarse-filter the dilution and/or waste water through a nylon sieve having 2- to 4-mm mesh openings to remove debris and/or break up large floating or suspended solids. Because filtration may increase the dissolved oxygen (DO) in the effluent, the DO should be checked both before and after filtering. Low dissolved oxygen concentrations will indicate a potential problem in performing the test. **Caution:** filtration may remove some toxicity.

8.8.3 If the samples must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved oxygen and nitrogen may become a problem. To avoid this problem, samples may be warmed slowly in open test containers. If DO is still above 100% saturation after warming to test temperature, samples should be aerated moderately (approximately 500 mL/min) for a few minutes using an airstone. If DO is below 4.0 mg/L after warming to test temperature, the solutions must be aerated moderately (approximately 500 mL/min) for a few minutes, using an airstone, until the DO is within the prescribed range (≥4.0 mg/L). **Caution:** avoid excessive aeration.

8.8.4 The DO concentration in the samples should be near saturation prior to use. Aeration may be used to bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH. However, aeration during collection, transfer, and preparation of samples should be minimized to reduce the loss of volatile chemicals.

8.8.4.1 Aeration during the test may alter the results and should be used only as a last resort to maintain the required DO. Aeration can reduce the apparent toxicity of the test solutions by stripping them of highly volatile toxic substances, or increase their toxicity by altering pH. However, the DO in the test solutions should not be allowed to fall below 4.0 mg/L.

8.8.4.2 In static tests (renewal or non-renewal), low DOs may commonly occur in the higher concentrations of wastewater. Aeration is accomplished by bubbling air through a pipet at a rate of 100 bubbles/min. If aeration is necessary, all test solutions must be aerated. It is advisable to monitor the DO closely during the first few hours of the test. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8 h after the test is started. Unless aeration is initiated during the first 8 h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test.

8.8.5 At a minimum, pH, conductivity, and total residual chlorine are measured in the undiluted effluent or receiving water, and pH and conductivity are measured in the dilution water.

8.8.5.1 It is recommended that total alkalinity and total hardness also be measured in the undiluted effluent test water, receiving water, and the dilution water.

8.8.6 Total ammonia is measured in effluent and receiving water samples where toxicity may be contributed by un-ionized ammonia (i.e., where total ammonia ≥ 5 mg/L). The concentration (mg/L) of un-ionized (free) ammonia in a sample is a function of temperature and pH, and is calculated using the percentage value obtained from Table 5, under the appropriate pH and temperature, and multiplying it by the concentration (mg/L) of total ammonia in the sample.

8.8.7 Effluents and receiving waters can be dechlorinated using 6.7 mg/L anhydrous sodium thiosulfate to reduce 1 mg/L chlorine (APHA, 1992). Note that the amount of thiosulfate required to dechlorinate effluents is greater than the amount needed to dechlorinate tap water (see Section 7, Dilution Water, Subsection 7.4.1). Since thiosulfate may contribute to sample toxicity, a thiosulfate control should be used in the test in addition to the normal dilution water control.

8.8.8 Mortality or impairment of growth or reproduction due to pH alone may occur if the pH of the sample falls outside the range of 6.0 - 9.0. Thus, the presence of other forms of toxicity (metals and organics) in the sample may be masked by the toxic effects of low or high pH. The question about the presence of other toxicants can be answered only by performing two parallel tests, one with an adjusted pH, and one without an adjusted pH. Freshwater samples are adjusted to pH 7.0 by adding 1N NaOH or 1N HCl dropwise, as required, being careful to avoid overadjustment.

TABLE 5. PERCENT UNIONIZED NH_3 IN AQUEOUS AMMONIA SOLUTIONS: TEMPERATURES 15-26°C AND pH 6.0-8.9[1]

pH	TEMPERATURE (°C)											
	15	16	17	18	19	20	21	22	23	24	25	26
6.0	0.0274	0.0295	0.0318	0.0343	0.0369	0.0397	0.0427	0.0459	0.0493	0.0530	0.0568	0.0610
6.1	0.0345	0.0372	0.0400	0.0431	0.0464	0.0500	0.0537	0.0578	0.0621	0.0667	0.0716	0.0768
6.2	0.0434	0.0468	0.0504	0.0543	0.0584	0.0629	0.0676	0.0727	0.0781	0.0901	0.0901	0.0966
6.3	0.0546	0.0589	0.0634	0.0683	0.0736	0.0792	0.0851	0.0915	0.0983	0.1134	0.1134	0.1216
6.4	0.0687	0.0741	0.0799	0.0860	0.0926	0.0996	0.107	0.115	0.124	0.133	0.143	0.153
6.5	0.0865	0.0933	0.1005	0.1083	0.1166	0.1254	0.135	0.145	0.156	0.167	0.180	0.193
6.6	0.109	0.117	0.127	0.136	0.147	0.158	0.170	0.182	0.196	0.210	0.226	0.242
6.7	0.137	0.148	0.159	0.171	0.185	0.199	0.214	0.230	0.247	0.265	0.284	0.305
6.8	0.172	0.186	0.200	0.216	0.232	0.250	0.269	0.289	0.310	0.333	0.358	0.384
6.9	0.217	0.234	0.252	0.271	0.292	0.314	0.338	0.363	0.390	0.419	0.450	0.482
7.0	0.273	0.294	0.317	0.342	0.368	0.396	0.425	0.457	0.491	0.527	0.566	0.607
7.1	0.343	0.370	0.399	0.430	0.462	0.497	0.535	0.575	0.617	0.663	0.711	0.762
7.2	0.432	0.466	0.502	0.540	0.581	0.625	0.672	0.722	0.776	0.833	0.893	0.958
7.3	0.543	0.586	0.631	0.679	0.731	0.786	0.845	0.908	0.975	1.05	1.12	1.20
7.4	0.683	0.736	0.793	0.854	0.918	0.988	1.061	1.140	1.224	1.31	1.41	1.51
7.5	0.858	0.925	0.996	1.07	1.15	1.24	1.33	1.43	1.54	1.65	1.77	1.89
7.6	1.08	1.16	1.25	1.35	1.45	1.56	1.67	1.80	1.93	2.07	2.21	2.37
7.7	1.35	1.46	1.57	1.69	1.82	1.95	2.10	2.25	2.41	2.59	2.77	2.97
7.8	1.70	1.83	1.97	2.12	2.28	2.44	2.62	2.82	3.02	3.24	3.46	3.71
7.9	2.13	2.29	2.46	2.65	2.85	3.06	3.28	3.52	3.77	4.04	4.32	4.62
8.0	2.66	2.87	3.08	3.31	3.56	3.82	4.10	4.39	4.70	5.03	5.38	5.75
8.1	3.33	3.58	3.85	4.14	4.44	4.76	5.10	5.46	5.85	6.25	6.68	7.14
8.2	4.16	4.47	4.80	5.15	5.52	5.92	6.34	6.78	7.25	7.75	8.27	8.82
8.3	5.18	5.56	5.97	6.40	6.86	7.34	7.85	8.39	8.96	9.56	10.2	10.9
8.4	6.43	6.90	7.40	7.93	8.48	9.07	9.69	10.3	11.0	11.7	12.5	13.3
8.5	7.97	8.54	9.14	9.78	10.45	11.16	11.90	12.7	13.5	14.4	15.2	16.2
8.6	9.83	10.5	11.2	12.0	12.8	13.6	14.5	15.5	16.4	17.4	18.5	19.5
8.7	12.07	12.9	13.8	14.7	15.6	16.6	17.6	18.7	19.8	21.0	22.2	23.4
8.8	14.7	15.7	16.7	17.8	18.9	20.0	21.2	22.5	23.7	25.1	26.4	27.8
8.9	17.9	19.0	20.2	21.4	22.7	24.0	25.3	26.7	28.2	29.6	31.1	32.6

[1] Table provided by Teresa Norberg-King, ERL, Duluth, Minnesota. Also see Emerson et al. (1975), Thurston et al. (1974), and USEPA (1985a).

8.9 PRELIMINARY TOXICITY RANGE-FINDING TESTS

8.9.1 USEPA Regional and State personnel generally have observed that it is not necessary to conduct a toxicity range-finding test prior to initiating a static, chronic, definitive toxicity test. However, when preparing to perform a static test with a sample of completely unknown quality, or before initiating a flow-through test, it is advisable to conduct a preliminary toxicity range-finding test.

8.9.2 A toxicity range-finding test ordinarily consists of a down-scaled, abbreviated static acute test in which groups of five organisms are exposed to several widely-spaced sample dilutions in a logarithmic series, such as

100%, 10.0%, 1.00%, and 0.100%, and a control, for 8-24 h. **Caution**: if the sample must also be used for the full-scale definitive test, the 36-h limit on holding time (see Subsection 8.5.4) must not be exceeded before the definitive test is initiated.

8.9.3 It should be noted that the toxicity (LC50) of a sample observed in a range-finding test may be significantly different from the toxicity observed in the follow-up chronic definitive test because: (1) the definitive test is longer; and (2) the test may be performed with a sample collected at a different time, and possibly differing significantly in the level of toxicity.

8.10 MULTI-CONCENTRATION (DEFINITIVE) EFFLUENT TOXICITY TESTS

8.10.1 The tests recommended for use in determining discharge permit compliance in the NPDES program are multi-concentration, or definitive, tests which provide (1) a point estimate of effluent toxicity in terms of an IC25, IC50, or LC50, or (2) a no-observed-effect-concentration (NOEC) defined in terms of mortality, growth, reproduction, and/or teratogenicity and obtained by hypothesis testing. The tests may be static renewal or static non-renewal.

8.10.2 The tests consist of a control and a minimum of five effluent concentrations. USEPA recommends the use of a ≥0.5 dilution factor for selecting effluent test concentrations. Effluent test concentrations of 6.25%, 12.5%, 25%, 50%, and 100% are commonly used, however, test concentrations should be selected independently for each test based on the objective of the study, the expected range of toxicity, the receiving water concentration, and any available historical testing information on the effluent. USEPA (2000a) provides additional guidance on choosing appropriate test concentrations.

8.10.3 When these tests are used in determining compliance with permit limits, effluent test concentrations should be selected to bracket the receiving water concentration. This may be achieved by selecting effluent test concentrations in the following manner: (1) 100% effluent, (2) [RWC + 100]/2, (3) RWC, (4) RWC/2, and (5) RWC/4. For example, where the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

8.10.4 If acute/chronic ratios are to be determined by simultaneous acute and short-term chronic tests with a single species, using the same sample, both types of tests must use the same test conditions, i.e., pH, temperature, water hardness, salinity, etc.

8.11 RECEIVING WATER TESTS

8.11.1 Receiving water toxicity tests generally consist of 100% receiving water and a control. The total hardness of the control should be comparable to the receiving water.

8.11.2 The data from the two treatments are analyzed by hypothesis testing to determine if test organism survival in the receiving water differs significantly from the control. Four replicates and 10 organisms per replicate are required for each treatment (see Summary of Test Conditions and Test Acceptability Criteria in the specific test method).

8.11.3 In cases where the objective of the test is to estimate the degree of toxicity of the receiving water, a multi-concentration test is performed by preparing dilutions of the receiving water, using a ≥ 0.5 dilution series, with a suitable control water.

SECTION 9

CHRONIC TOXICITY TEST ENDPOINTS AND DATA ANALYSIS

9.1 ENDPOINTS

9.1.1 The objective of chronic aquatic toxicity tests with effluents and pure compounds is to estimate the highest "safe" or "no-effect concentration" of these substances. For practical reasons, the responses observed in these tests are usually limited to hatchability, gross morphological abnormalities, survival, growth, and reproduction, and the results of the tests are usually expressed in terms of the highest toxicant concentration that has no statistically significant observed effect on these responses, when compared to the controls. The terms currently used to define the endpoints employed in the rapid, chronic and sub-chronic toxicity tests have been derived from the terms previously used for full life-cycle tests. As shorter chronic tests were developed, it became common practice to apply the same terminology to the endpoints. The terms used in this manual are as follows:

9.1.1.1 Safe Concentration - The highest concentration of toxicant that will permit normal propagation of fish and other aquatic life in receiving waters. The concept of a "safe concentration" is a biological concept, whereas the "no-observed-effect concentration" (below) is a statistically defined concentration.

9.1.1.2 No-Observed-Effect-Concentration (NOEC) - The highest concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle (short-term) test, that causes no observable adverse effects on the test organisms (i.e., the highest concentration of toxicant in which the values for the observed responses are not statistically significantly different from the controls). This value is used, along with other factors, to determine toxicity limits in permits.

9.1.1.3 Lowest-Observed-Effect-Concentration (LOEC) - The lowest concentration of toxicant to which organisms are exposed in a life-cycle or partial life-cycle (short-term) test, which causes adverse effects on the test organisms (i.e., where the values for the observed responses are statistically significantly different from the controls).

9.1.1.4 Effective Concentration (EC) - A point estimate of the toxicant concentration that would cause an observable adverse affect on a quantal, "all or nothing," response (such as death, immobilization, or serious incapacitation) in a given percent of the organisms, calculated by point estimation techniques. If the observable effect is death or immobility, the term, Lethal Concentration (LC), should be used (see Subsection 9.1.1.5). A certain EC or LC value might be judged from a biological standpoint to represent a threshold concentration, or lowest concentration that would cause an adverse effect on the observed response.

9.1.1.5 Lethal Concentration (LC) - The toxicant concentration that would cause death in a given percent of the test population. Identical to EC when the observed adverse effect is death. For example, the LC50 is the concentration of toxicant that would cause death in 50% of the test population.

9.1.1.6 Inhibition Concentration (IC) - The toxicant concentration that would cause a given percent reduction in a non-quantal biological measurement for the test population. For example, the IC25 is the concentration of toxicant that would cause a 25% reduction in mean young per female or in growth for the test population, and the IC50 is the concentration of toxicant that would cause a 50% reduction.

9.2 RELATIONSHIP BETWEEN ENDPOINTS DETERMINED BY HYPOTHESIS TESTING AND POINT ESTIMATION TECHNIQUES

9.2.1 If the objective of chronic aquatic toxicity tests with effluents and pure compounds is to estimate the highest "safe or no-effect concentration" of these substances, it is imperative to understand how the statistical endpoints of these tests are related to the "safe" or "no-effect" concentration. NOECs and LOECs are determined by hypothesis testing (Dunnett's Test, a t test with the Bonferroni adjustment, Steel's Many-one Rank Test, or the Wilcoxon Rank

Sum Test with the Bonferroni adjustment), whereas LCs, ICs, and ECs are determined by point estimation techniques (Probit Analysis, Spearman-Karber Method, Trimmed Spearman-Karber Method, Graphical Method or Linear Interpolation Method). There are inherent differences between the use of a NOEC or LOEC derived from hypothesis testing to estimate a "safe" concentration, and the use of a LC, EC, IC, or other point estimates derived from curve fitting, interpolation, etc.

9.2.2 Most point estimates, such as the LC, IC, or EC, are derived from a mathematical model that assumes a continuous dose-response relationship. By definition, any LC, IC, or EC value is an estimate of some amount of adverse effect. Thus the assessment of a "safe" concentration must be made from a biological standpoint rather than with a statistical test. In this instance, the biologist must determine some amount of adverse effect that is deemed to be "safe", in the sense that from a practical biological viewpoint it will not affect the normal propagation of fish and other aquatic life in receiving waters.

9.2.3 The use of NOECs and LOECs, on the other hand, assumes either (1) a continuous dose-response relationship, or (2) a non-continuous (threshold) model of the dose-response relationship.

9.2.3.1 In the case of a continuous dose-response relationship, it is also assumed that adverse effects that are not "statistically observable" are also not important from a biological standpoint, since they are not pronounced enough to test as statistically significant against some measure of the natural variability of the responses.

9.2.3.2 In the case of non-continuous dose-response relationships, it is assumed that there exists a true threshold, or concentration below which there is no adverse effect on aquatic life, and above which there is an adverse effect. The purpose of the statistical analysis in this case is to estimate as closely as possible where that threshold lies.

9.2.3.3 In either case, it is important to realize that the amount of adverse effect that is statistically observable (LOEC) or not observable (NOEC) is highly dependent on all aspects of the experimental design, such as the number of concentrations of toxicant, number of replicates per concentration, number of organisms per replicate, and use of randomization. Other factors that affect the sensitivity of the test include the choice of statistical analysis, the choice of an alpha level, and the amount of variability between responses at a given concentration.

9.2.3.4 Where the assumption of a continuous dose-response relationship is made, by definition some amount of adverse effect might be present at the NOEC, but is not great enough to be detected by hypothesis testing.

9.2.3.5 Where the assumption of a non-continuous dose-response relationship is made, the NOEC would indeed be an estimate of a "safe" or "no-effect" concentration if the amount of adverse effect that appears at the threshold is great enough to test as statistically significantly different from the controls in the face of all aspects of the experimental design mentioned above. If, however, the amount of adverse effect at the threshold were not great enough to test as statistically different, some amount of adverse effect might be present at the NOEC. In any case, the estimate of the NOEC with hypothesis testing is always dependent on the aspects of the experimental design mentioned above. For this reason, the reporting and examination of some measure of the sensitivity of the test (either the minimum significant difference or the percent change from the control that this minimum difference represents) is extremely important.

9.2.4 In summary, the assessment of a "safe" or "no-effect" concentration cannot be made from the results of statistical analysis alone, unless (1) the assumptions of a strict threshold model are accepted, and (2) it is assumed that the amount of adverse effect present at the threshold is statistically detectable by hypothesis testing. In this case, estimates obtained from a statistical analysis are indeed estimates of a "no-effect" concentration. If the assumptions are not deemed tenable, then estimates from a statistical analysis can only be used in conjunction with an assessment from a biological standpoint of what magnitude of adverse effect constitutes a "safe" concentration. In this instance, a "safe" concentration is not necessarily a truly "no-effect" concentration, but rather a concentration at which the effects are judged to be of no biological significance.

9.2.5 A better understanding of the relationship between endpoints derived by hypothesis testing (NOECs) and point estimation techniques (LCs, ICs, and ECs) would be very helpful in choosing methods of data analysis. Norberg-King (1991) reported that the IC25s were comparable to the NOECs for 23 effluent and reference toxicant data sets analyzed. The data sets included short-term chronic toxicity tests for the fathead minnow, *Pimephales promelas*, and the daphnid, *Ceriodaphnia dubia*. Birge et al. (1985) reported that LC1s derived from Probit Analysis of data from short-term embryo-larval tests with reference toxicants were comparable to NOECs for several organisms. Similarly, USEPA (1988d) reported that the IC25s were comparable to the NOECs for a set of daphnid, *Ceriodaphnia dubia*, chronic tests with a single reference toxicant. However, the scope of these comparisons was very limited, and sufficient information is not yet available to establish an overall relationship between these two types of endpoints, especially when derived from effluent toxicity test data.

9.3 **PRECISION**

9.3.1 HYPOTHESIS TESTS

9.3.1.1 When hypothesis tests are used to analyze toxicity test data, it is not possible to express precision in terms of a commonly used statistic. The results of the test are given in terms of two endpoints, the No-Observed- Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC). The NOEC and LOEC are limited to the concentrations selected for the test. The width of the NOEC-LOEC interval is a function of the dilution series, and differs greatly depending on whether a dilution factor of 0.3 or 0.5 is used in the test design. Therefore, **USEPA recommends the use of the ≥ 0.5 dilution factor** (see Section 4, Quality Assurance). It is not possible to place confidence limits on the NOEC and LOEC derived from a given test, and it is difficult to quantify the precision of the NOEC-LOEC endpoints between tests. If the data from a series of tests performed with the same toxicant, toxicant concentrations, and test species, were analyzed with hypothesis tests, precision could only be assessed by a qualitative comparison of the NOEC-LOEC intervals, with the understanding that maximum precision would be attained if all tests yielded the same NOEC-LOEC interval. In practice, the precision of results of repetitive chronic tests is considered acceptable if the NOECs vary by no more than one concentration interval above or below a central tendency. Using these guidelines, the "normal" range of NOECs from toxicity tests using a 0.5 dilution factor (two-fold difference between adjacent concentrations), would be four-fold.

9.3.2 POINT ESTIMATION TECHNIQUES

9.3.2.1 Point estimation techniques have the advantage of providing a point estimate of the toxicant concentration causing a given amount of adverse (inhibiting) effect, the precision of which can be quantitatively assessed (1) within tests by calculation of 95% confidence limits, and (2) across tests by calculating a standard deviation and coefficient of variation.

9.3.2.2 It should be noted that software used to calculate point estimates occasionally may not provide associated 95% confidence intervals. This situation may arise when test data do not meet specific assumptions required by the statistical methods, when point estimates are outside of the test concentration range, and when specific limitations imposed by the software are encountered. USEPA (2000a) provides guidance on confidence intervals under these circumstances.

9.4 **DATA ANALYSIS**

9.4.1 ROLE OF THE STATISTICIAN

9.4.1.1 The use of the statistical methods described in this manual for routine data analysis does not require the assistance of a statistician. However, the interpretation of the results of the analysis of the data from any of the toxicity tests described in this manual can become problematic because of the inherent variability and sometimes unavoidable anomalies in biological data. If the data appear unusual in any way, or fail to meet the necessary assumptions, a statistician should be consulted. Analysts who are not proficient in statistics are strongly advised to seek the assistance of a statistician before selecting the method of analysis and using any of the results.

9.4.1.2 The statistical methods recommended in this manual are not the only possible methods of statistical analysis. Many other methods have been proposed and considered. Certainly there are other reasonable and defensible methods of statistical analysis for this kind of toxicity data. Among alternative hypothesis tests some, like Williams' Test, require additional assumptions, while others, like the bootstrap methods, require computer-intensive computations. Alternative point estimation approaches most probably would require the services of a statistician to determine the appropriateness of the model (goodness of fit), higher order linear or nonlinear models, confidence intervals for estimates generated by inverse regression, etc. In addition, point estimation or regression approaches would require the specification by biologists or toxicologists of some low level of adverse effect that would be deemed acceptable or safe. The statistical methods contained in this manual have been chosen because they are (1) applicable to most of the different toxicity test data sets for which they are recommended, (2) powerful statistical tests, (3) hopefully "easily" understood by nonstatisticians, and (4) amenable to use without a computer, if necessary.

9.4.2 PLOTTING THE DATA

9.4.2.1 The data should be plotted, both as a preliminary step to help detect problems and unsuspected trends or patterns in the responses, and as an aid in interpretation of the results. Further discussion and plotted sets of data are included in the methods and the Appendices.

9.4.3 DATA TRANSFORMATIONS

9.4.3.1 Transformations of the data, (e.g., arc sine square root and logs), are used where necessary to meet assumptions of the proposed analyses, such as the requirement for normally distributed data.

9.4.4 INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

9.4.4.1 Statistical independence among observations is a critical assumption in all statistical analysis of toxicity data. One of the best ways to insure independence is to properly follow rigorous randomization procedures. Randomization techniques should be employed at the start of the test, including the randomization of the placement of test organisms in the test chambers and randomization of the test chamber location within the array of chambers. Discussions of statistical independence, outliers and randomization, and a sample randomization scheme, are included in Appendix A.

9.4.5 REPLICATION AND SENSITIVITY

9.4.5.1 The number of replicates employed for each toxicant concentration is an important factor in determining the sensitivity of chronic toxicity tests. Test sensitivity generally increases as the number of replicates is increased, but the point of diminishing returns in sensitivity may be reached rather quickly. The level of sensitivity required by a hypothesis test or the confidence interval for a point estimate will determine the number of replicates, and should be based on the objectives for obtaining the toxicity data.

9.4.5.2 In a statistical analysis of toxicity data, the choice of a particular analysis and the ability to detect departures from the assumptions of the analysis, such as the normal distribution of the data and homogeneity of variance, is also dependent on the number of replicates. More than the minimum number of replicates may be required in situations where it is imperative to obtain optimal statistical results, such as with tests used in enforcement cases or when it is not possible to repeat the tests. For example, when the data are analyzed by hypothesis testing, the nonparametric alternatives cannot be used unless there are at least four replicates at each toxicant concentration.

9.4.6 RECOMMENDED ALPHA LEVELS

9.4.6.1 The data analysis examples included in the manual specify an alpha level of 0.01 for testing the assumptions of hypothesis tests and an alpha level of 0.05 for the hypothesis tests themselves. These levels are

common and well accepted levels for this type of analysis and are presented as a recommended minimum significance level for toxicity test data analysis.

9.5 CHOICE OF ANALYSIS

9.5.1 The recommended statistical analysis of most data from chronic toxicity tests with aquatic organisms follows a decision process illustrated in the flowchart in Figure 2. An initial decision is made to use point estimation techniques (the Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, the Graphical Method, or Linear Interpolation Method) and/or to use hypothesis testing (Dunnett's Test, the t test with the Bonferroni adjustment, Steel's Many-one Rank Test, or the Wilcoxon Rank Sum Test with the Bonferroni adjustment). **NOTE: For the NPDES Permit Program, the point estimation techniques are the preferred statistical methods in calculating end points for effluent toxicity tests.** If hypothesis testing is chosen, subsequent decisions are made on the appropriate procedure for a given set of data, depending on the results of the tests of assumptions, as illustrated in the flowchart. A specific flow chart is included in the analysis section for each test.

9.5.2 Since a single chronic toxicity test might yield information on more than one parameter (such as survival, growth, and reproduction), the lowest estimate of a "no-observed-effect concentration" for any of the responses would be used as the "no-observed-effect concentration" for each test. It follows logically that in the statistical analysis of the data, concentrations that had a significant toxic effect on one of the observed responses would not be subsequently tested for an effect on some other response. This is one reason for excluding concentrations that have shown a statistically significant reduction in survival from a subsequent hypothesis test for effects on another parameter such as reproduction. A second reason is that the exclusion of such concentrations usually results in a more powerful and appropriate statistical analysis. In performing the point estimation techniques recommended in this manual, an all-data approach is used. For example, data from concentrations above the NOEC for survival are included in determining ICp estimates using the Linear Interpolation Method.

9.5.3 ANALYSIS OF GROWTH AND REPRODUCTION DATA

9.5.3.1 Growth data from the fathead minnow, *Pimephales promelas*, larval survival and growth test are analyzed using hypothesis testing or point estimation techniques according to the flowchart in Figure 2. The above mentioned growth data may also be analyzed by generating a point estimate with the Linear Interpolation Method. Data from effluent concentrations that have tested significantly different from the control for survival are excluded from further hypothesis tests concerning growth effects. Growth is defined as the dry weight per original number of test organisms when group weights are obtained. When analyzing the data using point estimation techniques, data from all concentrations are included in the analysis.

9.5.3.2 Reproduction data from the daphnid, *Ceriodaphnia dubia*, survival and reproduction test are analyzed using hypothesis testing or point estimation techniques according to the flowchart in Figure 2. In hypothesis testing, data from effluent concentrations that have significantly lower survival than the control, as determined by Fisher's Exact test, are not included in the hypothesis tests for reproductive effects. Data from all concentrations are included when using point estimation techniques.

9.5.4 ANALYSIS OF ALGAL GROWTH RESPONSE DATA

9.5.4.1 The growth response data from the green alga, *Selenastrum capricornutum*, toxicity test, after an appropriate transformation, if necessary, to meet the assumptions of normality and homogeneity of variance, may be analyzed by hypothesis testing according to the flowchart in Figure 2. Point estimates, such as the IC25 and IC50, would also be appropriate in analyzing algal growth data.

9.5.5 ANALYSIS OF MORTALITY DATA

9.5.5.1 Mortality data are analyzed by Probit Analysis, if appropriate, or other point estimation techniques (i.e., the Spearman-Karber Method, the Trimmed Spearman-Karber Method, or the Graphical Method) (see Appendices I-L and the discussion below). The mortality data can also be analyzed by hypothesis testing, after an arc sine square root transformation (see Appendix B-F), according to the flowchart in Figure 2.

9.5.5.2 Mortality data from the daphnid, *Ceriodaphnia dubia*, survival and reproduction test are analyzed by Fisher's Exact Test (Appendix G) prior to the analysis of the reproduction data. The mortality data may also be analyzed by Probit Analysis, if appropriate or other methods (see Subsection 9.5.5.1).

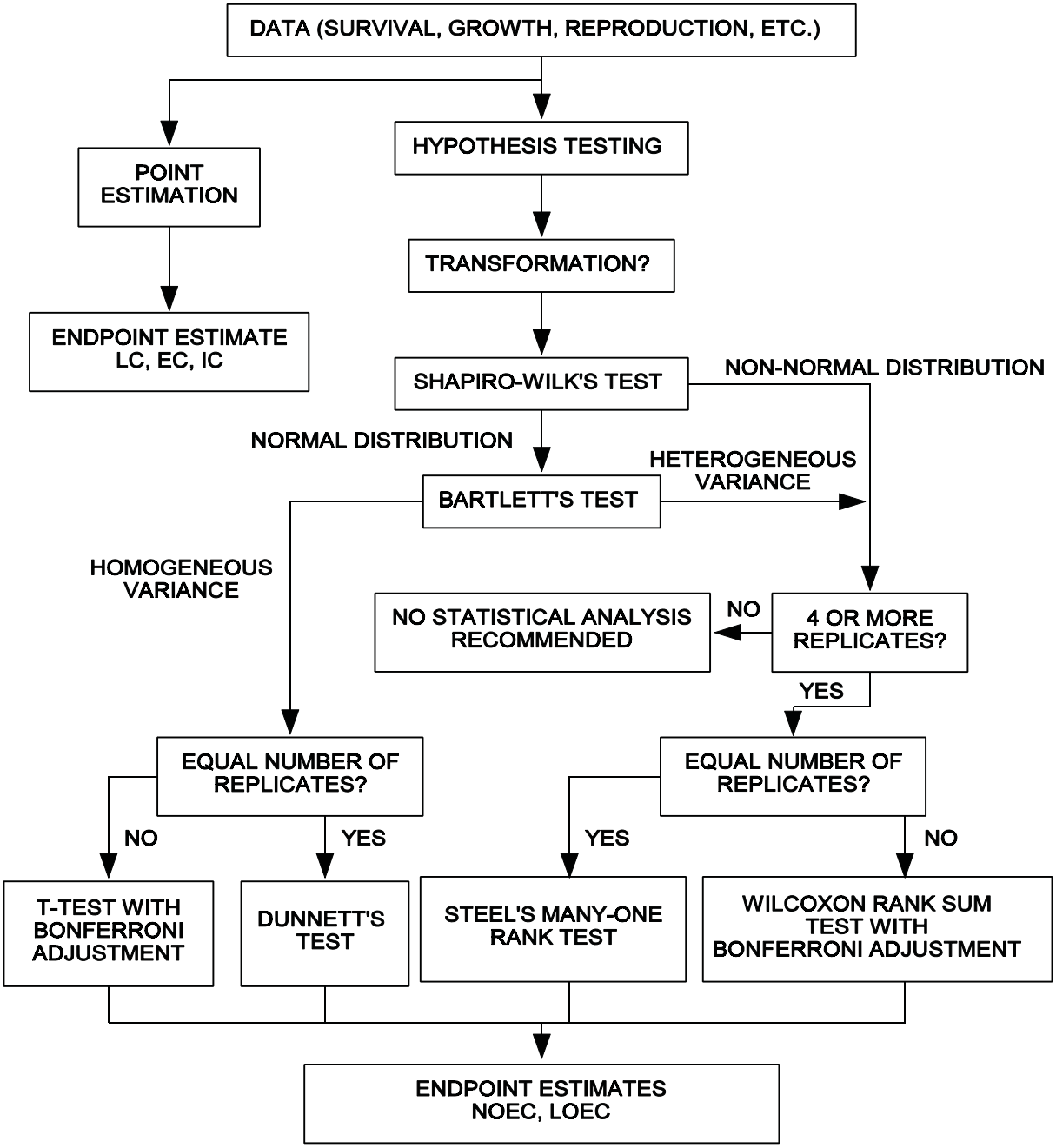

Figure 2. Flowchart for statistical analysis of test data

9.6 **HYPOTHESIS TESTS**

9.6.1 DUNNETT'S PROCEDURE

9.6.1.1 Dunnett's Procedure is used to determine the NOEC. The procedure consists of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison procedure for comparing each of the treatment means with the control mean, in a series of paired tests (see Appendix C). Use of Dunnett's Procedure requires at least three replicates per treatment to check the assumptions of the test. In cases where the numbers of data points (replicates) for each concentration are not equal, a t test may be performed with Bonferroni's adjustment for multiple comparisons (see Appendix D), instead of using Dunnett's Procedure.

9.6.1.2 The assumptions upon which the use of Dunnett's Procedure is contingent are that the observations within treatments are normally distributed, with homogeneity of variance. Before analyzing the data, these assumptions must be tested using the procedures provided in Appendix B.

9.6.1.3 If, after suitable transformations have been carried out, the normality assumptions have not been met, Steel's Many-one Rank Test should be used if there are four or more data points (replicates) per toxicant concentration. If the numbers of data points for each toxicant concentration are not equal, the Wilcoxon Rank Sum Test with Bonferroni's adjustment should be used (see Appendix F).

9.6.1.4 Some indication of the sensitivity of the analysis should be provided by calculating (1) the minimum difference between means that can be detected as statistically significant, and (2) the percent change from the control mean that this minimum difference represents for a given test.

9.6.1.5 A step-by-step example of the use of Dunnett's Procedure is provided in Appendix C.

9.6.2 T TEST WITH THE BONFERRONI ADJUSTMENT

9.6.2.1 A t test with Bonferroni's adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus Dunnett's Procedure is a more powerful test.

9.6.2.2 The assumptions upon which the use of the t test with Bonferroni's adjustment is contingent are that the observations within treatments are normally distributed, with homogeneity of variance. These assumptions must be tested using the procedures provided in Appendix B.

9.6.2.3 The estimate of the safe concentration derived from this test is reported in terms of the NOEC. A step-by-step example of the use of the t test with Bonferroni's adjustment is provided in Appendix D.

9.6.3 STEEL'S MANY-ONE RANK TEST

9.6.3.1 Steel's Many-one Rank Test is a multiple comparison procedure for comparing several treatments with a control. This method is similar to Dunnett's Procedure, except that it is not necessary to meet the assumption of normality. The data are ranked, and the analysis is performed on the ranks rather than on the data themselves. If the data are normally or nearly normally distributed, Dunnett's Procedure would be more sensitive (would detect smaller differences between the treatments and control). For data that are not normally distributed, Steel's Many-one Rank Test can be much more efficient (Hodges and Lehmann, 1956).

9.6.3.2 It is necessary to have at least four replicates per toxicant concentration to use Steel's test. Unlike Dunnett's procedure, the sensitivity of this test cannot be stated in terms of the minimum difference between treatment means and the control mean that can be detected as statistically significant.

9.6.3.3 The estimate of the safe concentration is reported as the NOEC. A step-by-step example of the use of Steel's Many-one Rank Test is provided in Appendix E.

9.6.4 WILCOXON RANK SUM TEST WITH THE BONFERRONI ADJUSTMENT

9.6.4.1 The Wilcoxon Rank Sum Test with the Bonferroni Adjustment is a nonparametric test for comparing treatments with a control. The data are ranked and the analysis proceeds exactly as in Steel's Test except that Bonferroni's adjustment for multiple comparisons is used instead of Steel's tables. When Steel's test can be used (i.e., when there are equal numbers of data points per toxicant concentration), it will be more powerful (able to detect smaller differences as statistically significant) than the Wilcoxon Rank Sum Test with Bonferroni's adjustment.

9.6.4.2 The estimate of the safe concentration is reported as the NOEC. A step-by-step example of the use of the Wilcoxon Rank Sum Test with Bonferroni Adjustment is provided in Appendix F.

9.6.5 A CAUTION IN THE USE OF HYPOTHESIS TESTING

9.6.5.1 If in the calculation of an NOEC by hypothesis testing, two tested concentrations cause statistically significant adverse effects, but an intermediate concentration did not cause statistically significant effects, the results should be used with extreme caution.

9.7 **POINT ESTIMATION TECHNIQUES**

9.7.1 PROBIT ANALYSIS

9.7.1.1 Probit Analysis is used to estimate the LC1, LC50, EC1, or EC50 and the associated 95% confidence interval. The analysis consists of adjusting the data for mortality in the control, and then using a maximum likelihood technique to estimate the parameters of the underlying log tolerance distribution, which is assumed to have a particular shape.

9.7.1.2 The assumption upon which the use of Probit Analysis is contingent is a normal distribution of log tolerances. If the normality assumption is not met, and at least two partial mortalities are not obtained, Probit Analysis should not be used. It is important to check the results of Probit Analysis to determine if use of the analysis is appropriate. The chi-square test for heterogeneity provides one good test of appropriateness of the analysis. The computer program (see Appendix I) checks the chi-square statistic calculated for the data set against the tabular value, and provides an error message if the calculated value exceeds the tabular value.

9.7.1.3 A discussion of Probit Analysis, and examples of computer program input and output, are found in Appendix I.

9.7.1.4 In cases where Probit Analysis is not appropriate, the LC50 and associated confidence interval may be estimated by the Spearman-Karber Method (Appendix J) or the Trimmed Spearman-Karber Method (Appendix K). If the test results in 100% survival and 100% mortality in adjacent treatments (all or nothing effect), the LC50 may be estimated using the Graphical Method (Appendix L).

9.7.2 LINEAR INTERPOLATION METHOD

9.7.2.1 The Linear Interpolation Method (see Appendix M) is a procedure to calculate a point estimate of the effluent or other toxicant concentration [Inhibition Concentration, (IC)] that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms. The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests.

9.7.2.2 Use of the Linear Interpolation Method is based on the assumptions that the responses (1) are monotonically non-increasing (the mean response for each higher concentration is less than or equal to the mean response for the previous concentration), (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. The assumption for piecewise linear response cannot be tested statistically, and no defined statistical procedure is provided to test the assumption for monotonicity. Where the observed means are not strictly monotonic by examination, they are adjusted by smoothing. In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean.

9.7.2.3 The inability to test the monotonicity and piecewise linear response assumptions for this method makes it difficult to assess when the method is, or is not, producing reliable results. Therefore, the method should be used with caution when the results of a toxicity test approach an "all or nothing" response from one concentration to the next in the concentration series, and when it appears that there is a large deviation from monotonicity. See Appendix M for a more detailed discussion of the use of this method and a computer program available for performing calculations.

SECTION 10

REPORT PREPARATION AND TEST REVIEW

10.1 REPORT PREPARATION

The following general format and content are recommended for the report:

10.1.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contract Laboratory (if the tests are performed under contract)
   a Name of firm
   b. Phone number
   c. Address
6. Objective of test

10.1.2 PLANT OPERATIONS

1. Product(s)
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Volume of waste flow (MGD, CFS, GPM)
8. Design flow of treatment facility at time of sampling

10.1.3 SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER

1. Effluent Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Mean daily discharge on sample collection date
   f. Lapsed time from sample collection to delivery
   g. Sample temperature when received at the laboratory

2. Receiving Water Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Streamflow (at time of sampling)
   f. Sample temperature when received at the laboratory
   g Lapsed time from sample collection to delivery

3. Dilution Water Samples
   a. Source

b. Collection date(s) and time(s)
c. Pretreatment
d. Physical and chemical characteristics

10.1.4 TEST METHODS

1. Toxicity test method used (title, number, source)
2. Endpoint(s) of test
3. Deviation(s) from reference method, if any, and the reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type and volume of test chambers
7. Volume of solution used per chamber
8. Number of organisms per test chamber
9. Number of replicate test chambers per treatment
10. Acclimation of test organisms (temperature mean and range)
11. Test temperature (mean and range)
12. Specify if aeration was needed
13. Feeding frequency, and amount and type of food
14. Specify if (and how) pH control measures were implemented

10.1.5 TEST ORGANISMS

1. Scientific name and how determined
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)
7. Taxonomic key used for species identification

10.1.6 QUALITY ASSURANCE

1. Reference toxicant used routinely; source
2. Date and time of most recent reference toxicant test, test results, and current control chart
3. Dilution water used in reference toxicant test
4. Results (NOEC or, where applicable, LOEC, LC50, EC50, IC25 and/or IC50); report percent minimum significant difference (PMSD) calculated for sublethal endpoints determined by hypothesis testing in reference toxicant test
5. Physical and chemical methods used

10.1.7 RESULTS

1. Provide raw toxicity data in tabular form, including daily records of affected organisms in each concentration (including controls) and replicate, and in graphical form (plots of toxicity data)
2. Provide table of LC50s, NOECs, IC25, IC50, etc. (as required in the applicable NPDES permit)
3. Indicate statistical methods used to calculate endpoints
4. Provide summary table of physical and chemical data
5. Tabulate QA data
6. Provide percent minimum significant difference (PMSD) calculated for sublethal endpoints

10.1.8 CONCLUSIONS AND RECOMMENDATIONS

1. Relationship between test endpoints and permit limits
2. Actions to be taken

10.2 TEST REVIEW

10.2.1 Test review is an important part of an overall quality assurance program (Section 4) and is necessary for ensuring that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the regulatory authority.

10.2.2 SAMPLING AND HANDLING

10.2.2.1 The collection and handling of samples are reviewed to verify that the sampling and handling procedures given in Section 8 were followed. Chain-of-custody forms are reviewed to verify that samples were tested within allowable sample holding times (Subsection 8.5.4). Any deviations from the procedures given in Section 8 should be documented and described in the data report (Subsection 10.1).

10.2.3 TEST ACCEPTABILITY CRITERIA

10.2.3.1 Test data are reviewed to verify that test acceptability criteria (TAC) requirements for a valid test have been met. Any test not meeting the minimum test acceptability criteria is considered invalid. All invalid tests must be repeated with a newly collected sample.

10.2.4 TEST CONDITIONS

10.2.4.1 Test conditions are reviewed and compared to the specifications listed in the summary of test condition tables provided for each method. Physical and chemical measurements taken during the test (e.g., temperature, pH, and DO) also are reviewed and compared to specified ranges. Any deviations from specifications should be documented and described in the data report (Subsection 10.1).

10.2.4.2 The summary of test condition tables presented for each method identify test conditions as required or recommended. For WET test data submitted under NPDES permits, all required test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample. Deviations from recommended test conditions must be evaluated on a case-by-case basis to determine the validity of test results. Deviations from recommended test conditions may or may not invalidate a test result depending on the degree of the departure and the objective of the test. The reviewer should consider the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or accepting a test result as valid. For example, if dissolved oxygen is measured below 4.0 mg/L in one test chamber, the reviewer should consider whether any observed mortality in that test chamber corresponded with the drop in dissolved oxygen.

10.2.4.3 Whereas slight deviations in test conditions may not invalidate an individual test result, test condition deviations that continue to occur frequently in a given laboratory may indicate the need for improved quality control in that laboratory.

10.2.5 STATISTICAL METHODS

10.2.5.1 The statistical methods used for analyzing test data are reviewed to verify that the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report. Statistical methods other than those recommended in the statistical flowcharts may be appropriate (see Subsection 9.4.1.2), however, the laboratory must document the use of and provide the rationale for the use of any alternate statistical method. In all cases (flowchart recommended

methods or alternate methods), reviewers should verify that the necessary assumptions are met for the statistical method used.

10.2.6 CONCENTRATION-RESPONSE RELATIONSHIPS

10.2.6.1 The concept of a concentration-response, or more classically, a dose-response relationship is "the most fundamental and pervasive one in toxicology" (Casarett and Doull, 1975). This concept assumes that there is a causal relationship between the dose of a toxicant (or concentration for toxicants in solution) and a measured response. A response may be any measurable biochemical or biological parameter that is correlated with exposure to the toxicant. The classical concentration-response relationship is depicted as a sigmoidal shaped curve, however, the particular shape of the concentration-response curve may differ for each coupled toxicant and response pair. In general, more severe responses (such as acute effects) occur at higher concentrations of the toxicant, and less severe responses (such as chronic effects) occur at lower concentrations. A single toxicant also may produce multiple responses, each characterized by a concentration-response relationship. A corollary of the concentration-response concept is that every toxicant should exhibit a concentration-response relationship, given that the appropriate response is measured and given that the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent possesses toxicity and in identifying anomalous test results.

10.2.6.2 The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. USEPA (2000a) provides guidance on evaluating concentration-response relationships to assist in determining the validity of WET test results. All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to USEPA guidance on the evaluation of concentration-response relationships (USEPA, 2000a). This guidance provides review steps for 10 different concentration-response patterns that may be encountered in WET test data. Based on the review, the guidance provides one of three determinations: that calculated effect concentrations are reliable and should be reported, that calculated effect concentrations are anomalous and should be explained, or that the test was inconclusive and the test should be repeated with a newly collected sample. It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Data from some tests may suggest consultation with professional toxicologists and/or regulatory officials. Tests that exhibit unexpected concentration-response relationships also may indicate a need for further investigation and possible retesting.

10.2.7 REFERENCE TOXICANT TESTING

10.2.7.1 Test review of a given effluent or receiving water test should include review of the associated reference toxicant test and current control chart. Reference toxicant testing and control charting is required for documenting the quality of test organisms (Subsection 4.7) and ongoing laboratory performance (Subsection 4.16). The reviewer should verify that a quality control reference toxicant test was conducted according to the specified frequency required by the permitting authority or recommended by the method (e.g., monthly). The test acceptability criteria, test conditions, concentration-response relationship, and test sensitivity of the reference toxicant test are reviewed to verify that the reference toxicant test conducted was a valid test. The results of the reference toxicant test are then plotted on a control chart (see Subsection 4.16) and compared to the current control chart limits (± 2 standard deviations).

10.2.7.2 Reference toxicant tests that fall outside of recommended control chart limits are evaluated to determine the validity of associated effluent and receiving water tests (see Subsection 4.16). An out of control reference toxicant test result does not necessarily invalidate associated test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increasing test organism sensitivity or toward decreasing test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test. More frequent and/or concurrent reference toxicant testing may be advantageous if recent problems (e.g., invalid tests, reference toxicant test results outside of control chart limits, reduced health of organism cultures, or increased within-test variability) have been identified in testing.

10.2.8 TEST VARIABILITY

10.2.8.1 The within-test variability of individual tests should be reviewed. Excessive within-test variability may invalidate a test result and warrant retesting. For evaluating within-test variability, reviewers should consult EPA guidance on upper and lower percent minimum significant difference (PMSD) bounds (USEPA, 2000b).

10.2.8.2 When NPDES permits require sublethal hypothesis testing endpoints from Methods 1000.0,1002.0, or 1003.0 (e.g., growth or reproduction NOECs and LOECs), within-test variability must be reviewed and variability criteria must be applied as described in this section (10.2.8.2). When the methods are used for non-regulatory purposes, the variability criteria herein are recommended but are not required, and their use (or the use of alternative variability criteria) may depend upon the intended uses of the test results and the requirements of any applicable data quality objectives and quality assurance plan.

10.2.8.2.1 To measure test variability, calculate the percent minimum significant difference (PMSD) achieved in the test. The PMSD is the smallest percentage decrease in growth or reproduction from the control that could be determined as statistically significant in the test. The PMSD is calculated as 100 times the minimum significant difference (MSD) divided by the control mean. The equation and examples of MSD calculations are shown in Appendix C. PMSD may be calculated legitimately as a descriptive statistic for within-test variability, even when the hypothesis test is conducted using a non-parametric method. The PMSD bounds were based on a representative set of tests, including tests for which a non-parametric method was required for determining the NOEC or LOEC. The conduct of hypothesis testing to determine test results should follow the statistical flow charts provided for each method. That is, when test data fail to meet assumptions of normality or heterogeneity of variance, a non-parametric method (determined following the statistical flowchart for the method) should be used to calculate test results, but the PMSD may be calculated as described above (using parametric methods) to provide a measure of test variability.

10.2.8.2.2 Compare the PMSD measured in the test with the upper PMSD bound variability criterion listed in Table 6. When the test PMSD exceeds the upper bound, the variability among replicates is unusually large for the test method. Such a test should be considered insufficiently sensitive to detect toxic effects on growth or reproduction of substantial magnitude. A finding of toxicity at a particular concentration may be regarded as trustworthy, but a finding of "no toxicity" or "no statistically significant toxicity" at a particular concentration should not be regarded as a reliable indication that there is no substantial toxic effect on growth or reproduction at that concentration.

10.2.8.2.3 If the PMSD measured for the test is less than or equal to the upper PMSD bound variability criterion in Table 6, then the test's variability measure lies within normal bounds and the effect concentration estimate (e.g., NOEC or LOEC) would normally be accepted unless other test review steps raise serious doubts about its validity.

10.2.8.2.4 If the PMSD measured for the test exceeds the upper PMSD bound variability criterion in Table 6, then one of the following two cases applies (10.2.8.2.4.1, 10.2.8.2.4.2).

10.2.8.2.4.1 If toxicity is found at the permitted receiving water concentration (RWC) based upon the value of the effect concentration estimate (NOEC or LOEC), then the test shall be accepted and the effect concentration estimate may be reported, unless other test review steps raise serious doubts about its validity.

10.2.8.2.4.2 If toxicity is not found at the permitted RWC based upon the value of the effect concentration estimate (NOEC or LOEC) and the PMSD measured for the test exceeds the upper PMSD bound, then the test shall not be accepted, and a new test must be conducted promptly on a newly collected sample.

10.2.8.2.5 To avoid penalizing laboratories that achieve unusually high precision, lower PMSD bounds shall also be applied when a hypothesis test result (e.g., NOEC or LOEC) is reported. Lower PMSD bounds, which are based on the 10^{th} percentiles of national PMSD data, are presented in Table 6. The 10^{th} percentile PMSD represents a practical limit to the sensitivity of the test method because few laboratories are able to achieve such precision on a

regular basis and most do not achieve it even occasionally. In determining hypothesis test results (e.g., NOEC or LOEC), a test concentration shall not be considered toxic (i.e., significantly different from the control) if the relative difference from the control is less than the lower PMSD bounds in Table 6. See USEPA, 2000b for specific examples of implementing lower PMSD bounds.

10.2.8.3 To assist in reviewing within-test variability, EPA recommends maintaining control charts of PMSDs calculated for successive effluent tests (USEPA, 2000b). A control chart of PMSD values characterizes the range of variability observed within a given laboratory, and allows comparison of individual test PMSDs with the laboratory's typical range of variability. Control charts of other variability and test performance measures, such as the MSD, standard deviation or CV of control responses, or average control response, also may be useful for reviewing tests and minimizing variability. The log of PMSD will provide an approximately normal variate useful for control charting.

TABLE 6. VARIABILITY CRITERIA (UPPER AND LOWER PMSD BOUNDS) FOR SUBLETHAL HYPOTHESIS TESTING ENDPOINTS SUBMITTED UNDER NPDES PERMITS.[1]

Test Method	Endpoint	Lower PMSD Bound	Upper PMSD Bound
Method 1000.0, Fathead Minnow Larval Survival and Growth Test	growth	12	30
Method 1002.0, *Ceriodaphnia dubia* Survival and Reproduction Test	reproduction	13	47
Method 1003.0, *Selenastrum capricornutum* Growth Test	growth	9.1	29

[1] Lower and upper PMSD bounds were determined from the 10th and 90th percentile, respectively, of PMSD data from EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

SECTION 11

TEST METHOD

FATHEAD MINNOW, *PIMEPHALES PROMELAS,* LARVAL SURVIVAL AND GROWTH TEST METHOD 1000.0

11.1 SCOPE AND APPLICATION

11.1.1 This method estimates the chronic toxicity of effluents and receiving water to the fathead minnow, *Pimephales promelas*, using newly hatched larvae in a seven-day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

11.1.2 Daily observations on mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

11.1.3 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

11.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly degradable or highly volatile toxicants present in the source may not be detected in the test.

11.1.5 This test method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

11.2 SUMMARY OF METHOD

11.2.1 Fathead minnow, *Pimephales promelas*, larvae are exposed in a static renewal system for seven days to different concentrations of effluent or to receiving water. Test results are based on the survival and weight of the larvae.

11.3. INTERFERENCES

11.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment and Supplies).

11.3.2 Adverse effects of low dissolved oxygen (DO) concentrations, high concentrations of suspended and/or dissolved solids, and extremes of pH, alkalinity, or hardness, may mask the presence of toxic substances.

11.3.3 Improper effluent sampling and sample handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.3.4 Pathogenic and/or predatory organisms in effluent samples or receiving water that is used for dilution may affect test organism survival and confound test results. When pathogen interference is suggested by observation (11.3.4.1) and data evaluations (11.3.4.2) and confirmed by parallel testing (11.3.4.4), steps should be taken to minimize pathogen interference to the extent that test results are not confounded by mortality due to pathogens. Pathogen control techniques that do not require modification of effluent samples, such as use of the modified test design described in Subsection 11.3.4.5, are recommended for controlling pathogen interference. Upon approval by the regulatory authority, analysts also may use additional pathogen control techniques that require sample modification (11.3.4.6) provided that parallel testing of altered and unaltered samples further confirms the presence

of pathogen interference and demonstrates successful pathogen control (11.3.4.6).

11.3.4.1 A typical indication that pathogen interference has occurred in a WET test is when test organisms exhibit "sporadic mortality". This sporadic morality phenomenon is characterized by an unexpected concentration-response relationship (i.e., effects that do not increase with increasing effluent concentration) and organism survival that varies greatly among replicates and among effluent dilutions (USEPA, 2000a). The observed sporadic mortality among replicates may occur in receiving water controls, lower effluent concentrations, and occasionally in full-strength effluent on day 3 or day 4 of the chronic test. When sporadic mortality occurs, a fungal growth may appear directly on the fish, especially in the gill area. The fungus has not been definitively identified, but the fungal growth appears to be compatible with *Saprolegnea sp.* (Downey *et al.*, 2000). Microbiological evaluations on receiving waters, the fish, and the food indicated the ubiquitous nature of pathogenic organisms (e.g., *Flexibacter spp*., *Aeromonas hydrophila*), and eradicating them from the test through the decontamination of the fish and their food has not been practical (Geis *et al.*, 2000).

11.3.4.2 When pathogen interference is suspected, a series of data evaluations are required. The test data must be reviewed to determine a cause for any unexpected concentration-response pattern and subsequently to determine the validity of calculated results (USEPA, 2000a). USEPA (2000a) provides guidance on reviewing concentration-response relationships including specific response patterns that may indicate pathogen effects. Each treatment (including the control) should be evaluated for an unusually high mortality response and unevenness of mortalities among replicates. Within-treatment coefficient of variation (CVs) for survival of >40% in effluent or receiving water treatments but relatively small for control replicates in a standard reconstituted water may be an indication of pathogen interference. Receiving water controls from improper preparation or collection also should be evaluated.

11.3.4.3 Because of the ubiquitous nature of the pathogens or predatory organisms, all test equipment, glassware, and pipettes must be kept clean and dry when not in use. Use of separate glassware, pipettes, and siphons for each concentration is recommended to minimize cross contaminating replicates of all treatments. Care also should be taken to properly clean test chambers by removing excess food, dead fish larvae, and other debris prior to daily renewal (see Subsection 11.10.7). When proper laboratory hygiene and filtration through a 2-4 mm mesh opening (Subsection 8.8.2) do not eliminate the sporadic mortality, the analyst should determine the source and confirm pathogen interference using parallel testing (11.3.4.4).

11.3.4.4 Parallel tests should be conducted using reconstituted water and receiving water as diluents with the effluent to confirm that the test results are due to pathogen interference and to determine the source of pathogens in the test. This determination is an important step in controlling pathogen interference. When the dilution water exhibits the interference (i.e., pathogen interference is not observed in the test using reconstituted laboratory water for dilution), reconstituted laboratory water instead of receiving waters should be used to eliminate the interference. However, if receiving water is required, the analyst may modify the test design to control pathogen interference (Subsection 11.3.4.5) or treat the dilution water prior to testing to remove the interference (Subsection 11.3.4.6). If pathogen interference is due to pathogens in the effluent (i.e., pathogen interference is still observed in the test using reconstituted laboratory water for dilution), it is recommended that the analyst modify the test design to control pathogen interference (Subsection 11.3.4.5). Upon approval by the regulatory authority, analysts also may use various sample sterilization techniques to control pathogen interference (11.3.4.6) provided that parallel testing of altered and unaltered samples further confirms the presence of pathogen interference and demonstrates successful pathogen control.

11.3.4.5 When data evaluation indicates that sporadic mortality has occurred as described in Subsections 11.3.4.1 - 11.3.4.2, the test design can be modified as described below to control pathogen interference. The use of 2 fish per 20 ml in each 1 ounce plastic cup test solution or 2 fish per 50 ml in each 4 ounce plastic cup can be used rather than 10 fish per test chamber. The total number of fish tested remains unchanged (i.e., 40 per treatment). At test initiation, for each test concentration and replicate, the test cups must be labeled to easily recombine the fish to the original replicate at the end of the test. For example, for replicate A, each of the five plastic test cups would be identified as subreplicate A1, A2, A3, A4, and A5 repeating the pattern for subsequent replicates (e.g., for replicate B, each cup would be identified as subreplicate B1, B2, B3, B4, and B5). At test termination, all test organisms

from the five A subreplicates are combined for a survival and weight determination. Document the recombination of replicates in records.

11.3.4.5.1 All test chambers must be randomized using a template for randomization or by using a table of random numbers. Test chambers are randomized once at the beginning of the test (see Subsection 11.10.2.3). When using templates, a number of different templates should be prepared, so that the same template is not used for every test. Randomization procedures must be documented with daily records.

11.3.4.5.2 When adding or transferring the larvae to test chambers, the amount of excess water added to the chambers should be kept to a minimum to avoid unnecessary dilution of the test concentrations. The fish in each test chamber should be fed 0.1 mL of a concentrated suspension of newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4 h intervals, or 0.15 mL should be fed twice daily at an interval of 6 h. (NOTE: to prevent low dissolved oxygen levels, the amount of food added to cups should be adjusted to account for the modified test design that uses smaller test chambers). Dead test organisms should be removed as soon as they are observed.

11.3.4.5.3 Fish are transferred to new or clean test chambers daily. At the time of the daily renewal of the test solutions, the fish are transferred to a new test chamber containing fresh test solution using a pipette which has at least a 5mm bore diameter. Separate pipettes should be used for each treatment. Water transfer is kept to a minimum by allowing the fish to swim out of the pipette into the new test chamber. Any potential injury to individual fish should be recorded on the test sheets.

11.3.4.5.4 At test termination, the surviving larvae in each chamber must be counted and all subreplicates within a replicate (e.g., A1, A2, A3, A4, and A5) combined. For example, all test cups (within a treatment) labeled A would be combined for a survival and dry weight determination.

11.3.4.6 When parallel testing has confirmed pathogen interference, the regulatory authority may allow modifications of the effluent samples or receiving water diluent to remove or inactivate the pathogens (Subsection 11.3.4.6.1 - 11.3.4.6.4). Techniques that control pathogen interference without modifying the effluent sample (11.3.4.5) are recommended, but they may not always be able to minimize pathogen interference to the extent that test results are not confounded by mortality due to pathogens. Therefore, regulatory authorities may allow appropriate pathogen control techniques (including those that modify the effluent sample) on a case-by-case basis. TIE approaches (USEPA, 1991b; USEPA, 1992) and the following procedures (Subsection 11.3.4.6.1 - 11.3.4.6.4) can be used alone or in combination to ascertain the adverse influence on tests caused by pathogens. Prior to routine use of pathogen control techniques that modify the sample, the effects of pathogenic bacteria and the effectiveness of the selected pathogen control technique must be confirmed by parallel and simultaneous testing of the technique with altered and unaltered samples.

11.3.4.6.1 Use of ultra-violet light to irradiate the sample. The rate of pumping specified by the manufacturer of the apparatus should be used (provided that adequate disinfection is achieved), and the life of the UV light source must follow manufacturers' recommendations and be documented. For example, one liter of water can be irradiated for 20 min using an 8 watt UV light (Aquatic Ecosystems, Apopka, FL) prior to use each day of the test. Light sources have limited lifetimes and their effectiveness will decrease with age. The delivery pump and the light source should be on the same electrical circuit to ensure that when power is interrupted both terminate operation. QA/QC procedures should be put into place to assure that the light source is on at the beginning and at the end of the procedure. Treatment of the large volumes of water necessary for test dilution also may be impractical. Caution: Since the effluent or receiving water samples must be passed through the UV sterilizer and then test treatments prepared, there may be potential effects of UV light on the sample. UV exposure may increase or decrease toxicity from other pollutants in the sample. UV treatment is known to cause photoactivation of some organic compounds, which may increase toxicity. UV treatment also is known to cause the photochemical breakdown of certain organic compounds, which could decrease toxicity (if the parent compound is toxic) or increase toxicity (if reaction products are toxic). These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. The effectiveness of UV for

sterilization may decrease with turbid or stained samples. Bacteria can escape exposure by being lodged in crevices of particulate matter in the sample. All toxicity tests using a sterilized sample must include a blank preparation consisting of similarly sterilized laboratory water.

11.3.4.6.2 Ultra-filtration through a 0.22 µm pore diameter filter (such as Gelman Suprocap®) may be conducted on sample aliquots before daily use. Samples may need to be filtered through a glass fiber filter prior to the 0.22 µm filter. This is time consuming and volume restricted. Treatment of the large volumes of water necessary for test dilution may be impractical. Caution: Since the effluent or receiving water samples must be passed through the filter, the effect of filtering must be evaluated. Filtration can remove toxicity if toxic components of the sample are bound to particles (USEPA, 1991b; 1992). The removal of suspended solids also may influence the bioavailability of chemical pollutants. These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. The removal of toxicity by filtration must be evaluated for each sample by testing samples before and after filtration. All toxicity tests using a sterilized sample also must include a blank preparation consisting of similarly sterilized reconstituted laboratory water.

11.3.4.6.3 Use of chlorination and dechlorination. In some cases, pathogens can survive the chlorination/ dechlorination process and the pathogenic effects may increase due to lack of competition from other organisms. Sufficient data must be collected and documented to determine the effective dosage required. Caution: Chlorination of effluent samples could cause unknown effects on the sample. Chlorination could increase or decrease sample toxicity by oxidizing organic compounds or forming chlorination by-products. These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. Toxicity tests conducted with the addition of chlorine and subsequent dechlorination (USEPA, 1991b; 1992) to either effluent or receiving water samples also must include a blank preparation consisting of similarly treated laboratory water.

11.3.4.6.4 Use of antibiotics. The addition of wide spectrum antibiotics has been effective in removing the pathogen effect (Downey *et al.*, 2000). Antibacterial treatment such as those commonly used in aquaculture or home aquarium maintenance (e.g., oxytetracycline, chloramphenicol, and actinomycin) may be effective. Sufficient data must be collected to determine the effective dosage required. Caution: While antibiotics are effective, easy to use, inexpensive, and readily available, the antibiotic treatment may alter the sample in unknown or undesirable ways and may make the sample too cloudy. Large volumes of a sample may need to be treated. These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. All toxicity tests using antibiotic treatments also must include treatment blanks of similarly prepared laboratory water.

11.3.5 Food added during the test may sequester metals and other toxic substances and confound test results. Daily renewal of solutions, however, will reduce the probability of reduction of toxicity caused by feeding.

11.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 11.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 11.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

11.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 11.3.6.2.

The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample upon completion of collection (as measured on an aliquot removed from the sample container).

11.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ± 0.2 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ± 0.2 pH units in pH-controlled tests (USEPA, 1992).

11.3.6.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

11.3.6.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 11.3.6.1.1).

11.3.6.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 11.3.6.2) is applied routinely to subsequent testing of the effluent.

11.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992); or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, CO_2 is injected to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, CO_2 is injected to maintain the test pH at the pH of the sample upon completion of collection. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

11.4 **SAFETY**

11.4.1 See Section 3, Health and Safety.

11.5 **APPARATUS AND EQUIPMENT**

11.5.1 Fathead minnow and brine shrimp culture units -- see USEPA, 1985a and USEPA, 2002a. This test requires 240-360 larvae. It is preferable to obtain larvae from an in-house fathead minnow culture unit. If it is not feasible to culture fish in-house, embryos or newly hatched larvae can be shipped in well oxygenated water in insulated containers.

11.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L.

11.5.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.5.4 Environmental chamber or equivalent facility with temperature control (25 ± 1°C).

11.5.5 Water purification system -- MILLIPORE MILLI-Q®, deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

11.5.6 Balance -- analytical, capable of accurately weighing to 0.00001 g.

11.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus fish.

11.5.8 Test chambers -- four borosilicate glass or non-toxic disposable plastic test chambers are required for each concentration and control. Test chambers may be 1 L, 500 mL or 250 mL beakers, 500 mL plastic cups, or fabricated rectangular (0.3 cm thick) glass chambers, 15 cm x 7.5 cm x 7.5 cm. To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic (6 mm thick).

11.5.9 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions. 5.10

11.5.10 Volumetric pipets -- Class A, 1-100 mL.

11.5.11 Serological pipets -- 1-10 mL, graduated.

11.5.12 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

11.5.13 Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring larvae.

11.5.14 Wash bottles -- for rinsing small glassware and instrument electrodes and probes.

11.5.15 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

11.5.16 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

11.5.17 Thermometers, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, 1979b) -- to calabrate laboratory themometers.

11.5.18 Meters, pH, DO, and specific conductivity -- for routine physical and chemical measurements.

11.5.19 Drying oven -- 50-105° C range for drying larvae.

11.6 **REAGENTS AND CONSUMABLE MATERIALS**

11.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.6.2 Data sheets (one set per test) -- for recording data.

11.6.3 Vials, marked -- 24 per test, containing 4% formalin or 70% ethanol to preserve larvae (optional).

11.6.4 Weighing boats, aluminum -- 24 per test.

11.6.5 Tape, colored -- for labeling test chambers.

11.6.6 Markers, waterproof -- for marking containers, etc.

11.6.7 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA, 1979b.

11.6.8 Buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for instrument calibration (see USEPA Method 150.1, USEPA, 1979b).

11.6.9 Specific conductivity standards -- see USEPA Method 120.1, USEPA, 1979b.

11.6.10 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

11.6.11 Laboratory quality control samples and standards -- for calibration of the above methods.

11.6.12 Reference toxicant solutions (see Section 4, Quality Assurance).

11.6.13 Ethanol (70%) or formalin (4%) -- for use as a preservative for the fish larvae.

11.6.14 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

11.6.15 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

11.6.16 Brine Shrimp, *Artemia*, Nauplii -- for feeding cultures and test organisms

11.6.16.1 Newly-hatched *Artemia* nauplii are used as food (see USEPA, 2002a) for fathead minnow, *Pimephales promelas*, larvae in toxicity tests and frozen brine shrimp and flake food are used in the maintenance of continuous stock cultures. Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are currently preferred because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size.

11.6.16.2 Each new batch of brine shrimp, *Artemia*, cysts must be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (see Leger et al., 1985; Leger et al., 1986) against known suitable reference cysts by performing a side by side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be a previously tested and acceptable batch of cysts. A

sample of newly-hatched *Artemia* nauplii from each new batch of cysts should be chemically analyzed. The *Artemia* cysts should not be used if the concentration of total organochlorine exceeds 0.15 µg/g wet weight or the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight. (For analytical methods see USEPA, 1982).

11.6.16.3 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or a solution prepared by adding 35.0 g uniodized salt (NaCl) or artificial sea salts to 1 L deionized water, to a 2-L separatory funnel, or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24-h at 27°C. (Hatching time varies with incubation temperature and the geographic strain of *Artemia* used) (see USEPA, 1991b; USEPA, 2002a and ASTM, 1993).
3. After 24 h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic, and will concentrate at the bottom of the funnel if it is covered for 5-10 min. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 min without aeration.
4. Drain the nauplii into a beaker or funnel fitted with a ≤ 150 µm Nitex® or stainless steel screen, and rinse with deionized water, or equivalent, before use.

11.6.16.4 Testing *Artemia* nauplii as food for toxicity test organisms.

11.6.16.4.1 The primary criterion for acceptability of each new supply of brine shrimp cysts is the ability of the nauplii to support good survival and growth of the fathead minnow larvae (see Subsection 11.12). The larvae used to evaluate the suitability of the brine shrimp nauplii must be of the same geographical origin, species, and stage of development as those used routinely in the toxicity tests. Sufficient data to detect differences in survival and growth should be obtained by using three replicate test vessels, each containing a minimum of 15 larvae, for each type of food.

11.6.16.4.2 The feeding rate and frequency, test vessels, volume of control water, duration of the test, and age of the nauplii at the start of the test, should be the same as used for the routine toxicity tests.

11.6.16.4.3 Results of the brine shrimp nutrition assay, where there are only two treatments, can be evaluated statistically by use of a t test. The "new" food is acceptable if there are no statistically significant differences in the survival and growth of the larvae fed the two sources of nauplii.

11.6.17 TEST ORGANISMS, FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

11.6.17.1 Newly hatched fish less than 24 h old should be used for the test. If organisms must be shipped to the testing site, fish up to 48 h old may be used, all hatched within a 24-h window.

11.6.17.2 If the fish are kept in a holding tank or container, most of the water should be siphoned off to concentrate the fish. The fish are then transferred one at a time randomly to the test chambers until each chamber contains ten fish. Alternately, fish may be placed one or two at a time into small beakers or plastic containers until they each contain five fish. Three (minimum of two) of these beakers/plastic containers are then assigned to randomly-arranged control and exposure chambers.

11.6.17.2.1 The fish are transferred directly to the test vessels or intermediate beakers/plastic containers, using a large-bore, fire-polished glass tube (6 mm to 9 mm I.D. X 30 cm long) equipped with a rubber bulb, or a large volumetric pipet with tip removed and fitted with a safety type bulb filler. The glass or plastic containers should only contain a small volume of dilution water.

11.6.17.2.2 It is important to note that larvae should not be handled with a dip net. Dipping small fish with a net may result in damage to the fish and cause mortality.

11.6.17.3 The test is conducted with a minimum of four test chambers at each toxicant concentration and control. Fifteen (minimum of ten) embryos are placed in each replicate test chamber. Thus 60 (minimum of 40) fish are exposed at each test concentration.

11.6.17.4 Sources of organisms

11.6.17.4.1 Fathead minnows, *Pimephales promelas*, may be obtained from commercial biological supply houses. Fish obtained from outside sources for use as brood stock or in toxicity tests may not always be of suitable age and quality. Fish provided by supply houses should be guaranteed to be of (1) the correct species, (2) disease free, (3) in the requested age range, and (4) in good condition. This can be done by providing the record of the date on which the eggs were laid and hatched, and information on the sensitivity of contemporary fish to reference toxicants.

11.6.17.5 Inhouse Sources of Fathead Minnows, *Pimephales promelas*

11.6.17.5.1 Problems in obtaining suitable fish from outside laboratories can be avoided by developing an inhouse laboratory culture facility. Fathead minnows, *Pimephales promelas*, can be easily cultured in the laboratory from eggs to adults in static, recirculating, or flow-through systems. The larvae, juveniles, and adult fish should be kept in 60 L (15 gal) or 76 L (20 gal) rearing tanks supplied with reconstituted water, dechlorinated tap water, or natural water. The water should be analyzed for toxic metals and organics quarterly (see Section 4, Quality Assurance).

11.6.17.5.1.1 If a static or recirculating system is used, it is necessary to equip each tank with an outside activated carbon filter system, similar to those sold for tropical fish hobbyists (or one large activated carbon filter system for a series of tanks) to prevent the accumulation of toxic metabolic wastes (principally nitrite and ammonia) in the water.

11.6.17.5.2 Flow-through systems require large volumes of water and may not be feasible in some laboratories. The culture tanks should be shielded from extraneous disturbances using opaque curtains, and should be isolated from toxicity testing activities to prevent contamination.

11.6.17.5.3 To avoid the possibility of inbreeding of the inhouse brood stock, fish from an outside source should be introduced yearly into the culture unit.

11.6.17.5.4 Dissolved oxygen -- The DO concentration in the culture tanks should be maintained near saturation, using gentle aeration with 15 cm air stones if necessary. Brungs (1971), in a carefully controlled long-term study, found that the growth of fathead minnows was reduced significantly at all dissolved oxygen concentrations below 7.9 mg/L. Soderberg (1982) presented an analytical approach to the re-aeration of flowing water for culture systems.

11.6.17.5.5 Culture Maintenance

11.6.17.5.5.1 Adequate procedures for culture maintenance must be followed to avoid poor water quality in the culture system. The spawning and brood stock culture tanks should be kept free of debris (excess food, detritus, waste, etc.) by siphoning the accumulated materials (such as dead brine shrimp nauplii or cysts) from the bottom of the tanks daily with a glass siphon tube attached to a plastic hose leading to the floor drain. The tanks are more thoroughly cleaned as required. Algae, mostly diatoms and green algae, growing on the glass of the spawning tanks are left in place, except for the front of the tank, which is kept clean for observation. To avoid excessive build-up of algal growth, the walls of the tanks are periodically scraped. The larval culture tanks are cleaned once or twice a week to reduce the mass of fungus growing on the bottom of the tank.

11.6.17.5.5.2 Activated charcoal and floss in the tank filtration systems should be changed weekly, or more often if needed. Culture water may be maintained by preparation of reconstituted water or use of dechlorinated tap water. Distilled or deionized water is added as needed to compensate for evaporation.

11.6.17.5.5.3 Before new fish are placed in tanks, salt deposits are removed by scraping or with 5% acid solution, the tanks are washed with detergent, sterilized with a hypochlorite solution, and rinsed well with hot tap water and then with laboratory water.

11.6.17.5.6 Obtaining Embryos for Toxicity Tests

11.6.17.5.6.1 Embryos can be shipped to the laboratory from an outside source or obtained from adults held in the laboratory as described below.

11.6.17.5.6.2 For breeding tanks, it is convenient to use 60 L (15 gal) or 76 L (20 gal) aquaria. The spawning unit is designed to simulate conditions in nature conducive to spawning, such as water temperature and photoperiod. Spawning tanks must be held at a temperature of 25 ± 2°C. Each aquarium is equipped with a heater, if necessary, a continuous filtering unit, and spawning substrates. The photoperiod for the culture system should be maintained at 16 h light and 8 h darkness. For the spawning tanks, this photoperiod must be rigidly controlled. A convenient photoperiod is 5:00 AM to 9:00 PM. Fluorescent lights should be suspended about 60 cm above the surface of the water in the brood and larval tanks. Both DURATEST® and cool-white fluorescent lamps have been used, and produce similar results. An illumination level of 50 to 100 ft-c is adequate.

11.6.17.5.6.3 To simulate the natural spawning environment, it is necessary to provide substrates (nesting territories) upon which the eggs can be deposited and fertilized, and which are defended and cared for by the males. The recommended spawning substrates consist of inverted half-cylinders, 7.6 cm × 7.6 cm (3 in × 3 in) of Schedule 40 PVC pipe. The substrates should be placed equi-distant from each other on the bottom of the tanks.

11.6.17.5.6.4 To establish a breeding unit, 15-20 pre-spawning adults six to eight months old are taken from a "holding" or culture tank and placed in a 76-L spawning tank. At this point, it is not possible to distinguish the sexes. However, after less than a week in the spawning tank, the breeding males will develop their distinct coloration and territorial behavior, and spawning will begin. As the breeding males are identified, all but two are removed, providing a final ratio of 5-6 females per male. The excess spawning substrates are used as shelter by the females.

11.6.17.5.6.5 Sexing of the fish to ensure a correct female/male ratio in each tank can be a problem. However, the task usually becomes easier as experience is gained (Flickinger, 1966). Sexually mature females usually have large bellies and a tapered snout. The sexually mature males are usually distinguished by their larger overall size, dark vertical color bands, and the spongy nuptial tubercles on the snout. Unless the males exhibit these secondary breeding characteristics, no reliable method has been found to distinguish them from females. However, using the coloration of the males and the presence of enlarged urogenital structures and other characteristics of the females, the correct selection of the sexes can usually be achieved by trial and error.

11.6.17.5.6.6 Sexually immature males are usually recognized by their aggressive behavior and partial banding. These undeveloped males must be removed from the spawning tanks because they will eat the eggs and constantly harass the mature males, tiring them and reducing the fecundity of the breeding unit. Therefore, the fish in the spawning tanks must be carefully checked periodically for extra males.

11.6.17.5.6.7 A breeding unit should remain in their spawning tank about four months. Thus, each brood tank or unit is stocked with new spawners about three times a year. However, the restocking process is rotated so that at any one time the spawning tanks contain different age groups of brood fish.

11.6.17.5.6.8 Fathead minnows spawn mostly in the early morning hours. They should not be disturbed except for a morning feeding (8:00 AM) and daily examination of substrates for eggs in late morning or early afternoon. In nature, the male protects, cleans, and aerates the eggs until they hatch. In the laboratory, however, it is necessary to remove the eggs from the tanks to prevent them from being eaten by the adults, for ease of handling, for purposes of recording embryo count and hatchability, and for the use of the newly hatched young fish for toxicity tests.

11.6.17.5.6.9 Daily, beginning six to eight hours after the lights are turned on (11:00 AM - 1:00 PM), the substrates in the spawning tanks are each lifted carefully and inspected for embryos. Substrates without embryos are immediately returned to the spawning tank. Those with embryos are immersed in clean water in a collecting tray, and replaced with a clean substrate. A daily record is maintained of each spawning site and the estimated number of embryos on the substrate.

11.6.17.5.6.10 Three different methods are described for embryo incubation.

1. Incubation of Embryos on the Substrates: Several (2-4) substrates are placed on end in a circular pattern (with the embryos on the innerside) in 10 cm of water in a tray. The tray is then placed in a constant temperature water bath, and the embryos are aerated with a 2.5 cm airstone placed in the center of the circle. The embryos are examined daily, and the dead and fungused embryos are counted, recorded, and removed with forceps. At an incubation temperature of 25°C, 50% hatch occurs in five days. At 22°C embryos incubated on aerated tiles require 7 days for 50% hatch.

2. Incubation of Embryos in a Separatory Funnel: The embryos are removed from the substrates with a rolling action of the index finger ("rolled off") (Gast and Brungs, 1973), their total volume is measured, and the number of embryos is calculated using a conversion factor of approximately 430 embryos/mL. The embryos are incubated in about 1.5 L of water in a 2 L separatory funnel maintained in a water bath. The embryos are stirred in the separatory funnel by bubbling air from the tip of a plastic micro-pipette placed at the bottom, inside the separatory funnel. During the first two days, the embryos are taken from the funnel daily, those that are dead and fungused are removed, and those that are alive are returned to the separatory funnel in clean water. The embryos hatch in four days at a temperature of 25°C. However, usually on day three the eyed embryos are removed from the separatory funnel and placed in water in a plastic tray and gently aerated with an air stone. Using this method, the embryos hatch in five days. Hatching time is greatly influenced by the amount of agitation of the embryos and the incubation temperature. If on day three the embryos are transferred from the separatory funnel to a static, unaerated container, a 50% hatch will occur in six days (instead of five) and a 100% hatch will occur in seven days. If the culture system is operated at 22°C, embryos incubated on aerated tiles require seven days for 50% hatch.

3. Incubation in Embryo Incubation Cups: The embryos are "rolled off" the substrates, and the total number is estimated by determining the volume. The embryos are then placed in incubation cups attached to a rocker arm assembly (Mount, 1968). Both flow-through and static renewal incubation have been used. On day one, the embryos are removed from the cups and those that are dead and fungused are removed. After day one only dead embryos are removed from the cups. During the incubation period, the eggs are examined daily for viability and fungal growth, until they hatch. Unfertilized eggs, and eggs that have become infected by fungus, should be removed with forceps using a table top magnifier-illuminator. Non-viable eggs become milky and opaque, and are easily recognized. The non-viable eggs are very susceptible to fungal infection, which may then spread throughout the egg mass. Removal of fungus should be done quickly, and the substrates should be returned to the incubation tanks as rapidly as possible so that the good eggs are not damaged by desiccation. Hatching takes four to five days at an optimal temperature of 25°C. Hatching can be delayed several (two to four) days by incubating at lower temperatures. A large plastic tank receiving recirculating water from a temperature control unit, can be used as a water bath for incubation of embryos.

11.6.17.5.6.11 Newly-hatched larvae are transferred daily from the egg incubation apparatus to small rearing tanks, using a large bore pipette, until the hatch is complete. New rearing tanks are set up on a daily basis to separate fish by age group. Approximately 1500 newly hatched larvae are placed in a 60-L (15 gal) or 76-L (20 gal) all-glass aquarium for 30 days. A density of 150 fry per liter is suitable for the first four weeks. The water temperature in the rearing tanks is allowed to follow ambient laboratory temperatures of 20-25°C, but sudden, extreme variations in temperature must be avoided.

11.6.17.5.7 Food and Feeding

11.6.17.5.7.1 The amount of food and feeding schedule affects both growth and egg production. The spawning fish and pre-spawners in holding tanks usually are fed all the adult frozen brine shrimp and tropical fish flake food or dry commercial fish food (No. l or No. 2 granules) that they can eat (*ad libitum*) at the beginning of the work day and in the late afternoon (8:00 AM and 4:00 PM). The fish are fed twice a day (twice a day with dry food and once a day with adult shrimp) during the week and once a day on weekends.

11.6.17.5.7.2 Fathead minnow larvae are fed freshly-hatched brine shrimp (*Artemia*) nauplii twice daily until they are four weeks old. Utilization of older (larger) brine shrimp nauplii may result in starvation of the young fish because they are unable to ingest the larger food organisms (see Subsection 11.6.16 or USEPA, 2002a for instructions on the preparation of brine shrimp nauplii).

11.6.17.5.7.3 Fish older than four weeks are fed frozen brine shrimp and commercial fish starter (#l and #2), which is ground fish meal enriched with vitamins. As the fish grow, larger pellet sizes are used, as appropriate. (Starter, No. 1 and N. 2 granules, U.S. Fish and Wildlife Service Formulation Specification Diet SD9-30). Newly hatched brine shrimp nauplii, and frozen adult brine shrimp are fed to the fish cultures in volumes based on age, size, and number of fish in the tanks.

11.6.17.5.7.4 Fish in the larval tanks (from hatch to 30 days old) are fed commercial starter fish food at the beginning and end of the work day, and newly hatched brine shrimp nauplii (from the brine shrimp culture unit) once a day, usually mid-morning and mid-afternoon.

11.6.17.5.7.5 Attempts should be made to avoid introducing *Artemia* cysts and empty shells when the brine shrimp nauplii are fed to the fish larvae. Some of the mortality of the larval fish observed in cultures could be caused from the ingestion of these materials.

11.6.17.5.8 Disease Control

11.6.17.5.8.1 Fish are observed daily for abnormal appearance or behavior. Bacterial or fungal infections are the most common diseases encountered. However, if normal precautions are taken, disease outbreaks will rarely, if ever, occur. Hoffman and Mitchell (1980) have put together a list of some chemicals that have been used commonly for fish diseases and pests.

11.6.17.5.8.2 In aquatic culture systems where filtration is utilized, the application of certain antibacterial agents should be used with caution. A treatment with a single dose of antibacterial drugs can interrupt nitrate reduction and stop nitrification for various periods of time, resulting in changes in pH, and in ammonia, nitrite and nitrate concentrations (Collins et al., 1976). These changes could cause the death of the culture organisms.

11.6.17.5.8.3 Do not transfer equipment from one tank to another without first disinfecting tanks and nets. If an outbreak of disease occurs, any equipment, such as nets, airlines, tanks, etc., which has been exposed to diseased fish should be disinfected with sodium hypochlorite. Also to avoid the contamination of cultures or spread of disease, each time nets are used to remove live or dead fish from tanks, they are first sterilized with sodium hypochlorite or formalin, and rinsed in hot tap water. Before a new lot of fish is transferred to culture tanks, the tanks are cleaned and sterilized as described above.

11.6.17.5.8.4 It is recommended that chronic toxicity tests be performed monthly with a reference toxicant. Newly hatched fathead minnow larvae less than 24 h old are used to monitor the chronic toxicity of the reference toxicant to the test fish produced by the culture unit (see Section 4, Quality Assurance).

11.6.17.5.9 Record Keeping

11.6.17.5.9.1 Records, kept in a bound notebook, include: (l) type of food and time of feeding for all fish tanks; (2) time of examination of the tiles for embryos, the estimated number of embryos on the tile, and the tile position number; (3) estimated number of dead embryos and embryos with fungus observed during the embryonic development stages; (4) source of all fish; (5) daily observation of the condition and behavior of the fish; and (6) dates and results of reference toxicant tests performed (see Section 4, Quality Assurance).

11.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

11.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

11.8 CALIBRATION AND STANDARDIZATION

11.8.1 See Section 4, Quality Assurance.

11.9 QUALITY CONTROL

11.9.1 See Section 4, Quality Assurance.

11.10 TEST PROCEDURES

11.10.1 TEST SOLUTIONS

11.10.1.1 Receiving Waters

11.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 250 mL, and 400 mL for chemical analyses, would require approximately 1.5 L or more of sample per test per day.

11.10.1.2 Effluents

11.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as the dilution factor is increased beyond 0.5, and declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.**

11.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions should be added at the lower range of effluent concentrations.

11.10.1.2.3 The volume of effluent required for daily renewal of four replicates per concentration, each containing 250 mL of test solution, is approximately 2.5 L. Sufficient test solution (approximately 1500 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses at the high, medium, and low test concentrations. If the sample is used for more than one daily renewal of test solutions, the volume must be increased proportionately.

11.10.1.2.4 Tests should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity

tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.10.1.2.5 Just prior to test initiation (approximately 1 h) the temperature of sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature and maintained at that temperature during the addition of dilution water.

11.10.1.2.6 The DO of the test solutions should be checked prior to the test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If any solution has a DO concentration below 4.0 mg/L, all of the solutions and the control must be gently aerated.

11.10.1.3 Dilution Water

11.10.1.3.1 Dilution water may be uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other uncontaminated natural water (see Section 7, Dilution Water).

11.10.2 START OF THE TEST

11.10.2.1 Label the test chambers with a marking pen. Use of color-coded tape to identify each treatment and replicate is helpful. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) must have a minimum of four replicates.

11.10.2.2 Tests performed in laboratories that have in-house fathead minnow breeding cultures should use larvae less than 24 h old. When eggs or larvae must be shipped to the test site from a remote location, it may be necessary to use larvae older than 24 h because of the difficulty in coordinating test organism shipments with field operations. However, in the latter case, the larvae must not be more than 48 h old at the start of the test and must all be within 24 h of the same age.

11.10.2.3 Randomize the position of test chambers at the beginning of the test (see Appendix A). Maintain the chambers in this configuration throughout the test. Preparation of a position chart may be helpful.

11.10.2.4 The larvae are pooled and placed one or two at a time into each randomly arranged test chamber or intermediate container in sequential order, until each chamber contains 15 (minimum of 10) larvae, for a total of 60 larvae (minimum of 40) for each concentration (see Appendix A). The test organisms should come from a pool of larvae consisting of at least three separate spawnings. The amount of water added to the chambers when transferring the larvae should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

11.10.2.4.1 The chambers may be placed on a light table to facilitate counting the larvae.

11.10.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

11.10.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The water temperature in the test chambers should be maintained at 25 ± 1°C.

11.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

11.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO concentrations. The DO concentrations should be measured in the new solutions at the start of the test (Day 0) and before daily renewal of the test solutions on subsequent days. The DO concentrations should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all concentrations and the control should be aerated.

The aeration rate should not exceed 100 bubbles/min, using a pipet with an orifice of approximately 1.5 mm, such as a 1-mL, KIMAX® serological pipet, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue physical stress to the fish.

11.10.5 FEEDING

11.10.5.1 The fish in each test chamber are fed 0.1 g (approximately 700 to 1000) of a concentrated suspension of newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals or, as a minimum, 0.15 g are fed twice daily at an interval of 6 h. Equal amounts of nauplii must be added to each replicate chamber to reduce variability in larval weight. Sufficient numbers of nauplii should be provided to assure that some remain alive in the test chambers for several hours, but not in excessive amounts which will result in depletion of DO below acceptable levels (below 4.0 mg/L).

11.10.5.2 The feeding schedule will depend on when the test solutions are renewed. If the test is initiated after 12:00 PM, the larvae may be fed only once the first day. On following days, the larvae normally would be fed at the beginning of the work day, at least 2 h before test solution renewal, and at the end of the work day, after test solution renewal. However, if the test solutions are changed at the beginning of the work day, the first feeding would be after test solution renewal in the morning, and the remaining feeding(s) would be at the appropriate intervals. The larvae are not fed during the final 12 h of the test.

11.10.5.3 The nauplii should be rinsed with freshwater to remove salinity before use (see USEPA, 2002a). At feeding time pipette about 5 mL (5 g) of concentrated newly hatched brine shrimp nauplii into a 120 mesh nylon net or plastic cup with nylon mesh bottom. Slowly run freshwater through the net or rinse by immersing the cup in a container of fresh water several times. Resuspend the brine shrimp in 10 mL of fresh water in a 30 mL beaker or simply set the cup of washed brine shrimp in ¼ inch of fresh water so that the cup contains about 10 mL of water. Allow the container to set for a minute or two to allow dead nauplii and empty cysts to settle or float to the surface before collecting the brine shrimp from just below the surface in a pipette for feeding. Distribute 2 drops (0.1 g) of the brine shrimp to each test chamber. If the survival rate in any test chamber falls below 50%, reduce the feeding in that chamber to 1 drop of brine shrimp at each subsequent feeding.

11.10.6 OBSERVATIONS DURING THE TEST

11.10.6.1 Routine Chemical and Physical Determinations

11.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period in at least one test chamber at each test concentration and in the control.

11.10.6.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test chamber at each test concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test vessels at least at the end of the test to determine the temperature variation in the environmental chamber.

11.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

11.10.6.1.4 Conductivity, alkalinity and hardness are measured in each new sample (100% effluent or receiving water) and in the control.

11.10.6.1.5 Record all the measurements on the data sheet (Figure 1)

11.10.6.2 Routine Biological Observations

11.10.6.2.1 The number of live larvae in each test chamber are recorded daily (Figure 2) , and the dead larvae are discarded.

11.10.6.2.2 Protect the larvae from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of dead larvae, carefully. Make sure the larvae remain immersed during the performance of these operations.

Discharger:______________________________ Test Dates:__________________________

Location:________________________________ Analyst:____________________________

Conc:		Day							
		1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day							
		1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day							
		1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, larval survival and growth test. Routine chemical and physical determinations.

Discharger:__________________________________ Test Dates:____________________________

Location:___________________________________ Analyst:______________________________

Conc:		Day							
		1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day							
		1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day							
		1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, larval survival and growth test. Routine chemical and physical determinations (CONTINUED).

Discharger:________________________ Test Dates:____________________

Location:________________________ Analyst:____________________

No. Surviving Organisms

Conc:	Rep. No.	Day							
Control:		1	2	3	4	5	6	7	Remarks
Conc:									
Conc:									
Conc:									
Conc:									
Conc:									
Conc:									
Conc:									

Comments:

Figure 2. Survival data for the fathead minnow, *Pimephales promelas*, larval survival and growth test.

11.10.7 DAILY CLEANING OF TEST CHAMBERS

11.10.7.1 Before the daily renewal of test solutions, uneaten and dead *Artemia*, dead fish larvae, and other debris are removed from the bottom of the test chambers with a siphon hose. Alternately, a large pipet (50 mL) fitted with a rubber bulb can be used. Because of their small size during the first few days of the tests, larvae are easily drawn into the siphon tube or pipet when cleaning the test chambers. By placing the test chambers on a light box, inadvertent removal of larvae can be greatly reduced because they can be more easily seen. If the water siphoned from the test chambers is collected in a white plastic tray, the larvae caught up in the siphon can be retrieved and returned to the chambers. Any incidence of removal of live larvae from the test chambers during cleaning, and subsequent return to the chambers, should be noted in the records.

11.10.8 TEST SOLUTION RENEWAL

11.10.8.1 Freshly prepared solutions are used to renew the tests daily immediately after cleaning the test chambers. For on-site toxicity studies, fresh effluent or receiving water samples should be collected daily, and no more than 24 h should elapse between collection of the samples and their use in the tests (see Section 8, Effluent and Receiving Water Sampling, Sample Holding, and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples are collected, preferably on days one, three, and five. Maintain the samples in the refrigerator at 0-6ºC until used.

11.10.8.2 For test solution renewal, the water level in each chamber is lowered to a depth of 7 to 10 mm, which leaves 15 to 20% of the test solution. New test solution (250 mL) should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence and possible injury to the larvae.

11.10.9 TERMINATION OF THE TEST

11.10.9.1 The test is terminated after seven days of exposure. At test termination, dead larvae are removed and discarded. The surviving larvae in each test chamber (replicate) are counted and immediately prepared as a group for dry weight determination, or are preserved as a group in 70% ethanol or 4% formalin. Preserved organisms are dried and weighed within 7 days. For safety, formalin should be used under a hood.

11.10.9.2 For immediate drying and weighing, place live larvae onto a 500 µm mesh screen in a large beaker to wash away debris that might contribute to the dry weight. Each group of larvae is rinsed with deionized water to remove food particles, transferred to a tared weighing boat that has been properly labeled, and dried at 60ºC, for 24 h or at 100ºC for a minimum of 6 h. Immediately upon removal from the drying oven, the weighing boats are placed in a dessicator until weighed, to prevent the absorption of moisture from the air. All weights should be measured to the nearest 0.01 mg and recorded on data sheets (Figure 3). Subtract tare weight to determine the dry weight of the larvae in each replicate. For each test chamber, divide the final dry weight by the number of original larvae in the test chamber to determine the average individual dry weight and record on the data sheet (Figure 3). For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptability criteria (See Section 11.11). Average weights should be expressed to the nearest 0.001 mg.

11.10.9.3 Prepare a summary table as illustrated in Figure 4.

11.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

11.11.1 A summary of test conditions and test acceptability criteria is presented in Table 1.

Discharge:_____ Test Date(s): _____ Drying Temperature (°C): _____
Location: _____ Weighing Date:_____ Drying Time (h): _____
Analyst: _____

Conc:	Rep. No.	A Wgt. of tare (mg)	B Dry wgt: tare and larvae (mg)	B-A Total dry wgt of larvae (mg)	C No. of original larvae (mg)	(B-A)/C Mean dry wgt of larvae (mg)	Remarks
Control							
Conc:							
Conc:							
Conc:							
Conc:							
Conc:							

Figure 3. Weight data for the fathead minnow, *Pimephales promelas*, larval survival and growth test. From USEPA (1989a).

Discharger: _____ Test Dates: _____

Location: _____ Analyst: _____

TREATMENT	CONTROL					
NO. LIVE LARVAE						
SURVIVAL (%)						
MEAN DRY WGT OF LARVAE (MG) ± SD						
TEMPERATURE RANGE (°C)						
DISSOLVED OXYGEN RANGE (MG/L)						
HARDNESS						
CONDUCTIVITY						

COMMENTS:

Figure 4. Summary data for the fathead minnow, *Pimephales promelas*, larval survival and growth test.

TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAL SURVIVAL AND GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1000.0)[1]

1.	Test type:	Static renewal (required)
2.	Temperature (°C):	25 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
3.	Light quality:	Ambient laboratory illumination (recommended)
4.	Light intensity:	10-20 µE/m^2/s (50-100 ft-c)(ambient laboratory levels) (recommended)
5.	Photoperiod:	16 h light, 8 h darkness (recommended)
6.	Test chamber size:	500 mL (recommended minimum)
7.	Test solution volume:	250 mL (recommended minimum)
8.	Renewal of test solutions:	Daily (required)
9.	Age of test organisms:	Newly hatched larvae less than 24 h old. If shipped, not more than 48 h old, 24 h range in age (required)
10.	No. larvae per test chamber:	10 (recommended)
11.	No. replicate chambers per concentration:	4 (required minimum)
12.	No. larvae per concentration:	40 (required minimum)
13.	Source of food:	Newly hatched *Artemia* nauplii (less than 24 h old) (required)
14.	Feeding regime:	On days 0-6, feed 0.1 g newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals or, as a minimum, 0.15 g twice daily at 6-h intervals (at the beginning of the work day prior to renewal, and at the end of the work day following renewal). Sufficient nauplii are added to provide an excess. (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAL SURVIVAL AND GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1000.0) (CONTINUED)

15.	Cleaning:	Siphon daily, immediately before test solution renewal (required)
16.	Aeration:	None, unless DO concentration falls below 4.0 mg/L. Rate should not exceed 100 bubbles/minimum (recommended)
17.	Dilution water:	Uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals, or DMW (see Section 7, Dilution Water) (available options)
18.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Water: 100% receiving water (or minimum of 5) and a control (recommended)
19.	Dilution factor:	Effluents: ≥ 0.5 (recommended) Receiving waters: None or ≥ 0.5 (recommended)
20.	Test duration:	7 days (required)
21.	Endpoints:	Survival and growth (weight) (required)
22.	Test acceptability criteria:	80% or greater survival in controls; average dry weight per surviving organism in control chambers equals or exceeds 0.25 mg (required)
23.	Sampling requirements:	For on-site tests, samples collected daily, and used within 24 h of the time they are removed from the sampling device; For off-site tests, a minimum of three samples (e.g., collected on days one, three and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
24.	Sample volume required:	2.5 L/day (recommended)

11.12 **ACCEPTABILITY OF TEST RESULTS**

11.12.1 For the test results to be acceptable, survival in the controls must be at least 80%. The average dry weight per surviving control larvae at the end of the test must equal or exceed 0.25 mg.

11.13 **DATA ANALYSIS**

11.13.1 GENERAL

11.13.1.1 Tabulate and summarize the data. A sample set of survival and growth response data is shown in Table 2.

TABLE 2. SUMMARY OF SURVIVAL AND GROWTH DATA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAE EXPOSED TO A REFERENCE TOXICANT FOR SEVEN DAYS[1]

NaPCP Conc. (µg/L)	Proportion of Survival in Replicate Chambers A	B	C	D	Mean Prop. Surv	Avg Dry Wgt (mg) In Replicate Chambers A	B	C	D	Mean Dry Wgt (mg)
0	1.0	1.0	0.9	0.9	0.95	0.711	0.662	0.646	0.690	0.677
32	0.8	0.8	1.0	0.8	0.85	0.517	0.501	0.723	0.560	0.575
64	0.9	1.0	1.0	1.0	0.975	0.602	0.669	0.694	0.676	0.660
128	0.9	0.9	0.8	1.0	0.90	0.566	0.612	0.410	0.672	0.565
256	0.7	0.9	1.0	0.5	0.775	0.455	0.502	0.606	0.254	0.454
512	0.4	0.3	0.4	0.2	0.325	0.143	0.163	0.195	0.099	0.150

[1] Four replicates of 10 larvae each.

11.13.1.2 The endpoints of toxicity tests using the fathead minnow, *Pimephales promelas*, larvae are based on the adverse effects on survival and growth. The LC50, the IC25, and the IC50 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for survival and growth are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50, IC25 and IC50. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for survival and growth, but included in the estimation of the LC50, IC25, and IC50. See the Appendices for examples of the manual computations, and examples of data input and program output.

11.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

11.13.2 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW, *PIMEPHALES PROMELAS*, SURVIVAL DATA

11.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 5 and 6. The response used in the analysis is the proportion of animals surviving in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the LC50, EC50, and IC endpoints. Concentrations at which there is no survival in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC, EC, and LC endpoints.

11.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

11.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

11.13.2.4 Probit Analysis (Finney, 1971; see Appendix I) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, or the Graphical Method may be used (see Appendices I-L).

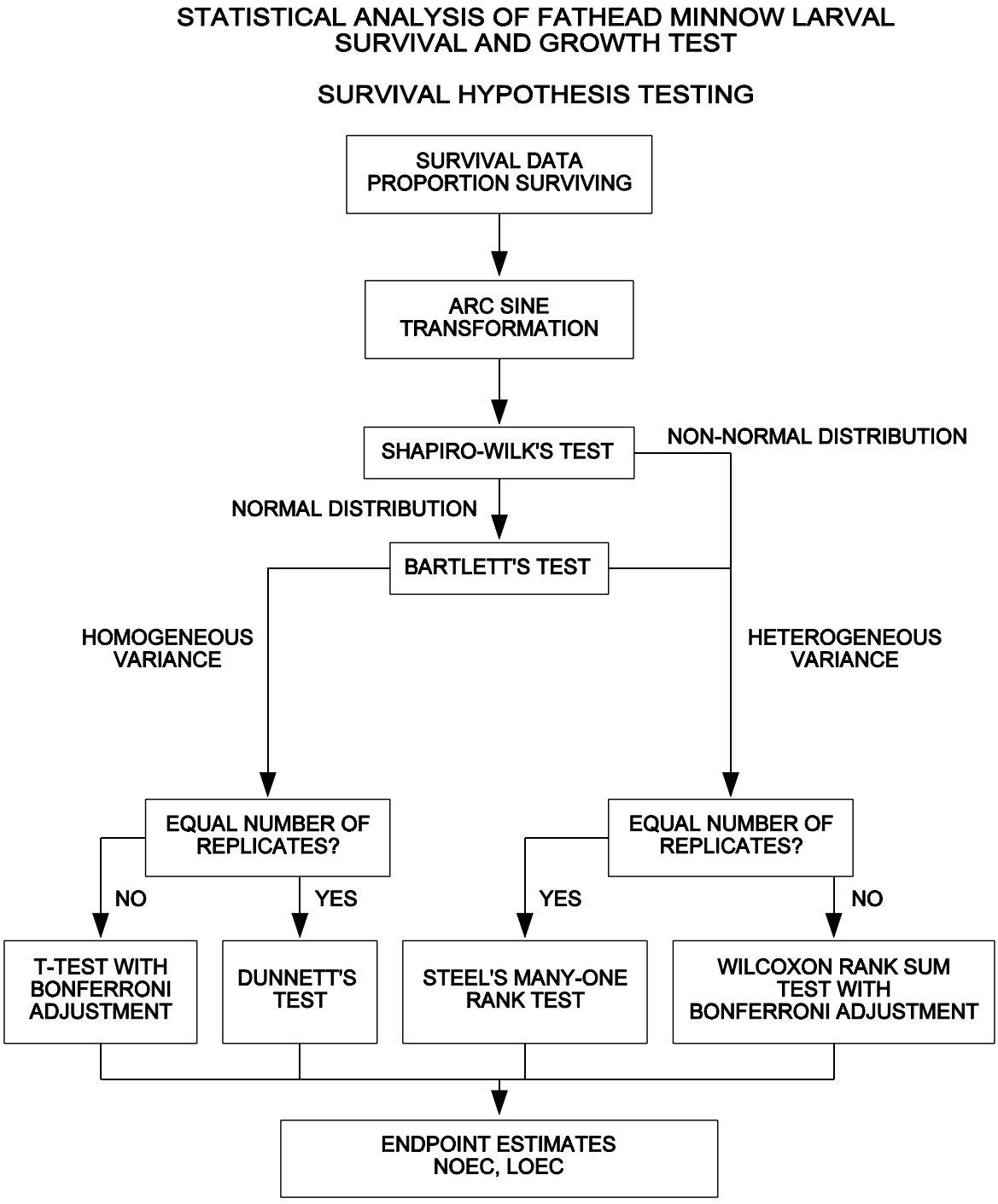

Figure 5. Flowchart for statistical analysis of the fathead minnow, *Pimephales promelas*, larval survival data by hypothesis testing.

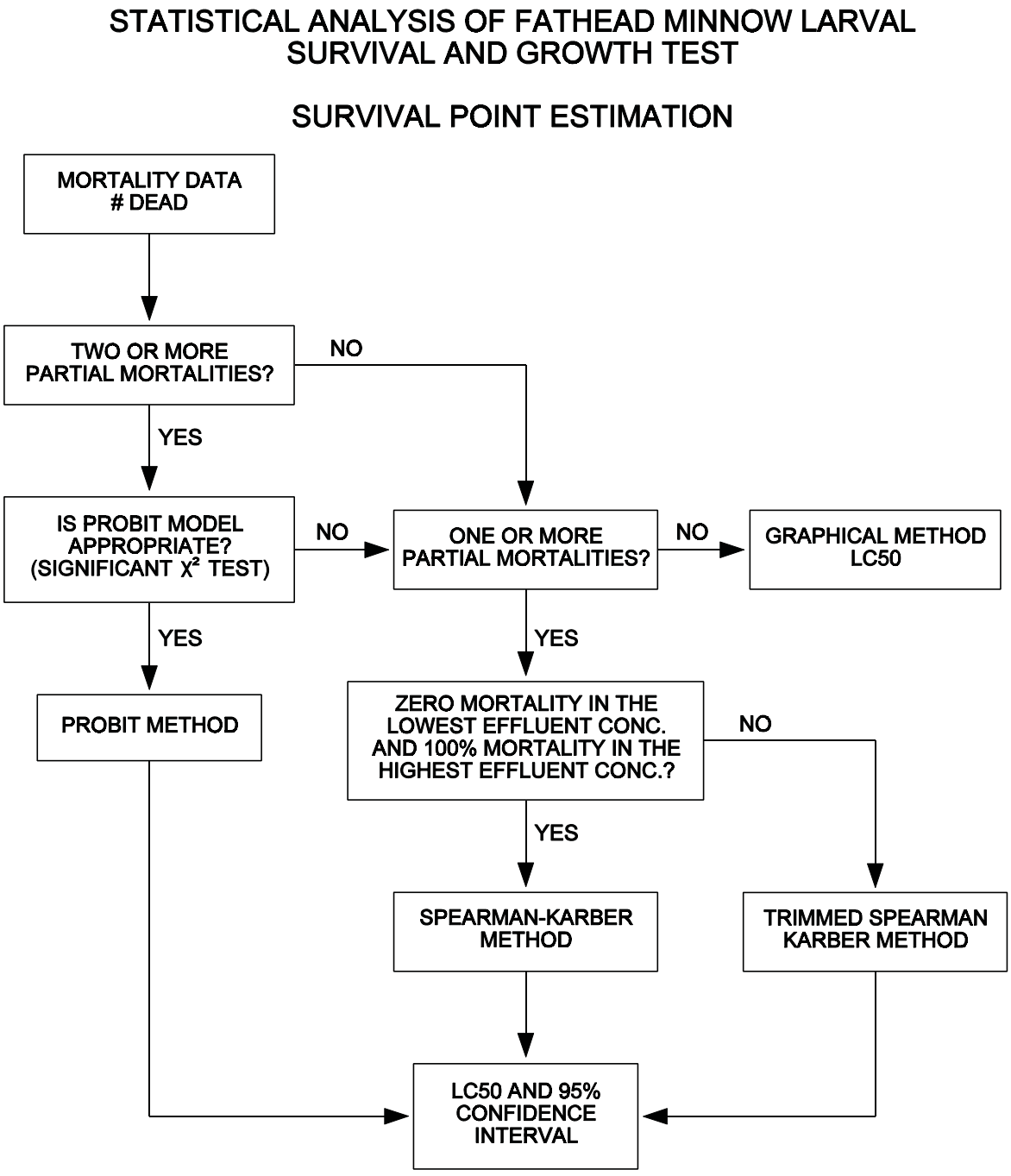

Figure 6. Flowchart for statistical analysis of the fathead minnow, *Pimephales promelas,* larval survival data by point estimation.

11.13.2.5 Example of Analysis of Survival Data

11.13.2.5.1 This example uses the survival data from the Fathead Minnow Larval Survival and Growth Test (Table 2). The proportion surviving in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each toxicant concentration and control are listed in Table 3. A plot of the survival proportions is provided in Figure 7.

TABLE 3. FATHEAD MINNOW, *PIMEPHALES PROMELAS*, SURVIVAL DATA

			NaPCP Concentration (µg/L)				
	Replicate	Control	32	64	128	256	512
RAW	A	1.0	0.8	0.9	0.9	0.7	0.4
	B	1.0	0.8	1.0	0.9	0.9	0.3
	C	0.9	1.0	1.0	0.8	1.0	0.4
	D	0.9	0.8	1.0	1.0	0.5	0.2
ARC SINE TRANS-FORMED	A	1.412	1.107	1.249	1.249	0.991	0.685
	B	1.412	1.107	1.412	1.249	1.249	0.580
	C	1.249	1.412	1.412	1.107	1.412	0.685
	D	1.249	1.107	1.412	1.412	0.785	0.464
Mean($\bar{Y}_i$)		1.330	1.183	1.371	1.254	1.109	0.604
S_i^2		0.0088	0.0232	0.0066	0.0155	0.0768	0.0111
i		1	2	3	4	5	6

11.13.2.6 Test for Normality

11.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 4.

11.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

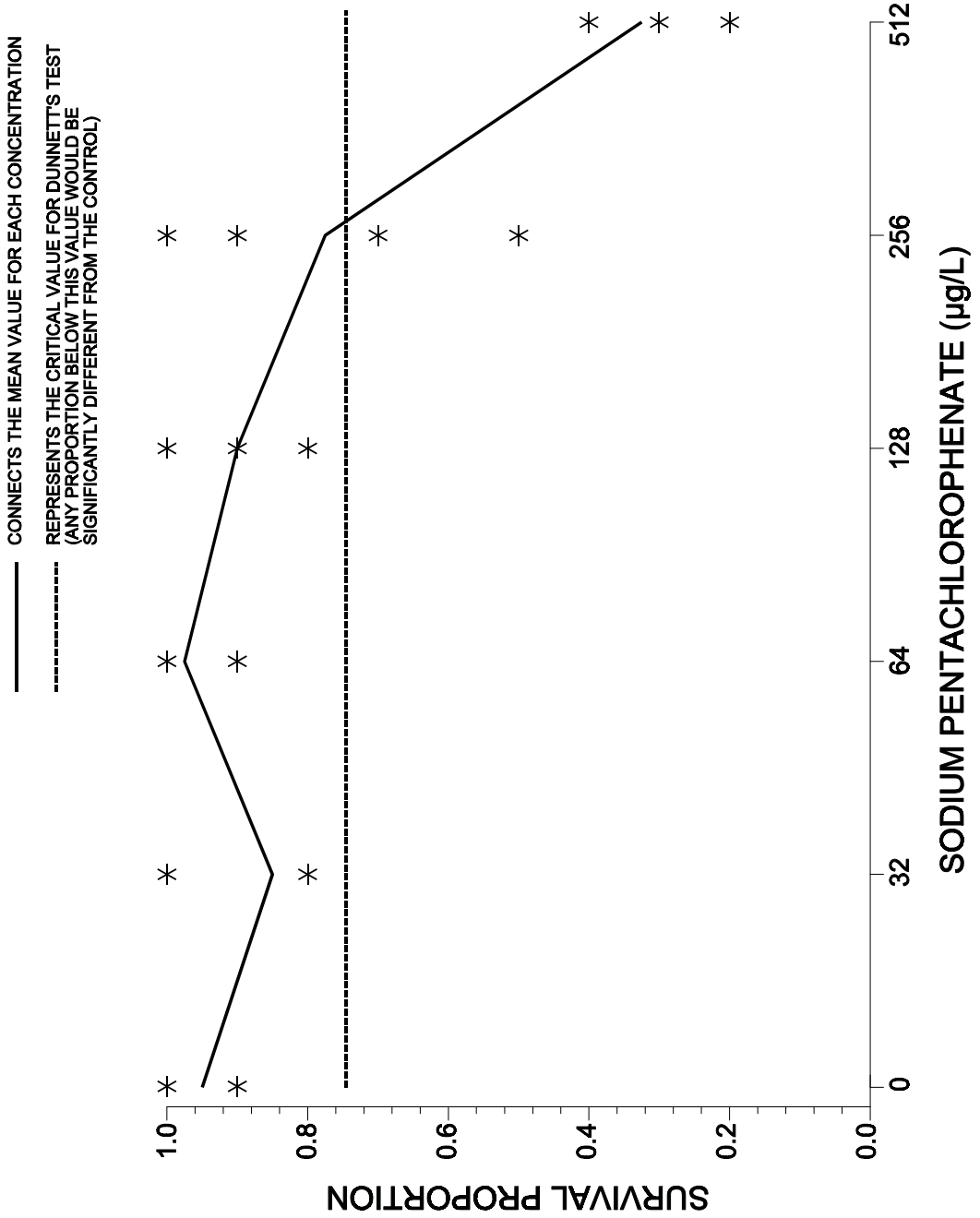

Figure 7. Plot of survival proportion data in Table 3.

TABLE 4. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	NaPCP Concentration (µg/L) 32	64	128	256	512
A	0.082	-0.076	-0.122	-0.005	-0.118	0.081
B	0.082	-0.076	0.041	-0.005	0.140	-0.024
C	-0.081	0.229	0.041	-0.147	0.303	0.081
D	-0.081	-0.076	0.041	0.158	-0.324	-0.140

11.13.2.6.3 For this set of data: n = 24

$$\bar{X} = \frac{1}{24}(0.000) = 0.000$$

D = 0.4265

11.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 5.

TABLE 5. ORDERED CENTERED OBSERVATIONS FOR THE SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.324	13	-0.005
2	-0.147	14	0.041
3	-0.140	15	0.041
4	-0.122	16	0.041
5	-0.118	17	0.081
6	-0.081	18	0.081
7	-0.081	19	0.082
8	-0.076	20	0.082
9	-0.076	21	0.140
10	-0.076	22	0.158
11	-0.024	23	0.229
12	-0.005	24	0.303

11.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 24 and k = 12. The a_i values are listed in Table 6.

TABLE 6. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.627	$X^{(24)} - X^{(1)}$
2	0.3098	0.376	$X^{(23)} - X^{(2)}$
3	0.2554	0.298	$X^{(22)} - X^{(3)}$
4	0.2145	0.262	$X^{(21)} - X^{(4)}$
5	0.1807	0.200	$X^{(20)} - X^{(5)}$
6	0.1512	0.163	$X^{(19)} - X^{(6)}$
7	0.1245	0.162	$X^{(18)} - X^{(7)}$
8	0.0997	0.157	$X^{(17)} - X^{(8)}$
9	0.0764	0.117	$X^{(16)} - X^{(9)}$
10	0.0539	0.117	$X^{(15)} - X^{(10)}$
11	0.0321	0.065	$X^{(14)} - X^{(11)}$
12	0.0107	0.000	$X^{(13)} - X^{(12)}$

1.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 6. For the data in this example,

$$W = \frac{1}{0.4265}(0.6444)^2 = 0.974$$

11.13.2.6.7 The decision rule for this test is to compare W as calculated in Section 13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 24 observations is 0.884. Since W = 0.974 is greater than the critical value, conclude that the data are normally distributed.

11.13.2.7 Test for Homogeneity of Variance

11.13.2.7.1 The test used to examine whether the variation in mean proportion surviving is the same across all toxicant concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i)\ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each toxicant concentration and control, $V_i = (n_i - 1)$

n_i = the number of replicates for concentration i

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + (3(p-1))^{-1}[\sum_{i=1}^{P} \frac{1}{V_i} - (\sum_{i=1}^{P} V_i)^{-1}]$$

11.13.2.7.2 For the data in this example (see Table 3), all toxicant concentrations including the control have the same number of replicates ($n_i = 4$ for all i). Thus, $V_i = 3$ for all i.

11.13.2.7.3 Bartlett's statistic is therefore:

$$B = [(18)\ln(0.0236) - 3\sum_{i=1}^{P}\ln(S_i^2)]/1.1296$$

$$= [18(-3.7465) - 3(-24.7516)]/1.1296$$

$$= 6.8178/1.1296$$

$$= 6.036$$

11.13.2.7.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test (from a table of chi-square distribution), at a significance level of 0.01 with five degrees of freedom, is 15.086. Since B = 6.036 is less than the critical value of 15.086, conclude that the variances are not different.

11.13.2.8 Dunnett's Procedure

11.13.2.8.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 7.

TABLE 7. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number toxicant concentrations including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$ Between Sum of Squares

$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the proportion surviving for toxicant concentration i in test chamber j)

11.13.2.8.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 4$

N = 24

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 5.322$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 4.733$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 5.485$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 5.017$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 4.437$
$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} = 2.414$

$$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 27.408$$

$$SSB = \sum_{i=1}^{P} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$

$$= \frac{1}{4}(131.495) - \frac{(27.408)^2}{24} = 1.574$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$

$$= 33.300 - \frac{(27.408)^2}{24} = 2.000$$

$$SSW = SST - SSB = 2.000 - 1.574 = 0.4260$$

S_B^2 = SSB/(p-1) = 1.574/(6-1) = 0.3150

S_W^2 = SSW/(N-p) = 0.426/(24-6) = 0.024

11.13.2.8.3 Summarize these calculations in the ANOVA table (Table 8).

TABLE 8. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	5	1.574	0.315
Within	18	0.426	0.024
Total	23	2.002	

11.13.2.8.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean proportion surviving for concentration i

$\bar{Y}_1$ = mean proportion surviving for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

11.13.2.8.5 Table 9 includes the calculated t values for each concentration and control combination. In this example, comparing the 32 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.330 - 1.183)}{[0.155\sqrt{(1/4) + (1/4)}]} = 1.341$$

TABLE 9. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.341
64	3	-0.374
128	4	0.693
256	5	2.016
512	6	6.624

11.13.2.8.6 Since the purpose of this test is to detect a significant reduction in proportion surviving, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 18 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.41. The mean proportion surviving for concentration i is considered significantly less than the mean proportion surviving for the control if t_i is greater than the critical value. Since t_6 is greater than 2.41, the 512 µg/L concentration has significantly lower survival than the control. Hence the NOEC and the LOEC for survival are 256 µg/L and 512 µg/L, respectively.

11.13.2.8.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be detected statistically may be calculated.

$$MSD = d\ S_w\sqrt{(1/n_1) + (1/n)}$$

Where:

d = the critical value for Dunnett's procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

11.13.2.8.8 In this example:

$$MSD = 2.41\,(0.155)\sqrt{(1/4) + (1/4)}$$

$$= 2.41\ (0.155)(0.707)$$

$$= 0.264$$

11.13.2.8.9 The MSD (0.264) is in transformed units. To determine the MSD in terms of percent survival, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

   1.330 - 0.264 = 1.066

2. Obtain the untransformed values for the control mean and the difference calculated in 1.

   $[\text{Sine}\ (\ 1.330)\ ]^2 = 0.943$

   $[\text{Sine}\ (\ 1.066)\ ]^2 = 0.766$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from 2.

   $MSD_u = 0.943 - 0.766 = 0.177$

11.13.2.8.10 Therefore, for this set of data, the minimum difference in mean proportion surviving between the control and any toxicant concentration that can be detected as statistically significant is 0.177.

11.13.2.8.11 This represents a decrease in survival of 19% from the control.

11.13.2.9 Calculation of the LC50

11.13.2.9.1 The data used for the Probit Analysis is summarized in Table 10. To perform the Probit Analysis, run the USEPA Probit Analysis Program. An example of the program input and output is supplied in Appendix I.

TABLE 10. DATA FOR PROBIT ANALYSIS

	NaPCP Concentration (µg/L)					
	Control	32	64	128	256	512
Number Dead	2	6	1	4	9	27
Number Exposed	40	40	40	40	40	40

11.13.2.9.2 For this example, the chi-square test for heterogeneity was not significant, thus Probit Analysis appears appropriate for this data.

11.13.2.9.3 Figure 8 shows the output data for the Probit Analysis of the data in Table 10 using the USEPA Probit Program.

11.13.3 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW, *PIMEPHALES PROMELAS,* GROWTH DATA

11.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 9. The response used in the statistical analysis is mean weight per original organism for each replicate. Because this measurement is based on the number of original organisms exposed (rather than the number surviving), the measured response is a combined survival and growth endpoint that can be termed biomass. An IC estimate can be calculated for the growth data via a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain the NOEC for growth. Concentrations above the NOEC for survival are excluded from the hypothesis test for growth effects.

11.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

11.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

Probit Analysis of Fathead Minnow Larval Survival Data

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	40	2	0.0500	0.0000
32.0000	40	6	0.1500	0.0779
64.0000	40	1	0.0250	-.0577
128.0000	40	4	0.1000	0.0237
256.0000	40	9	0.2250	0.1593
512.0000	40	27	0.6750	0.6474

Chi - Square for Heterogeneity (calculated) = 4.522
Chi - Square for Heterogeneity
(Tabular value at 0.05 level) = 7.815

Probit Analysis of Fathead Minnow Larval Survival Data

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
LC/EC 1.00	127.637	34.590	195.433
LC/EC 50.00	422.696	345.730	531.024

Figure 8. Output for USEPA Probit Analysis Program, Version 1.5

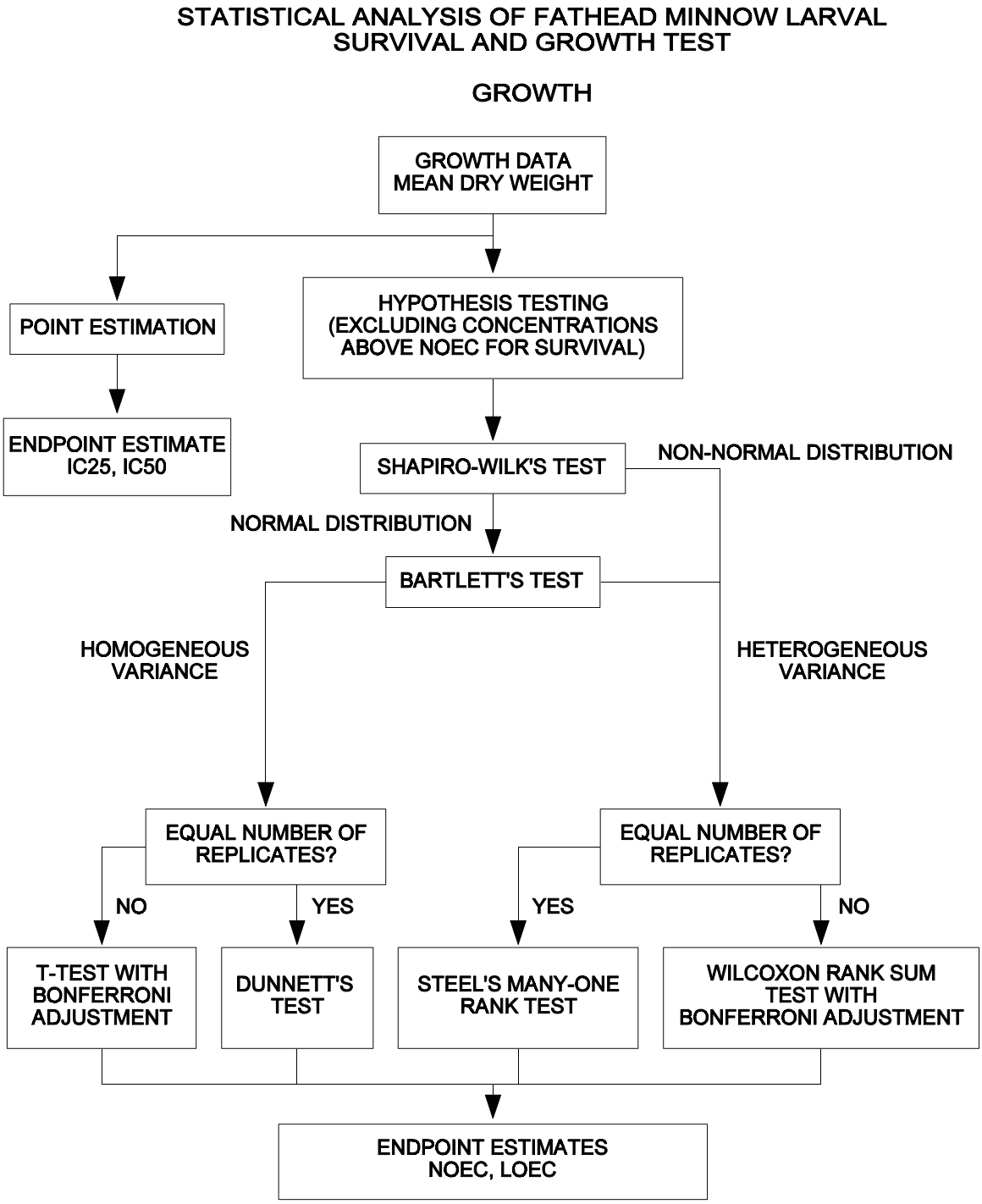

Figure 9. Flowchart for statistical analysis of fathead minnow, *Pimephales promelas,* larval growth data.

11.13.3.4 The data, mean and variance of the observations at each concentration including the control are listed in Table 11. A plot of the weight data for each treatment is provided in Figure 10. Since there is significant mortality in the 512 µg/L concentration, its effect on growth is not considered.

TABLE 11. FATHEAD MINNOW, *PIMEPHALES PROMELAS,* GROWTH DATA

		NaPCP Concentration (µg/L)				
Replicate	Control	32	64	128	256	512
A	0.711	0.517	0.602	0.566	0.455	-
B	0.662	0.501	0.669	0.612	0.502	-
C	0.646	0.723	0.694	0.410	0.606	-
D	0.690	0.560	0.676	0.672	0.254	-
Mean($\bar{Y}_i$)	0.677	0.575	0.660	0.565	0.454	-
S_i^2	0.00084	0.01032	0.00162	0.01256	0.0218	-
i	1	2	3	4	5	6

11.13.3.5 Test for Normality

11.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 12.

TABLE 12. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.034	-0.058	-0.058	0.001	0.001
B	-0.015	-0.074	0.009	0.047	0.048
C	-0.031	0.148	0.034	-0.155	0.152
D	0.013	-0.015	0.016	0.107	-0.200

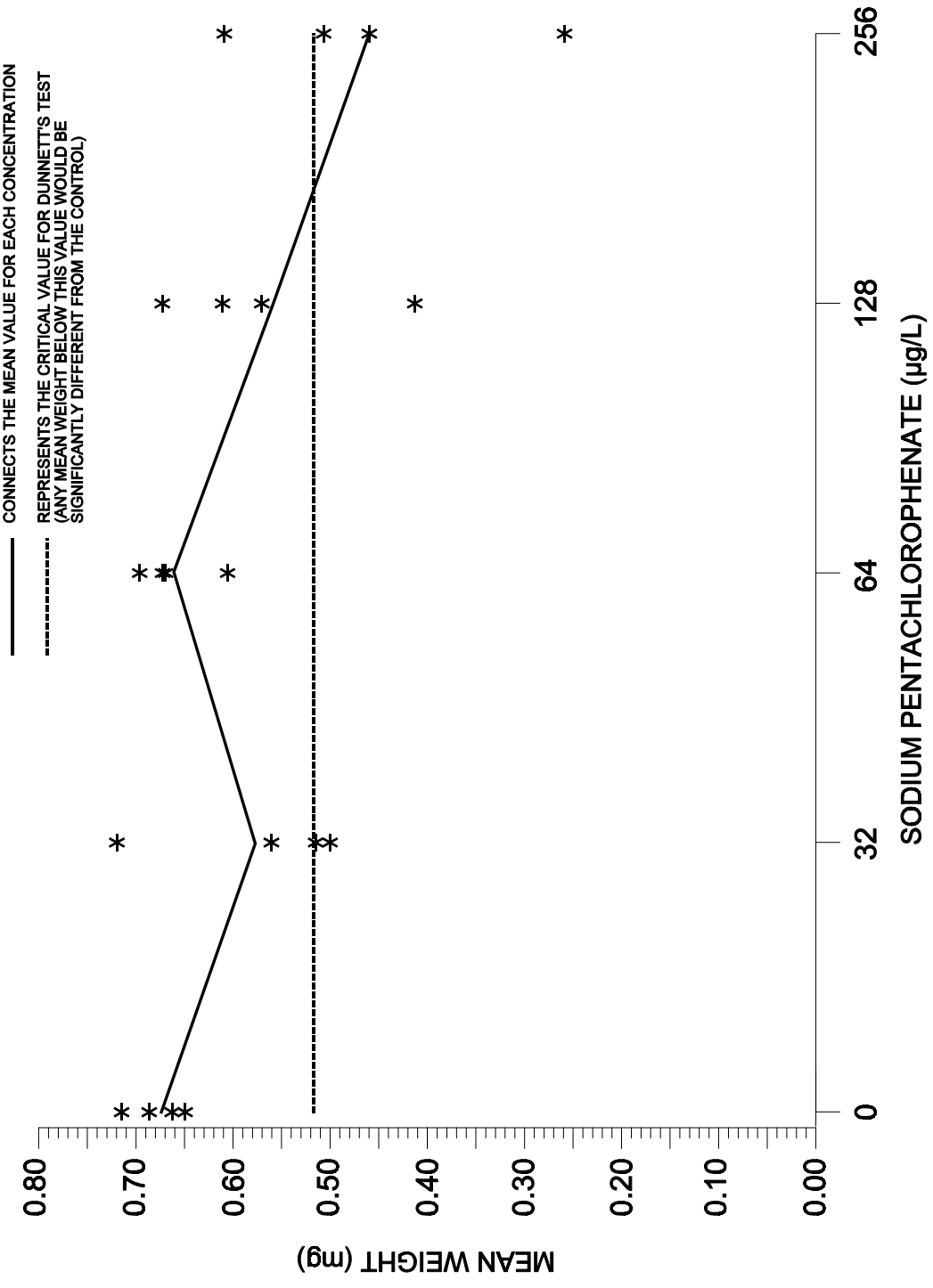

Figure 10. Plot of weight data from fathead minnow, *Pimephales promelas*, larval survival and growth test for point estimate testing.

11.13.3.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

For this set of data, n = 20

$$\bar{X} = \frac{1}{20}(0.004) = 0.000$$

D = 0.1414

11.13.3.5.3 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 13.

TABLE 13. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.200	11	0.009
2	-0.155	12	0.013
3	-0.074	13	0.016
4	-0.058	14	0.034
5	-0.058	15	0.034
6	-0.031	16	0.047
7	-0.015	17	0.048
8	-0.015	18	0.107
9	0.001	19	0.148
10	0.001	20	0.152

11.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table 14.

TABLE 14. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.352	$X^{(20)} - X^{(1)}$
2	0.3211	0.303	$X^{(19)} - X^{(2)}$
3	0.2565	0.181	$X^{(18)} - X^{(3)}$
4	0.2085	0.106	$X^{(17)} - X^{(4)}$
5	0.1686	0.105	$X^{(16)} - X^{(5)}$
6	0.1334	0.065	$X^{(15)} - X^{(6)}$
7	0.1013	0.049	$X^{(14)} - X^{(7)}$
8	0.0711	0.031	$X^{(13)} - X^{(8)}$
9	0.0422	0.012	$X^{(12)} - X^{(9)}$
10	0.0140	0.008	$X^{(11)} - X^{(10)}$

11.13.3.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}[\sum_{i=1}^{k} a_i(X^{(n-i+1)} - X^{(i)})]^2$$

the differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 14. For this set of data:

$$W = \frac{1}{0.1414}(0.3666)^2 = 0.9505$$

11.13.3.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 20 observations (n) is 0.868. Since W = 0.9505 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

11.13.3.6 Test for Homogeneity of Variance

11.13.3.6.1 The test used to examine whether the variation in mean dry weight is the same across all toxicant concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i)\ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each toxicant concentration and control, $V_i = (n_i - 1)$

n_i = the number of replicates for concentration i.

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + (3(p-1))^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

11.13.3.6.2 For the data in this example, (see Table 11) all toxicant concentrations including the control have the same number of replicates ($n_i = 4$ for all i). Thus, $V_i = 3$ for all i.

11.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(15)\ln(0.00947) - 3\sum_{i=1}^{P} \ln(S_i^2)]/1.133$$

$$= [15(-5.9145) - 3(-26.2842]/1.133$$

$$= 8.8911/1.133$$

$$= 7.847$$

11.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with four degrees of freedom, is 13.277. Since B = 7.847 is less than the critical value of 13.277, conclude that the variances are not different.

11.13.3.7 Dunnett's Procedure

11.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 15.

TABLE 15. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number toxicant concentrations including the control

N = total number of observations $n_1 + n_2 ... + n_p$

n_i = number of observations in concentration i

$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$ Between Sum of Squares

$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean dry weight of the fish for toxicant concentration i in test chamber j)

11.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 4$

N = 20

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 2.709$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 2.301$

$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 2.641$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.260$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 1.817$
$G = T_1 + T_2 + T_3 + T_4 + T_5 = 11.728$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$

$$= \frac{1}{4}(28.017) - \frac{(11.728)^2}{20} = 0.1270$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 7.146 - \frac{(11.728)^2}{20} = 0.2687$$

$SSW = SST - SSB = 0.2687 - 0.1270 = 0.1417$

$S_B^2 = SSB/(p-1) = 0.1270/(5-1) = 0.0318$

$S_W^2 = SSW/(N-p) = 0.041/(20-5) = 0.0094$

11.13.3.7.3 Summarize these calculations in the ANOVA table (Table 16).

TABLE 16. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	4	0.1270	0.0318
Within	15	0.1417	0.0094
Total	19	0.2687	

11.13.3.7.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean dry weight for toxicant concentration i

$\bar{Y}_1$ = mean dry weight for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

11.13.3.7.5 Table 17 includes the calculated t values for each concentration and control combination. In this example, comparing the 32 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(0.677 - 0.575)}{[0.097\sqrt{(1/4) + (1/4)}]} = 1.487$$

TABLE 17. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.487
64	3	0.248
128	4	1.632
256	5	3.251

11.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean weight, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 15 degrees of freedom for error and four concentrations (excluding the control) the critical value is 2.36. The mean weight for concentration "*i*" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Since t_5 is greater than 2.36, the 256 µg/L concentration had significantly lower growth than the control. Hence the NOEC and the LOEC for growth are 128 µg/L and 256 µg/L, respectively.

11.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\ S_w\sqrt{(1/n_1) + (1/n)}$$

Where: d = the critical value for the Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

11.13.3.7.8 In this example:

$$MSD = 2.36(0.052)\sqrt{(1/4) + (1/4)}$$

$$= 2.36\ (0.097)\ (0.707)$$

$$= 0.162$$

11.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.162 mg.

11.13.3.7.10 This represents a 24% reduction in mean weight from the control.

11.13.3.8 Calculation of the IC

11.13.3.8.1 The growth data in Table 2 modified to be mean weights per original number of fish are utilized in this example. As seen in Table 2 and Figure 11, the observed means are not monotonically non-increasing with respect to concentration (the mean response for each higher concentration is not less than or equal to the mean response for the previous concentration, and the responses between concentrations do not follow a linear trend). Therefore, the means are smoothed prior to calculating the IC. In the following discussion, the observed means are represented by $\bar{Y}_i$ and the smoothed means by M_i.

11.13.3.8.2 Starting with the control mean, $\bar{Y}_1 = 0.677$, we see that $\bar{Y}_1 > \bar{Y}_2$. Set $M_1 = \bar{Y}_1$ Comparing $\bar{Y}_2$ to $\bar{Y}_3$, $\bar{Y}_2 < \bar{Y}_3$.

11.13.3.8.3 Calculate the smoothed means:

$$M_2 = M_3 = (\bar{Y}_2 + \bar{Y}_3)/2 = 0.618$$

11.13.3.8.4 For the remaining observed means, $M_3 > \bar{Y}_4 > \bar{Y}_5 > \bar{Y}_6$. Thus, M_4 becomes $\bar{Y}_4$, M_5 becomes $\bar{Y}_5$ etc., for the remaining concentrations. Table 18 contains the smoothed means, and Figure 11 provides a plot of the smoothed concentration response curve.

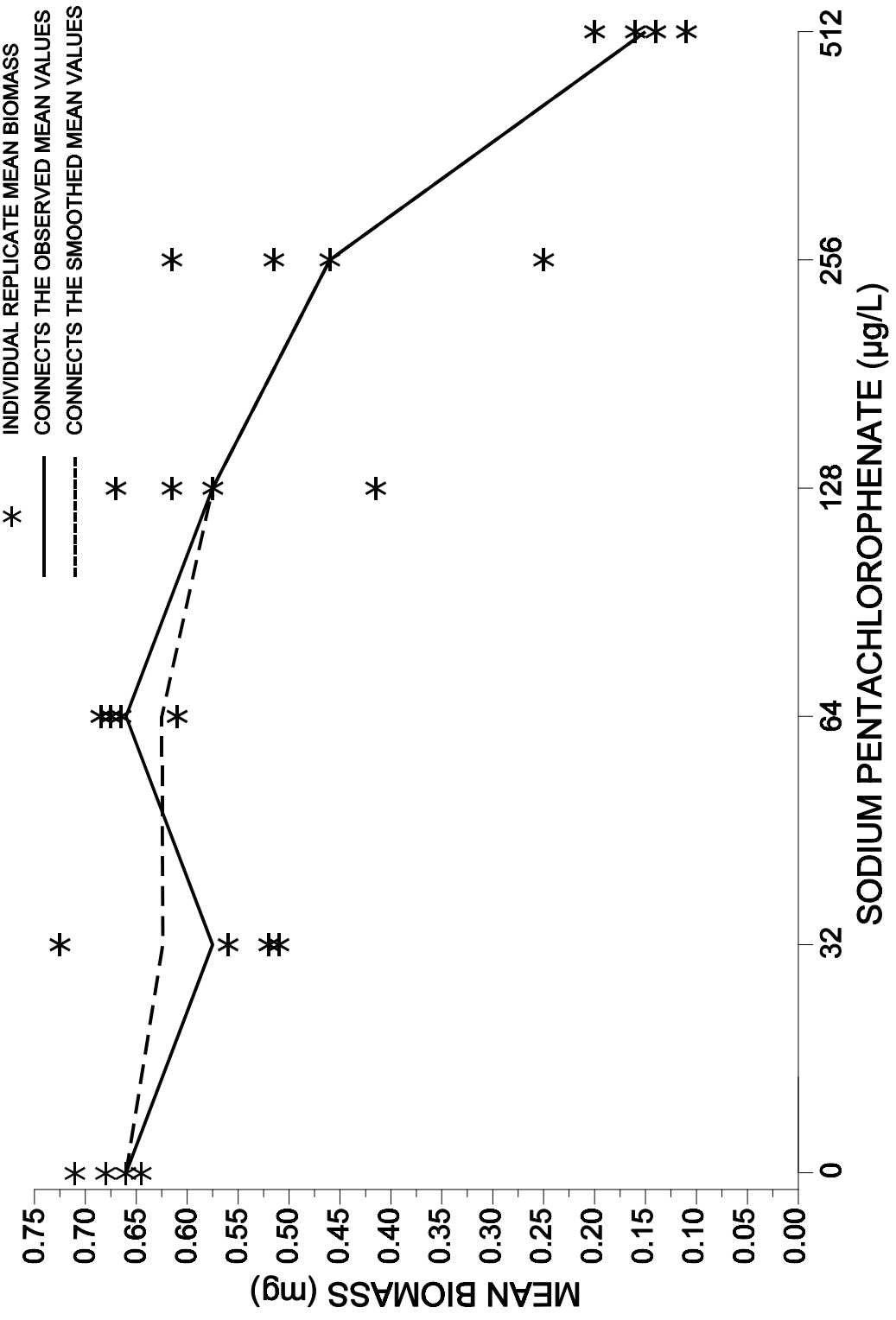

Figure 11. Plot of raw data, observed means, and smoothed means for the fathead minnow, *Pimephales promelas*, growth data in Tables 2 and 18.

TABLE 18. FATHEAD MINNOW, *PIMEPHALES PROMELAS*, MEAN GROWTH RESPONSE AFTER SMOOTHING

NaPCP Conc (µg/L)	i	Response means, $\bar{Y}_i$ (mg)	Smoothed means, M_i (mg)
Control	1	0.677	0.677
32	2	0.575	0.618
64	3	0.660	0.618
128	4	0.565	0.565
256	5	0.454	0.454
512	6	0.150	0.150

11.13.3.8.5 An IC25 and an IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in weight, compared to the controls, would result in a mean dry weight of 0.508 mg, where $M_1(1 - p/100) = 0.677(1 - 25/100)$. A 50% reduction in weight, compared to the controls, would result in a mean weight of 0.339 mg, where $M_1(1 - p/100) = 0.677(1 - 50/100)$. Examining the smoothed means and their associated concentrations (Table 18), the response 0.508 mg is bracketed by $C_4 = 128$ µg/L and $C_5 = 256$ µg/L. For the 50% reduction (0.339 mg), the response (0.339 µg) is bracketed by $C_5 = 256$ µg/L and $C_6 = 512$ µg/L.

11.13.3.8.6 Using the equation in Section 4.2 from Appendix M, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 128 + [0.677(1 - 25/100) - 0.565]\frac{(256 - 128)}{(0.454 - 0.565)}$$

$$= 194 \text{ µg/L}$$

11.13.3.8.7 Using the equation in Section 4.2 of Appendix M the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC50 = 256 + [0.677(1 - 50/100) - 0.454]\frac{(512 - 256)}{(0.150 - 0.454)}$$

$$= 353 \text{ µg/L}$$

11.13.3.8.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 193.9503 µg/L. The empirical 95% confidence interval for the true mean was (54.9278 µg/L, 340.6617 µg/L). The computer program output for the IC25 for this data set is shown in Figure 12.

11.13.3.8.9 When the ICPIN program was used to analyze this set of data for the IC50, requesting 80 resamples, the estimate of the IC50 was 353.2884 µg/L. The empirical 95% confidence interval for the true mean was 208.4723 µg/L and 418.5276 µg/L. The computer program output is shown in Figure 13.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	32	64	128	256	512
Response 1	0.711	0.517	0.602	0.566	0.455	0.143
Response 2	0.662	0.501	0.669	0.612	0.502	0.163
Response 3	0.646	0.723	0.694	0.410	0.606	0.195
Response 4	0.690	0.560	0.676	0.672	0.254	0.099

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: NaPCP
Test Start Date: Example Test Ending Date:
Test Species: Fathead minnows
Test Duration: 7-d
DATA FILE: fhmanual.icp
OUTPUT FILE: fhmanual.i25

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	0.677	0.029	0.677
2	4	32.000	0.575	0.102	0.618
3	4	64.000	0.660	0.040	0.618
4	4	128.000	0.565	0.112	0.565
5	4	256.000	0.454	0.148	0.454
6	4	512.000	0.150	0.040	0.150

The Linear Interpolation Estimate: 193.9503 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 186.4935 Standard Deviation: 52.6094
Original Confidence Limits: Lower: 107.0613 Upper: 285.6449
Expanded Confidence Limits: Lower: 54.9278 Upper: 340.6617
Resampling time in Seconds: 1.81 Random Seed: 1272173518

Figure 12. ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	32	64	128	256	512
Response 1	0.711	0.517	0.602	0.566	0.455	0.143
Response 2	0.662	0.501	0.669	0.612	0.502	0.163
Response 3	0.646	0.723	0.694	0.410	0.606	0.195
Response 4	0.690	0.560	0.676	0.672	0.254	0.099

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: NaPCP
Test Start Date: Example Test Ending Date:
Test Species: Fathead minnows
Test Duration: 7-d
DATA FILE: fhmanual.icp
OUTPUT FILE: fhmanual.i50

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	0.677	0.029	0.677
2	4	32.000	0.575	0.102	0.618
3	4	64.000	0.660	0.040	0.618
4	4	128.000	0.565	0.112	0.565
5	4	256.000	0.454	0.148	0.454
6	4	512.000	0.150	0.040	0.150

The Linear Interpolation Estimate: 353.2884 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 345.1108 Standard Deviation: 37.0938
Original Confidence Limits: Lower: 262.7783 Upper: 394.0629
Expanded Confidence Limits: Lower: 208.4723 Upper: 418.5276
Resampling time in Seconds: 1.87 Random Seed: 1126354766

Figure 13. ICPIN program output for the IC50.

11.14 PRECISION AND ACCURACY

11.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 11.14.1.1 and 11.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

11.14.1.1 Single-Laboratory Precision

11.14.1.1.1 Information on the single-laboratory precision of the fathead minnow larval survival and growth test is presented in Table 19. The range of NOECs was only two concentration intervals, indicating good precision.

11.14.1.1.2 EPA evaluated within-laboratory precision of the Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test using a database of routine reference toxicant test results from 19 laboratories (USEPA, 2000b). The database consisted of 205 reference toxicant tests conducted in 19 laboratories using a variety of reference toxicants including: cadmium, chromium, copper, potassium chloride, sodium chloride, sodium pentachlorophenate, and sodium dodecyl sulfate. Among the 19 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 26% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 21%; and in 75% of laboratories, the within-laboratory CV was less than 38%.

TABLE 19. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAL SURVIVAL AND GROWTH TEST, USING NAPCP AS A REFERENCE TOXICANT[1,2]

Test	NOEC (µg/L)	LOEC (µg/L)	Chronic Value (µg/L)
1	256	512	362
2	128	256	181
3	256	512	362
4	128	256	181
5	128	256	181
n:	5	5	5
Mean:	NA	NA	253.4

[1] From Pickering, 1988.
[2] For a discussion of the precision of data from chronic toxicity tests, (see Section 4, Quality Assurance).

11.14.1.2 Multilaboratory Precision

11.14.1.2.1 A multilaboratory study of Method 1000.0 described in the first edition of this manual (USEPA, 1985e), was performed using seven blind samples over an eight month period (DeGraeve et. al., 1988). In this study, each of the 10 participating laboratories was to conduct two tests simultaneous with each sample, each test having two replicates of 10 larvae for each of five concentrations and the control. Of the 140 tests planned, 135 were completed. Only nine of the 135 tests failed to meet the acceptance criterion of 80% survival in the controls. Of the 126 acceptable survival NOECs reported, an average of 41% were median values, and 89% were within one concentration interval of the median (Table 20). For the growth (weight) NOECs, an average of 32% were at the median, and 84% were within one concentration interval of the median (Table 21). Using point estimate techniques, the precision (CV) of the IC50 was 19.5% for the survival data and 19.8% for the growth data. If the mean weight acceptance criterion of 0.25 mg for the surviving control larvae, which is included in this revised edition of the method, had applied to the test results of the interlaboratory study, one third of the 135 tests would have failed to meet the test criteria (Norberg-King, personal communication and 1989 memorandum; DeGraeve et al., 1991).

11.14.1.2.2 In 2000, EPA conducted an interlaboratory variability study of the Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 27 participant laboratories tested 3 or 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Of the 101 Fathead Minnow Larval Survival and Growth tests conducted in this study, 98.0% were successfully completed and met the required test acceptability criteria. Of 24 tests that were conducted on blank samples, none showed false positive results for survival endpoints, and only one resulted in false positive results for the growth endpoint, yielding a false positive rate of 4.35%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 22 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 20.9% for IC25 results. Table 23 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned four concentrations for the reference toxicant sample type and two concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 97.2%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively. For the growth endpoint, NOEC values spanned five concentrations for the reference toxicant sample type and four concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 86.1%, 91.7%, and 76.9% for the reference toxicant, effluent, and receiving water sample types, respectively.

11.14.2 ACCURACY

11.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 20. COMBINED FREQUENCY DISTRIBUTION FOR SURVIVAL NOECs FOR ALL LABORATORIES[1]

Sample	NOEC Frequency (%) Distribution					
	Tests with Two Reps			Tests with Four Reps		
	Median	± 1[2]	> 2[3]	Median	± 1[2]	> 2[3]
1. Sodium Pentachlorophenate (A)	35	53	12	57	29	14
2. Sodium Pentachlorophenate (B)	42	42	16	56	44	0
3. Potassium Dichromate (A)	47	47	6	75	25	0
4. Potassium Dichromate (B)	41	41	18	50	50	0
5. Refinery Effluent 301	26	68	6	78	22	0
6. Refinery Effluent 401	37	53	10	56	44	0
7. Utility Waste 501	56	33	11	56	33	11

[1] From DeGraeve et al., 1988.
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.

TABLE 21. COMBINED FREQUENCY DISTRIBUTION FOR WEIGHT NOECs FOR ALL LABORATORIES[1]

	NOEC Frequency (%) Distribution					
	Tests with Two Reps			Tests with Four Reps		
Sample	Median	± 1[2]	> 2[3]	Median	± 1[2]	> 2[3]
1. Sodium Pentachlorophenate (A)	59	41	0	57	43	0
2. Sodium Pentachlorophenate (B)	37	63	0	22	45	33
3. Potassium Dichromate (A)	35	47	18	88	0	12
4. Potassium Dichromate (B)	12	47	41	63	25	12
5. Refinery Effluent 301	35	53	12	75	25	0
6. Refinery Effluent 401	37	47	16	33	56	11
7. Utility Waste 501	11	61	28	33	56	11

[1] From DeGraeve et al., 1988.
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.

TABLE 22. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	10.0	17.2	19.9
	Effluent	19.1	12.9	23.1
	Receiving water	-	-	19.8
	Average	14.6	15.0	20.9

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

[3] The within-laboratory component of variability for duplicate samples tested at the same time in the same laboratory.

[4] The between-laboratory component of variability for duplicate samples tested at different laboratories..

[5] The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 23. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	50%	75.0	22.2	2.78
	Effluent	12.5%	76.9	23.1	0.00
	Receiving water	25%	69.2	30.8	0.00
Growth NOEC	Reference toxicant	50%	58.3	27.8	13.9
	Effluent	12.5%	66.7	25.0	8.33
	Receiving water	12.5%	30.8	46.1	23.1

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.

SECTION 12

TEST METHOD

FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST METHOD 1001.0

12.1 SCOPE AND APPLICATION

12.1.1 This method estimates the chronic toxicity of whole effluents and receiving water to the fathead minnow, *Pimephales promelas*, using embryos in a seven-day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms. The test is useful in screening for teratogens because organisms are exposed during embryonic development.

12.1.2 Daily observations on mortality make it possible to also calculate the acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

12.1.3 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

12.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly degradable and highly volatile toxicants, in the source may not be detected in the test.

12.1.5 This test method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

12.2 SUMMARY OF METHOD

12.2.1 Fathead minnow, *Pimephales promelas*, embryos are exposed in a static renewal system to different concentrations of effluent or to receiving water for seven days, starting shortly after fertilization of the eggs. Test results are based on the total frequency of both mortality and gross morphological deformities (terata).

12.3 INTERFERENCES

12.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

12.3.2 Adverse effects of low dissolved oxygen (DO), high concentrations of suspended and/or dissolved solids, and extremes of pH may mask the presence of toxic substances.

12.3.3 Improper effluent sampling and sample handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.3.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival and confound test results.

12.3.5 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with

increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 12.3.5.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 12.3.5.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

12.3.5.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 12.3.5.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample upon completion of collection (as measured on an aliquot removed from the sample container).

12.3.5.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ±0.2 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ± 0.2 pH units in pH-controlled tests (USEPA, 1992).

12.3.5.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

12.3.5.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 12.3.6.1.1).

12.3.5.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 12.3.5.2) is applied routinely to subsequent testing of the effluent.

12.3.5.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992); or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents.

If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, CO_2 is injected to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, CO_2 is injected to maintain the test pH at the pH of the sample upon completion of collection. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

12.4 **SAFETY**

12.4.1 See Section 3, Health and Safety.

12.5 **APPARATUS AND EQUIPMENT**

12.5.1 Fathead minnow and brine shrimp culture units -- See Section 11, Fathead Minnow, *Pimephales Promelas*, Larval Survival and Growth Test, and USEPA, 2002a. To test effluent toxicity on-site or in the laboratory, sufficient numbers of newly fertilized eggs must be available, preferably from a laboratory fathead minnow culture unit. If necessary, embryos can be shipped in well oxygenated water in insulated containers. In cases where shipping is necessary, up to 48-h old embryos may be used for the test.

12.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L or more.

12.5.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.5.4 Environmental chamber or equivalent facility with temperature control (25 ± 1 °C).

12.5.5 Water purification system -- MILLIPORE MILLI-Q®, deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

12.5.6 Balance -- analytical, capable of accurately weighing to 0.00001 g.

12.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of material to be weighed.

12.5.8 Test chambers -- four borosilicate glass or disposable, non-toxic plastic labware, per test solution, such as: 500-mL beakers; 100 mm x 15 mm or 100 mm x 20 mm glass or disposable polystyrene Petri dishes; or 12-cm OD, stackable "Carolina" culture dishes. The chambers should be covered with safety glass plates or sheet plastic during the test to avoid potential contamination from the air and excessive evaporation of the test solutions during the test.

12.5.9 Dissecting microscope, or long focal length magnifying lens, hand or stand supported -- for examining embryos and larvae in the test chambers.

12.5.10 Light box, microscope lamp, or flashlight -- for illuminating chambers during examination and observation of embryos and larvae.

12.5.11 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL, for making test solutions.

12.5.12 Volumetric pipets -- Class A, 1-100 mL.

12.5.13 Serological pipets -- 1-10 mL, graduated.

12.5.14 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

12.5.15 Droppers, and glass tubing with fire polished edges, 2-mm ID -- for transferring embryos, and 4-mm ID -- for transferring larvae.

12.5.16 Wash bottles -- for washing embryos from substrates and containers and for rinsing small glassware and instrument electrodes and probes.

12.5.17 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

12.5.18 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

12.5.19 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA 1979b) -- to calibrate laboratory thermometers.

12.5.20 Meters, pH, DO, and specific conductivity -- for routine physical and chemical measurements.

12.6 **REAGENTS AND CONSUMABLE MATERIALS**

12.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling and Sample Preparation for Toxicity Tests).

12.6.2 Data sheets (one set per test) -- for recording data.

12.6.3 Tape, colored -- for labelling test chambers.

12.6.4 Markers, waterproof -- for marking containers, etc.

12.6.5 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA 1979b.

12.6.6 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA 1979b), or reagents -- for modified Winkler analysis.

12.6.7 Standard pH buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for instrument calibration (see USEPA Method 150.1, USEPA 1979b).

12.6.8 Specific conductivity standards -- see USEPA Method 120.1, USEPA 1979b.

12.6.9 Laboratory quality control samples and standards -- for calibration of the above methods.

12.6.10 Reference toxicant solutions -- see Section 4, Quality Assurance.

12.6.11 Reagent water -- defined as distilled or deionized water which does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

12.6.12 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

12.6.13 TEST ORGANISMS, FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

12.6.13.1 Fathead minnow embryos, less than 36-h old, are used for the test. The test is conducted with four (minimum of three) test chambers at each toxicant concentration and control. Fifteen (minimum of ten) embryos are placed in each replicate test chamber. Thus 60 (minimum of 30) embryos are exposed at each test concentration and 360 (minimum of 180) embryos would be needed for a test consisting of five effluent concentrations and a control.

12.6.13.2 Sources of Organisms

12.6.13.2.1 It is recommended that the embryos be obtained from inhouse cultures or other local sources if at all possible, because it is often difficult to ship the embryos so that they will be less than 36 h old for beginning the test. Receipt of embryos via Express Mail, air express, or other carrier, from a reliable outside source is an acceptable alternative, but they must not be over 48 h old when used to begin the test.

12.6.13.2.2 Culturing methods for fathead minnows, *Pimephales promelas*, are described in Section 6, Section 11 and in USEPA, 2002a.

12.6.13.2.3 Fish obtained from outside sources (see Section 5, Facilities, Equipment, and Supplies) such as commercial biological supply houses for use as brood stock should be guaranteed to be (1) of the correct species, (2) disease free, (3) in the requested age range, and (4) in good condition. This can be done by providing the record of the date on which the eggs were laid and hatched, and information on the sensitivity of the contemporary fish to reference toxicants.

12.6.13.3 Obtaining Embryos for Toxicity Tests from Inhouse Cultures.

12.6.13.3.1 Spawning substrates with the newly-spawned, fertilized embryos are removed from the spawning tanks or ponds, and the embryos are separated from the spawning substrate by using the index finger and rolling the embryos gently with a circular movement of the finger (see Gast and Brungs, 1973). The embryos are then combined and washed from the spawning substrate onto a 400 µm NITEX® screen, sprayed with a stream of deionized water to remove detritus and food particles, and back-washed with dilution water into a crystallizing dish for microscopic examination. Damaged and infertile eggs are discarded.

12.6.13.3.2 The embryos from three or more spawns are pooled in a single container to provide a sufficient number to conduct the tests. These embryos may be used immediately to start a test inhouse or may be transported for use at a remote location. When transportation is required, embryos should be taken from the substrates within 12 h of spawning. This permits off-site tests to be started with less than 36-h old embryos. Embryos should be transported or shipped in clean, opaque, insulated containers, in well aerated or oxygenated fresh culture or dilution water, and should be protected from extremes of temperature and any other stressful conditions during transport. Instantaneous changes of water temperature when embryos are transferred from culture unit water to test dilution water, or from transport container water to on-site test dilution water, should be less than 2°C. Sudden changes in pH, dissolved ions, osmotic strength, and DO should be avoided.

12.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

12.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

12.8 CALIBRATION AND STANDARDIZATION

12.8.1 See Section 4, Quality Assurance.

12.9 **QUALITY CONTROL**

12.9.1 See Section 4, Quality Assurance.

12.10 **TEST PROCEDURES**

12.10.1 TEST SOLUTIONS

12.10.1.1 Receiving Waters

12.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 100 mL, and 400 mL for chemical analysis, would require approximately one liter, or more, of sample per test day.

12.10.1.2 Effluents

12.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5 and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.**

12.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions should be added at the lower range of effluent concentrations.

12.10.1.2.3 The volume of effluent required for daily renewal of four replicates per concentration, each containing 100 mL of test solution, is 1.5 L. Sufficient test solution (approximately 1000 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses. If the sample is used for more than one daily renewal of test solutions, the volume must be increased proportionately.

12.10.1.2.4 Tests should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for the off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.10.1.2.5 Just prior to test initiation (approximately 1 h) the temperature of sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature and maintained at that temperature during the addition of dilution water.

12.10.1.2.6 The DO of the test solutions should be checked prior to test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If any solution has a DO below 4.0 mg/L, all of the solutions and the control must be gently aerated.

12.10.1.3 Dilution Water

12.10.1.3.1 Dilution water may be uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other uncontaminated natural water (see Section 7, Dilution Water).

12.10.1.3.2 If the hardness of the test solutions (including the control) does not equal or exceed 25 mg/L as $CaCO_3$, it may be necessary to adjust the hardness by adding reagents for synthetic softwater as listed in Table 3, Section 7. In this case parallel tests should be conducted, one with the hardness adjusted and one unadjusted.

12.10.2 START OF THE TEST

12.10.2.1 Label the test chambers with a marking pen and use color-coded tape to identify each treatment and replicate. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) should have four (minimum of three) replicates.

12.10.2.2 Tests performed in laboratories that have inhouse fathead minnow breeding cultures must initiate tests with embryos less than 36 h old. When the embryos must be shipped to the test site from a remote location, it may be necessary to use embryos older than 36 h because of the difficulty of coordinating test organism shipments with field operations. However, in the latter case, the embryos must not be more than 48 h old at the start of the test and should all be within 24 h of the same age.

12.10.2.3 Randomize the position of the test chambers at the beginning of the test (see Appendix A). Maintain the chambers in this configuration throughout the test. Preparation of a position chart may be helpful.

12.10.2.4 The test organisms should come from a pool of embryos consisting of at least three separate spawnings. Gently agitate and mix the embryos to be used in the test in a large container so that eggs from different spawns are thoroughly mixed.

12.10.2.5 Using a small bore (2 mm ID) glass tube, the embryos are placed one or two at a time into each randomly arranged test chamber or intermediate container in sequential order, until each chamber contains 15 (minimum of 10) embryos, for a total of 60 (minimum of 30) embryos for each concentration (see Appendix A). The amount of water added to the chambers when transferring the embryos to the compartments should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

12.10.2.6 After the embryos have been distributed to each test chamber, examine and count them. Remove and discard damaged or infertile eggs and replace with new undamaged embryos. Placing the test chambers on a light table may facilitate examining and counting the embryos.

12.10.3 LIGHT, PHOTOPERIOD AND TEMPERATURE

12.10.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The water temperature in the test chambers should be maintained at 25 ± 1 °C.

12.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

12.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO concentrations. The DO concentrations should be measured in the new solutions at the start of the test (Day 0) and before daily renewal of the new solutions on subsequent days. The DO concentrations should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all concentrations and the control should be aerated. The aeration rate should not exceed 100 bubbles/min, using a pipet with an orifice of approximately 1.5 mm, such as a l-mL KIMAX® serological Pipet, or equivalent. Care should be taken to ensure that turbulence resulting from the aeration does not cause undue physical stress to the embryos.

12.10.5 FEEDING

12.10.5.1 Feeding is not required.

12.10.6 OBSERVATIONS DURING THE TEST

12.10.6.1 Routine Chemical and Physical Determinations

12.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period in at least one test chamber at each test concentrations and in the control.

12.10.6.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test chamber at each test concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test vessels, at least at the end of the test, to determine temperature variation in the environmental chamber.

12.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

12.10.6.1.4 Conductivity, alkalinity and hardness are measured in each new sample (100% effluent or receiving water) and in the control.

12.10.6.2 Record all the measurements on the data sheet (Figure 1).

12.10.6.3 Routine Biological Observations

12.10.6.3.1 At the end of the first 24 h of exposure, before renewing the test solutions, examine the embryos. Remove the dead embryos (milky colored and opaque) and record the number (Figure 2). If the rate of mortality (including those with fungal infection) exceeds 20% in the control chambers, or if excessive non-concentration-related mortality occurs, terminate the test and start a new test with new embryos.

12.10.6.3.2 At 25°C, hatching may begin on the fourth day. After hatching begins, count the number of dead and live embryos and the number of hatched, dead, live, and deformed larvae, daily. Deformed larvae are those with gross morphological abnormalities such as lack of appendages, lack of fusiform shape (non-distinct mass), lack of mobility, a colored, beating heart in an opaque mass, or other characteristics that preclude survival. Count and remove dead embryos and larvae as previously discussed and record the numbers for all of the test observations (Figure 2). Upon hatching, deformed larvae are counted as dead.

12.10.6.3.3 Protect the embryos and larvae from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of dead organisms carefully. Make sure that the test organisms remain immersed during the performance of the above operations.

12.10.7 DAILY CLEANING OF TEST CHAMBERS

12.10.7.1 Since feeding is not required, test chambers are not cleaned daily unless accumulation of particulate matter at the bottom of the chambers causes a problem.

12.10.8 TEST SOLUTION RENEWAL

12.10.8.1 Freshly prepared solutions are used to renew the tests daily. For on-site toxicity studies, fresh effluent or receiving water samples should be collected daily, and no more than 24 h should elapse between collection of the samples and their use in the tests (see Section 8, Effluent and Receiving Water Sampling, Sample Handling and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples are collected, preferably on days one, three, and five. Maintain the samples in the refrigerator at 0-6°C until used.

12.10.8.2 The test solutions are renewed immediately after removing dead embryos and/or larvae. During the daily renewal process, the water level in each chamber is lowered to a depth of 7 to 10 mm, which leaves 15 to 20% of the test solution. New test solution should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence and possible injury to the embryos or larvae.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Routine chemical and physical determinations.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Routine chemical and physical determinations (CONTINUED)

Discharger:________________________ Test Dates:________________________

Location:________________________ Analyst:________________________

____

Conc:	Rep. No.	Condition of Embryo/larvae	Day						
			1	2	3	4	5	6	7
Control	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							

Figure 2. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Survival and terata data.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Conc:	Rep. No.	Condition of Embryo/larvae	Day						
			1	2	3	4	5	6	7
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							

Comments:

Figure 2. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Survival and terata data (CONTINUED).

12.10.9 TERMINATION OF THE TEST

12.10.9.1 The test is terminated after seven days of exposure. Count the number of surviving, dead, and deformed larvae, and record the numbers of each (Figure 2). The deformed larvae are treated as dead in the analysis of the data. Keep a separate record of the total number and percent of deformed larvae for use in reporting the teratogenicity of the test solution.

12.10.9.2 Prepare a summary of the data as illustrated in Figure 3.

12.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

12.11.1 A summary of test conditions and test acceptability criteria is presented in Table 1.

12.12 **ACCEPTABILITY OF TEST RESULTS**

12.12.1 For the test results to be acceptable, survival in the controls must be at least 80%.

Discharger: _____ Test Dates: _____

Location: _____ Analyst: _____

Treatment	Control					
No. dead embryos and larvae						
No. terata						
Total mortality (dead and deformed)						
Total mortality (%)						
Terata (%)						
Hatch (%)						

Comments:

Figure 3. Summary data for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test.

TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS,* EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1001.0)[1]

1.	Test type:	Static renewal (required)
2.	Temperature:	25 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
3.	Light quality:	Ambient laboratory illumination (recommended)
4.	Light intensity:	10-20 μE/m^2/s or 50-100 ft-c (ambient laboratory levels) (recommended)
5.	Photoperiod:	16 h light, 8 h dark (recommended)
6.	Test chamber size:	150 mL (recommended minimum)
7.	Test solution volume:	70 mL (recommended minimum)
8.	Renewal of test solutions:	Daily (required)
9.	Age of test organisms:	Less than 36-h old embryos (Maximum of 48-h if shipped) (required)
10.	No. embryos per test chamber:	15 (recommended) 10 (required minimum)
11.	No. replicate test chambers per concentration:	4 (recommended) 3 (required minimum)
12.	No. embryos per concentration:	60 (recommended) 30 (required minimum)
13.	Feeding regime:	Feeding not required
14.	Aeration:	None unless DO falls below 4.0 mg/L (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS,* EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1001.0) (CONTINUED)

15. Dilution water:	Uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or DMW (see Section 7, Dilution Water). The hardness of the test solutions should equal or exceed 25 mg/L ($CaCO_3$) to ensure hatching success (available options)
16. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving waters: 100% receiving water (or minimum of 5) and a control (recommended)
17. Dilution factor:	Effluents: ≥ 0.5 (recommended) Receiving waters: None, or ≥ 0.5 (recommended)
18. Test duration:	7 days (required)
19. Endpoint:	Combined mortality (dead and deformed organisms) (required)
20. Test acceptability criteria:	80% or greater survival in controls (required)
21. Sampling requirements:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
22. Sample volume required:	1.5 to 2.5 L/day depending on the volume of test solutions used (recommended)

12.13 **DATA ANALYSIS**

12.13.1 GENERAL

12.13.1.1 Tabulate and summarize the data (Figure 3).

12.13.1.2 The endpoints of this toxicity test are based on total mortality, combined number of dead embryos, and dead and deformed larvae. The EC1 is calculated using Probit Analysis (Finney, 1971; see Appendix I). Separate analyses are performed for the estimation of LOEC and NOEC endpoints and for the estimation of the EC1 endpoint. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the EC1 endpoint. See the Appendices for examples of the manual computations and examples of data input and output for the computer programs.

12.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

12.13.2 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY DATA

12.13.2.1 Formal statistical analysis of the total mortality data is outlined on the flowchart in Figure 4. The response used in the analysis is the total mortality proportion in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the EC endpoint. Concentrations at which there is 100% total mortality in all of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the EC1 endpoint.

12.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

12.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

12.13.2.4 Probit Analysis (Finney, 1971) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined.

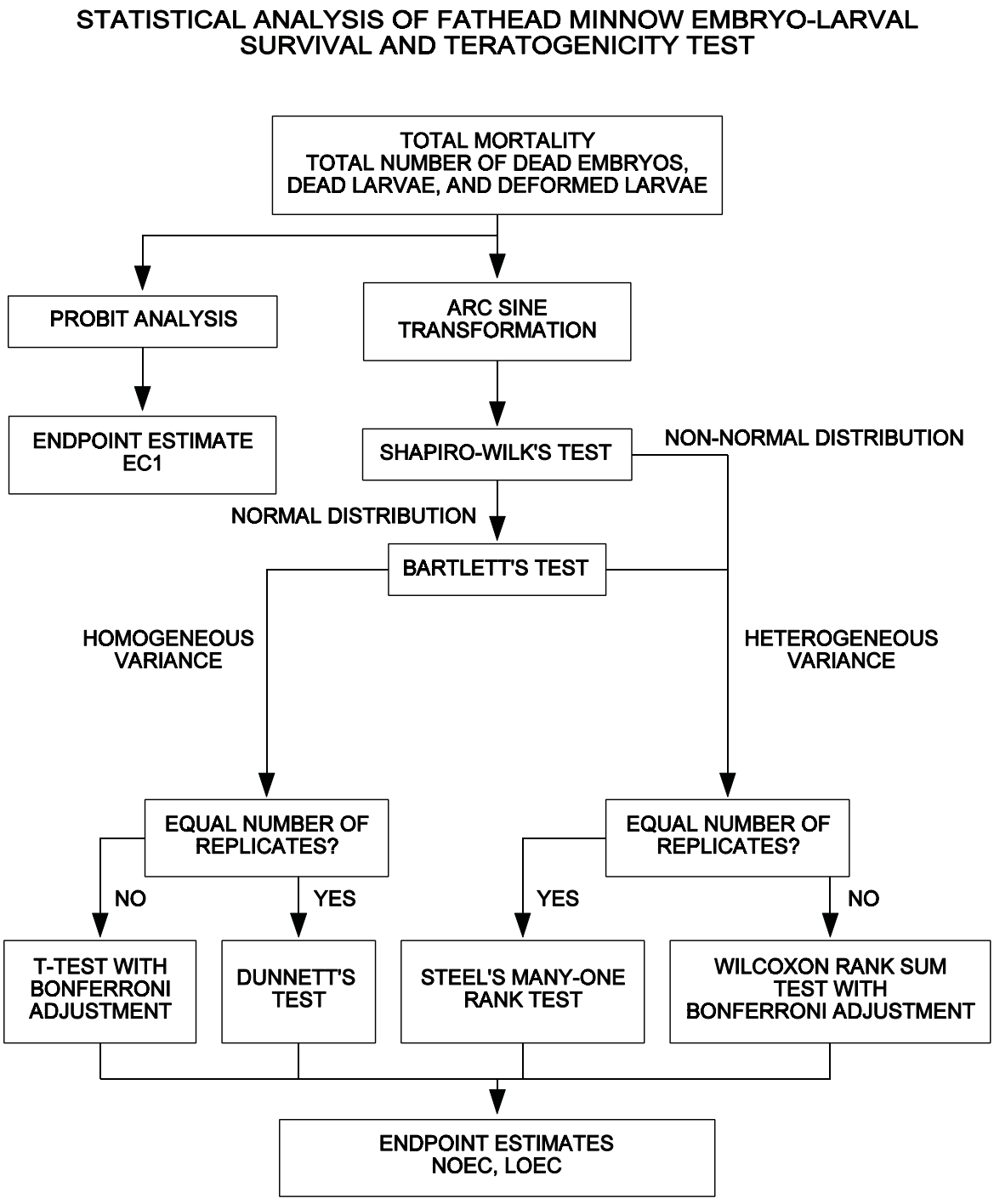

Figure 4. Flowchart for statistical analysis of fathead minnow, *Pimephales promelas*, embryo-larval data.

12.13.2.5 The data for this example are listed in Table 2. Total mortality, expressed as a proportion (combined total number of dead embryos, dead larvae and deformed larvae divided by the number of embryos at start of test), is the response of interest. The total mortality proportion in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and control are listed in Table 3. A plot of the data is provided in Figure 5. Since there is 100% total mortality in replicates for the 50.0% concentration, it is not included in this statistical analysis and is considered a qualitative mortality effect.

TABLE 2. DATA FROM FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL TOXICITY TEST WITH GROUND WATER EFFLUENT

Effluent Conc. (%)	No. Eggs at Start	Dead at Test Termination		Deformed at Test Termination		Dead + Deformed at Termination	
		No.	%	No.	%	No.	%
Control	10	0	0	0	0	0	0
	10	2	20	0	0	2	20
	10	0	0	0	0	0	0
	10	1	10	0	0	1	10
3.125	10	0	0	0	0	0	0
	10	0	0	1	10	1	10
	10	0	0	0	0	0	0
	10	1	10	0	0	1	10
6.25	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	0	0	1	10	1	10
12.5	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	1	10	0	0	1	10
25.0	10	1	10	9	90	10	100
	10	2	20	8	80	10	100
	10	2	20	8	80	10	100
	10	1	10	4	40	5	50
50.0	10	4	40	6	60	10	100
	10	3	30	7	70	10	100
	10	5	50	5	50	10	100
	10	3	30	7	70	10	100

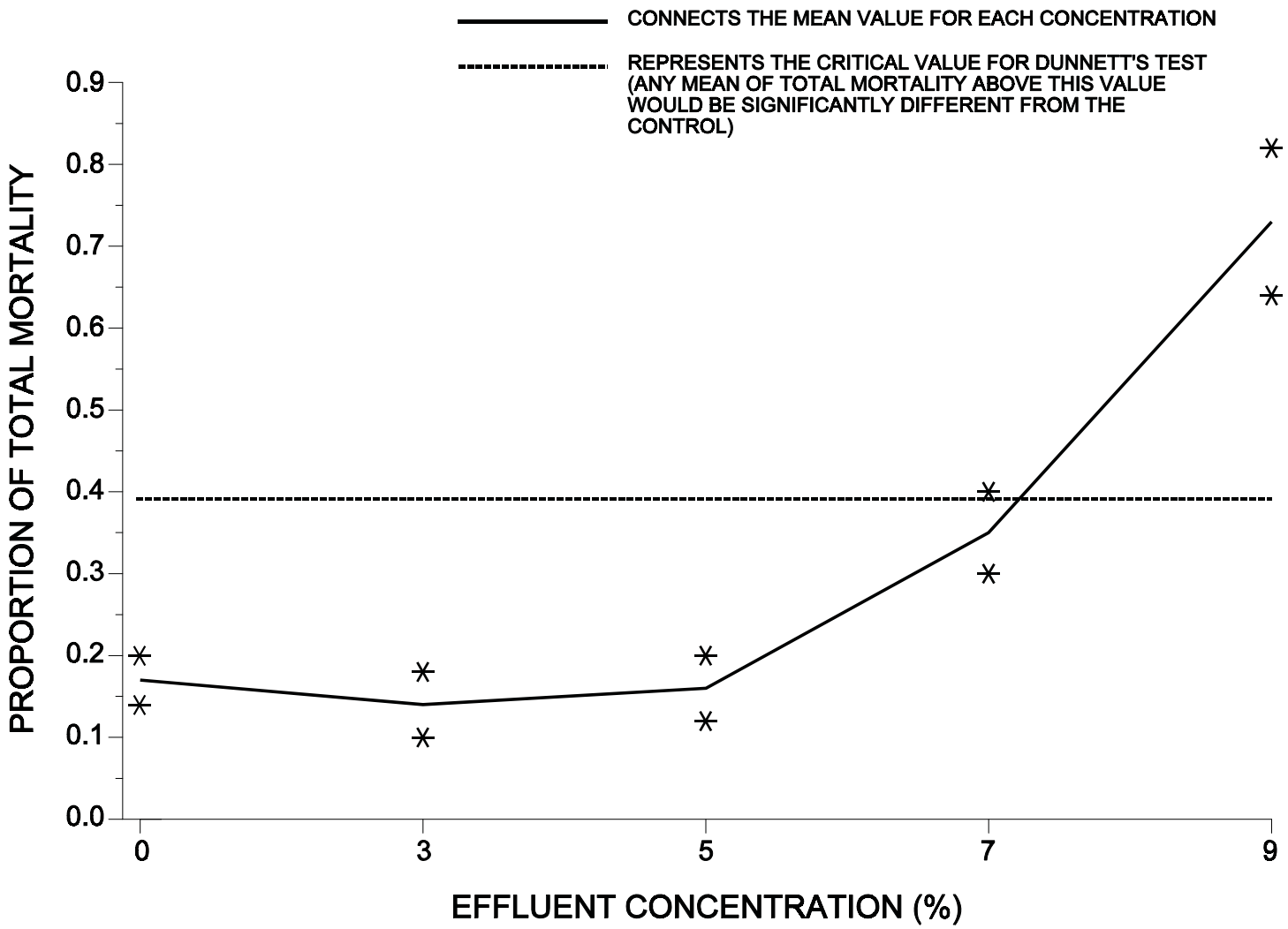

Figure 5. Plot of fathead minnow, *Pimephales promelas*, total mortality data from the embryo-larval test.

TABLE 3. FATHEAD MINNOW, *PIMEPHALES PROMELAS,* EMBRYO-LARVAL TOTAL MORTALITY DATA

			Effluent Concentration (%)				
	Replicate	Control	3.125	6.25	12.5	25.0	50.0
RAW	A	0.00	0.00	0.00	0.00	1.00	1.00
	B	0.20	0.10	0.00	0.00	1.00	1.00
	C	0.00	0.00	0.00	0.00	1.00	1.00
	D	0.10	0.10	0.10	0.10	0.50	1.00
ARC SINE	A	0.159	0.159	0.159	0.159	1.412	-
TRANS-	B	0.464	0.322	0.159	0.159	1.412	-
FORMED	C	0.159	0.159	0.159	0.159	1.412	-
	D	0.322	0.322	0.322	0.322	0.785	-
Mean($\bar{Y}_i$)		0.276	0.241	0.200	0.200	1.255	
S_i^2		0.022	0.009	0.007	0.007	0.098	
i		1	2	3	4	5	

12.13.2.6 Test for Normality

12.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 4.

TABLE 4. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Effluent Concentration (%)				
Replicate	Control	3.125	6.25	12.5	25.0	50.0
A	-0.117	-0.082	-0.041	-0.041	0.157	-
B	0.188	0.081	-0.041	-0.041	0.157	-
C	-0.117	0.081	-0.041	-0.041	0.157	-
D	0.046	-0.082	0.122	0.122	-0.470	-

12.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

12.13.2.6.3 For this set of data, $n = 20$

$$\bar{X} = \frac{1}{20}(-0.003) = 0.000$$

$$D = 0.4261$$

12.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 5.

TABLE 5. ORDERED CENTERED OBSERVATIONS FOR THE SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.470	11	-0.041
2	-0.117	12	0.046
3	-0.117	13	0.081
4	-0.082	14	0.081
5	-0.082	15	0.122
6	-0.041	16	0.122
7	-0.041	17	0.157
8	-0.041	18	0.157
9	-0.041	19	0.157
10	-0.041	20	0.188

12.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table 6.

TABLE 6. COEFFICIENTS AND DIFFERENCES FOR THE SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.658	$X^{(20)} - X^{(1)}$
2	0.3211	0.274	$X^{(19)} - X^{(2)}$
3	0.2565	0.274	$X^{(18)} - X^{(3)}$
4	0.2085	0.239	$X^{(17)} - X^{(4)}$
5	0.1686	0.204	$X^{(16)} - X^{(5)}$
6	0.1334	0.163	$X^{(15)} - X^{(6)}$
7	0.1013	0.122	$X^{(14)} - X^{(7)}$
8	0.0711	0.122	$X^{(13)} - X^{(8)}$
9	0.0422	0.087	$X^{(12)} - X^{(9)}$
10	0.0140	0.000	$X^{(11)} - X^{(10)}$

12.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 6. For the data in this example,

$$W = \frac{1}{0.4261}(0.6004)^2$$
$$= 0.846$$

12.13.2.6.7 The decision rule for this test is to compare W as calculated in Section 13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 20 observations is 0.868. Since W = 0.846 is less than the critical value, conclude that the data are not normally distributed.

12.13.2.6.8 Since the data do not meet the assumption of normality, Steel's Many-one Rank Test will be used to analyze the total mortality data.

12.13.2.7 Steel's Many-one Rank Test

12.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ..., 8) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

12.13.2.7.2 An example of assigning ranks to the combined data for the control and 3.125% effluent concentration is given in Table 7. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 8. The control group ranks are next summed for each effluent concentration pairing, as shown in Table 9.

TABLE 7. ASSIGNING RANKS TO THE CONTROL AND 3.125% EFFLUENT CONCENTRATION FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Mortality	Effluent Concentration (%)
2.5	0.159	Control
2.5	0.159	Control
2.5	0.159	3.125
2.5	0.159	3.125
6	0.322	Control
6	0.322	3.125
6	0.322	3.125
8	0.464	Control

TABLE 8. TABLE OF RANKS FOR STEEL'S MANY-ONE RANK TEST

			Effluent Concentration (%)							
Repl.	Control		3.125		6.25		12.5		25.0	
A	0.159	(2.5,3,3,1.5)	0.159	(2.5)	0.159	(3)	0.159	(3)	1.412	(7)
B	0.464	(8,8,8,4)	0.322	(6)	0.159	(3)	0.159	(3)	1.412	(7)
C	0.159	(2.5,3,3,1.5)	0.159	(2.5)	0.159	(3)	0.159	(3)	1.412	(7)
D	0.322	(6,6.5,6.5,3)	0.322	(6)	0.322	(3)	0.159	(3)	0.785	(5)

TABLE 9. RANK SUMS

Effluent Concentration (%)	Control Rank Sum
3.125	19
6.25	20.5
12.5	20.5
25.0	10

12.13.2.7.3 For this example, we want to determine if the total mortality in any of the effluent concentrations is significantly higher than the total mortality in the control. If this occurs, the rank sum of the control would be significantly less than the rank sum at that concentration. Thus we are only concerned with comparing the control rank sum for each pairing with the various effluent concentrations with some "minimum" or critical rank sum, at or below which the concentration total mortality would be considered significantly greater than the control. At a signficance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control) and four replicates per concentration is 10 (see Table 5, Appendix E).

12.13.2.7.4 Since the control rank sum for the 25.0% effluent concentration pairing is equal to the critical value, the total proportion mortality in the 25.0% concentration is considered significantly greater than that in the control. Since no other rank sums are less than or equal to the critical value, no other concentrations have signficantly higher total proportion mortality than the control. Hence the NOEC is 12.5% and the LOEC is 25.0%.

12.13.2.8 Calculation of the LC50

12.13.2.8.1 The data used for the Probit Analysis is summarized in Table 10. To perform the Probit Analysis, run the USEPA Probit Analysis Program. An example of the program input and output is supplied in Appendix I.

12.13.2.8.2 For this example, the chi-square test for heterogeneity was not significant. Thus Probit Analysis appears appropriate for this data.

12.13.2.8.3 Figure 6 shows the output data for the Probit Analysis of the data from Table 10 using the USEPA Probit Program.

TABLE 10. DATA FOR PROBIT ANALYSIS

		Effluent Concentration (%)				
	Control	3.125	6.25	12.5	25.0	50.0
Number Dead	3	1	0	1	6	15
Number Exposed	40	40	40	40	40	40

12.14 **PRECISION AND ACCURACY**

12.14.1 PRECISION

12.14.1.1 Single-laboratory Precision

12.14.1.1.1 Data shown in Tables 11 and 12 indicate that the precision of the embryo-larval survival and teratogenicity test, expressed as the relative standard deviation (or coefficient of variation, CV) of the LC1 values, was 62% for cadmium (Table 11) and 41% for Diquat (Table 12).

12.14.1.1.2 Precision data are also available from four embryo-larval survival and teratogenicity tests on trickling filter pilot plant effluent (Table 13). Although the data could not be analyzed by Probit Analysis, the NOECs and LOECs obtained using Dunnett's Procedure were the same for all four tests, 7% and 11% effluent, respectively, indicating maximum precision in terms of the test design.

12.14.1.2 Multilaboratory Precision

12.14.1.2.1 Data on the multilaboratory precision of this test are not yet available.

12.14.2 ACCURACY

12.14.2.1 The accuracy of toxicity tests cannot be determined.

USEPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

Probit Analysis of Fathead Minnow Embryo-Larval Survival
and Teratogenicity Data

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	20	2	0.1000	0.0000
0.5000	20	2	0.1000	0.0174
1.0000	20	1	0.0500	-.0372
2.0000	20	4	0.2000	0.1265
4.0000	20	16	0.8000	0.7816
8.0000	20	20	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 0.441
Chi - Square for Heterogeneity (tabular value) = 7.815

Probit Analysis of Fathead Minnow Embryo-Larval Survival
and Teratogenicity Data

Estimated LC/EC Values and Confidence Limits

Point		Exposure Conc.	Lower 95% Confidence	Upper Limits
LC/EC	1.00	1.346	0.453	1.922
LC/EC	50.00	3.018	2.268	3.672

Figure 6. Output for USEPA Probit Program, Version 1.5.

TABLE 11. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST, USING CADMIUM AS A REFERENCE TOXICANT[1,2]

Test	LC1[3] (mg/L)	95% Confidence Limits	NOEC[4] (mg/L)
1	0.014	0.009 - 0.018	0.012
2	0.006	0.003 - 0.010	0.012
3	0.005	0.003 - 0.009	0.013
4	0.003	0.002 - 0.004	0.011
5	0.006	0.003 - 0.009	0.012
N	5		5
Mean	0.0068		NA
SD	0.0042		
CV(%)	62		NA

1 Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Bioassessment and Ecotoxicology Branch, EMSL, USEPA, Cincinnati, OH.

2 Cadmium chloride was used as the reference toxicant. The nominal concentrations, expressed as cadmium (mg/L), were: 0.01, 0.032, 0.100, 0.320, and 1.000. The dilution water was reconstituted water with a hardness of 100 mg/L as calcium carbonate, and a pH of 7.8.

3 Determined by Probit Analysis.

4 Highest no-observed-effect concentration determined by independent statistical analysis (2X2 Chi-square Fisher's Exact Test). NOEC range of 0.011 - 0.013 represents a difference of one exposure concentration.

TABLE 12. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL, SURVIVAL AND TERATOGENICITY TOXICITY TEST, USING DIQUAT AS A REFERENCE TOXICANT[1,2]

Test	LC1[3] (mg/L)	95% Confidence Limits
1	0.58	0.32 - 0.86
2	2.31	--[4]
3	1.50	1.05 - 1.87
4	1.71	1.24 - 2.09
5	1.43	0.93 - 1.83
N	5	
Mean	1.51	
SD	0.62	
CV(%)	41.3	

[1] Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Bioassessment and Ecotoxicology Branch, EMSL, USEPA, Cincinnati, OH.

[2] The Diquat concentrations were determined by chemical analysis. The dilution water was reconstituted water with a hardness of 100 mg/L as calcium carbonate, and a pH of 7.8.

[3] Determined by Probit Analysis.

[4] Cannot be calculated.

TABLE 13. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY STATIC-RENEWAL TEST CONDUCTED WITH TRICKLING FILTER EFFLUENT[1,2,3]

Test No.	NOEC (% Effluent)	LOEC (% Effluent)
1	7	11
2	7	11
3	7	11
4	7	11

[1] Data provided by Timothy Neiheisel, Bioassessment and Ecotoxiology Branch, EMSL, USEPA, Cincinnati, OH.
[2] Effluent concentrations used: 3, 5, 7, 11 and 16%
[3] Maximum precision achieved in terms of NOEC-LOEC interval. For a discusssion of the precision of data from chronic toxicity tests (see Section 4, Quality Assurance).

SECTION 13

TEST METHOD

DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TEST METHOD 1002.0

13.1 SCOPE AND APPLICATION

13.1.1 This method measures the chronic toxicity of effluents and receiving water to the daphnid, *Ceriodaphnia dubia*, using less than 24 h old neonates during a three-brood (seven-day), static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

13.1.2 Daily observations on mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, and 96-h LC50s).

13.1.3 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

13.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly degradable or highly volatile toxicants in the source may not be detected in the test.

13.1.5 This test method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

13.2 SUMMARY OF METHOD

13.2.1 *Ceriodaphnia dubia* are exposed in a static renewal system to different concentrations of effluent, or to receiving water, until 60% or more of surviving control females have three broods of offspring. Test results are based on survival and reproduction. If the test is conducted as described, the surviving control organisms should produce 15 or more young in three broods. If these criteria are not met at the end of 8 days, the test must be repeated.

13.3 INTERFERENCES

13.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

13.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.3.3 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival and confound test results.

13.3.4 The amount and type of natural food in the effluent or dilution water may confound test results.

13.3.5 Food added during the test may sequester metals and other toxic substances and confound test results. Daily renewal of solutions, however, will reduce the probability of reduction of toxicity caused by feeding.

13.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 13.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 13.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

13.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 13.3.6.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the initial pH of the sample upon completion of collection (as measured on an aliquot removed from the sample container).

13.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ±0.2 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ±0.2 pH units in pH-controlled tests (USEPA, 1992).

13.3.6.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

13.3.6.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 13.3.6.1.1).

13.3.6.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 13.3.6.2) is applied routinely to subsequent testing of the effluent.

13.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992);

or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, CO_2 is injected to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, CO_2 is injected to maintain the test pH at the pH of the sample upon completion of collection. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

13.4 **SAFETY**

13.4.1 See Section 3, Health and Safety.

13.5 **APPARATUS AND EQUIPMENT**

13.5.1 *Ceriodaphnia* and algal culture units -- See *Ceriodaphnia* and algal culturing methods below and algal culturing methods in Section 14 and USEPA, 2002a.

13.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, capable of collecting a 24-h composite sample of 5 L or more.

13.5.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.5.4 Environmental chambers, incubators, or equivalent facilities with temperature control (25 ± 1 °C).

13.5.5 Water purification system -- MILLIPORE MILLI-Q®, deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

13.5.6 Balance -- analytical, capable of accurately weighing 0.00001 g.

13.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the material to be weighed.

13.5.8 Test chambers -- 10 test chambers are required for each concentration and control. Test chambers such as 30-mL borosilicate glass beakers or disposable polystyrene cups are recommended because they will fit in the viewing field of most stereoscopic microscopes. The glass beakers and plastic cups are rinsed thoroughly with dilution water before use. To avoid potential contamination from the air and excessive evaporation of the test solutions during the test, the test vessels should be covered with safety glass plates or sheet plastic (6 mm thick).

13.5.9 Mechanical shaker or magnetic stir plates -- for algal cultures.

13.5.10 Light meter -- with a range of 0-200 $\mu E/m^2/s$ (0-1000 ft-c).

13.5.11 Fluorometer (optional) -- equipped with chlorophyll detection light source, filters, and photomultiplier tube (Turner Model 110 or equivalent).

13.5.12 UV-VIS spectrophotometer (optional) -- capable of accommodating 1-5 cm cuvettes.

13.5.13 Cuvettes for spectrophotometer -- 1-5 cm light path.

13.5.14 Electronic particle counter (optional) -- Coulter Counter, ZBI, or equivalent, with mean cell (particle) volume determination.

13.5.15 Microscope with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condensor, and light source (inverted or conventional microscope) -- for determining sex and verifying identification.

13.5.16 Dissecting microscope, stereoscopic, with zoom objective, magnification to 50X -- for examining and counting the neonates in the test vessels.

13.5.17 Counting chamber -- Sedgwick-Rafter, Palmer-Maloney, or hemocytometer.

13.5.18 Centrifuge (optional) -- plankton, or with swing-out buckets having a capacity of 15-100 mL.

13.5.19 Centrifuge tubes -- 15-100 mL, screw-cap.

13.5.20 Filtering apparatus -- for membrane and/or glass fiber filters.

13.5.21 Racks (boards) -- to hold test chambers. It is convenient to use a piece of styrofoam insulation board, 50 cm x 30 cm x 2.5 cm (20 in x 12 in x 1 in), drilled to hold 60 test chambers, in six rows of 10 (see Figure 1).

13.5.22 Light box -- for illuminating organisms during examination.

13.5.23 Volumetric flasks and graduated cylinders -- class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL, for culture work and preparation of test solutions.

13.5.24 Pipettors, adjustable volume repeating dispensers -- for feeding. Pipettors such as the Gilson REPETMAN®, Eppendorf, Oxford, or equivalent, provide a rapid and accurate means of dispensing small volumes (0.1 mL) of food to large numbers of test chambers.

13.5.25 Volumetric pipets -- class A, 1-100 mL.

13.5.26 Serological pipets -- 1-10 mL, graduated.

13.5.27 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

13.5.28 Disposable polyethylene pipets, droppers, and glass tubing with fire-polished edges, ≥ 2mm ID -- for transferring organisms.

13.5.29 Wash bottles -- for rinsing small glassware and instrument electrodes and probes.

13.5.30 Thermometer, glass or electronic, laboratory grade, -- for measuring water temperatures.

13.5.31 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

13.5.32 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA 1979b) -- to calibrate laboratory thermometers.

13.5.33 Meters, DO, pH, and specific conductivity -- for routine physical and chemical measurements.

13.6 **REAGENTS AND CONSUMABLE MATERIALS**

13.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.6.2 Data sheets (one set per test) -- for recording the data.

13.6.3 Vials, marked -- for preserving specimens for verification (optional).

13.6.4 Tape, colored -- for labeling test vessels.

13.6.5 Markers, waterproof -- for marking containers.

13.6.6 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA, 1979b.

13.6.7 Buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for instrument calibration check (see USEPA Method 150.1, USEPA, 1979b).

13.6.8 Specific conductivity standards -- see USEPA Method 120.1, USEPA, 1979b.

13.6.9 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

13.6.10 Laboratory quality control samples and standards -- for calibration of the above methods.

13.6.11 Reference toxicant solutions -- see Section 4, Quality Assurance.

13.6.12 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

13.6.13 Effluent, surface water, and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

13.6.14 Trout chow, yeast, and CEROPHYL® food (or substitute food) -- for feeding the cultures and test organisms.

13.6.14.1 Digested trout chow, or substitute flake food (TETRAMIN®, BIORIL®, or equivalent), is prepared as follows:

1. Preparation of trout chow or substitute flake food requires one week. Use starter or No. 1 pellets prepared according to current U.S. Fish and Wildlife Service specifications.
2. Add 5.0 g of trout chow pellets or substitute flake food to 1 L of MILLI-Q® water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation is replaced during digestion. Because of the offensive odor usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.
3. At the end of digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (i.e., NITEX® 110 mesh). Combine with equal volumes of supernatant from CEROPHYLL® and yeast preparations (below). The supernatant can be used fresh, or frozen until use. Discard the sediment.

13.6.14.2 Yeast is prepared as follows:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S® Yeast, Lake State Kosher Certified Yeast, or equivalent, to 1 L of MILLI-Q® water.
2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.
3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow (above) and CEROPHYLL® preparations (below). Discard excess material.

13.6.14.3 CEROPHYLL® is prepared as follows:

1. Place 5.0 g of dried, powdered, cereal or alfalfa leaves, or rabbit pellets, in a blender. Cereal leaves, CEROPHYLL®, or equivalent are available from commercial sources. Dried, powdered, alfalfa leaves may be obtained from health food stores, and rabbit pellets are available at pet shops.
2. Add 1 L of MILLI-Q® water.
3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.
4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard excess material.

13.6.14.4 Combined yeast-cerophyl-trout chow (YCT) is mixed as follows:

1. Thoroughly mix equal (approximately 300 mL) volumes of the three foods as described above.
2. Place aliquots of the mixture in small (50 mL to 100 mL) screw-cap plastic bottles and freeze until needed.
3. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is used for a maximum of two weeks. Do not store frozen over three months.
4. It is advisable to measure the dry weight of solids in each batch of YCT before use. The food should contain 1.7-1.9 g solids/L. Cultures or test solutions should contain 12-13 mg solids/L.

13.6.15 Algal food -- for feeding the cultures and test organisms.

13.6.15.1 Algal Culture Medium is prepared as follows:

1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.
2. Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L, mix well. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.
3. Immediately filter the medium through a 0.45 µm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.
4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.
5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 mL MILLI-Q® WATER	
1. MACRONUTRIENTS			
A.	$MgCl_2 \cdot 6H_2O$	6.08	g
	$CaCl_2 \cdot 2H_2O$	2.20	g
	$NaNO_3$	12.75	g
B.	$MgSO_4 \cdot 7H_2O$	7.35	g
C.	K_2HPO_4	0.522	g
D.	$NaHCO_3$	7.50	g
2. MICRONUTRIENTS			
	H_3BO_3	92.8	mg
	$MnCl_2 \cdot 4H_2O$	208.0	mg
	$ZnCl_2$	1.64	mg[1]
	$FeCl_3 \cdot 6H_2O$	79.9	mg
	$CoCl_2 \cdot 6H_2O$	0.714	mg[2]
	$Na_2MoO_4 \cdot 2H_2O$	3.63	mg[3]
	$CuCl_2 \cdot 2H_2O$	0.006	mg[4]
	$Na_2EDTA \cdot 2H_2O$	150.0	mg
	Na_2SeO_4	1.196	mg[5]

[1] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[2] $CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[3] $Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[4] $CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock 2, micronutrients.

[5] Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

MACRONUTRIENT	CONCENTRATION (mg/L)	ELEMENT	CONCENTRATION (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

MICRONUTRIENT	CONCENTRATION (µg/L)	ELEMENT	CONCENTRATION (µg/L)
H_3BO_3	185.0	B	32.5
$MnCl_2 \cdot 4H_2O$	416.0	Mn	115.0
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160.0	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300.0	--	----
Na_2SeO_4	2.39	Se	0.91

13.6.15.2 Algal Cultures

13.6.15.2.1 See Section 6, Test Organisms, for information on sources of "starter" cultures of *Selenastrum capricornutum, S. minutum*, and *Chlamydomonas reinhardti.*

13.6.15.2.2 Two types of algal cultures are maintained: "stock" cultures, and "food" cultures.

13.6.15.2.2.1 Establishing and Maintaining Stock Cultures of Algae:

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring one milliliter to each of several 250-mL culture flasks containing 100 mL algal culture medium (prepared as described above). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.
2. The stock cultures are used as a source of algae to initiate "food" cultures for *Ceriodaphnia dubia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *Ceriodaphnia dubia* cultures and tests. Stock culture volume may be rapidly "scaled up" to several liters, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.
3. Culture temperature is not critical. Stock cultures may be maintained at 25°C in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
4. Cultures are mixed twice daily by hand.
5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three milliliters of 7-day old algal stock culture, containing approximately 1.5 X 10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 10,000-30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.
6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6-12 months if stored in the dark at 4°C. It is advisable to prepare new stock cultures from "starter" cultures obtained from established outside sources of organisms (see Section 6, Test Organisms) every four to six months.

13.6.15.2.2.2 Establishing and Maintaining "Food" Cultures of Algae:

1. "Food" cultures are started seven days prior to use for *Ceriodaphnia dubia* cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5 X 10^6 cells/mL, are added to each liter of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30,000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are terminated in 7-10 days. A one-month supply of algal food can be grown at one time, and stored in the refrigerator.
2. Food cultures may be maintained at 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$ or 400 ft-c).
3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by hand. If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be exercised to prevent the culture temperature from rising more than 2-3°C.

13.6.15.2.3 Preparing Algal Concentrate for Use as *Ceriodaphnia dubia* Food:

1. An algal concentrate containing 3.0 to 3.5 X 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for at least three weeks and siphoning off the supernatant.
2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer (see Section 14, Green Alga, *Selenastrum capricornutum* Growth Test), and used to determine the dilution (or further concentration) required to achieve a final cell count of 3.0 to 3.5 X 10^7/mL.
3. Assuming a cell density of approximately 1.5 X 10^6 cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7-10 day culture will provide 4.5 X 10^9 algal cells. This number of cells would provide approximately 150 mL of algal cell concentrate (1500 feedings at 0.1 mL/feeding) for use as food. This would be enough algal food for four *Ceriodaphnia dubia* tests.
4. Algal concentrate may be stored in the refrigerator for one month.

13.6.15.3 Food Quality

13.6.15.3.1 USEPA recommends Fleishmann's® yeast, Cerophyll®, trout chow, and *Selenastrum capricornutum* as the preferred *Ceriodaphnia dubia* food combination. This recommendation is based on extensive data developed by many laboratories which indicated high *Ceriodaphnia dubia* survival and reproduction in culturing and testing. The use of substitute food(s) is acceptable only after side-by-side tests are conducted to determine that the quality of the substitute food(s) is equal to the USEPA recommended food combination based on survival and reproduction of *Ceriodaphnia dubia*.

13.6.15.3.2 The quality of food prepared with newly acquired supplies of yeast, trout chow, dried cereal leaves, algae, and/or any substitute food(s) should be determined in side-by-side comparisons of *Ceriodaphnia dubia* survival and reproduction, using the new food and food of known, acceptable quality, over a seven-day period in control medium.

13.6.16 TEST ORGANISMS, DAPHNIDS, *CERIODAPHNIA DUBIA*

13.6.16.1 Cultures of test organisms should be started at least three weeks before the brood animals are needed, to ensure an adequate supply of neonates for the test. Only a few individuals are needed to start a culture because of their prolific reproduction.

13.6.16.2 Neonates used for toxicity tests must be obtained from individually cultured organisms. Mass cultures may be maintained, however, to serve as a reserve source of organisms for use in initiating individual cultures and in case of loss of individual cultures.

13.6.16.3 Starter animals may be obtained from commercial sources and may be shipped in polyethylene bottles. Approximately 40 animals and 3 mL of food are placed in a l-L bottle filled full with culture water for shipment. Animals received from an outside source should be transferred to new culture media gradually over a period of 1-2 days to avoid mass mortality.

13.6.16.4 It is best to start the cultures with one animal, which is sacrificed after producing young, mounted on a microscope slide, and retained as a permanent slide mount to facilitate identification and permit future reference. The species identification of the stock culture should be verified by preparing slide mounts, regardless of the number of animals used to start the culture. The following procedure is recommended for making slide mounts of *Ceriodaphnia dubia* (modified from Beckett and Lewis, 1982):

1. Pipet the animal onto a watch glass.
2. Reduce the water volume by withdrawing excess water with the pipet.

3. Add a few drops of carbonated water (club soda or seltzer water) or 70% ethanol to relax the specimen so that the post-abdomen is extended. (Optional: with practice, extension of the postabdomen may be accomplished by putting pressure on the cover slip).
4. Place a small amount (one to three drops) of mounting medium on a glass microscope slide. The recommended mounting medium is CMCP-9/10 Medium, prepared by mixing two parts of CMCP-9 with one part of CMCP-10 stained with enough acid fuchsin dye to color the mixture a light pink. For more viscosity and faster drying, CMC-10 stained with acid fuchsin may be used.
5. Using forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.
6. Cover with a 12 mm round cover slip and exert minimum pressure to remove any air bubbles trapped under the cover slip. Slightly more pressure will extend the postabdomen.
7. Allow mounting medium to dry.
8. Make slide permanent by placing varnish around the edges of the coverslip.
9. Identify to species (see Pennak, 1978; Pennak, 1989; and Berner, 1986).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

13.6.16.5 Mass Culture

13.6.16.5.1 Mass cultures are used only as a "backup" reservoir of organisms.

13.6.16.5.2 One-liter or 2-L glass beakers, crystallization dishes, "battery jars," or aquaria may be used as culture vessels. Vessels are commonly filled to three-fourths capacity. Cultures are fed daily. Four or more cultures are maintained in separate vessels and with overlapping ages to serve as back-up in case one culture is lost due to accident or other unanticipated problems, such as low DO concentrations or poor quality of food or laboratory water.

13.6.16.5.3 Mass cultures which will serve as a source of brood organisms for individual culture should be maintained in good condition by frequent renewal with new culture medium at least twice a week for two weeks. At each renewal, the adult survival is recorded, and the offspring and the old medium are discarded. After two weeks, the adults are also discarded, and the culture is re-started with neonates in fresh medium. Using this schedule, 1-L cultures will produce 500 to 1000 neonate *Ceriodaphnia dubia* each week.

13.6.16.6 Individual Culture

13.6.16.6.1 Individual cultures are used as the immediate source of neonates for toxicity tests.

13.6.16.6.2 Individual organisms are cultured in 15 mL of culture medium in 30-mL (1 oz) plastic cups or 30-mL glass beakers. One neonate is placed in each cup. It is convenient to place the cups in the same type of board used for toxicity tests (see Figure 1).

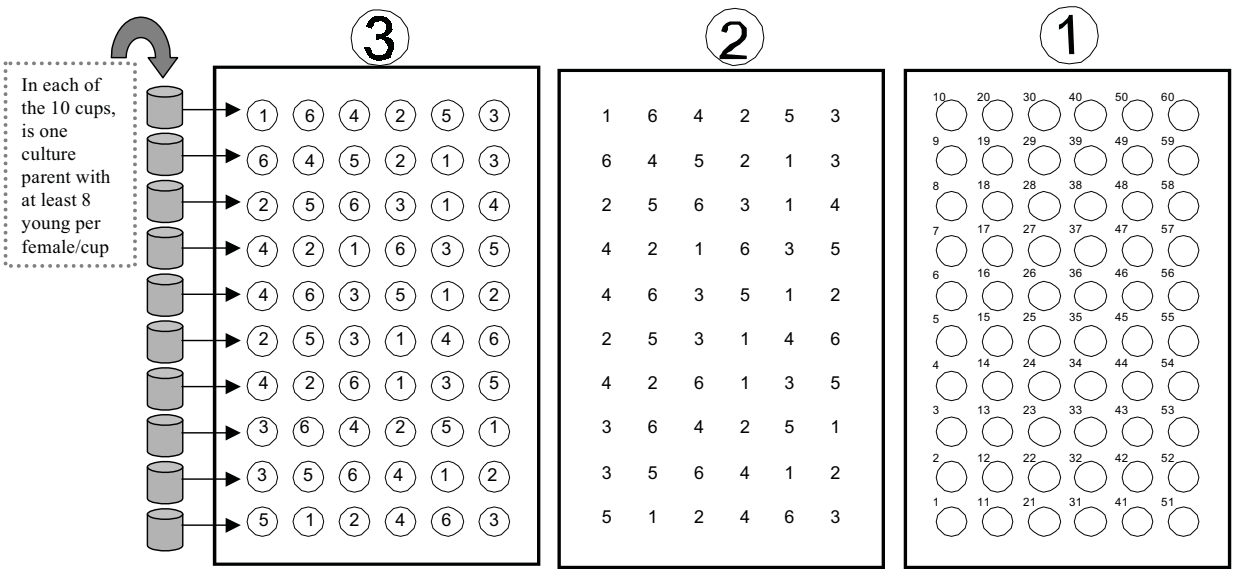

Figure 1. Examples of a test board and randomizing template: 1) test board with positions for six columns of ten replicate test chambers with each position numbered for recording results on data sheets, 2) cardboard randomizing template prepared by randomly drawing numbers (1-6) for each position in a row across the board, and 3) test board (1) placed on top of the randomizing template (2) for the purpose of assigning the position of test treatments (1-6) within each block (row on the test board). Following placement of test chambers, test organisms are allocated using blocking by known parentage. Test organisms from a single brood cup are distributed to each treatment within a given block (row on the test board).

13.6.16.6.3 Organisms are fed daily (see Subsection 13.6.16.9) and are transferred to fresh medium a minimum of three times a week, typically on Monday, Wednesday, and Friday. On the transfer days, food is added to the new medium immediately before or after the organisms are transferred.

13.6.16.6.4 To provide cultures of overlapping ages, new boards are started weekly, using neonates from adults which produce at least eight young in their third or fourth brood. These adults can be used as sources of neonates until 14 days of age. A minimum of two boards are maintained concurrently to provide backup supplies of organisms in case of problems.

13.6.16.6.5 Cultures which are properly maintained should produce at least 20 young per adult in three broods (seven days or less). Typically, 60 adult females (one board) will produce more than the minimum number of neonates (120) required for two tests.

13.6.16.6.6 Records should be maintained on the survival of brood organisms and number of offspring at each renewal. Greater than 20% mortality of adults, or less than an average of 20 young per female would indicate problems, such as poor quality of culture media or food. Cultures that do not meet these criteria should not be used as a source of test organisms.

13.6.16.7 Culture Medium

13.6.16.7.1 Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW is recommended as a standard culture medium (see Section 7, Dilution Water).

13.6.16.8 Culture Conditions

13.6.16.8.1 The daphnid, *Ceriodaphnia dubia,* should be cultured at a temperature of 25 ± 1 °C.

13.6.16.8.2 Day/night cycles prevailing in most laboratories will provide adequate illumination for normal growth and reproduction. A photoperiod of 16-h of light and 8-h of darkness is recommended. Light intensity should be 10-20 $\mu E/m^2/s$ or 50 to 100 ft-c.

13.6.16.8.3 Clear, double-strength safety glass or 6 mm plastic panels are placed on the culture vessels to exclude dust and dirt, and reduce evaporation.

13.6.16.8.4 The organisms are delicate and should be handled as carefully and as little as possible so that they are not unnecessarily stressed. They are transferred with a pipet of approximately 2-mm bore, taking care to release the animals under the surface of the water. Any organism that is injured during handling should be discarded.

13.6.16.9 Food and Feeding

13.6.16.9.1 Feeding the proper amount of the right food is extremely important in *Ceriodaphnia dubia* culturing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food which may reduce the toxicity of the test solutions, clog the animal's filtering apparatus, or greatly decrease the DO concentration and increase mortality. A combination of Yeast, CEROPHYLL®, and Trout chow (YCT), along with the unicellular green alga, *Selenastrum capricornutum*, will provide suitable nutrition if fed daily.

13.6.16.9.2 Other algal species (such as *S. minutum* or *Chlamydomonas reinhardti*), other substitute food combinations (such as Flake Fish Food), or different feeding rates may be acceptable as long as performance criteria are met and side-by-side comparison tests confirm acceptable quality (see Subsection 13.6.15.3).

13.6.16.9.3 Cultures should be fed daily to maintain the organisms in optimum condition so as to provide maximum reproduction. Stock cultures which are stressed because they are not adequately fed may produce low

numbers of young, large numbers of males, and/or ephippial females. Also, their offspring may produce few young when used in toxicity tests.

13.6.16.9.4 Feed as follows:

1. If YCT is frozen, remove a bottle of food from the freezer 1h before feeding time, and allow to thaw.
2. YCT food mixture and algal concentrates should both be thoroughly mixed by shaking before dispensing.
3. Mass cultures are fed daily at the rate of 7 mL YCT and 7 mL algae concentrate/L culture.
4. Individual cultures are fed at the rate of 0.1 mL YCT and 0.1 mL algae concentrate per 15 mL culture.
5. Return unused YCT food mixture and algae concentrate to the refrigerator. Do not re-freeze YCT. Discard unused portion after two weeks.

13.6.16.10 It is recommended that chronic toxicity tests be performed monthly with a reference toxicant. Daphnid, *Ceriodaphnia dubia,* neonates less than 24 h old, and all within 8 h of the same age are used to monitor the chronic toxicity of the reference toxicant to the *Ceriodaphnia dubia* produced by the culture unit (see Section 4, Quality Assurance).

13.6.16.11 Record Keeping

13.6.16.11.1 Records, kept in a bound notebook, include (1) source of organisms used to start the cultures, (2) type of food and feeding times, (3) dates culture were thinned and restarted, (4) rate of reproduction in individual cultures, (5) daily observations of the condition and behavior of the organisms in the cultures, and (6) dates and results of reference toxicant tests performed (see Section 4, Quality Assurance).

13.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

13.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

13.8 CALIBRATION AND STANDARDIZATION

13.8.1 See Section 4, Quality Assurance.

13.9 QUALITY CONTROL

13.9.1 See Section 4, Quality Assurance.

13.10 TEST PROCEDURES

13.10.1 TEST SOLUTIONS

13.10.1.1 Receiving Waters

13.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 µm NITEX® filter and compared without dilution, against a control. For a test consisting of single receiving water and control, approximately 600 mL of sample would be required for each test, assuming 10 replicates of 15 mL, and sufficient additional sample for chemical analysis.

13.10.1.2 Effluents

13.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5, and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.**

13.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions should be added at the lower range of effluent concentrations.

13.10.1.2.3 The volume of effluent required for daily renewal of 10 replicates per concentration, each containing 15 mL of test solution, with a dilution series of 0.5, is approximately 1 L/day. A volume of 15 mL of test solution is adequate for the organisms, and will provide a depth in which it is possible to count the animals under a stereomicroscope with a minimum of re-focusing. Ten test chambers are used for each effluent dilution and for the control. Sufficient test solution (approximately 550 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses at the high, medium, and low test concentrations.

13.10.1.2.4 Tests should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.10.1.2.5 Just prior to test initiation (approximately one h) the temperature of sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature and maintained at that temperature during the preparation of the test solutions.

13.10.1.2.6 The DO of the test solutions should be checked prior to test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If any solution has a DO concentration below 4.0 mg/L, all the solutions and the control must be gently aerated.

13.10.1.3 Dilution Water

13.10.1.3.1 Dilution water may be uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other uncontaminated natural water (see Section 7, Dilution Water).

13.10.2 START OF THE TEST

13.10.2.1 Label the test chambers with a marking pen. Use of color-coded tape to identify each treatment and replicate is helpful. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) must have ten replicates.

13.10.2.2 The test chambers must be randomly assigned to a board using a template (Figure 1) or by using random numbers (see Appendix A). Randomizing the position of test chambers as described in Figure 1 (or equivalent) will assist in assigning test organisms using blocking by known parentage (Subsection 13.10.2.4). A number of different templates should be prepared, and the template used for each test should be identified on the data sheet. The same template must not be used for every test.

13.10.2.3 Neonates less than 24 h old, and all within 8 h of the same age, are required to begin the test. The neonates must be obtained from individual cultures using brood boards, as described above in Subsection 13.6.16.6,

Individual Culture (also see Section 6, Test Organisms). Neonates must be taken only from adults in individual cultures that have eight or more young in their third or subsequent broods. These adults can be used as brood stock until they are 14 days old. If the neonates are held more than one or two hours before using in the test, they should be fed (0.1 mL YCT and 0.1 mL algal concentrate/15 mL of media). Record the age range of test organisms, source, and feeding of neonates on test data sheets.

13.10.2.4 Ten brood cups, each with 8 or more young, are randomly selected from a brood board for use in setting up a test. To start the test, neonates from these ten brood cups are distributed to each test chamber in the test board (one per test chamber). Test organisms must be assigned to test chambers using a block randomization procedure, such that offspring from a single female are distributed evenly among the treatments, appearing once in every test concentration. This arrangement is referred to as "blocking by known parentage". The technique used to achieve blocking by known parentage should be recorded in the test data report. One effective technique is to block randomize the test board as described in Figure 1 and transfer one neonate from the first brood cup to each of the six test chambers in the first row on the test board. One neonate from the second brood cup is then transferred to each of the six test chambers in the second row on the test board. This process is continued until each of the 60 test chambers contains one neonate. The set of six test chambers (one for each test treatment) containing organisms derived from a single female parent is referred to as a block. When using the technique described in Figure 1, each row of the test board will represent a block.

13.10.2.4.1 The brood cups and test chambers may be placed on a light table to facilitate counting the neonates. However, care must be taken to avoid temperature increase due to heat from the light table.

13.10.2.4.2 Following the allocation of test organisms to the test board, additional neonates might remain in the ten brood cups that were selected for test setup. These additional neonates may be discarded, used as future culture organisms if needed, or used to start additional tests (provided that at least 6 neonates remain and these neonates continue to meet test organism age requirements).

13.10.2.5 Blocking by known parentage allows the performance of each test organism to be tracked to its parent culture organism. This technique ensures that any brood effects (i.e., differences in test organism fecundity or sensitivity attributable to the source of parentage) are evenly distributed among the test treatments. Also, by knowing the parentage of each test organism, blocks consisting largely of males can be omitted from all test treatments at the end of the test (see Subsection 13.13.1.4), decreasing variability among replicates.

13.10.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

13.10.3.1 The light quality and intensity should be at ambient laboratory levels, approximately 10-20 $\mu E/m^2/s$, or 50 to 100 ft-c, with a photoperiod of 16 h of light and 8 h of darkness.

13.10.3.2 It is critical that the test water temperature be maintained at 25 ± 1°C to obtain three broods in seven days.

13.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

13.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO concentrations. The DO concentrations should be measured in the new solutions at the start of the test (Day 0) and before daily renewal of the test solutions on subsequent days. The DO concentration should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). Aeration is generally not practical during the daphnid, *Ceriodaphnia dubia*, test. If the DO in the effluent and/or dilution water is low, aerate gently before preparing the test solutions. The aeration rate should not exceed 100 bubbles/min using a pipet with an orifice of approximately 1.5 mm, such as a 1 ml KIMAX® serological pipet, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue physical stress to the organisms.

13.10.5 FEEDING

13.10.5.1 The organisms are fed when the test is initiated, and daily thereafter. Food is added to the fresh medium immediately before or immediately after the adults are transferred. Each feeding consists of 0.1 mL YCT and 0.1 mL *Selenastrum capricornutum* concentrate/15 mL test solution (0.1 mL of algal concentrate containing 3.0-3.5 X 10^7 cells/mL will provide 2-2.3 X 10^5 cells/mL in the test chamber).

13.10.5.2 The YCT and algal suspension can be added accurately to the test chambers by using automatic pipettors, such as Gilson, Eppendorf, Oxford, or equivalent.

13.10.6 OBSERVATIONS DURING THE TEST

13.10.6.1 Routine Chemical and Physical Determinations

13.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period in at least one test chamber at each test concentration and in the control.

13.10.6.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test chamber at each test concentration and in the control. Temperature should be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in sufficient number of test vessels at least at the end of the test to determine the temperature variation in the environmental chamber.

13.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

13.10.6.1.4 Conductivity, alkalinity and hardness are measured in each new sample (100% effluent or receiving water) and in the control.

13.10.6.1.5 Record the data on data sheet (Figure 2).

13.10.6.2 Routine Biological Observations

13.10.6.2.1 Three or four broods are usually obtained in the controls in a 7-day test conducted at 25 ± 1°C. A brood is a group of offspring released from the female over a short period of time when the carapace is discarded during molting. In the controls, the first brood of two-to-five young is usually released on the third or fourth day of the test. Successive broods are released every 30 to 36 h thereafter. The second and third broods usually consist of eight to 20 young each. The total number of young produced by a healthy control organism in three broods often exceeds 30 per female. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test.

13.10.6.2.2 The release of a brood may be inadvertently interrupted during the daily transfer of organisms to fresh test solutions, resulting in a split in the brood count between two successive days. For example, four neonates of a brood of five might be released on Day 3, just prior to test solution renewal, and the fifth released just after renewal, and counted on Day 4. Partial broods, released over a two-day period, should be counted as one brood.

13.10.6.2.3 Each day, the live adults are transferred to fresh test solutions, and the numbers of live young are recorded (see data form, Figure 3). The young can be counted with the aid of a stereomicroscope with substage lighting. Place the test chambers on a light box over a strip of black tape to aid in counting the neonates. The young are discarded after counting.

13.10.6.2.4 Some of the effects caused by toxic substances include, (1) a reduction in the number of young produced, (2) young may develop in the brood pouch of the adults, but may not be released during the exposure

period, and (3) partially or fully developed young may be released, but are all dead at the end of the 24-h period. Such effects should be noted on the data sheets (Figure 3).

13.10.6.2.5 Protect the daphnids, *Ceriodaphnia dubia*, from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and transfer of females carefully. Make sure the females remain immersed during the performance of these operations.

13.10.7 DAILY PREPARATION OF TEST CHAMBERS

13.10.7.1 The test is started (Day 0) with new disposable polystyrene cups or precleaned 30-mL borosilicate glass beakers that are labeled and color-coded with tape. Each following day, a new set of plastic cups or precleaned glass beakers is prepared, labeled, and color-coded with tape similar to the original set. New solutions are placed in the new set of test chambers, and the test organisms are transferred from the original test chambers to the new ones with corresponding labels and color-codes. Each day, previously used glass beakers are recleaned (see Section 5, Facilities, Equipment, and Supplies) for the following day, and previously used plastic cups are discarded.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 2. Data form for the daphnid, *Ceriodaphnia dubia,* survival and reproduction test. Routine chemical and physical determinations.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 2. Data form for the daphnid, *Ceriodaphnia dubia,* survival and reproduction test. Routine chemical and physical determinations (CONTINUED).

13.10.8 TEST SOLUTION RENEWAL

13.10.8.1 Freshly prepared solutions are used to renew the test daily. For on-site toxicity studies, fresh effluent or receiving water samples should be collected daily, and no more than 24 h should elapse between collection of the samples and their use in the tests (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples are collected, preferably on days one, three, and five. No more than 36 h should elapse between collection of the sample and the first use in the test. Maintain the samples in the refrigerator at 0-6°C until used.

13.10.8.2 New test solutions are prepared daily, and the test organisms are transferred to the freshly prepared solutions using a small-bore (2 mm) glass or polyethylene dropper or pipet. The animals are released under the surface of the water so that air is not trapped under the carapace. Organisms that are dropped or injured are discarded.

13.10.9 TERMINATION OF THE TEST

13.10.9.1 Tests should be terminated when 60% or more of the surviving control females have produced their third brood, or at the end of 8 days, whichever occurs first. Because of the rapid rate of development of *Ceriodaphnia dubia*, at test termination all observations on organism survival and numbers of offspring should be completed within two hours. An extension of more than a few hours in the test period would be a significant part of the brood production cycle of the animals, and could result in additional broods. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test.

13.10.9.2 Count the young, conduct required chemical measurements, and complete the data sheets (Figure 3).

13.10.9.3 Any animal not producing young should be examined to determine if it is a male (Berner, 1986). In most cases, the animal will need to be placed on a microscope slide before examining (see Subsection 13.6.16.4).

13.10.9.3.1 In general, the occurrence of males in healthy, well-maintained individual cultures is rare. In interlaboratory testing of the *Ceriodaphnia dubia* Survival and Reproduction Test, males were identified in only 7% (9 of 126 tests) of tests conducted (USEPA, 2001a). The number of males identified in these tests ranged from 1 to 12. In five tests containing a large number of males (4-12), laboratories conducting those tests also noted that organism cultures were experiencing or recovering from some stress. Since male production in cladoceran populations is generally associated with conditions of environmental stress (Pennak, 1989), culture conditions should be examined whenever males are identified in a test.

13.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

13.11.1 A summary of test conditions and test acceptability criteria is presented in Table 3.

13.12 ACCEPTABILITY OF TEST RESULTS

13.12.1 For the test results to be acceptable, at least 80% of all control organisms must survive, and 60% of surviving control females must produce at least three broods, with an average of 15 or more young per surviving female.

Discharger:______________________
Location: ______________________
Date Sample Collected: ___________

Analyst:______________________________
Test Start-Date/Time: __________________
Test Start-Date/time: ___________________

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

Figure 3. Data form for the daphnid, *Ceriodaphnia dubia*, survival and reproduction test. Daily summary of data.

Discharger:______________________
Location: ______________________
Date Sample Collected: ___________

Analyst:______________________________
Test Start-Date/Time: __________________
Test Start-Date/time: ___________________

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

Figure 3. Data form for the daphnid, *Ceriodaphnia dubia*, survival and reproduction test. Daily summary of data (CONTINUED).

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1002.0)[1]

1.	Test type:	Static renewal (required)
2.	Temperature (°C):	25 ± 1°C (recommended) Test temperatures should not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
3.	Light quality:	Ambient laboratory illumination (recommended)
4.	Light intensity:	10-20 μE/m^2/s, or 50-100 ft-c (ambient laboratory levels) (recommended)
5.	Photoperiod:	16 h light, 8 h dark (recommended)
6.	Test chamber size:	30 mL (recommended minimum)
7.	Test solution volume:	15 mL (recommended minimum)
8.	Renewal of test solutions:	Daily (required)
9.	Age of test organisms:	Less than 24 h; and all released within a 8-h period (required)
10.	No. neonates per test chamber:	1 Assigned using blocking by known parentage (Subsection 13.10.2.4) (required)
11.	No. replicate test chambers per concentration:	10 (required minimum)
12.	No. neonates per test concentration:	10 (required minimum)
13.	Feeding regime:	Feed 0.1 mL each of YCT and algal suspension per test chamber daily (recommended)
14.	Cleaning:	Use freshly cleaned glass beakers or new plastic cups daily (recommended)
15.	Aeration:	None (recommended)
16.	Dilution water:	Uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or DMW (see Section 7, Dilution Water) (available options)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1002.0) (CONTINUED)

17.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Water: 100% receiving water (or minimum of 5) and a control (recommended)
18.	Dilution factor:	Effluents: ≥ 0.5 (recommended) Receiving Waters: None or ≥ 0.5 (recommended)
19.	Test duration:	Until 60% or more of surviving control females have three broods (maximum test duration 8 days) (required)
20.	Endpoints:	Survival and reproduction (required)
21.	Test acceptability criteria:	80% or greater survival of all control organisms and an average of 15 or more young per surviving female in the control solutions. 60% of surviving control females must produce three broods (required)
22.	Sampling requirements:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
23.	Sample volume required:	1 L/day (recommended)

13.13 **DATA ANALYSIS**

13.13.1 GENERAL

13.13.1.1 Tabulate and summarize the data. A sample set of survival and reproduction data is listed in Table 4.

TABLE 4. SUMMARY OF SURVIVAL AND REPRODUCTION DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, EXPOSED TO AN EFFLUENT FOR SEVEN DAYS

Effluent Concentration (%)	No. of Young per Adult Replicate										No. Live Adults
	1	2	3	4	5	6	7	8	9	10	
Control	27	30	29	31	16	15	18	17	14	27	10
1.56	32	35	32	26	18	29	27	16	35	13	10
3.12	39	30	33	33	36	33	33	27	38	44	10
6.25	27	34	36	34	31	27	33	31	33	31	10
12.5	10	13	7	7	7	10	10	16	12	2	10
25.0	0	0	0	0	0	0	0	0	0	0	2

13.13.1.2 The endpoints of toxicity tests using the daphnid, *Ceriodaphnia dubia*, are based on the adverse effects on survival and reproduction. The LC50, the IC25, the IC50 and the EC50 are calculated using point estimation techniques, and LOEC and NOEC values for survival and reproduction are obtained using a hypothesis test approach such as Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962), Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50, IC25, IC50 and EC50. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for reproduction, but included in the estimation of the LC50, IC25, IC50, and EC50. See the Appendices for examples of the manual computations, program listings, and examples of data input and program output.

13.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

13.13.1.4 At the end of the test, if 50% or more of the surviving organisms in a block are identified as males, the entire block must be excluded from data analysis for the reproduction endpoint (i.e., calculation of the reproduction NOEC and IC25 as described in Subsection 13.13.3), but may be used in the analysis of the survival endpoint (i.e., calculation of the survival NOEC and LC50 as described in Subsection 13.13.2). For blocks having fewer than 50% of surviving organisms identified as males, the males (not the entire block) must be excluded from the analysis of reproduction (i.e., calculation of the reproduction NOEC and IC25 as described in Subsection 13.13.3), but may be used in the analysis of survival (i.e., calculation of the survival NOEC and LC50 as described in Subsection 13.13.2). Note that the exclusion of males from the analysis of reproduction may create unequal sample sizes among the concentrations, influencing the statistical methods chosen for analysis of reproduction (Figure 6). Determinations regarding test acceptability criteria for survival and reproduction (Subsection 13.12) must be made prior to exclusion of any blocks. In addition to these test acceptability criteria, if fewer than eight replicates in the

control remain after excluding males and blocks with 50% or more of surviving organisms identified as males, the test is invalid and must be repeated with a newly collected sample.

13.13.2 EXAMPLE OF ANALYSIS OF THE DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL DATA

13.13.2.1 Formal statistical analysis of the survival data is outlined on the flowchart in Figure 4. The response used in the analysis is the number of animals surviving at each test concentration. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the EC50, LC50, IC25, or IC50 endpoints. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the LC, EC, and IC endpoints.

13.13.2.2 Fisher's Exact Test is used to determine the NOEC and LOEC endpoints. It provides a conservative test of the equality of any two survival proportions assuming only the independence of responses from a Bernoulli (binomial) population. Additional information on Fisher's Exact Test is provided in Appendix G.

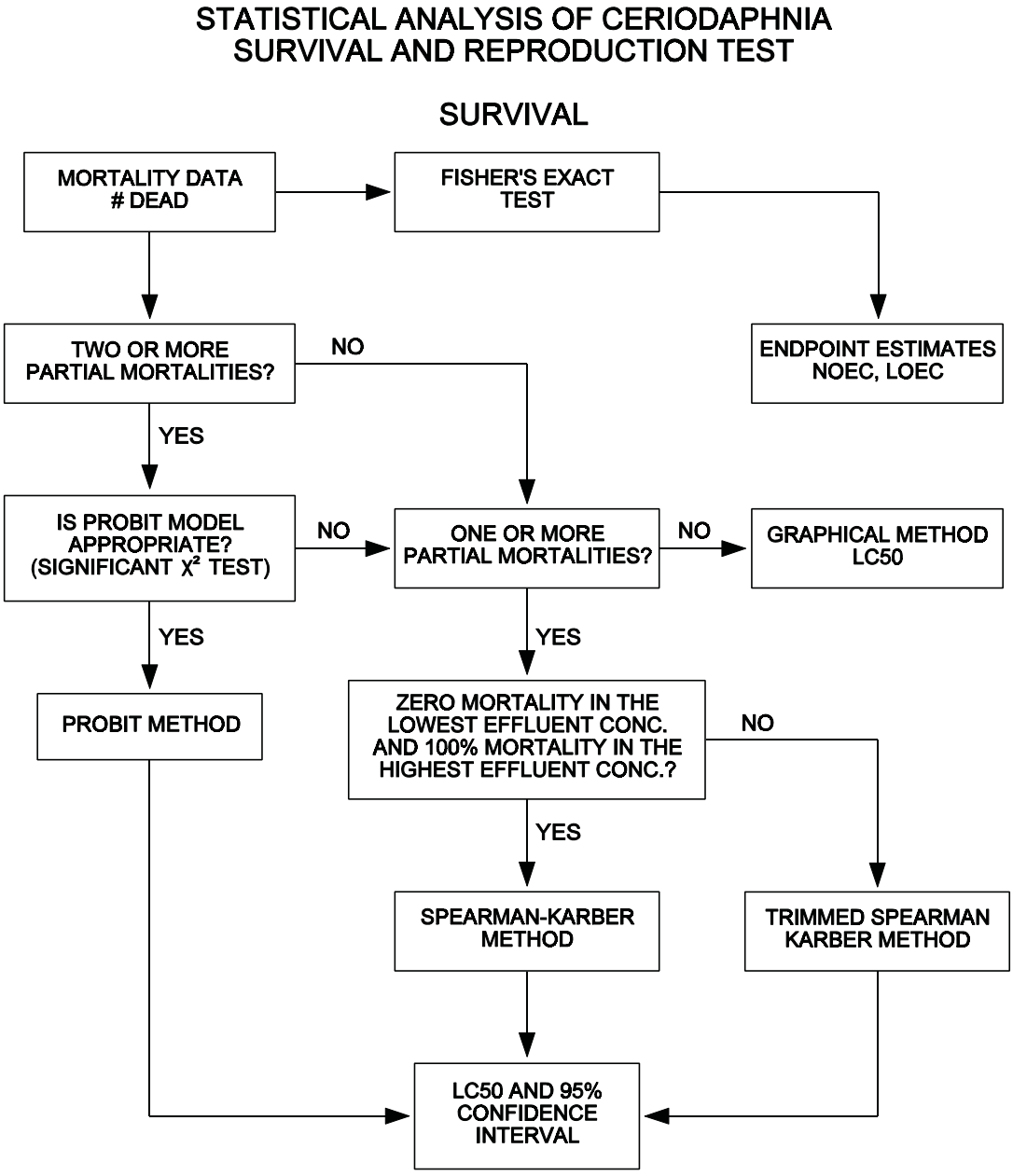

Figure 4. Flowchart for statistical analysis of the daphnid, *Ceriodaphnia dubia*, survival data.

13.13.2.3 Probit Analysis (Finney, 1971; Appendix I) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total number dead at a given concentration is the response.

13.13.2.4 Example of Analysis of Survival Data

13.13.2.4.1 The data in Table 4 will be used to illustrate the analysis of survival data from the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test. As can be seen from the data in Table 4, there were no deaths in the 1.56%, 3.12%, 6.25%, and 12.5% concentrations. These concentrations are obviously not different from the control in terms of survival. This leaves only the 25% effluent concentration to be tested statistically for a difference in survival from the control.

13.13.2.5 Fisher's Exact Test

13.13.2.5.1 The basis for Fisher's Exact Test is a 2x2 contingency table. From the 2x2 table prepared by comparing the control and the effluent concentration, determine statistical significance by looking up a value in the table provided in Appendix G (Table G.5). However, to use this table the contingency table must be arranged in the format illustrated in Table 5.

TABLE 5. FORMAT OF THE 2x2 CONTINGENCY TABLE

	Number of		Number of
	Successes	Failures	Observations
Condition 1	a	A - a	A
Condition 2	b	B - b	B
Total	a + b	[(A+B) - a - b]	A + B

13.13.2.5.2 Arrange the table so that the total number of observations for row one is greater than or equal to the total for row two ($A \geq B$). Categorize a success such that the proportion of successes for row one is greater than or equal to the proportion of successes for row two ($a/A \geq b/B$). For these data, a success may be 'alive' or 'dead' whichever causes $a/A \geq b/B$. The test is then conducted by looking up a value in the table of significance levels of b and comparing it to the b value given in the contingency table. The table of significance levels of b is included in Appendix G, Table G.5. Enter Table G.5 in the section for A, subsection for B, and the line for a. If the b value of the contingency table is equal to or less than the integer in the column headed 0.05 in Table G.5, then the survival proportion for the effluent concentration is significantly different from that of the control. A dash or absence of entry in Table G.5 indicates that no contingency table in that class is significant.

13.13.2.5.3 To compare the control and the effluent concentration of 25%, the appropriate contingency table for the test is given in Table 6.

13.13.2.5.4 Since $10/10 \geq 3/10$, the category 'alive' is regarded as a success. For A = 10, B = 10 and, a = 10, under the column headed 0.05, the value from Table G.5 is b = 6. Since the value of b (b = 3) from the contingency table (Table 6), is less than the value of b (b = 6) from Table G.5 in Appendix G, the test concludes that the proportion

surviving in the 25% effluent concentration is significantly different from the control. Thus the NOEC for survival is 12.5% and the LOEC is 25%.

TABLE 6. 2x2 CONTIGENCY TABLE FOR CONTROL AND 25% EFFLUENT

	Number of		
	Alive	Dead	Number of Observations
Condition 1	10	0	10
Condition 2	3	7	10
Total	13	7	20

13.13.2.6 Calculation of the LC50

13.13.2.6.1 The data used for the Trimmed Spearman-Karber Method are summarized in Table 7. To perform the Trimmed Spearman-Karber Method, run the USEPA Trimmed Spearman-Karber Program. An example of the program input and output is supplied in Appendix J.

TABLE 7. DATA FOR TRIMMED SPEARMAN-KARBER ANALYSIS

	Effluent Concentration (%)					
	Control	1.56	3.12	6.25	12.5	25.0
Number Dead	0	0	0	0	0	8
Number Exposed	10	10	10	10	10	10

13.13.2.6.2 For this example, with only one partial mortality, Trimmed Spearman-Karber analysis appears appropriate for this data.

13.13.2.6.3 Figure 5 shows the output for the Trimmed Spearman-Karber Analysis of the data in Table 7 using the USEPA Program.

13.13.3 EXAMPLE OF ANALYSIS OF THE DAPHNID, *CERIODAPHNIA DUBIA,* REPRODUCTION DATA

13.13.3.1 Formal statistical analysis of the reproduction data is outlined on the flowchart in Figure 6. The response used in the statistical analysis is the number of young produced per adult female, which is determined by taking the total number of young produced until either the time of death of the adult or the end of the experiment, whichever comes first. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test. An animal that dies before

producing young, if it has not been identified as a male, would be included in the analysis with zero entered as the number of young produced. The subsequent calculation of the mean number of live young produced per adult female for each toxicant concentration provides a combined measure of the toxicant's effect on both mortality and reproduction. An IC estimate can be calculated for the reproduction data using a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC for reproduction. Concentrations above the NOEC for survival are excluded from the hypothesis test for reproduction effects.

13.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested using the Shapiro Wilk's Test for normality, and Bartlett's Test for homogeneity of variance. If either of these tests fails, a nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

13.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

13.13.3.4 The data, mean, and variance of the observations at each concentration including the control are listed in Table 8. A plot of the number of young per adult female for each concentration is provided in Figure 7. Since there is significant mortality in the 25% effluent concentration, its effect on reproduction is not considered.

TABLE 8. THE DAPHNID, *CERIODAPHNIA DUBIA,* REPRODUCTION DATA

		Effluent Concentration (%)			
Replicate	Control	1.56	3.12	6.25	12.5
1	27	32	39	27	10
2	30	35	30	34	13
3	29	32	33	36	7
4	31	26	33	34	7
5	16	18	36	31	7
6	15	29	33	27	10
7	18	27	33	33	10
8	17	16	27	31	16
9	14	35	38	33	12
10	27	13	44	31	2
Mean $\overline{Y}_I$	22.4	26.3	34.6	31.7	9.4
S_i^2	48.0	64.0	23.4	8.7	15.1
i	1	2	3	4	5

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 1 TEST NUMBER: 2 DURATION: 7 Days
TOXICANT: effluent
SPECIES: *Ceriodaphnia dubia*

RAW DATA: Concentration (%)	Number Exposed	Mortalities
.00	10	0
1.25	10	0
3.12	10	0
6.25	10	0
12.5	10	0
25.0	10	8

SPEARMAN-KARBER TRIM: 20.00 %

SPEARMAN-KARBER ESTIMATES: LC50: 19.28
95% CONFIDENCE LIMITS
ARE NOT RELIABLE.

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

Figure 5. Output for USEPA Trimmed Spearman-Karber program.

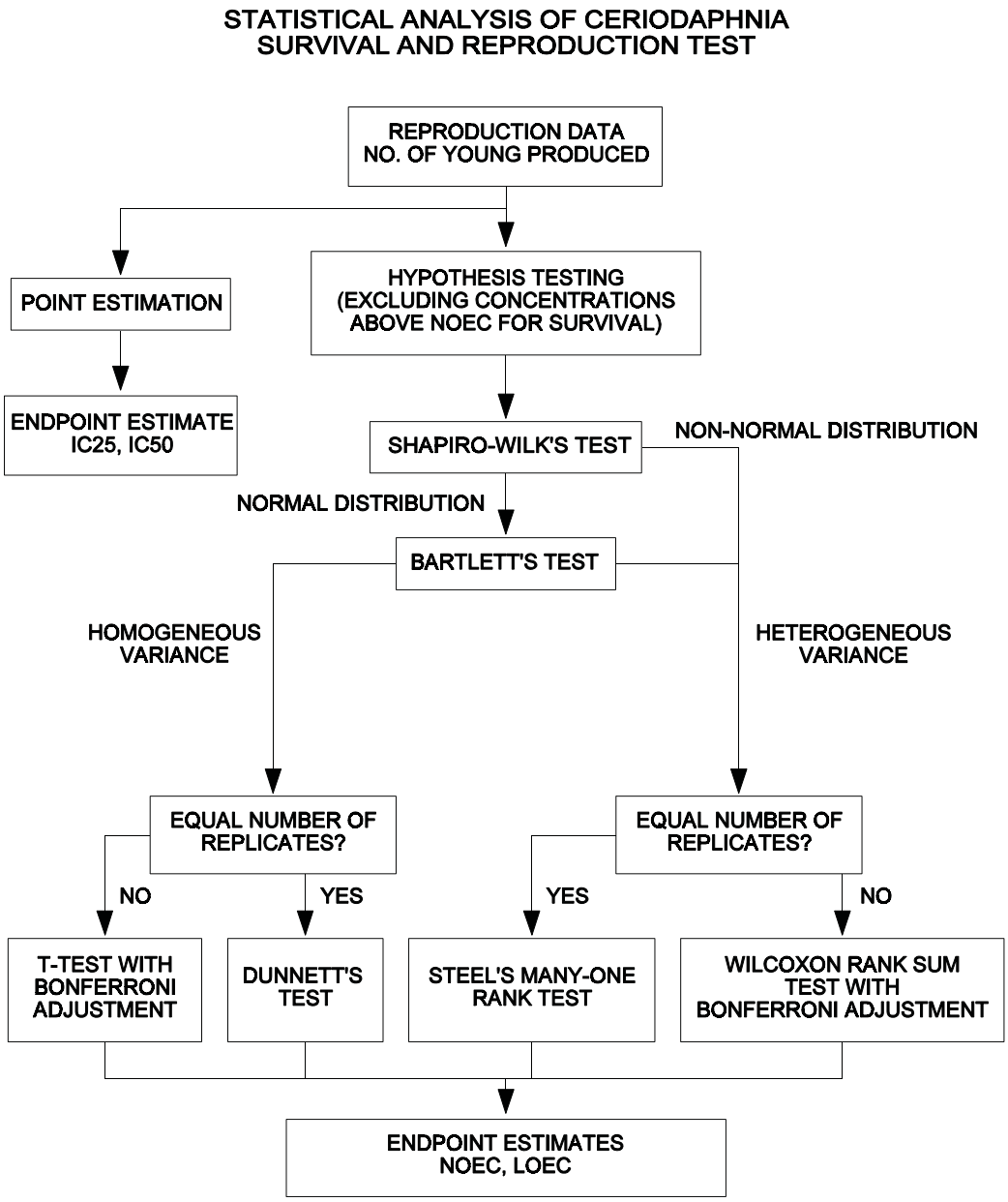

Figure 6. Flowchart for the statistical analysis of the daphnid, *Ceriodaphnia dubia*, reproduction data.

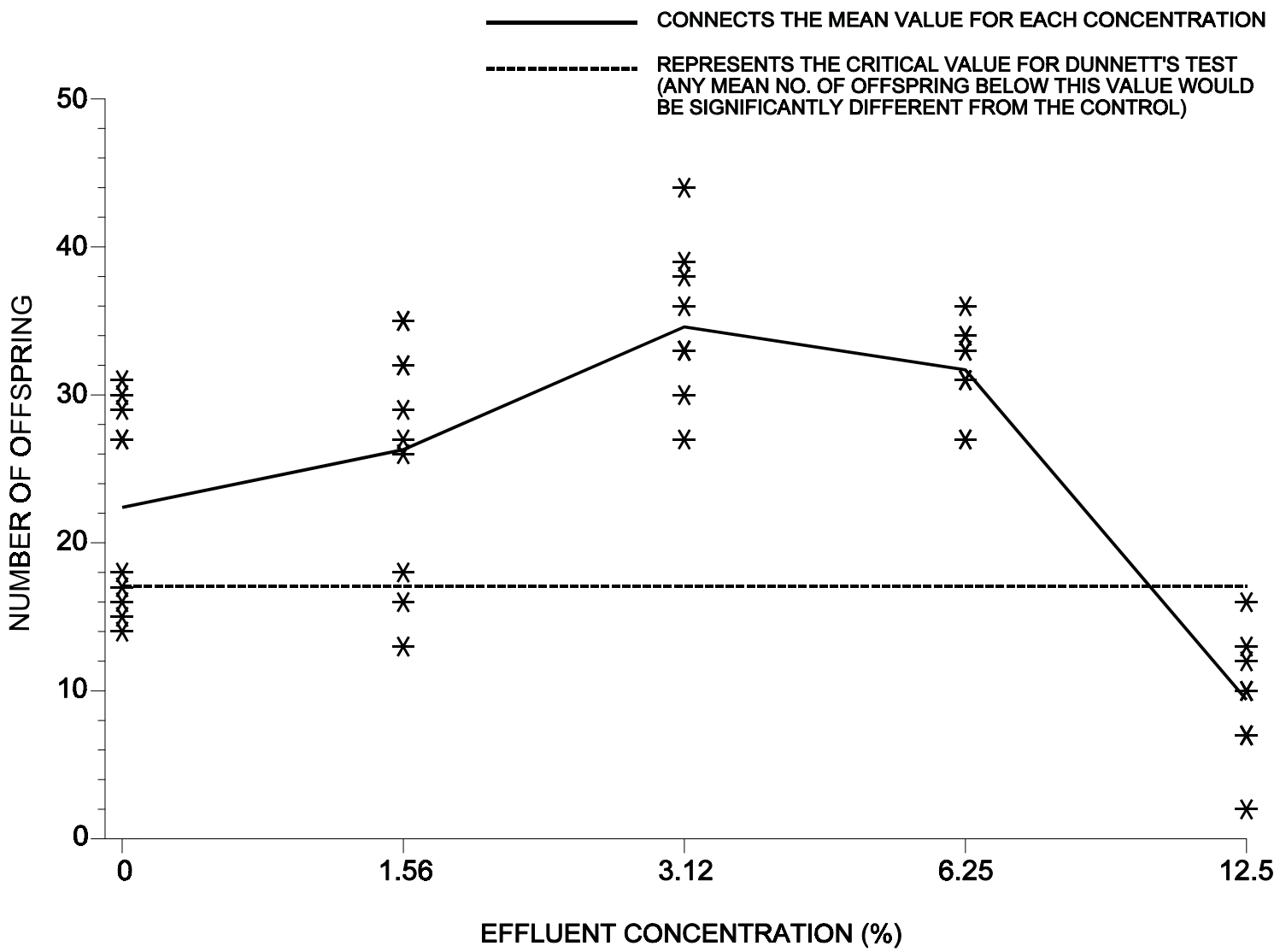

Figure 7. Plot of number of young per adult female from a daphnid, *Ceriodaphnia dubia*, survival and reproduction test.

13.13.3.5 Test for Normality

13.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 9.

TABLE 9. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Effluent Concentration (%)			
Replicate	Control	1.56	3.12	6.25	12.5
1	4.6	5.7	4.4	-4.7	0.6
2	7.6	8.7	-4.6	2.3	3.6
3	6.6	5.7	-1.6	4.3	-2.4
4	8.6	-0.3	-1.6	2.3	-2.4
5	-6.4	-8.3	1.4	-0.7	-2.4
6	-7.4	2.7	-1.6	-4.7	0.6
7	-4.4	0.7	-1.6	1.3	0.6
8	-5.4	-10.3	-7.6	-0.7	6.6
9	-8.4	8.7	3.4	1.3	2.6
10	4.6	-13.3	9.4	-0.7	-7.4

13.13.3.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations.

For this set of data,

$n = 50$

$\bar{X} = \frac{1}{50}(0.0) = 0.0$

$D = 1433.4$

13.13.3.5.3 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq ... \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 10.

13.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, ..., a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 50, k = 25. The a_i values are listed in Table 11.

13.13.3.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 11.

For this set of data:

$$W = \frac{1}{1433.4}(37.3)^2 = 0.97$$

TABLE 10. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-13.3	26	0.6
2	-10.3	27	0.6
3	-8.4	28	0.7
4	-8.3	29	1.3
5	-7.6	30	1.3
6	-7.4	31	1.4
7	-7.4	32	2.3
8	-6.4	33	2.3
9	-5.4	34	2.6
10	-4.7	35	2.7
11	-4.7	36	3.4
12	-4.6	37	3.6
13	-4.4	38	4.3
14	-2.4	39	4.4
15	-2.4	40	4.6
16	-2.4	41	4.6
17	-1.6	42	5.7
18	-1.6	43	5.7
19	-1.6	44	6.6
20	-1.6	45	6.6
21	-0.7	46	7.6
22	-0.7	47	8.6
23	-0.7	48	8.7
24	-0.3	49	8.7
25	0.6	50	9.4

TABLE 11. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.3751	22.7	$X^{(50)} - X^{(1)}$
2	0.2574	19.0	$X^{(49)} - X^{(2)}$
3	0.2260	17.1	$X^{(48)} - X^{(3)}$
4	0.2032	16.9	$X^{(47)} - X^{(4)}$
5	0.1847	15.2	$X^{(46)} - X^{(5)}$
6	0.1691	14.0	$X^{(45)} - X^{(6)}$
7	0.1554	14.0	$X^{(44)} - X^{(7)}$
8	0.1430	12.1	$X^{(43)} - X^{(8)}$
9	0.1317	11.1	$X^{(42)} - X^{(9)}$
10	0.1212	9.3	$X^{(41)} - X^{(10)}$
11	0.1113	9.3	$X^{(40)} - X^{(11)}$
12	0.1020	9.0	$X^{(39)} - X^{(12)}$
13	0.0932	8.7	$X^{(38)} - X^{(13)}$
14	0.0846	6.0	$X^{(37)} - X^{(14)}$
15	0.0764	5.8	$X^{(36)} - X^{(15)}$
16	0.0685	5.1	$X^{(35)} - X^{(16)}$
17	0.0608	4.2	$X^{(34)} - X^{(17)}$
18	0.0532	3.9	$X^{(33)} - X^{(18)}$
19	0.0459	3.9	$X^{(32)} - X^{(19)}$
20	0.0386	3.0	$X^{(31)} - X^{(20)}$
21	0.0314	2.0	$X^{(30)} - X^{(21)}$
22	0.0244	2.0	$X^{(29)} - X^{(22)}$
23	0.0174	1.4	$X^{(28)} - X^{(23)}$
24	0.0104	0.9	$X^{(27)} - X^{(24)}$
25	0.0035	0.0	$X^{(26)} - X^{(25)}$

13.13.3.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 50 observations (n) is 0.930. Since W = 0.97 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

13.13.3.6 Test for Homogeneity of Variance

13.13.3.6.1 The test used to examine whether the variation in number of young produced is the same across all effluent concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i)\ln \overline{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, $V_i = (n_i - 1)$

p = number of levels of effluent concentration and control

n_i = the number of replicates for concentration i

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + (3(p-1))^{-1}[\sum_{i=1}^{P} \frac{1}{V_i} - (\sum_{i=1}^{P} V_i)^{-1}]$$

13.13.3.6.2 For the data in this example (see Table 8), all effluent concentrations including the control have the same number of replicates (n_i = 10 for all i). Thus, V_i = 9 for all i.

13.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(45)\ln(31.8) - 9\sum_{i=1}^{P} \ln(S_i^2)]/1.04$$

= [45(3.46) - 9(16.061)]/1.04

= 11.15/1.04

= 10.72

13.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with four degrees of freedom, is 13.3. Since B = 10.7 is less than the critical value of 13.3, conclude that the variances are not different.

13.13.3.7 Dunnett's Procedure

13.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 12.

TABLE 12. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	= SSB/(p-1)
Within	N - p	SSW	= SSW/(N-p)
Total	N - 1	SST	

Where: p = number effluent concentrations including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$ Between Sum of Squares

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the number of young produced by female j in effluent concentration i)

13.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 10$

$N = 50$

$T_1 = Y_{11} + Y_{12} + \ldots + Y_{110} = 224$
$T_2 = Y_{21} + Y_{22} + \ldots + Y_{210} = 263$

$T_3 = Y_{31} + Y_{32} + \ldots + Y_{310} = 346$
$T_4 = Y_{41} + Y_{42} + \ldots + Y_{410} = 317$
$T_5 = Y_{51} + Y_{52} + \ldots + Y_{510} = 94$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 1244$

$$SSB = \sum_{i=1}^{P} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$

$$t_2 = \frac{(22.4 - 26.3)}{[5.64\sqrt{(\frac{1}{10}) + (\frac{1}{10})}]} = -1.55$$

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$

$$= 36{,}272 - \frac{(1244)^2}{50} = 5321.28$$

$SSW = SST - SSB = 5321.28 - 3887.88 = 1433.40$

$S_B^2 = SSB/(p-1) = 3887.88/(5-1) = 971.97$

$S_W^2 = SSW/(N-p) = 1433.40/(50-5) = 31.85$

13.13.3.7.3 Summarize these calculations in an ANOVA table (Table 13).

TABLE 13. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	4	3887.88	971.97
Within	45	1433.40	31.85
Total	49	5321.28	

13.13.3.7.4 To perform the individual comparisons, calculate the t statistic for each concentration and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(\frac{1}{n_1}) + (\frac{1}{n_i})}}$$

Where: $\bar{Y}_i$ = mean number of young produced for effluent concentration i

$\bar{Y}_1$ = mean number of young produced for the control

S_W = square root of within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

Since we are looking for a decrease in reproduction from the control, the mean for concentration i is subtracted from the control mean in the t statistic above. However, if we were looking for an increased response over the control, the control mean would be subtracted from the mean at a concentration.

13.13.3.7.5 Table 14 includes the calculated t values for each concentration and control combination. In this example, comparing the 1.56% concentration with the control the calculation is as follows:

$$t_2 = \frac{(22.4 - 26.3)}{[5.64\sqrt{(\frac{1}{10}) + (\frac{1}{10})}]} = -1.55$$

TABLE 14. CALCULATED T VALUES

Effluent Concentration (%)	i	t_i
1.56	2	-1.55
3.12	3	-4.84
6.25	4	-3.69
12.5	5	5.16

13.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean reproduction, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. Since an entry for 45 degrees of freedom for error is not provided in the table, the entry for 40 degrees of freedom for error, an alpha level of 0.05 and four concentrations (excluding the control) will be used, 2.23. The mean reproduction for concentration "i" is considered significantly less than the mean reproduction for the control if t_i is greater than the critical value.

Since t_5 is greater than 2.23, the 12.5% concentration has significantly lower reproduction than the control. Hence the NOEC and the LOEC for reproduction are 6.25% and 12.5%, respectively.

13.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\,S_w\sqrt{(\frac{1}{n_1})+(\frac{1}{n})}$$

Where: d = the critical value for the Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

13.13.3.7.8 In this example:

$$MSD = 2.23(5.64)\sqrt{(\frac{1}{10})+(\frac{1}{10})}$$

$$= 2.23\ (5.64)\ (0.447)$$

$$= 5.62$$

13.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 5.62.

13.13.3.7.10 This represents a 25% decrease in mean reproduction from the control.

13.13.3.8 Calculation of the IC

13.13.3.8.1 The reproduction data in Table 4 are utilized in this example. As can be seen from Figure 8, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

13.13.3.8.2 Starting with the observed control mean, $\overline{Y}_1$= 22.4, and the observed mean for the lowest effluent concentration,$\overline{Y}_2$= 26.3, we see that $\overline{Y}_1$ is less than $\overline{Y}_2$.

13.13.3.8.3 Calculate the smoothed means:

$M_1 = M_2 = (\overline{Y}_1+\overline{Y}_2)/2 = 24.35$

13.13.3.8.4 Since $\overline{Y}_3$= 34.6 is larger than M_2, average $\overline{Y}_3$ with the previous concentrations:

$$M_1 = M_2 = M_3 = (M_1 + M_2 + \overline{Y}_3)/3 = 27.7.$$

13.13.3.8.5 Additionally, $\overline{Y}_4$ = 31.7 is larger than M_3, and is pooled with the first three means. Thus:

$$(M_1 + M_2 + M_3 + \overline{Y}_4)/4 = 28.7 = M_1 = M_2 = M_3 = M_4$$

13.13.3.8.6 Since $M_4 > \overline{Y}_5$ = 9.4, set M_5 = 9.4. Likewise, $M_5 > \overline{Y}_6$ = 0, and M_6 becomes 0. Table 15 contains the smoothed means and Figure 8 gives a plot of the smoothed means and the interpolated response curve.

TABLE 15. DAPHNID, *CERIODAPHNIA DUBIA*, REPRODUCTION MEAN RESPONSE AFTER SMOOTHING

Response Effluent Conc. (%)	i	Smoothed Means, Y_i (young/female)	Means, M_i (young/female)
Control	1	22.4	28.75
1.56	2	26.3	28.75
3.12	3	34.6	28.75
6.25	4	31.7	28.75
12.5	5	9.4	9.40
25.0	6	0.0	0.00

13.13.3.8.7 Estimates of the IC25 and IC50 can be calculated using the Linear Interpolation Method. A 25% reduction in reproduction, compared to the controls, would result in a mean reproduction of 21.56 young per adult, where $M_1(1 - p/100) = 28.75(1 - 25/100)$. A 50% reduction in reproduction, compared to the controls, would result in a mean reproduction of 14.38 young per adult, where $M_1(1 - p/100) = 28.75(1 - 50/100)$. Examining the smoothed means and their associated concentrations (Table 15), the two effluent concentrations bracketing 21.56 young per adult are C_4 = 6.25% effluent and C_5 = 12.5% effluent. The two effluent concentrations bracketing a response of 14.38 young per adult are also C_4 = 6.25% and C_5 = 12.5%.

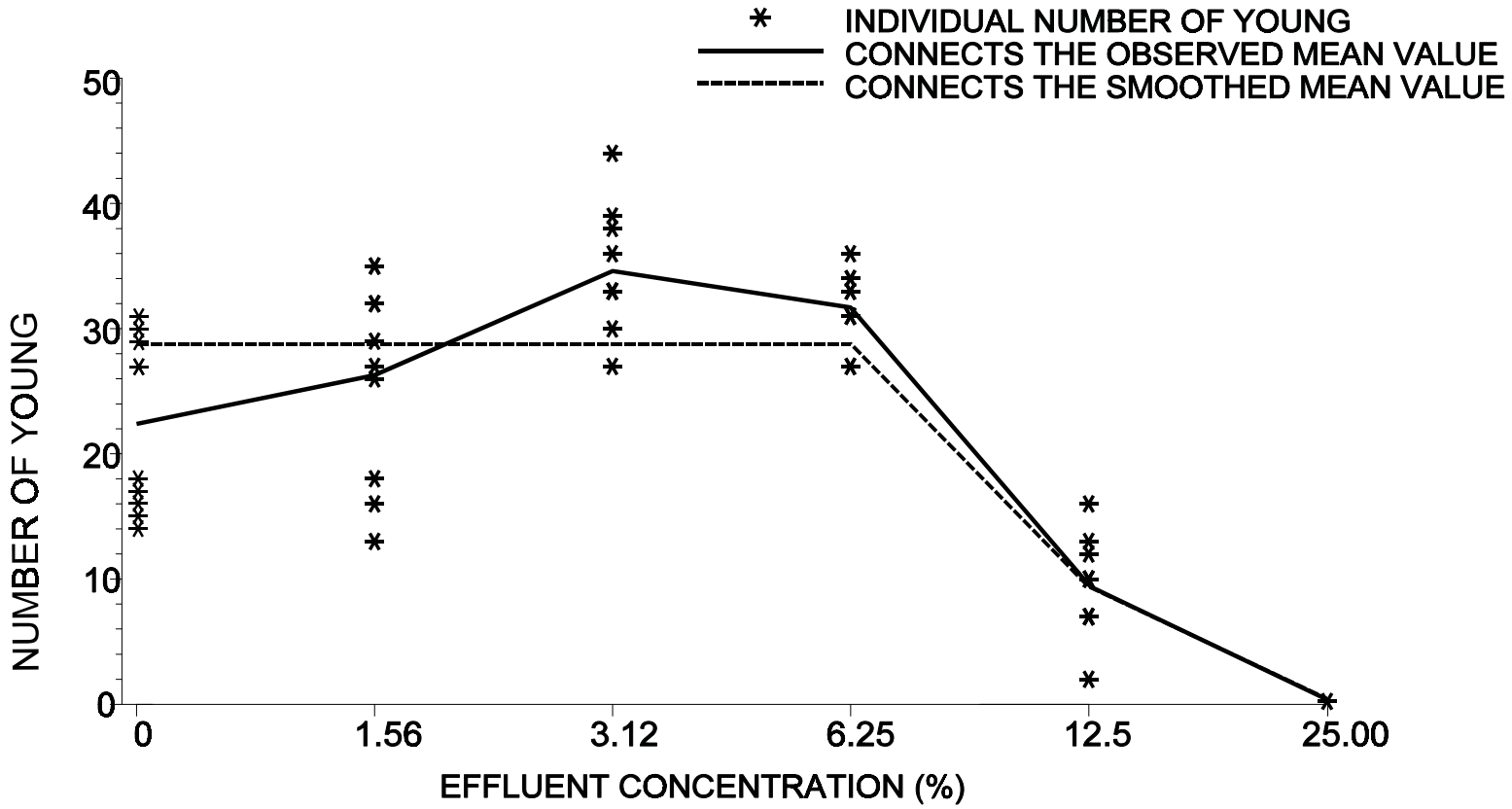

Figure 8. Plot of raw data, observed means, and smoothed means for the daphnid, *Ceriodaphnia dubia*, reproductive data.

13.13.3.8.8 Using equation from Section 4.2 in Appendix M, the estimate of the IC25 is as follows:

$$ICp = C_j + [M_1(1 - \frac{p}{100}) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 6.25 + [28.75(1 - \frac{25}{100}) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

$$= 8.57\% \text{ effluent}$$

13.13.3.8.9 The estimate of the IC50 is as follows:

$$ICp = C_j + [M_1(1 - \frac{p}{100}) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC50 = 6.25 + [28.75(1 - \frac{50}{100}) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

$$= 10.89\% \text{ effluent}$$

13.13.3.8.10 When the ICPIN program was used to analyze this data set for the IC25, requesting 80 resamples, the estimate of the IC25 was 8.5715% effluent. The empirical 95% confidence interval for the true mean was 8.3112% and 9.0418% effluent. The computer output for this data set is provided in Figure 9.

13.13.3.8.11 When the ICPIN program was used to analyze this data set for the IC50, requesting 80 resamples, the estimate of the IC50 was 10.8931% effluent. The empirical 95% confidence interval for the true mean was 10.4373% and 11.6269% effluent. The computer output for this data set is provided in Figure 10.

```
Conc. ID             1         2         3         4         5         6
-------------------------------------------------------------------------
Conc. Tested         0      1.56      3.12      6.25      12.5      25.0
-------------------------------------------------------------------------
Response  1         27        32        39        27        10         0
Response  2         30        35        30        34        13         0
Response  3         29        32        33        36         7         0
Response  4         31        26        33        34         7         0
Response  5         16        18        36        31         7         0
Response  6         15        29        33        27        10         0
Response  7         18        27        33        33        10         0
Response  8         17        16        27        31        16         0
Response  9         14        35        38        33        12         0
Response 10         27        13        44        31         2         0
-------------------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Example   Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration:             7-d
DATA FILE: cdmanual.icp
OUTPUT FILE: cdmanual.i25
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates               %       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1         10             0.000         22.400      6.931     28.750
  2         10             1.560         26.300      8.001     28.750
  3         10             3.120         34.600      4.835     28.750
  4         10             6.250         31.700      2.946     28.750
  5         10            12.500          9.400      3.893      9.400
  6         10            25.000          0.000      0.000      0.000
-------------------------------------------------------------------------
The Linear Interpolation Estimate:     8.5715   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:   8.5891 Standard Deviation:     0.1831
Original Confidence Limits:   Lower:     8.3112 Upper:     9.0418
Resampling time in Seconds:     2.53  Random Seed: -641671986
```

Figure 9. Example of ICPIN program output for the IC25.

```
Conc. ID             1         2         3         4         5         6
-------------------------------------------------------------------------
Conc. Tested         0      1.56      3.12      6.25      12.5      25.0
-------------------------------------------------------------------------
Response  1         27        32        39        27        10         0
Response  2         30        35        30        34        13         0
Response  3         29        32        33        36         7         0
Response  4         31        26        33        34         7         0
Response  5         16        18        36        31         7         0
Response  6         15        29        33        27        10         0
Response  7         18        27        33        33        10         0
Response  8         17        16        27        31        16         0
Response  9         14        35        38        33        12         0
Response 10         27        13        44        31         2         0
-------------------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Example   Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration:             7-d
DATA FILE: cdmanual.icp
OUTPUT FILE: cdmanual.i50
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates               %       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1         10             0.000         22.400      6.931     28.750
  2         10             1.560         26.300      8.001     28.750
  3         10             3.120         34.600      4.835     28.750
  4         10             6.250         31.700      2.946     28.750
  5         10            12.500          9.400      3.893      9.400
  6         10            25.000          0.000      0.000      0.000
-------------------------------------------------------------------------
The Linear Interpolation Estimate:    10.8931   Entered P Value: 50
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:  10.9316 Standard Deviation:     0.3357
Original Confidence Limits:   Lower:    10.4373 Upper:    11.6269
Resampling time in Seconds:     2.58  Random Seed: 172869646
```

Figure 10. Example of ICPIN program output for the IC50.

13.14 PRECISION AND ACCURACY

13.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 13.14.1.1 and 13.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

13.14.1.1 Single-Laboratory Precision

13.14.1.1.1 Information on the single-laboratory precision of the daphnid, *Ceriodaphnia dubia*, survival and reproduction test is based on the NOEC and LOEC values from nine tests with the reference toxicant sodium pentachlorophenate (NaPCP) is provided in Table 16. The NOECs and LOECs of all tests fell in the same concentration range, indicating maximum possible precision. Table 17 gives precision data for the IC25 and IC50 values for seven tests with the reference toxicant NaPCP. Coefficient of variation was 41% for the IC25 and 28% for the IC50.

13.14.1.1.2 Ten sets of data from six laboratories met the acceptability criteria, and were statistically analyzed using nonparametric procedures to determine NOECs and LOECs.

13.14.1.1.3 EPA evaluated within-laboratory precision of the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test using a database of routine reference toxicant test results from 33 laboratories (USEPA, 2000b). The database consisted of 393 reference toxicant tests conducted in 33 laboratories using a variety of reference toxicants including: cadmium, copper, potassium chloride, sodium chloride, and sodium pentachlorophenate. Among the 33 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 27% for the IC25 reproduction endpoint. In 25% of laboratories, the within-laboratory CV was less than 17%; and in 75% of laboratories, the within-laboratory CV was less than 45%.

TABLE 16: SINGLE LABORATORY PRECISION OF THE DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TEST, USING NAPCP AS A REFERENCE TOXICANT[1,2]

Test	NOEC (mg/L)	LOEC (mg/L)	Chronic Value (mg/L)
1[3]	0.25	0.50	0.35
2[4]	0.20	0.60	0.35
3	0.20	0.60	0.35
4[5]	0.30	0.60	0.42
5	0.30	0.60	0.42
6	0.30	0.60	0.42
7	0.30	0.60	0.42
8	0.30	0.60	0.42
9	0.30	0.60	0.42

1 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

2 Data from Tests performed by Philip Lewis, Aquatic Biology Branch, EMSL-Cincinnati, OH. Tests were conduted in reconstituted hard water (hardness = 180 mg $CaC0_3$/L; pH - 8.1).

3 Concentrations used in Test 1 were: 0.03, 0.06, 0.12, 0.25, 0.50, 1.0 mg NaPCP/L.

4 Concentrations used in Tests 2 and 3 were: 0.007, 0.022, 0.067, 0.020, 0.60 mg NaPCP/L.

5 Concentrations used in Tests 4 through 9 were: 0.0375, 0.075, 0.150, 0.30, 0.60 mg NaPCP/L.

TABLE 17. THE DAPHNID, *CERIODAPHNIA DUBIA,* SEVEN-DAY SURVIVAL AND REPRODUCTION TEST PRECISION FOR A SINGLE LABORATORY USING NAPCP AS THE REFERENCE TOXICANT (USEPA, 1991a)

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)
19	0.30	0.3754	0.4508
46A	0.20	0.0938	0.2608
46B	0.20	0.2213	0.2879
49	0.20	0.2303	0.2912
55	0.20	0.2306	0.3177
56	0.10	0.2241	0.2827
n	7	7	7
Mean	NA	0.2157	0.2953
CV(%)	NA	41.1	27.9

13.14.1.2 Multilaboratory Precision

13.14.1.2.1 A multilaboratory study was performed by the Aquatic Biology Branch, EMSL-Cincinnati in 1985e, involving a total of 11 analysts in 10 different laboratories (Neiheisel et. al., 1988; USEPA, 1988e). Each analyst performed one-to-three seven-day tests using aliquots of a copper-spiked effluent sample, for a total of 25 tests. The tests were performed on the same day in all participating laboratories, using a pre-publication draft of Method 1002.0. The NOECs and LOECs for these tests were within one concentration interval which, with a dilution factor of 0.5, is equivalent to a two-fold range in concentration (Table 18).

13.14.1.2.2 A second multilaboratory study of Method 1002.0 (using the first edition of this manual; USEPA, 1985c), was coordinated by Battelle, Columbus Division, and involved 11 participating laboratories (Table 19) (DeGraeve et al., 1989). All participants used 10% DMW (10% PERRIER® Water) as the culture and dilution water, and used their own formulation of food for culturing and testing the *Ceriodaphnia dubia*. Each laboratory was to conduct at least one test with each of eight blind samples. Each test consisted of 10 replicates of one organism each for five toxicant concentrations and a control. Of the 116 tests planned, 91 were successfully initiated, and 70 (77%) met the survival and reproduction criteria for acceptability of the results (80% survival and nine young per initial female). If the reproduction criteria of 15 young/female, used in this edition of the method, had been applied to the results of the interlaboratory study, 22 additional tests would have been unacceptable. The overall precision (CV) of the test was 27% for the survival data (7-day LC50s) and 37.5% and 39.0% for the reproduction data (IC50s and IC25s, respectively).

13.14.1.2.3 In 2000, EPA conducted an interlaboratory variability study of the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 34 participant laboratories tested 3 or 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Of the 122 *Ceriodaphnia dubia* Survival and Reproduction tests conducted in this study, 82.0% were successfully completed and met the required test acceptability criteria. Of 27 tests that were conducted on blank samples, none showed false positive results for survival endpoints, and only one resulted in false positive results for the growth endpoint, yielding a false positive rate of 3.70%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 20 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 35.0% for IC25 results. Table 21 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned three concentrations for the reference toxicant and effluent sample types and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 97.2%, 91.3%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively. For the growth endpoint, NOEC values spanned five concentrations for the reference toxicant sample type, three concentrations for the effluent sample type, and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 83.3%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

13.14.2 ACCURACY

13.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 18. INTERLABORATORY PRECISION FOR THE DAPHNID, *CERIODAPHNIA DUBIA,* SURVIVAL AND REPRODUCTION TEST WITH COPPER SPIKED EFFLUENT (USEPA, 1988e)

		Endpoints (% Effluent)			
		Reproduction		Survival	
Analyst	Test	NOEC	LOEC	NOEC	LOEC
3	1	12	25	25	50
4	1	6	12	12	25
4	2	6	12	25	50
5	1	6	12	12	25
5	2	12	25	12	25
6	1	12	25	25	50
6	2	6	12	25	50
10	1	6	12	12	25
10	2	6	12	12	25
11	1	12	25	25	50

TABLE 19. INTERLABORATORY PRECISION DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, SUMMARIZED FOR EIGHT REFERENCE TOXICANTS AND EFFLUENTS (USEPA, 1991a)

Test Material	Mean IC50	CV%	Mean IC25	CV%
Sodium chloride	1.34	29.9	1.00	34.3
Industrial	3.6	83.3	3.2	78.1
Sodium chloride	0.96	57.4	0.09	44.4
Pulp and Paper	60.0	28.3	47.3	27.0
Potassium dichromate	35.8	30.8	23.4	32.7
Pulp and Paper	70.2	7.5	55.7	12.2
Potassium dichromate	53.2	25.9	29.3	46.8
Industrial	69.8	37.0	67.3	36.7
n		8		8
Mean		37.5		39.0
Standard Deviation		23.0		19.1

TABLE 20. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	-	-	-
	Effluent	17.4	27.6	32.6
	Receiving water	-	-	37.4
	Average	17.4	27.6	35.0

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the reference toxicant sample type a majority of the results were outside of the test concentration range, so precision estimates were not calculated. For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

3 The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

4 The between-laboratory component of variability for duplicate samples tested at different laboratories.

5 The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 21. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	100%	97.2	0.00	2.78
	Effluent	25%	65.2	26.1	8.70
	Receiving water	25%	90.0	10.0	0.00
Growth NOEC	Reference toxicant	100%	72.2	11.1	16.7
	Effluent	12.5%	70.8	29.2	0.00
	Receiving water	25%	70.0	30.0	0.00

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.

[3] Percent of values two or more concentration intervals above or below the median.

SECTION 14

TEST METHOD

GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, GROWTH TEST METHOD 1003.0

14.1 SCOPE AND APPLICATION

14.1.1 This method measures the chronic toxicity of effluents and receiving water to the freshwater green alga, *Selenastrum capricornutum*, in a four-day static test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

14.1.2 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

14.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly degradable or highly volatile toxicants present in the source may not be detected in the test.

14.1.4 This test method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

14.1.5 This test is very versatile because it can also be used to identify wastewaters which are biostimulatory and may cause nuisance growths of algae, aquatic weeds, and other organisms at higher trophic levels.

14.2 SUMMARY OF METHOD

14.2.1 A green alga, *Selenastrum capricornutum,* population is exposed in a static system to a series of concentrations of effluent, or to receiving water, for 96 h. The response of the population is measured in terms of changes in cell density (cell counts per mL), biomass, chlorophyll content, or absorbance.

14.3 INTERFERENCES

14.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

14.3.2 Adverse effects of high concentrations of suspended and/or dissolved solids, color, and extremes of pH may mask the presence of toxic substances.

14.3.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.3.4 Pathogenic organisms and/or planktivores in the dilution water and effluent may affect test organism survival and growth, and confound test results.

14.3.5 Nutrients in the effluent or dilution water may confound test results.

14.4 SAFETY

14.4.1 See Section 3, Safety and Health.

14.5 **APPARATUS AND EQUIPMENT**

14.5.1 Laboratory *Selenastrum capricornutum* culture unit -- see culturing methods below and USEPA, 2002a. To test effluent toxicity, sufficient numbers of log-phase-growth organisms must be available.

14.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L or more.

14.5.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.5.4 Environmental chamber, incubator, or equivalent facility -- with "cool-white" fluorescent illumination (86 ± 8.6 $\mu E/m^2/s$, 400 ± 40 ft-c, or 4306 lux) and temperature control (25 ± 1 °C).

14.5.5 Mechanical shaker -- capable of providing orbital motion at the rate of 100 cycles per minute (cpm).

14.5.6 Light meter -- with a range of 0-200 $\mu E/m^2/s$ (0-1000 ft-c).

14.5.7 Water purification system -- MILLIPORE MILLI-Q®, deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

14.5.8 Balance -- analytical, capable of accurately weighing 0.00001 g.

14.5.9 Reference weights, class S -- for checking performance of balance.

14.5.10 Volumetric flasks and graduated cylinders -- class A, 10-1000 mL, borosilicate glass, for culture work and preparation of test solutions.

14.5.11 Volumetric pipets -- class A, 1-100 mL.

14.5.12 Serological pipets -- 1-10 mL, graduated.

14.5.13 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

14.5.14 Wash bottles -- for rinsing small glassware, instrument electrodes, and probes.

14.5.15 Test chambers -- four 125 or 250 mL borosilicate, Erlenmeyer flasks, with foam plugs or stainless steel or Shumadzu closures. For special glassware cleaning requirements (see Section 5, Facilities, Equipment, and Supplies).

14.5.16 Culture chambers -- 1-4 L borosilicate, Erlenmeyer flasks.

14.5.17 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

14.5.18 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

14.5.19 Thermometer, National Bureau of Standards Certified, (see USEPA Method 170.1, USEPA, 1979b) -- to calibrate laboratory thermometers.

14.5.20 Meters, pH and specific conductivity -- for routine physical and chemical measurements.

14.5.21 Tissue grinder -- for chlorophyll extraction.

14.5.22 Fluorometer (Optional) -- equipped with chlorophyll detection light source, filters, and photomultiplier tube (Turner Model 110 or equivalent).

14.5.23 UV-VIS spectrophotometer -- capable of accommodating 1-5 cm cuvettes.

14.5.24 Cuvettes for spectrophotometer -- 1-5 cm light path.

14.5.25 Electronic particle counter (Optional) -- Coulter Counter, Model ZBI, or equivalent, with mean cell (particle) volume determination.

14.5.26 Microscope -- with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condenser, and light source (inverted or conventional microscope).

14.5.27 Counting chamber -- Sedgwick-Rafter, Palmer-Maloney, or hemocytometer.

14.5.28 Centrifuge -- with swing-out buckets having a capacity of 15-100 mL.

14.5.29 Centrifuge tubes -- 15-100 mL, screw-cap.

14.5.30 Filtering apparatus -- for membrane and/or glass fiber filters.

14.6 **REAGENTS AND CONSUMABLE MATERIALS**

14.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.6.2 Data sheets (one set per test) -- for recording data.

14.6.3 Tape, colored -- for labeling test chambers.

14.6.4 Markers, waterproof -- for marking containers, etc.

14.6.5 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA, 1979b.

14.6.6 Buffers pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) for instrument calibration (see USEPA Method 150.1, USEPA, 1979b).

14.6.7 Specific conductivity standards (see USEPA Method 120.1, USEPA, 1979b).

14.6.8 Standard particles -- such as chicken or turkey fibroblasts or polymer microspheres, 5.0 ± 0.03 µm diameter, 65.4 μm^3 volume, for calibration of electronic particle counters.

14.6.9 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

14.6.10 Laboratory quality control samples and standards -- for calibration of the above methods.

14.6.11 Reference toxicant solutions -- see Section 4, Quality Assurance.

14.6.12 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

14.6.13 Effluent or receiving water and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Testing.

14.6.14 Acetone -- pesticide-grade or equivalent.

14.6.15 Dilute (10%) hydrochloric acid -- carefully add 10 mL of concentrated HCl to 90 mL of MILLI-Q® water.

14.6.16 TEST ORGANISMS, GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*

14.6.16.1 *Selenastrum capricornutum*, a unicellular coccoid green alga, is the test organism. The genus and species name of this organism was formally changed to *Pseudokirchneriella subcapitata* (Hindak, 1990), however, the method manual will continue to refer to *Selenastrum capricornutum* to maintain consistency with previous versions of the method.

14.6.16.2 Algal Culture Medium is prepared as follows:

14.6.16.2.1 Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.

14.6.16.2.2 Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L, mix well, and adjust the pH to 7.5 ± 0.1, using 0.1N NaOH or HCl, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.

14.6.16.2.3 Immediately filter the pH-adjusted medium through a 0.45 µm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.

14.6.16.2.4 If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels. If a 0.22 µg filter is used no sterilization is needed.

14.6.16.2.5 Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

14.6.16.2.6 When prepared according to Table 1, the micronutrient stock solution contains ethylenediaminetetraacetic acid (EDTA). EPA requires the addition of EDTA to nutrient stock solutions when conducting the *Selenastrum capricornutum* Growth Test and submitting data under NPDES permits. The use of EDTA improves test method performance by reducing the incidence of false positives and increasing test method precision. In interlaboratory testing of split samples analyzed with and without the addition of EDTA, false positive rates were 0.00% with EDTA and 33.3% without EDTA (USEPA, 2001a). Interlaboratory variability, expressed as the CV for IC25 values, was 34.3% with EDTA and 58.5% without EDTA (USEPA, 2001a). While the addition of EDTA improves test performance, EPA also cautions that the addition of EDTA may cause the *Selenastrum capricornutum* Growth Test to underestimate the toxicity of metals. Regulatory authorities should consider this possibility when selecting test methods for monitoring effluents that are suspected to contain metals. As recommended in EPA's Technical Support Document for Water Quality-Based Toxics Control (USEPA, 1991a), the most sensitive of at least three test species from different phyla should be used for monitoring the toxicity of effluents.

TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 mL MILLI-Q® WATER	
1.	MACRONUTRIENTS		
A.	$MgCl_2 \cdot 6H_2O$	6.08	g
	$CaCl_2 \cdot 2H_2O$	2.20	g
	$NaNO_3$	12.75	g
B.	$MgSO_4 \cdot 7H_2O$	7.35	g
C	K_2HPO_4	0.522	g
D.	$NaHCO_3$	7.50	g
2.	MICRONUTRIENTS		
	H_3BO_3	92.8	mg
	$MnCl_2 \cdot 4H_2O$	208.0	mg
	$ZnCl_2$	1.64	mg[1]
	$FeCl_3 \cdot 6H_2O$	79.9	mg
	$CoCl_2 \cdot 6H_2O$	0.714	mg[2]
	$Na_2MoO_4 \cdot 2H_2O$	3.63	mg[3]
	$CuCl_2 \cdot 2H_2O$	0.006	mg[4]
	$Na_2EDTA \cdot 2H_2O$	150.0	mg
	Na_2SeO_4	1.196	mg[5]

[1] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[2] $CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[3] $Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[4] $CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock 2, micronutrients.

[5] Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

MACRONUTRIENT	CONCENTRATION (mg/L)	ELEMENT	CONCENTRATION (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

MICRONUTRIENT	CONCENTRATION (µg/L)	ELEMENT	CONCENTRATION (µg/L)
H_3BO_3	185.0	B	32.5
$MnCl_2 \cdot 4H_2O$	416.0	Mn	115.0
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160.0	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300.0	--	----
Na_2SeO_4	2.39	Se	0.91

14.6.16.3 Stock Algal Cultures

14.6.16.3.1 See Section 6, Test Organisms, for information on sources of "starter" cultures of the green alga, *Selenastrum capricornutum*.

14.6.16.3.2 Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring 1 mL to a culture flask containing control algal culture medium (prepared as described above). The volume of stock culture medium initially prepared will depend upon the number of test flasks to be inoculated later from the stock, or other planned uses, and may range from 25 mL in a 125 mL flask to 2 L in a 4-L flask. The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.

14.6.16.3.3 Maintain the stock cultures at 25 ± 1°C, under continuous "Cool-White" fluorescent lighting of 86 ± 8.6 $\mu E/m^2/s$ (400 ± 40 ft-c). Shake continuously at 100 cpm or twice daily by hand.

14.6.16.3.4 Transfer 1 to 2 mL of stock culture weekly to 50 - 100 mL of new culture medium to maintain a continuous supply of "healthy" cells for tests. Aseptic techniques should be used in maintaining the algal cultures, and extreme care should be exercised to avoid contamination. Examine the stock cultures with a microscope for contaminating microorganisms at each transfer.

14.6.16.3.5 Viable unialgal culture material may be maintained for long periods of time if placed in a refrigerator at 4°C.

14.6.16.4 It is recommended that chronic toxicity tests be performed monthly with a reference toxicant. Algal cells four to seven days old are used to monitor the chronic toxicity (growth) of the reference toxicant to the algal stock produced by the culture unit (see Section 4, Quality Assurance, Subsection 4.17).

14.6.16.5 Record Keeping

14.6.16.5.1 Records, kept in a bound notebook, include (1) dates culture media was prepared, (2) source of "starter" cultures, (3) date stock cultures were started, (4) cell density in stock cultures, and (5) dates and results of reference toxicant tests performed (see Section 4, Quality Assurance).

14.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

14.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

14.8 CALIBRATION AND STANDARDIZATION

14.8.1 See Section 4, Quality Assurance.

14.9 QUALITY CONTROL

14.9.1 See Section 4, Quality Assurance.

14.10 TEST PROCEDURES

14.10.1 TEST SOLUTIONS

14.10.1.1 Receiving Waters

14.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 µm NITEX® filter and compared

without dilution against a control. Using four replicate chambers per test, each containing 100 mL and 400 mL for chemical analyses, would require approximately 1 L or more of sample for the test.

14.10.1.2 Effluents

14.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5 and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends using a ≥ 0.5 dilution factor**.

14.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions should be added at the lower range of the effluent concentrations.

14.10.1.2.3 The volume of effluent required for the test is 1 to 2 L. Sufficient test solution (approximately 900 or 1500 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses at the high, medium, and low test concentrations. There is no daily renewal of test solution.

14.10.1.2.4 Tests should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be usedfor the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.10.1.2.5 Just prior to test initiation (approximately 1 h) the temperature of sufficient quantity of the sample to make test solutions should be adjusted to the test temperature and maintained at that temperature during the addition of dilution water.

14.10.1.2.6 The DO of the test solutions should be checked prior to test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If or any solution has a DO concentration below 4.0 mg/L, all of the solutions and the control must be gently aerated.

14.10.1.2.7 Effluents may be toxic and/or nutrient poor. "Poor" growth in an algal toxicity test, therefore, may be due to toxicity or nutrient limitation, or both. To eliminate false negative results due to low nutrient concentrations, 1 mL of each stock nutrient solution is added per liter of effluent prior to use in preparing the test dilutions. Thus, all test treatments and controls will contain at a minimum the concentration of nutrients in the stock culture medium.

14.10.1.2.8 If samples contain volatile substances, the test sample should be added below the surface of the dilution water towards the bottom of the test container through an appropriate delivery tube.

14.10.1.3 Dilution Water

14.10.1.3.1 Dilution water may be stock culture medium, any uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other natural water (see Section 7, Dilution Water). However, if water other than the stock culture medium is used for dilution water, 1 mL of each stock nutrient solution should be added per liter of dilution water. Natural waters used as dilution water must be filtered through a prewashed filter, such as a GF/A, GF/C, or equivalent filter, that provides 0.45 µm particle size retention.

14.10.1.3.2 If the growth of the algae in the test solutions is to be measured with an electronic particle counter, the effluent and dilution water must be filtered through a GF/A or GF/C filter, or other filter providing 0.45 µm particle

size retention, and checked for "background" particle count before it is used in the test. Glass-fiber filters generally provide more rapid filtering rates and greater filtrate volume before plugging.

14.10.1.4 Preparation of Inoculum

14.10.1.4.1 The inoculum is prepared no more than 2 to 3 h prior to the beginning of the test, using *Selenastrum capricornutum* harvested from a four- to-seven-day stock culture. Each milliliter of inoculum must contain enough cells to provide an initial cell density of approximately 10,000 cells/mL (± 10%) in the test flasks. Assuming the use of 250 mL flasks, each containing 100 mL of test solution, the inoculum must contain 1,000,000 cells/mL.

14.10.1.4.2 Estimate the volume of stock culture required to prepare the inoculum. As an example, if the four-to-seven-day-old stock culture used as the source of the inoculum has a cell density of 2,000,000 cells/mL, a test employing 24 flasks, each containing 100 mL of test medium and inoculated with a total of 1,000,000 cells, would require 24,000,000 cells or 15 mL of stock solution (24,000,000/2,000,000) to provide sufficient inoculum. It is advisable to prepare a volume 20% to 50% in excess of the minimum volume required, to cover accidental loss in transfer and handling.

14.10.1.4.3 Prepare the inoculum as follows:

1. Centrifuge 15 mL of stock culture at 1000 x g for 5 min. This volume will provide a 50% excess in the number of cells.
2. Decant the supernatant and resuspend the cells in 10 mL of control medium.
3. Repeat the centrifugation and decantation step, and resuspend the cells in 10 mL control medium.
4. Mix well and determine the cell density in the algal concentrate. Some cells will be lost in the concentration process.
5. Determine the density of cells (cells/mL) in the stock culture (for this example, assume 2,000,000 per mL).
6. Calculate the required volume of stock culture as follows:

$$\text{Volume (mL) of Stock Culture Required} = \frac{\text{Number test flasks to be used} \times \text{Volume of test Solutions/flask} \times \text{10,000 cells/mL}}{\text{Cell density (cells/mL) in the stock culture}}$$

$$= \frac{24 \text{ flasks} \times 100 \text{ mL/flask} \times 10{,}000 \text{ cells/mL}}{2{,}000{,}000 \text{ cells/mL}}$$

$$= 12.0 \text{ mL Stock Culture}$$

7. Dilute the cell concentrate as needed to obtain a cell density of 1,000,000 cells/mL, and check the cell density in the final inoculum.
8. The volume of the algal inoculum should be considered in calculating the dilution of toxicant in the test flasks.

14.10.2 START OF THE TEST

14.10.2.1 Label the test chambers with a marking pen and use the color-coded tape to identify each treatment and replicate. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) should have a minimum of four replicates.

14.10.2.2 Randomize the position of the test flasks at the beginning of the test (see Appendix A). Preparation of a position chart may be helpful.

14.10.2.3 The test begins when the algae are added to the test flasks. Mix the inoculum well, and add 1 mL to the test solution in each randomly arranged flask. Make a final check of the cell density in three of the test solutions at time "zero" (within 2 h of the inoculation).

14.10.2.3.1 Alkalinity, hardness, and conductivity are measured at the beginning of the test in the high, medium, and low effluent concentrations and control before they are dispensed to the test chambers and the data recorded on the data sheet (Figure 1).

Discharger:_____ Test Dates: _____
Location:_____ Analyst: _____

		Effluent Concentration							
Parameter	Control								Remarks
Temperature									
pH									
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 1. Data form for the green alga, *Selenastrum capricornutum*, growth test. Routine chemical and physical determinations.

14.10.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

14.10.3.1 Test flasks are incubated under continuous illumination at 86 ± 8.6 µE/m^2/s (400 ± 40 ft-c), at 25 ± 1 °C, and should be shaken continuously at 100 cpm on a mechanical shaker or twice daily by hand. Flask positions in the incubator should be randomly rotated each day to minimize possible spatial differences in illumination and temperature on growth rate. If it can be verified that test specifications are met at all positions, this need not be done.

14.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

14.10.4.1 Because of the continuous illumination of the test flasks, DO concentration should never be a problem during the test and no aeration will be required.

14.10.5 OBSERVATIONS DURING THE TEST

14.10.5.1 Routine Chemical and Physical Determinations

14.10.5.1.1 Temperature should be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be checked in a sufficient number of test vessels at least at the end of the test to determine variability in the environmental chamber.

14.10.5.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test flask at each concentration and in the control.

14.10.5.1.3 Record all the measurements on the data sheet (Figure 1).

14.10.5.2 Biological Observations

14.10.5.2.1 Toxic substances in the test solutions may degrade or volatilize rapidly, and the inhibition in algal growth may be detectable only during the first one or two days in the test. It may be desirable, therefore, to determine the algal growth response daily. Otherwise, biological observations are not required until the test is terminated and the test solutions are not renewed during the test period.

14.10.6 TERMINATION OF THE TEST

14.10.6.1 The test is terminated 96 h after initiation. The algal growth in each flask is measured by one of the following methods: (a) cell counts, (b) chlorophyll content, or (c) turbidity (light absorbance).

14.10.6.2 Cell counts

14.10.6.2.1 Automatic Particle Counters

14.10.6.2.1.1 Several types of automatic electronic and optical particle counters are available for use in the rapid determination of cell density (cells/mL) and mean cell volume (MCV) in μm^3/cell. The Coulter Counter is widely used and is discussed in detail in USEPA (1978b).

14.10.6.2.1.2 If biomass data are desired for algal growth potential measurements, a Model ZM Coulter Counter is used. However, the instrument must be calibrated with a reference sample of particles of known volume.

14.10.6.2.1.3 When the Coulter Counter is used, an aliquot (usually 1 mL) of the test culture is diluted 10X to 20X with a l% sodium chloride electrolyte solution, such as ISOTON®, to facilitate counting. The resulting dilution is counted using an aperture tube with a 100-µm diameter aperture. Each cell (particle) passing through the aperture causes a voltage drop proportional to its volume. Depending on the model, the instrument stores the information on the number of particles and the volume of each, and calculates the mean cell volume. The following procedure is used:

1. Mix the algal culture in the flask thoroughly by swirling the contents of the flask approximately six times in a clockwise direction, and then six times in the reverse direction; repeat the two-step process at least once.
2. At the end of the mixing process, stop the motion of the liquid in the flask with a strong brief reverse mixing action, and quickly remove 1 mL of cell culture from the flask with a sterile pipet.
3. Place the aliquot in a counting beaker, and add 9 mL (or 19 mL) of electrolyte solution (such as Coulter ISOTON®).
4. Determine the cell density (and MCV, if desired).

14.10.6.2.2 Manual microscope counting method

14.10.6.2.2.1 Cell counts may be determined using a Sedgwick-Rafter, Palmer-Maloney, hemocytometer, inverted microscope, or similar methods. For details on microscope counting methods, see APHA (1992) and USEPA (1973). Whenever feasible, 400 cells per replicate are counted to obtain ± 10% precision at the 95% confidence level. This method has the advantage of allowing for the direct examination of the condition of the cells.

14.10.6.3 Chlorophyll Content

14.10.6.3.1 Chlorophyll may be estimated in-vivo fluorometrically, or in-vitro either fluorometrically or spectrophotometrically. In-vivo fluorometric measurements are recommended because of the simplicity and sensitivity of the technique and rapidity with which the measurements can be made (Rehnberg et al., 1982).

14.10.6.3.2 The in-vivo chlorophyll measurements are made as follows:

1. Adjust the "blank" reading of the fluorometer using the filtrate from an equivalent dilution of effluent filtered through a 0.45 µm particle retention filter.
2. Mix the contents of the test culture flask by swirling successively in opposite directions (at least three times), and remove 1 mL of culture from the flask with a sterile pipet.
3. Place the aliquot in a small disposable vial and record the fluorescence as soon as the reading stabilizes. (Do not allow the sample to stand in the instrument more than 1 min).
4. Discard the sample.

14.10.6.3.3 For additional information on chlorophyll measurement methods, (see APHA, 1992).

14.10.6.4 Turbidity (Absorbance)

14.10.6.4.1 A second rapid technique for growth measurement involves the use of a spectrophotometer to determine the turbidity, or absorbance, of the cultures at a wavelength of 750 nm. Because absorbance is a complex function of the volume, size, and pigmentation of the algae, it would be useful to construct a calibration curve to establish the relationship between absorbance and cell density.

14.10.6.4.2 The algal growth measurements are made as follows:

1. A blank is prepared as described for the fluorometric analysis.
2. The culture is thoroughly mixed as described above.
3. Sufficient sample is withdrawn from the test flask with a sterile pipet and transferred to a 1- to 5-cm cuvette.
4. The absorbance is read at 750 nm and divided by the light path length of the cuvette, to obtain an “absorbance-per-centimeter” value.
5. The 1-cm absorbance values are used in the same manner as the cell counts.

14.10.6.5 Record the data as indicated in Figure 2.

14.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

14.11.1 A summary of test conditions and test acceptability criteria is presented in Table 3.

14.12 ACCEPTABILITY OF TEST RESULTS

14.12.1 For the test results to be acceptable, the mean algal cell density in the control flasks must exceed 1 X 10^6 cells/mL at the end of the test, and the coefficient of variation (CV, calculated as standard deviation X 100 / mean) for algal cell density among the control replicates must not exceed 20%.

Discharger: _____ Test Dates: _____
Location: _____ Analyst: _____

Concentration	Cell Density Measurement Replicate 1	2	3	4	Treatment Mean	Comments
Control						
Conc:						
Conc:						
Conc:						
Conc:						
Conc:						

Comments:

Figure 2. Data form for the green alga, *Selenastrum capricornutum*, growth test, cell density determinations.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GREEN ALGA, *SELENASTRUM CAPRICORNUTUM,* GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1003.0)[1]

1.	Test type:	Static non-renewal (required)
2.	Temperature:	25 ± 1 °C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
3.	Light quality:	"Cool white" fluorescent lighting (recommended)
4.	Light intensity:	86 ± 8.6 µE/m^2/s (400 ± 40 ft-c or 4306 lux) (recommended)
5.	Photoperiod:	Continuous illumination (required)
6.	Test chamber size:	125 mL or 250 mL (recommended)
7.	Test solution volume:	50 mL or 100 mL[2] (recommended)
8.	Renewal of test solutions:	None (required)
9.	Age of test organisms:	4 to 7 days (required)
10.	Initial cell density in test chambers:	10,000 cells/mL (recommended)
11.	No. replicate chambers per concentration:	4 (required minimum)
12.	Shaking rate:	100 cpm continuous, or twice daily by hand (recommended)
13.	Aeration:	None (recommended)
14.	Dilution water:	Algal stock culture medium, enriched uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals, or DMW (see Section 7, Dilution Water) (available options)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] For tests not continuously shaken use 25 mL in 125 mL flasks and 50 mL in 250 mL flasks.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GREEN ALGA, *SELENASTRUM CAPRICORNUTUM,* GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1003.0) (CONTINUED)

15.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Water: 100% receiving water (or minimum of 5) and a control (recommended)
16.	Test dilution factor:	Effluents: ≥ 0.5 (recommended) Receiving Waters: None or ≥ 0.5 (recommended)
17.	Test duration:	96 h (required)
18.	Endpoint:	Growth (cell counts, chlorophyll fluorescence, absorbance, or biomass) (required)
19.	Test acceptability criteria:[3]	Mean cell density of at least 1 X 10^6 cells/mL in the controls; and variability (CV%) among control replicates less than or equal to 20% (required)
20.	Sampling requirements:	For on-site tests, one sample collected at test initiation, and used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
21.	Sample volume required:	1 or 2 L depending on test volume (recommended)

[3] If the test is conducted under non-NPDES applications (i.e., data are not submitted under NPDES permits) and used without EDTA in the nutrient stock solution, the test acceptability criteria are a mean cell density of at least 2 X 10^5 cells/mL in the controls, and variability (CV%) among control replicates less than or equal to 20%.

14.13 **DATA ANALYSIS**

14.13.1 GENERAL

14.13.1.1 Tabulate and summarize the data. A sample set of algal growth response data is shown in Table 4.

TABLE 4. GREEN ALGA, *SELENASTRUM CAPRICORNUTUM,* GROWTH RESPONSE DATA

			Toxicant Concentration (µg Cd/L)				
	Replicate	Control	5	10	20	40	80
	A	1209	1212	826	493	127	49.3
	B	1180	1186	628	416	147	40.0
	C	1340	1204	816	413	147	44.0
Log_{10}	A	3.082	3.084	2.917	2.693	2.104	1.693
Trans-	B	3.072	3.074	2.798	2.619	2.167	1.602
formed	C	3.127	3.081	2.912	2.616	2.167	1.643
	Mean($\bar{Y}_i$)	3.094	3.080	2.876	2.643	2.146	1.646

14.13.1.2 The endpoints of toxicity tests using the green alga, *Selenastrum capricornutum,* are based on the adverse effects on cell growth (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). The EC50, the IC25, and the IC50 are calculated using the point estimation techniques, and LOEC and NOEC values for growth are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the EC50, IC25, and IC50. See the Appendices for examples of the manual computations, and examples of data input and program output.

14.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

14.13.2 EXAMPLE OF ANALYSIS OF ALGAL GROWTH DATA

14.13.2.1 Formal statistical analysis of the growth data is outlined on the flowchart in Figure 3. The response used in the statistical analysis is the number of cells per milliliter per replicate. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 and IC50 endpoints.

14.13.2.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Tests, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

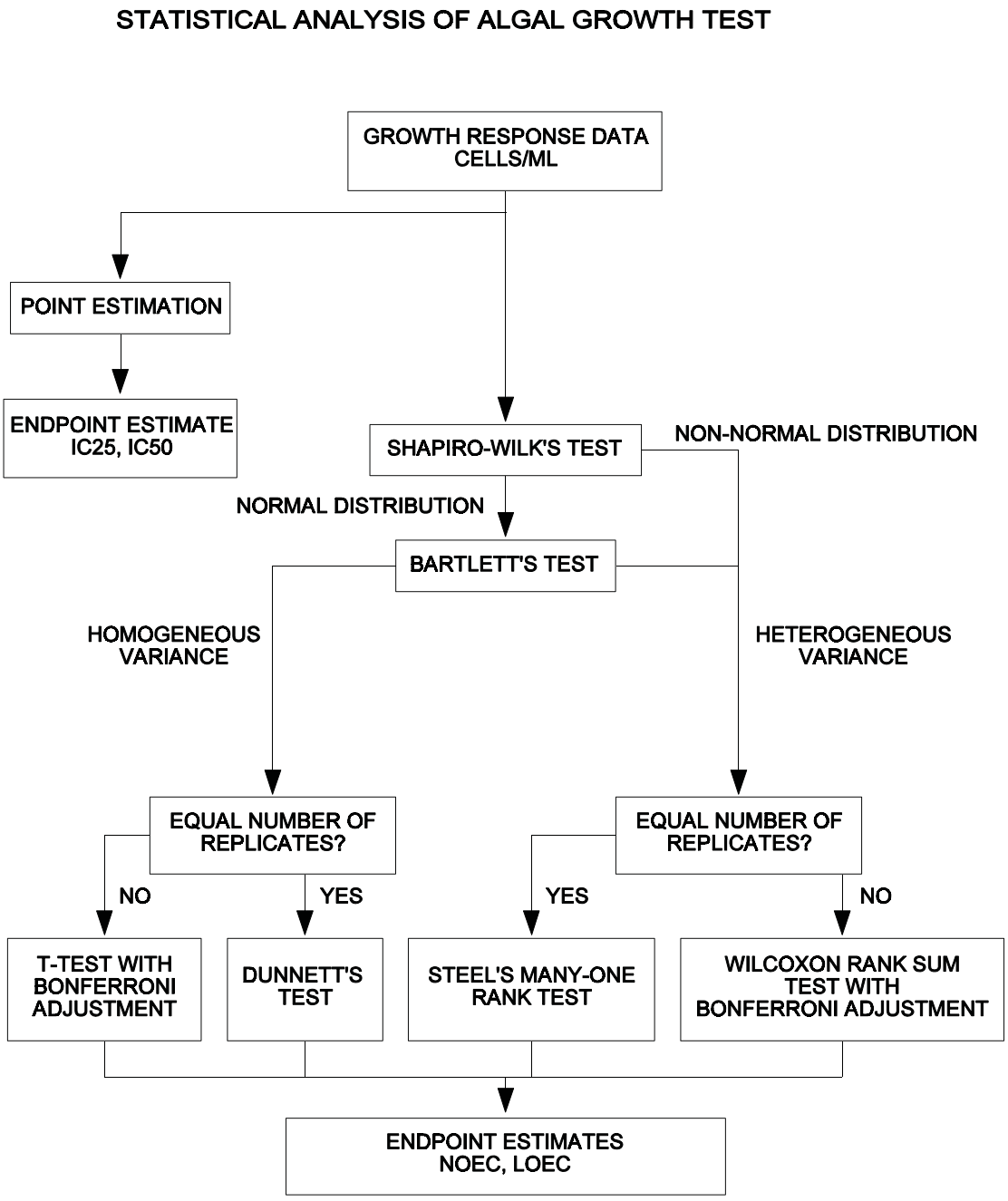

Figure 3. Flowchart for statistical analysis of the green alga, *Selenastrum capricornutum*, growth response data.

14.13.2.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

14.13.2.4 Data from an algal growth test with cadmium chloride will be used to illustrate the statistical analysis. The cell counts were $\log_{10}$ transformed in an effort to stabilize the variance for the ANOVA analysis. The raw data, $\log_{10}$ transformed data, mean and standard deviation of the observations at each concentration including the control are listed in Table 4. A plot of the $\log_{10}$ transformed cell counts for each treatment is provided in Figure 4.

14.13.2.5 Test for Normality

14.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 5.

TABLE 5. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Toxicant Concentration (µg Cd/L)					
Replicate	Control	5	10	20	40	80
A	-0.012	0.004	0.041	0.050	-0.042	0.047
B	-0.022	-0.006	-0.078	-0.024	0.021	-0.044
C	0.033	0.001	0.036	-0.027	0.021	-0.003

14.13.2.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

Where: X_i = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations.

For this set of data, n = 18

$$\overline{X} = \frac{1}{18}(0.000) = 0.000$$

D = 0.0214

14.13.2.5.3 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 6.

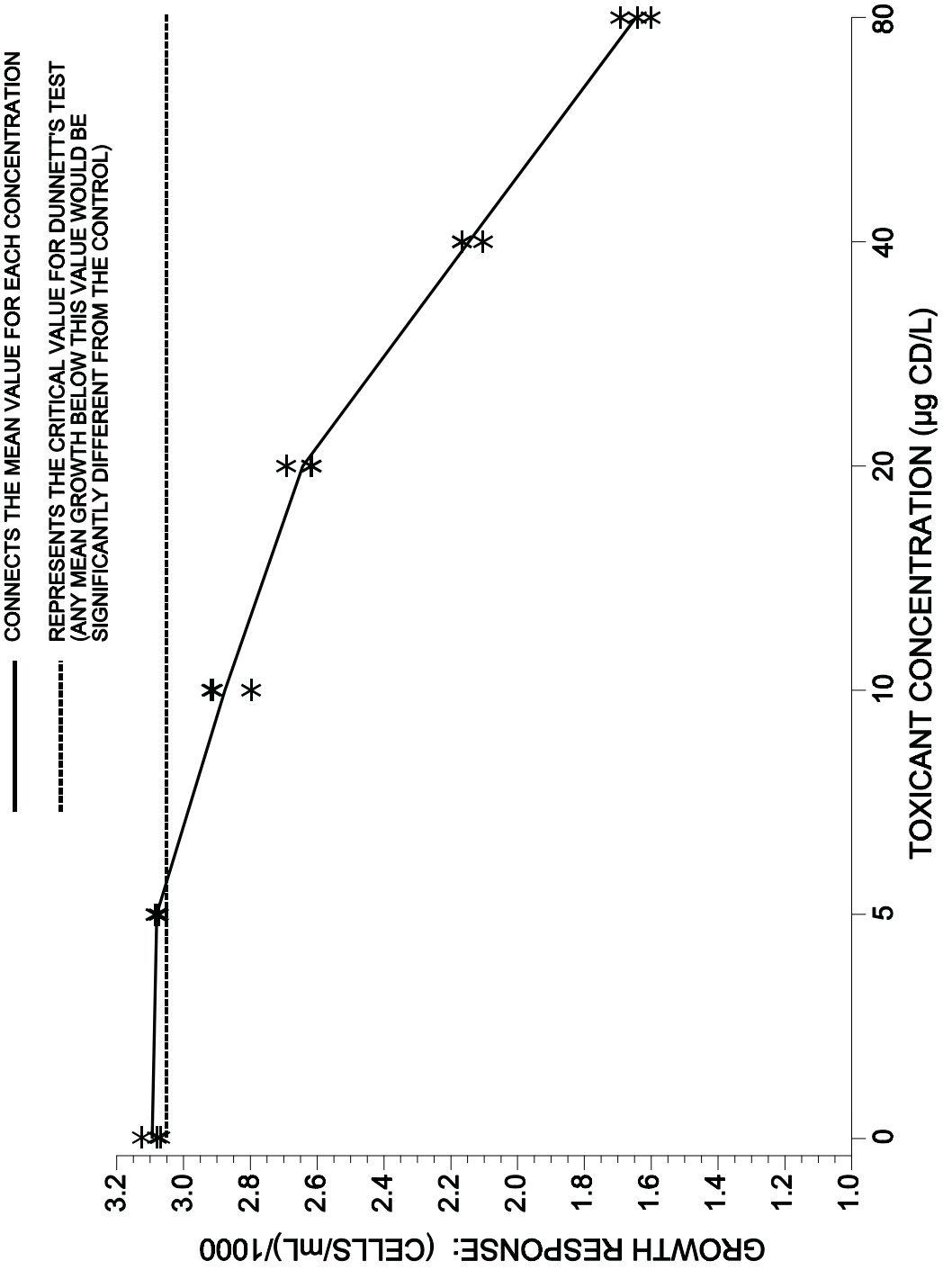

Figure 4. Plot of the $\log_{10}$ transformed cell count data from the green alga, *Selenastrum capricornutum*, growth response test in Table 4.

TABLE 6. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.078	10	0.001
2	-0.044	11	0.004
3	-0.042	12	0.021
4	-0.027	13	0.021
5	-0.024	14	0.033
6	-0.022	15	0.036
7	-0.012	16	0.041
8	-0.006	17	0.047
9	-0.003	18	0.050

14.13.2.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1 , a_2, ..., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 18 , k = 9. The a_i values are listed in Table 7.

14.13.2.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 7.

TABLE 7. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4886	0.128	$X^{(18)} - X^{(1)}$
2	0.3253	0.091	$X^{(17)} - X^{(2)}$
3	0.2553	0.083	$X^{(16)} - X^{(3)}$
4	0.2027	0.063	$X^{(15)} - X^{(4)}$
5	0.1587	0.057	$X^{(14)} - X^{(5)}$
6	0.1197	0.043	$X^{(13)} - X^{(6)}$
7	0.0837	0.033	$X^{(12)} - X^{(7)}$
8	0.0496	0.010	$X^{(11)} - X^{(8)}$
9	0.0163	0.004	$X^{(10)} - X^{(9)}$

For this set of data:

$$W = \frac{1}{0.0214}(0.1436)^2 = 0.964$$

14.13.2.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 18 observations (n) is 0.858. Since W = 0.964 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

14.13.2.6 Test for Homogeneity of Variance

14.13.2.6.1 The test used to examine whether the variation in mean cell count is the same across all toxicant concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i)\ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each toxicant concentration and control, $V_i = (n_i - 1)$

p = number of levels of toxicant concentration including the control

n_i = the number of replicates for concentration i

ln = $\log_e$

i = 1, 2, ..., p, where p is the number of concentrations including the control

$$\bar{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + (3(p-1))^{-1}[\sum_{i=1}^{P} \frac{1}{V_i} - (\sum_{i=1}^{P} V_i)^{-1}]$$

14.13.2.6.2 For the data in this example, (see Table 4) all toxicant concentrations including the control have the same number of replicates ($n_i = 3$ for all i). Thus, $V_i = 2$ for all i.

14.13.2.6.3 Bartlett's statistic is therefore:

$$B = \frac{[(12)\ln(0.0018) - 2\sum_{i=1}^{P} \ln(S_i^2)]}{1.194}$$

$$= [12(-6.3200) - 2(-41.9082)]/1.194$$

$$= 7.9764/1.194$$

$$= 6.6804$$

14.13.2.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with five degrees of freedom, is 15.09. Since B = 6.6804 is less than the critical value of 15.09, conclude that the variances are not different.

14.13.2.7 Dunnett's Procedure

14.13.2.7.1 To obtain an estimate of the pooled variance for Dunnett's Procedure, construct an ANOVA table as described in Table 8.

TABLE 8. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of toxicant concentrations including the control

N = total number of observations $n_1 + n_2 ... + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$ Between Sum of Squares

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the cell count for toxicant concentration i in test chamber j)

14.13.2.7.2 For the data in this example:

$$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 3$$

$$N = 18$$

$$T_1 = Y_{11} + Y_{12} + Y_{13} = 9.281$$
$$T_2 = Y_{21} + Y_{22} + Y_{23} = 9.239$$
$$T_3 = Y_{31} + Y_{32} + Y_{33} = 8.627$$
$$T_4 = Y_{41} + Y_{42} + Y_{43} = 7.928$$
$$T_5 = Y_{51} + Y_{52} + Y_{53} = 6.438$$
$$T_6 = Y_{61} + Y_{62} + Y_{63} = 4.938$$

$$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 46.451$$

$$SSB = \sum_{i=1}^{P} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$

$$= \frac{1}{3}(374.606) - \frac{(46.451)^2}{18} = 4.997$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$

$$= 124.890 - \frac{(46.451)^2}{18} = 5.018$$

$$SSW = SST - SSB = 5.018 - 4.997 = 0.0210$$

$$S_B^2 = SSB/(p-1) = 4.996/(6-1) = 0.9990$$

$$S_W^2 = SSW/(N-p) = 0.021/(18-6) = 0.0018$$

14.13.2.7.3 Summarize these calculations in the ANOVA table (Table 9).

TABLE 9. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	5	4.997	0.999
Within	12	0.021	0.0018
Total	17	5.017	

14.13.2.7.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(\frac{1}{n_1}) + (\frac{1}{n_i})}}$$

Where: $\bar{Y}_i$ = mean cell count for toxicant concentration i

$\bar{Y}_1$ = mean cell count for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

14.13.2.7.5 Table 10 includes the calculated t values for each concentration and control combination. In this example, comparing the 5 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(3.094 - 3.080)}{[0.0424\sqrt{(1/3)+(1/3)}]} = 0.405$$

TABLE 10. CALCULATED T VALUES

Toxicant Concentration (µg Cd/L)	i	t_i
5	2	0.405
10	3	6.300
20	4	13.035
40	5	27.399
80	6	41.850

14.13.2.7.6 Since the purpose of this test is to detect a significant reduction in mean cell count, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 12 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.50. The mean count for concentration i is considered significantly less than the mean count for the control if t_i is greater than the critical value. Since t_3, t_4, t_5 and t_6 are greater than 2.50, the 10, 20, 40 and 80 µg/L concentrations have significantly lower mean cell counts than the control. Hence the NOEC and the LOEC for the test are 5 µg/L and 10 µg/L, respectively.

14.13.2.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated.

$$MSD = d\,S_w\,\sqrt{(1/n_1)+(1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

14.13.2.7.8 In this example:

$$MSD = 2.50(0.0424)\sqrt{(1/3)+(1/3)}$$

$$= 2.50\ (0.0424)(0.8165)$$

$$= 0.086$$

14.13.2.7.9 The MSD (0.086) is in transformed units. An approximate MSD in terms of cell count per 100 mL may be calculated via the following conversion.

1. Subtract the MSD from the transformed control mean.

   3.094 - 0.086 = 3.008

2. Obtain the untransformed values for the control mean and the difference calculated in 1.

   $10^{(3.094)} = 1241.6$

   $10^{(3.008)} = 1018.6$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from 2.

   $MSU_u = 1241.6 - 1018.6 = 223$

14.13.2.7.10 Therefore, for this set of data, the minimum difference in mean cell count between the control and any toxicant concentration that can be detected as statistically significant is 223.

14.13.2.7.11 This represents a decrease in growth of 18% from the control.

14.13.2.8 Calculation of the ICp

14.13.2.8.1 The growth data in Table 4 are utilized in this example. Table 11 contains the means for each toxicant concentration. As can be seen, the observed means are monotonically non-increasing with respect to concentration. Therefore, it is not necessary to smooth the means prior to calculating the ICp. See Figure 5 for a plot of the response curve.

TABLE 11. ALGAL MEAN GROWTH RESPONSE AFTER SMOOTHING

Toxicant Concentration (µg Cd/L)	i	Response means, $\bar{Y}_i$ (cells/mL)	Smoothed mean, M_i (cells/mL)
Control	1	1243	1243
5	2	1201	1201
10	3	757	757
20	4	441	441
40	5	140	140
80	6	44	44

14.13.2.8.2 An IC25 and IC50 can be estimated using the Linear Interpolation Method (Appendix M). A 25% reduction in cell count, compared to the controls, would result in a mean count of 932 cells, where $M_1(1\text{-}p/100) = 1243(1\text{-}25/100)$. A 50% reduction in cell count, compared to the controls, would result in a mean count of 622 cells. Examining the means and their associated concentrations (Table 11), the response, 932 cells, is bracketed by $C_2 = 5$ µg Cd/L and $C_3 = 10$ µg Cd/L. The response, 622 cells, is bracketed by $C_3 = 10$ µg Cd/L and $C_4 = 20$ µg Cd/L.

14.13.2.8.3 Using the equation from section 4.2 of Appendix M, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C(j+1) - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 5 + [1243(1 - 25/100) - 1201]\frac{(10 - 5)}{(757 - 1201)}$$

$$= 8 \text{µg Cd/L.}$$

14.13.2.8.4 The IC50 estimate is 14 µg Cd/L:

$$IC25 = 6.25 + [28.75(1 - 25/100) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

$$IC50 = 10 + [1243(1 - 50/100) - 757]\frac{(20 - 10)}{(441 - 757)}$$

$$= 14 \text{ µg Cd/L.}$$

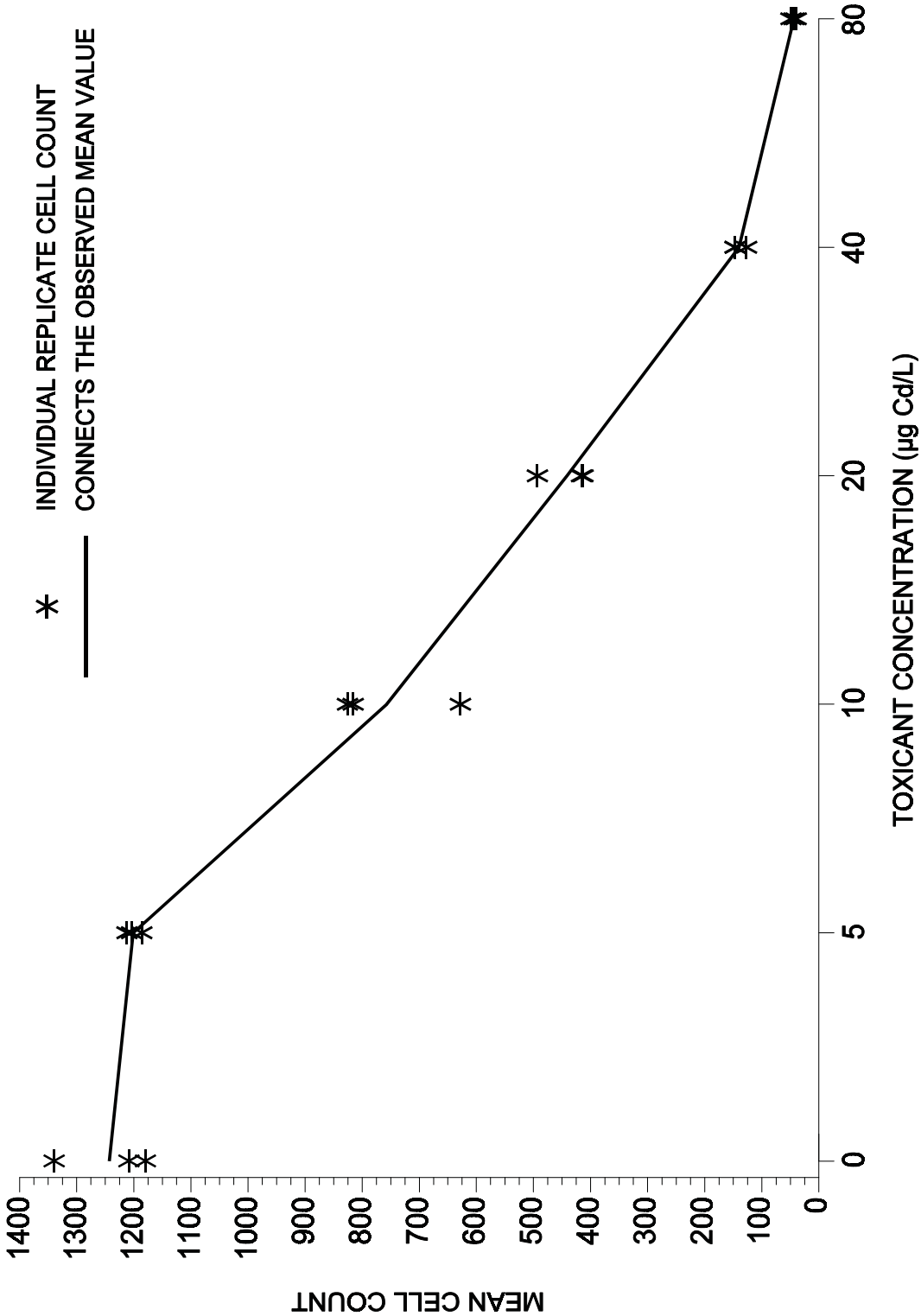

Figure 5. Plot of raw data and observed means for the green alga, *Selenastrum capricornutum*, growth data.

14.13.2.8.5 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 8.0227µg Cd/L. The empirical 95% confidence interval for the true mean was 6.4087 µg Cd/L and 10.0313 µg Cd/L. The ICPIN computer program output for the IC25 for this data set is shown in Figure 6.

14.13.2.8.6 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 14.2774 µg Cd/L. The empirical 95% confidence interval for the true mean was 9.7456 µg Cd/L and 18.5413 µg Cd/L. The computer program output for the IC50 for this data set is shown in Figure 7.

14.13.3 BIOSTIMULATION

14.13.3.1 Where the growth response in effluent (or surface water) exceeds growth in the control flasks, the percent stimulation, S(%), is calculated as shown below. Values which are significantly greater than the control indicate a possible degrading enrichment effect on the receiving water (Walsh et al., 1980):

$$S\ (\%)\ =\ \frac{T-C}{C}\ x\ 100$$

Where: T = Mean effluent or surface water response

C = Mean control response

14.14 **PRECISION AND ACCURACY**

14.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 14.14.1.1 and 14.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

14.14.1.1 Single-Laboratory Precison

14.14.1.1.1 Data from repetitive 96-h toxicity tests conducted with cadmium chloride as the reference toxicant, using medium containing EDTA, are shown in Table 12. The precision (CV) of the 10 EC50s was 10.2%.

14.14.1.1.2 EPA evaluated within-laboratory precision of the green alga, *Selenastrum capricornutum*, Growth Test using a database of routine reference toxicant test results from nine laboratories (USEPA, 2000b). The database consisted of 85 reference toxicant tests conducted in 9 laboratories using a variety of reference toxicants including: copper, sodium chloride, and zinc. Among the 9 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 26% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 25%; and in 75% of laboratories, the within-laboratory CV was less than 39%.

14.14.1.2 Multilaboratory Precision

14.14.1.2.1 In 2000, EPA conducted an interlaboratory variability study of the green alga, *Selenastrum capricornutum*, Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 11 participant laboratories tested 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a

municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Each sample was tested with and without the addition of EDTA. Of the 44 *Selenastrum capricornutum* Growth tests conducted with EDTA, 63.6% were successfully completed and met the required test acceptability criteria. Of the 44 tests conducted without EDTA, 65.9% were successfully completed and met the required test acceptability criteria. Of five tests that were conducted on blank samples with the addition of EDTA, none showed false positive results for the growth endpoint. Of 6 tests that were conducted on blank samples without the addition of EDTA, 2 showed false positive results for the growth endpoint, yielding a false positive rate of 33.3%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 13 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 34.3% and 58.5% for IC25 results in tests with EDTA and without EDTA, respectively. Table 14 shows the precision of growth NOEC endpoints for each sample type. NOEC values for tests with EDTA spanned three concentrations for the effluent sample type and four concentrations for the reference toxicant and receiving water sample types. NOEC values for tests without EDTA, spanned six concentrations for the reference toxicant sample type, four concentrations for the effluent sample type, and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median for tests conducted with EDTA was 85.7%, 100%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively. The percentage of values within one concentration of the median for tests conducted without EDTA was 40.0%, 50.0%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

14.14.2 ACCURACY

14.14.2.1 The accuracy of toxicity tests cannot be determined.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	5	10	20	40	80
Response 1	1209	1212	826	493	127	49.3
Response 2	1180	1186	628	416	147	40.0
Response 3	1340	1204	816	413	147	44.0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Cadmium
Test Start Date: Example Test Ending Date:
Test Species: Selenastrum capricornutum
Test Duration: 96 h
DATA FILE: scmanual.icp
OUTPUT FILE: scmanual.i25

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	1243.000	85.247	1243.000
2	3	5.000	1200.667	13.317	1200.667
3	3	10.000	756.667	111.541	756.667
4	3	20.000	440.667	45.347	440.667
5	3	40.000	140.333	11.547	140.333
6	3	80.000	44.433	4.665	44.433

The Linear Interpolation Estimate: 8.0227 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 8.1627 Standard Deviation: 0.4733
Original Confidence Limits: Lower: 7.2541 Upper: 8.9792
Expanded Confidence Limits: Lower: 6.4087 Upper: 10.0313
Resampling time in Seconds: 1.65 Random Seed: -1575623987

Figure 6. ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	5	10	20	40	80
Response 1	1209	1212	826	493	127	49.3
Response 2	1180	1186	628	416	147	40.0
Response 3	1340	1204	816	413	147	44.0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Cadmium
Test Start Date: Example Test Ending Date:
Test Species: Selenastrum capricornutum
Test Duration: 96 h
DATA FILE: scmanual.icp
OUTPUT FILE: scmanual.i50

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	1243.000	85.247	1243.000
2	3	5.000	1200.667	13.317	1200.667
3	3	10.000	756.667	111.541	756.667
4	3	20.000	440.667	45.347	440.667
5	3	40.000	140.333	11.547	140.333
6	3	80.000	44.433	4.665	44.433

The Linear Interpolation Estimate: 14.2774 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 14.2057 Standard Deviation: 1.1926
Original Confidence Limits: Lower: 12.1194 Upper: 16.3078
Expanded Confidence Limits: Lower: 9.7456 Upper: 18.5413
Resampling time in Seconds: 1.65 Random Seed: -1751550803

Figure 7. ICPIN program output for the IC50.

TABLE 12. SINGLE LABORATORY PRECISION OF THE GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, 96-H TOXICITY TESTS, USING THE REFERENCE TOXICANT CADMIUM CHLORIDE (USEPA, 1991a)

Test Number	EC_{50} (mg/L)
1	2.3
2	2.4
3	2.3
4	2.8
5	2.6
6	2.1
7	2.1
8	2.1
9	2.6
10	2.4
n	10.0
Mean	2.37
CV (%)	10.2

TABLE 13. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	10.9	20.8	23.5
(with EDTA)	Effluent	39.5	8.48	40.4
	Receiving water	-	-	38.9
	Average	25.2	14.6	34.3
IC25	Reference toxicant	25.6	83.6	87.5
(without EDTA)	Effluent	21.0	60.3	63.9
	Receiving water	-	-	24.1
	Average	23.3	72.0	58.5

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

3 The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

4 The between-laboratory component of variability for duplicate samples tested at different laboratories.

5 The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 14. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Growth NOEC (with EDTA)	Reference toxicant	25%	57.1	28.6	14.3
	Effluent	6.25%	42.9	57.1	0.00
	Receiving water	12.5%	28.6	57.1	14.3
Growth NOEC (without EDTA)	Reference toxicant	18.8%	-[4]	40.0	60.0
	Effluent	18.8%	-[4]	50.0	50.0
	Receiving water	6.25%	75.0	25.0	0.00

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).
2 Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
3 Percent of values two or more concentration intervals above or below the median.
4 The median NOEC fell between test concentrations, so no test results fell precisely on the median.

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APPENDICES

APPENDICES (CONTINUED)

APPENDIX A

INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

1. STATISTICAL INDEPENDENCE

1.1 Dunnett's Procedure and the t test with Bonferroni's adjustment are parametric procedures based on the assumptions that (1) the observations within treatments are independent and normally distributed, and (2) that the variance of the observations is homogeneous across all toxicant concentrations and the control. Of the three possible departures from the assumptions, non-normality, heterogeneity of variance, and lack of independence, those caused by lack of independence are the most difficult to resolve (see Scheffe, 1959). For toxicity data, statistical independence means that given knowledge of the true mean for a given concentration or control, knowledge of the error in any one actual observation would provide no information about the error in any other observation. Lack of independence is difficult to assess and difficult to test for statistically. It may also have serious effects on the true alpha or beta level. Therefore, it is of utmost importance to be aware of the need for statistical independence between observations and to be constantly vigilant in avoiding any patterned experimental procedure that might compromise independence. One of the best ways to help ensure independence is to follow proper randomization procedures throughout the test.

2. RANDOMIZATION

2.1 Randomization of the distribution of test organisms among test chambers and the arrangement of treatments and replicate chambers is an important part of conducting a valid test. The purpose of randomization is to avoid situations where test organisms are placed serially into test chambers, or where all replicates for a test concentration are located adjacent to one another, which could introduce bias into the test results.

2.2 An example of randomization of the distribution of test organisms among test chambers, and an example of randomization of arrangement of treatments and replicate chambers are described using the Fathead Minnow Larval Survival and Growth test. For the purpose of the example, the test design is as follows: five effluent concentrations are tested in addition to the control. The effluent concentrations are as follows: 6.25%, 12.5%, 25.0%, 50.0%, and 100.0%. There are four replicate chambers per treatment. Each replicate chamber contains ten fish.

2.3 RANDOMIZATION OF FISH TO REPLICATE CHAMBERS EXAMPLE

2.3.1 Consider first the random assignment of the fish to the replicate chambers. The first step is to label each of the replicate chambers with the control or effluent concentration and the replicate number. The next step is to assign each replicate chamber four double-digit numbers. An example of this assignment is provided in Table A.1. Note that the double digits 00 and 97 through 99 were not used.

TABLE A.1. RANDOM ASSIGNMENT OF FISH TO REPLICATE CHAMBERS EXAMPLE ASSIGNED NUMBERS FOR EACH REPLICATE CHAMBER

Assigned Numbers	Replicate Chamber
01, 25, 49, 73	Control, replicate chamber 1
02, 26, 50, 74	Control, replicate chamber 2
03, 27, 51, 75	Control, replicate chamber 3
04, 28, 52, 76	Control, replicate chamber 4
05, 29, 53, 77	6.25% effluent, replicate chamber 1
06, 30, 54, 78	6.25% effluent, replicate chamber 2
07, 31, 55, 79	6.25% effluent, replicate chamber 3
08, 32, 56, 80	6.25% effluent, replicate chamber 4
09, 33, 57, 81	12.5% effluent, replicate chamber 1
10, 34, 58, 82	12.5% effluent, replicate chamber 2
11, 35, 59, 83	12.5% effluent, replicate chamber 3
12, 36, 60, 84	12.5% effluent, replicate chamber 4
13, 37, 61, 85	25.0% effluent, replicate chamber 1
14, 38, 62, 86	25.0% effluent, replicate chamber 2
15, 39, 63, 87	25.0% effluent, replicate chamber 3
16, 40, 64, 88	25.0% effluent, replicate chamber 4
17, 41, 65, 89	50.0% effluent, replicate chamber 1
18, 42, 66, 90	50.0% effluent, replicate chamber 2
19, 43, 67, 91	50.0% effluent, replicate chamber 3
20, 44, 68, 92	50.0% effluent, replicate chamber 4
21, 45, 69, 93	100.0% effluent, replicate chamber 1
22, 46, 70, 94	100.0% effluent, replicate chamber 2
23, 47, 71, 95	100.0% effluent, replicate chamber 3
24, 48, 72, 96	100.0% effluent, replicate chamber 4

2.3.2 The random numbers used to carry out the random assignment of fish to replicate chambers are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double digit number. The first number read identifies the replicate chamber for the first fish taken from the tank. For the example, the first entry in row 2 was chosen as the starting position. The first number in this row is 37. According to Table A.1, this number corresponds to replicate chamber 1 of the 25.0% effluent concentration. Thus, the first fish taken from the tank is to be placed in replicate chamber 1 of the 25.0% effluent concentration.

2.3.3 The next step is to read the double digit number to the right of the first one. The second number identifies the replicate chamber for the second fish taken from the tank. Continuing the example, the second number read in row 2 of Table A.2 is 54. According to Table A.1, this number corresponds to replicate chamber 2 of the 6.25% effluent concentration. Thus, the second fish taken from the tank is to be placed in replicate chamber 2 of the 6.25% effluent concentration.

TABLE A.2. TABLE OF RANDOM NUMBERS (Dixon and Massey, 1983)

10	09	73	25	33	76	52	01	35	86	34	67	35	43	76	80	95	90	91	17	39	29	27	49	45
37	54	20	48	05	64	89	47	42	96	24	80	52	40	37	20	63	61	04	02	00	82	29	16	65
08	42	26	89	53	19	64	50	93	03	23	20	90	25	60	15	95	33	47	64	35	08	03	36	06
99	01	90	25	29	09	37	67	07	15	38	31	13	11	65	88	67	67	43	97	04	43	62	76	59
12	80	79	99	70	80	15	73	61	47	64	03	23	66	53	98	95	11	68	77	12	27	17	68	33
66	06	57	47	17	34	07	27	68	50	36	69	73	61	70	65	81	33	98	85	11	19	92	91	70
31	06	01	08	05	45	57	18	24	06	35	30	34	26	14	86	79	90	74	39	23	40	30	97	32
85	26	97	76	02	02	05	16	56	92	68	66	57	48	18	73	05	38	52	47	18	62	38	85	79
63	57	33	21	35	05	32	54	70	48	90	55	35	75	48	28	46	82	87	09	83	49	12	56	24
73	79	64	57	53	03	52	96	47	78	35	80	83	42	82	60	93	52	03	44	35	27	38	84	35
98	52	01	77	67	14	90	56	86	07	22	10	94	05	58	60	97	09	34	33	50	50	07	39	98
11	80	50	54	31	39	80	82	77	32	50	72	56	82	48	29	40	52	42	01	52	77	56	78	51
83	45	29	96	34	06	28	89	80	83	13	74	67	00	78	18	47	54	06	10	68	71	17	78	17
88	68	54	02	00	86	50	75	84	01	36	76	66	79	51	90	36	47	64	93	29	60	91	10	62
99	59	46	73	48	87	51	76	49	69	91	82	60	89	28	93	78	56	13	68	23	47	83	41	13
65	48	11	76	74	17	46	85	09	50	58	04	77	69	74	73	03	95	71	86	40	21	81	65	44
80	12	43	56	35	17	72	70	80	15	45	31	82	23	74	21	11	57	82	53	14	38	55	37	63
74	35	09	98	17	77	40	27	72	14	43	23	60	02	10	45	52	16	42	37	96	28	60	26	55
69	91	62	68	03	66	25	22	91	48	36	93	68	72	03	76	62	11	39	90	94	40	05	64	18
09	89	32	05	05	14	22	56	85	14	46	42	75	67	88	96	29	77	88	22	54	38	21	45	98
91	49	91	45	23	68	47	92	76	86	46	16	28	35	54	94	75	08	99	23	37	08	92	00	48
80	33	69	45	98	26	94	03	68	58	70	29	73	41	35	53	14	03	33	40	42	05	08	23	41
44	10	48	19	49	85	15	74	79	54	32	97	92	65	75	57	60	04	08	81	22	22	20	64	13
12	55	07	37	42	11	10	00	20	40	12	86	07	46	97	96	64	48	94	39	28	70	72	58	15
63	60	64	93	29	16	50	53	44	84	40	21	95	25	63	43	65	17	70	82	07	20	73	17	90
61	19	69	04	46	26	45	74	77	74	51	92	43	37	29	65	39	45	95	93	42	58	26	05	27
15	47	44	52	66	95	27	07	99	53	59	36	78	38	48	82	39	61	01	18	33	21	15	94	66
94	55	72	85	73	67	89	75	43	87	54	62	24	44	31	91	19	04	25	92	92	92	74	59	73
42	48	11	62	13	97	34	40	87	21	16	86	84	87	67	03	07	11	20	59	25	70	14	66	70
23	52	37	83	17	73	20	88	98	37	68	93	59	14	16	26	25	22	96	63	05	52	28	25	62
04	49	35	24	94	75	24	63	38	24	45	86	25	10	25	61	96	27	93	35	65	33	71	24	72
00	54	99	76	54	64	05	18	81	59	96	11	96	38	96	54	69	28	23	91	23	28	72	95	29
35	96	31	53	07	26	89	80	93	45	33	35	13	54	62	77	97	45	00	24	90	10	33	93	33
59	80	80	83	91	45	42	72	68	42	83	60	94	97	00	13	02	12	48	92	78	56	52	01	06
46	05	88	52	36	01	39	09	22	86	77	28	14	40	77	93	91	08	36	47	70	61	74	29	41
32	17	90	05	97	87	37	92	52	41	05	56	70	70	07	86	74	31	71	57	85	39	41	18	38
69	23	46	14	06	20	11	74	52	04	15	95	66	00	00	18	74	39	24	23	97	11	89	63	38
19	56	54	14	30	01	75	87	53	79	40	41	92	15	85	66	67	43	68	06	84	96	28	52	07
45	15	51	49	38	19	47	60	72	46	43	66	79	45	43	59	04	79	00	33	20	82	66	95	41
94	86	43	19	94	36	16	81	08	51	34	88	88	15	53	01	54	03	54	56	05	01	45	11	76
98	08	62	48	26	45	24	02	84	04	44	99	90	88	96	39	09	47	34	07	35	44	13	18	80
33	18	51	62	32	41	94	15	09	49	89	43	54	85	81	88	69	54	19	94	37	54	87	30	43
80	95	10	04	06	96	38	27	07	74	20	15	12	33	87	25	01	62	52	98	94	62	46	11	71
79	75	24	91	40	71	96	12	82	96	69	86	10	25	91	74	85	22	05	39	00	38	75	95	79
18	63	33	25	37	98	14	50	65	71	31	01	02	46	74	05	45	56	14	27	77	93	89	19	36
74	02	94	39	02	77	55	73	22	70	97	79	01	71	19	52	52	75	80	21	80	81	45	17	48
54	17	84	56	11	80	99	33	71	43	05	33	51	29	69	56	12	71	92	55	36	04	09	03	24
11	66	44	98	83	52	07	98	48	27	59	38	17	15	39	09	97	33	34	40	88	46	12	33	56
48	32	47	79	28	31	24	96	47	10	02	29	53	68	70	32	30	75	75	46	15	02	00	99	94
69	07	49	41	38	87	63	79	19	76	35	58	40	44	01	10	51	82	16	15	01	84	87	69	38

2.3.4 Continue in this fashion until all the fish have been randomly assigned to a replicate chamber. In order to fill each replicate chamber with ten fish, the assigned numbers will be used more than once. If a number is read from the table that was not assigned to a replicate chamber, then ignore it and continue to the next number. If a replicate chamber becomes filled and a number is read from the table that corresponds to it, then ignore that value and continue to the next number. The first ten random assignments of fish to replicate chambers for the example are summarized in Table A.3.

TABLE A.3. EXAMPLE OF RANDOM ASSIGNMENT OF FIRST TEN FISH TO REPLICATE CHAMBERS

Fish		Assignment	
First	fish taken from tank	25.0% effluent,	replicate chamber 1
Second	fish taken from tank	6.25% effluent,	replicate chamber 2
Third	fish taken from tank	50.0% effluent,	replicate chamber 4
Fourth	fish taken from tank	100.0% effluent,	replicate chamber 4
Fifth	fish taken from tank	6.25% effluent,	replicate chamber 1
Sixth	fish taken from tank	25.0% effluent,	replicate chamber 4
Seventh	fish taken from tank	50.0% effluent,	replicate chamber 1
Eighth	fish taken from tank	100.0% effluent,	replicate chamber 3
Ninth	fish taken from tank	50.0% effluent,	replicate chamber 2
Tenth	fish taken from tank	100.0% effluent,	replicate chamber 4

2.3.5 Four double-digit numbers were assigned to each replicate chamber (instead of one, two, or three double-digit numbers) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each replicate chamber: the first column of assigned numbers in Table A.1. Whenever the numbers 00 and 25 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

2.4 RANDOMIZATION OF REPLICATE CHAMBERS TO POSITIONS EXAMPLE

2.4.1 Next consider the random assignment of the 24 replicate chambers to positions within the water bath (or equivalent). Assume that the replicate chambers are to be positioned in a four row by six column rectangular array. The first step is to label the positions in the water bath. Table A.4 provides an example layout.

TABLE A.4 RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE LABELING THE POSITIONS WITHIN THE WATER BATH

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24

2.4.2 The second step is to assign each of the 24 positions four double-digit numbers. An example of this assignment is provided in Table A.5. Note that the double digits 00 and 97 through 99 were not used.

TABLE A.5. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE ASSIGNED NUMBERS FOR EACH POSITION

Assigned Numbers	Position
01, 25, 49, 73	1
02, 26, 50, 74	2
03, 27, 51, 75	3
04, 28, 52, 76	4
05, 29, 53, 77	5
06, 30, 54, 78	6
07, 31, 55, 79	7
08, 32, 56, 80	8
09, 33, 57, 81	9
10, 34, 58, 82	10
11, 35, 59, 83	11
12, 36, 60, 84	12
13, 37, 61, 85	13
14, 38, 62, 86	14
15, 39, 63, 87	15
16, 40, 64, 88	16
17, 41, 65, 89	17
18, 42, 66, 90	18
19, 43, 67, 91	19
20, 44, 68, 92	20
21, 45, 69, 93	21
22, 46, 70, 94	22
23, 47, 71, 95	23
24, 48, 72, 96	24

2.4.3 The random numbers used to carry out the random assignment of replicate chambers to positions are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double-digit number. The first number read identifies the position for the first replicate chamber of the control. For the example, the first entry in row 10 of Table A.2 was chosen as the starting position. The first number in this row was 73. According to Table A.5, this number corresponds to position 1. Thus, the first replicate chamber for the control will be placed in position 1.

2.4.4 The next step is to read the double-digit number to the right of the first one. The second number identifies the position for the second replicate chamber of the control. Continuing the example, the second number read in row 10 of Table A.2 is 79. According to Table A.5, this number corresponds to position 7. Thus, the second replicate chamber for the control will be placed in position 7.

2.4.5 Continue in this fashion until all the replicate chambers have been assigned to a position. The first four numbers read will identify the positions for the control replicate chambers, the second four numbers read will identify the positions for the lowest effluent concentration replicate chambers, and so on. If a number is read from the table that was not assigned to a position, then ignore that value and continue to the next number. If a number is repeated in Table A.2, then ignore the repeats and continue to the next number. The complete randomization of

replicate chambers to positions for the example is displayed in Table A.6.

TABLE A.6. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE ASSIGNMENT OF ALL 24 POSITIONS

Control	100.0%	6.25%	6.25%	6.25%	12.5%
Control	12.5%	Control	25.0%	12.5%	25.0%
100.0%	50.0%	100.0%	Control	100.0%	25.0%
50.0%	50.0%	25.0%	50.0%	12.5%	6.25%

2.4.6 Four double-digit numbers were assigned to each position (instead of one, two, or three) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each position: the first column of assigned numbers in Table A.5. Whenever the numbers 00 and 25 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

3. **OUTLIERS**

3.1 An outlier is an inconsistent or questionable data point that appears unrepresentative of the general trend exhibited by the majority of the data. Outliers may be detected by tabulation of the data, plotting, and by an analysis of the residuals. An explanation should be sought for any questionable data points. Without an explanation, data points should be discarded only with extreme caution. If there is no explanation, the analysis should be performed both with and without the outlier, and the results of both analyses should be reported.

3.2 Gentleman-Wilk's A statistic gives a test for the condition that the extreme observation may be considered an outlier. For a discussion of this, and other techniques for evaluating outliers, see Draper and John (1981).

APPENDIX B

VALIDATING NORMALITY AND HOMOGENEITY OF VARIANCE ASSUMPTIONS

1. INTRODUCTION

1.1 Dunnett's Procedure and the t test with Bonferroni's adjustment are parametric procedures based on the assumptions that the observations within treatments are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using these tests, to determine if they have been met. Tests for validating the assumptions are provided in the following discussion. If the tests fail (if the data do not meet the assumptions), a nonparametric procedure such as Steel's Many-one Rank Test may be more appropriate. However, the decision on whether to use parametric or nonparametric tests may be a judgment call, and a statistician should be consulted in selecting the analysis.

2. TEST FOR NORMAL DISTRIBUTION OF DATA

2.1 SHAPIRO-WILK'S TEST

2.1.1 One formal test for normality is the Shapiro-Wilk's Test (Conover, 1980). The test statistic is obtained by dividing the square of an appropriate linear combination of the sample order statistics by the usual symmetric estimate of variance. The calculated W must be greater than zero and less than or equal to one. This test is recommended for a sample size of 50 or less. If the sample size is greater than 50, the Kolmogorov "D" statistic (Stephens, 1974) is recommended. An example of the Shapiro-Wilk's test is provided below.

2.2 The example uses growth data from the Fathead Minnow Larval Survival and Growth Test. The same data are used in the discussion of the homogeneity of variance determination in Paragraph 3 and Dunnett's Procedure in Appendix C. The data, the mean and variance of the observations at each concentration, including the control, are listed in Table B.1.

TABLE B.1. FATHEAD LARVAL, *PIMEPHALES PROMELAS*, LARVAL GROWTH DATA (WEIGHT IN MG) FOR THE SHAPIRO-WILK'S TEST

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.711	0.646	0.669	0.629	0.650
B	0.662	0.626	0.669	0.680	0.558
C	0.718	0.723	0.694	0.513	0.606
D	0.767	0.700	0.676	0.672	0.508
Mean($\bar{Y}_i$)	0.714	0.674	0.677	0.624	0.580
S_i^2	0.0018	0.0020	0.0001	0.0059	0.0037
i	1	2	3	4	5

2.3 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are listed in Table B.2.

TABLE B.2. EXAMPLE OF SHAPIRO-WILK'S TEST: CENTERED OBSERVATIONS

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	-0.003	-0.028	-0.008	0.005	0.070
B	-0.052	-0.048	-0.008	0.056	-0.022
C	0.004	0.049	0.017	-0.111	0.026
D	0.053	0.026	-0.001	0.048	-0.072

2.4 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

Where: X_i = the centered observations and $\overline{X}$ is the overall mean of the centered observations. For this set of data, $\overline{X} = 0$, and D = 0.0412.

2.5 Order the centered observations from smallest to largest.

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations are listed in Table B.3.

TABLE B.3. EXAMPLE OF THE SHAPIRO-WILK'S TEST: ORDERED OBSERVATIONS

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.111	11	0.004
2	-0.072	12	0.005
3	-0.052	13	0.017
4	-0.048	14	0.026
5	-0.028	15	0.026
6	-0.022	16	0.048
7	-0.008	17	0.049
8	-0.008	18	0.053
9	-0.003	19	0.056
10	-0.001	20	0.070

2.6 From Table B.4, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$, where k is n/2 if n is even, and (n-1)/2 if n is odd. For the data in this example, n = 20, k = 10. The a_i values are listed in Table B.5.

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (Conover, 1980)

i \ n	Number of Observations 2	3	4	5	6	7	8	9	10
1	0.7071	0.7071	0.6872	0.6646	0.6431	0.6233	0.6052	0.5888	0.5739
2	-	0.0000	0.1667	0.2413	0.2806	0.3031	0.3164	0.3244	0.3291
3	-	-	-	0.0000	0.0875	0.1401	0.1743	0.1976	0.2141
4	-	-	-	-	-	0.0000	0.0561	0.0947	0.1224
5	-	-	-	-	-	-	-	0.0000	0.0399

i \ n	Number of Observations 11	12	13	14	15	16	17	18	19	20
1	0.5601	0.5475	0.5359	0.5251	0.5150	0.5056	0.4968	0.4886	0.4808	0.4734
2	0.3315	0.3325	0.3325	0.3318	0.3306	0.3209	0.3273	0.3253	0.3232	0.3211
3	0.2260	0.2347	0.2412	0.2460	0.2495	0.2521	0.2540	0.2553	0.2561	0.2565
4	0.1429	0.1586	0.1707	0.1802	0.1878	0.1939	0.1988	0.2027	0.2059	0.2085
5	0.0695	0.0922	0.1099	0.1240	0.1353	0.1447	0.1524	0.1587	0.1641	0.1686
6	0.0000	0.0303	0.0539	0.0727	0.0880	0.1005	0.1109	0.1197	0.1271	0.1334
7	-	-	0.0000	0.0240	0.0433	0.0593	0.0725	0.0837	0.0932	0.1013
8	-	-	-	-	0.0000	0.0196	0.0359	0.0496	0.0612	0.0711
9	-	-	-	-	-	-	0.0000	0.0163	0.0303	0.0422
10	-	-	-	-	-	-	-	-	0.0000	0.0140

i \ n	Number of Observations 21	22	23	24	25	26	27	28	29	30
1	0.4643	0.4590	0.4542	0.4493	0.4450	0.4407	0.4366	0.4328	0.4291	0.4254
2	0.3185	0.3156	0.3126	0.3098	0.3069	0.3043	0.3018	0.2992	0.2968	0.2944
3	0.2578	0.2571	0.2563	0.2554	0.2543	0.2533	0.2522	0.2510	0.2499	0.2487
4	0.2119	0.2131	0.2139	0.2145	0.2148	0.2151	0.2152	0.2151	0.2150	0.2148
5	0.1736	0.1764	0.1787	0.1807	0.1822	0.1836	0.1848	0.1857	0.1864	0.1870
6	0.1399	0.1443	0.1480	0.1512	0.1539	0.1563	0.1584	0.1601	0.1616	0.1630
7	0.1092	0.1150	0.1201	0.1245	0.1283	0.1316	0.1346	0.1372	0.1395	0.1415
8	0.0804	0.0878	0.0941	0.0997	0.1046	0.1089	0.1128	0.1162	0.1192	0.1219
9	0.0530	0.0618	0.0696	0.0764	0.0923	0.0876	0.0923	0.0965	0.1002	0.1036
10	0.0263	0.0368	0.0459	0.0539	0.0610	0.0672	0.0728	0.0778	0.0822	0.0862
11	0.0000	0.0122	0.0228	0.0321	0.0403	0.0476	0.0540	0.0598	0.0650	0.0697
12	-	-	0.0000	0.0107	0.0200	0.0284	0.0358	0.0424	0.0483	0.0537
13	-	-	-	-	0.0000	0.0094	0.0178	0.0253	0.0320	0.0381
14	-	-	-	-	-	-	0.0000	0.0084	0.0159	0.0227
15	-	-	-	-	-	-	-	-	0.0000	0.0076

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO WILK'S TEST (CONTINUED)

i \ n	Number of Observations									
	31	32	33	34	35	36	37	38	39	40
1	0.4220	0.4188	0.4156	0.4127	0.4096	0.4068	0.4040	0.4015	0.3989	0.3964
2	0.2921	0.2898	0.2876	0.2854	0.2834	0.2813	0.2794	0.2774	0.2755	0.2737
3	0.2475	0.2462	0.2451	0.2439	0.2427	0.2415	0.2403	0.2391	0.2380	0.2368
4	0.2145	0.2141	0.2137	0.2132	0.2127	0.2121	0.2116	0.2110	0.2104	0.2098
5	0.1874	0.1878	0.1880	0.1882	0.1883	0.1883	0.1883	0.1881	0.1880	0.1878
6	0.1641	0.1651	0.1660	0.1667	0.1673	0.1678	0.1663	0.1686	0.1689	0.1691
7	0.1433	0.1449	0.1463	0.1475	0.1487	0.1496	0.1505	0.1513	0.1520	0.1526
8	0.1243	0.1265	0.1284	0.1301	0.1317	0.1331	0.1344	0.1356	0.1366	0.1376
9	0.1066	0.1093	0.1118	0.1140	0.1160	0.1179	0.1196	0.1211	0.1225	0.1237
10	0.0899	0.0931	0.0961	0.0988	0.1013	0.1036	0.1056	0.1075	0.1092	0.1108
11	0.0739	0.0777	0.0812	0.0844	0.0873	0.0900	0.0924	0.0947	0.0967	0.0986
12	0.0585	0.0629	0.0669	0.0706	0.0739	0.0770	0.0798	0.0824	0.0848	0.0870
13	0.0435	0.0485	0.0530	0.0572	0.0610	0.0645	0.0677	0.0706	0.0733	0.0759
14	0.0289	0.0344	0.0395	0.0441	0.0484	0.0523	0.0559	0.0592	0.0622	0.0651
15	0.0144	0.0206	0.0262	0.0314	0.0361	0.0404	0.0444	0.0481	0.0515	0.0546
16	0.0000	0.0068	0.0131	0.0187	0.0239	0.0287	0.0331	0.0372	0.0409	0.0444
17	-	-	0.0000	0.0062	0.0119	0.0172	0.0220	0.0264	0.0305	0.0343
18	-	-	-	-	0.0000	0.0057	0.0110	0.0158	0.0203	0.0244
19	-	-	-	-	-	-	0.0000	0.0053	0.0101	0.0146
20	-	-	-	-	-	-	-	-	0.0000	0.0049

i \ n	Number of Observations									
	41	42	43	44	45	46	47	48	49	50
1	0.3940	0.3917	0.3894	0.3872	0.3850	0.3830	0.3808	0.3789	0.3770	0.3751
2	0.2719	0.2701	0.2684	0.2667	0.2651	0.2635	0.2620	0.2604	0.2589	0.2574
3	0.2357	0.2345	0.2334	0.2323	0.2313	0.2302	0.2291	0.2281	0.2271	0.2260
4	0.2091	0.2085	0.2078	0.2072	0.2065	0.2058	0.2052	0.2045	0.2038	0.2032
5	0.1876	0.1874	0.1871	0.1868	0.1865	0.1862	0.1859	0.1855	0.1851	0.1847
6	0.1693	0.1694	0.1695	0.1695	0.1695	0.1695	0.1695	0.1693	0.1692	0.1691
7	0.1531	0.1535	0.1539	0.1542	0.1545	0.1548	0.1550	0.1551	0.1553	0.1554
8	0.1384	0.1392	0.1398	0.1405	0.1410	0.1415	0.1420	0.1423	0.1427	0.1430
9	0.1249	0.1259	0.1269	9,1278	0.1286	0.1293	0.1300	0.1306	0.1312	0.1317
10	0.1123	0.1136	0.1149	0.1160	9.1170	0.1180	0.1189	0.1197	0.1205	0.1212
11	0.1004	0.1020	0.1035	0.1049	0.1062	0.1073	0.1085	0.1095	0.1105	0.1113
12	0.0891	0.0909	0.0927	0.0943	0.0959	0.0972	0.0986	0.0998	0.1010	0.1020
13	0.0782	0.0804	0.0824	0.0842	0.0860	0.0876	0.0892	0.0906	0.0919	0.0932
14	0.0677	0.0701	0.0724	0.0745	0.0765	0.0783	0.0801	0.0817	0.0832	0.0846
15	0.0575	0.0602	0.0628	0.0651	0.0673	0.0694	0.0713	0.0731	0.0748	0.0764
16	0.0476	0.0506	0.0534	0.0560	0.0584	0.0607	0.0628	0.0648	0.0667	0.0685
17	0.0379	0.0411	0.0442	0.0471	0.0497	0.0522	0.0546	0.0568	0.0588	0.0608
18	0.0283	0.0318	0.0352	0.0383	0.0412	0.0439	0.0465	0.0489	0.0511	0.0532
19	0.0188	0.0227	0.0263	0.0296	0.0328	0.0357	0.0385	0.0411	0.0436	0.0459
20	0.0094	0.0136	0.0175	0.0211	0.0245	0.0277	0.0307	0.0335	0.0361	0.0386
21	0.0000	0.0045	0.0087	0.0126	0.0163	0.0197	0.0229	0.0259	0.0288	0.0314
22	-	-	0.0000	0.0042	0.0081	0.0118	0.0153	0.0185	0.0215	0.0244
23	-	-	-	-	0.0000	0.0039	0.0076	0.0111	0.0143	0.0174
24	-	-	-	-	-	-	0.0000	0.0037	0.0071	0.0104
25	-	-	-	-	-	-	-	-	0.0000	0.0035

2.7 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table B.5.

2.8 The decision rule for this test is to compare the critical value from Table B.6 to the computed W. If the computed value is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 20 observations (n) is 0.868. The calculated value, 0.959, is not less than the critical value. Therefore, conclude that the data are normally distributed.

TABLE B.5. EXAMPLE OF THE SHAPIRO-WILK'S TEST: TABLE OF COEFFICIENTS AND DIFFERENCES

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.181	$X^{(20)} - X^{(1)}$
2	0.3211	0.128	$X^{(19)} - X^{(2)}$
3	0.2565	0.105	$X^{(18)} - X^{(3)}$
4	0.2085	0.097	$X^{(17)} - X^{(4)}$
5	0.1686	0.076	$X^{(16)} - X^{(5)}$
6	0.1334	0.048	$X^{(15)} - X^{(6)}$
7	0.1013	0.034	$X^{(14)} - X^{(7)}$
8	0.0711	0.025	$X^{(13)} - X^{(8)}$
9	0.0422	0.008	$X^{(12)} - X^{(9)}$
10	0.0140	0.005	$X^{(11)} - X^{(10)}$

2.9 In general, if the data fail the test for normality, a transformation such as to log values may normalize the data. After transforming the data, repeat the Shapiro-Wilk's Test for normality.

2.10 KOLMOGOROV "D" TEST

2.10.1 A formal two-sided test for normality is the Kolmogorov "D" Test. The test statistic is calculated by obtaining the difference between the cumulative distribution function estimated from the data and the standard normal cumulative distribution function for each standardized observation. This test is recommended for a sample size greater than 50. If the sample size is less than or equal to 50, then the Shapiro Wilk's Test is recommended. An example of the Kolmogorov "D" test is provided below.

2.10.2 The example uses reproduction data from the daphnid, *Ceriodaphnia dubia,* Survival and Reproduction Test. The observed data and the mean of the observations at each concentration, including the control, are listed in Table B.7.

2.10.3 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations for the example are listed in Table B.8.

TABLE B.6. QUANTILES OF THE SHAPIRO-WILK'S TEST STATISTIC (Conover, 1980)

n	0.01	0.02	0.05	0.10	0.50	0.90	0.95	0.98	0.99
3	0.753	0.756	0.767	0.789	0.959	0.998	0.999	1.000	1.000
4	0.687	0.707	0.748	0.792	0.935	0.987	0.992	0.996	0.997
5	0.686	0.715	0.762	0.806	0.927	0.979	0.986	0.991	0.993
6	0.713	0.743	0.788	0.826	0.927	0.974	0.981	0.986	0.989
7	0.730	0.760	0.803	0.838	0.928	0.972	0.979	0.985	0.988
8	0.749	0.778	0.818	0.851	0.932	0.972	0.978	0.984	0.987
9	0.764	0.791	0.829	0.859	0.935	0.972	0.978	0.984	0.986
10	0.781	0.806	0.842	0.869	0.938	0.972	0.978	0.983	0.986
11	0.792	0.817	0.850	0.876	0.940	0.973	0.979	0.984	0.986
12	0.805	0.828	0.859	0.883	0.943	0.973	0.979	0.984	0.986
13	0.814	0.837	0.866	0.889	0.945	0.974	0.979	0.984	0.986
14	0.825	0.846	0.874	0.895	0.947	0.975	0.980	0.984	0.986
15	0.835	0.855	0.881	0.901	0.950	0.975	0.980	0.984	0.987
16	0.844	0.863	0.887	0.906	0.952	0.976	0.981	0.985	0.987
17	0.851	0.869	0.892	0.910	0.954	0.977	0.981	0.985	0.987
18	0.858	0.874	0.897	0.914	0.956	0.978	0.982	0.986	0.988
19	0.863	0.879	0.901	0.917	0.957	0.978	0.982	0.986	0.988
20	0.868	0.884	0.905	0.920	0.959	0.979	0.983	0.986	0.988
21	0.873	0.888	0.908	0.923	0.960	0.980	0.983	0.987	0.989
22	0.878	0.892	0.911	0.926	0.961	0.980	0.984	0.987	0.989
23	0.881	0.895	0.914	0.928	0.962	0.981	0.984	0.987	0.989
24	0.884	0.898	0.916	0.930	0.963	0.981	0.984	0.987	0.989
25	0.888	0.901	0.918	0.931	0.964	0.981	0.985	0.988	0.989
26	0.891	0.904	0.920	0.933	0.965	0.982	0.985	0.988	0.989
27	0.894	0.906	0.923	0.935	0.965	0.982	0.985	0.988	0.990
28	0.896	0.908	0.924	0.936	0.966	0.982	0.985	0.988	0.990
29	0.898	0.910	0.926	0.937	0.966	0.982	0.985	0.988	0.990
30	0.900	0.912	0.927	0.939	0.967	0.983	0.985	0.988	0.990
31	0.902	0.914	0.929	0.940	0.967	0.983	0.986	0.988	0.990
32	0.904	0.915	0.930	0.941	0.968	0.983	0.986	0.988	0.990
33	0.906	0.917	0.931	0.942	0.968	0.983	0.986	0.989	0.990
34	0.908	0.919	0.933	0.943	0.969	0.983	0.986	0.989	0.990
35	0.910	0.920	0.934	0.944	0.969	0.984	0.986	0.989	0.990
36	0.912	0.922	0.935	0.945	0.970	0.984	0.986	0.989	0.990
37	0.914	0.924	0.936	0.946	0.970	0.984	0.987	0.989	0.990
38	0.916	0.925	0.938	0.947	0.971	0.984	0.987	0.989	0.990
39	0.917	0.927	0.939	0.948	0.971	0.984	0.987	0.989	0.991
40	0.919	0.928	0.940	0.949	0.972	0.985	0.987	0.989	0.991
41	0.920	0.929	0.941	0.950	0.972	0.985	0.987	0.989	0.991
42	0.922	0.930	0.942	0.951	0.972	0.985	0.987	0.989	0.991
43	0.923	0.932	0.943	0.951	0.973	0.985	0.987	0.990	0.991
44	0.924	0.933	0.944	0.952	0.973	0.985	0.987	0.990	0.991
45	0.926	0.934	0.945	0.953	0.973	0.985	0.988	0.990	0.991
46	0.927	0.935	0.945	0.953	0.974	0.985	0.988	0.990	0.991
47	0.928	0.936	0.946	0.954	0.974	0.985	0.988	0.990	0.991
48	0.929	0.937	0.947	0.954	0.974	0.985	0.988	0.990	0.991
49	0.929	0.937	0.947	0.955	0.974	0.985	0.988	0.990	0.991
50	0.930	0.938	0.947	0.955	0.974	0.985	0.988	0.990	0.991

TABLE B.7. *CERIODAPHNIA DUBIA* REPRODUCTION DATA FOR THE KOLMOGOROV "D" TEST

	Effluent Concentration (%)					
Replicate	Control	1.56	3.12	6.25	12.5	25.0
1	27	32	39	27	19	10
2	30	35	30	34	25	13
3	29	32	33	36	26	7
4	31	26	33	34	17	7
5	16	18	36	31	16	7
6	15	29	33	27	21	10
7	18	27	33	33	23	10
8	17	16	27	31	15	16
9	14	35	38	33	18	12
10	27	13	44	31	10	2
Mean	22.4	26.3	34.6	31.7	19.0	9.4

TABLE B.8. CENTERED OBSERVATIONS FOR KOLMOGOROV “D” EXAMPLE

	Effluent Concentration (%)					
Replicate	Control	1.56	3.12	6.25	12.5	25.0
1	4.6	5.7	4.4	-4.7	0.0	0.6
2	7.6	8.7	-4.6	2.3	6.0	3.6
3	6.6	5.7	-1.6	4.3	7.0	-2.4
4	8.6	-0.3	-1.6	2.3	-2.0	-2.4
5	-6.4	-8.3	1.4	-0.7	-3.0	-2.4
6	-7.4	2.7	-1.6	-4.7	2.0	0.6
7	-4.4	0.7	-1.6	1.3	4.0	0.6
8	-5.4	-10.3	-7.6	-0.7	-4.0	6.6
9	-8.4	8.7	3.4	1.3	-1.0	2.6
10	4.6	-13.3	9.4	-0.7	-9.0	-7.4

2.10.4 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation, and n denotes the total number of centered observations. The ordered observations for the example are listed in Table B.9.

TABLE B.9. EXAMPLE CALCULATION OF THE KOLMOGOROV "D" STATISTIC

i	$X^{(i)}$	z_i	p_i	D_i+	D_i-
1	-13.3	-2.51	0.0060	0.0107	0.0060
2	-10.3	-1.94	0.0262	0.0071	0.0095
3	-9.0	-1.70	0.0446	0.0054	0.0113
4	-8.4	-1.58	0.0571	0.0096	0.0071
5	-8.3	-1.57	0.0582	0.0251	-0.0085
6	-7.6	-1.43	0.0764	0.0236	-0.0069
7	-7.4	-1.40	0.0808	0.0359	-0.0192
8	-7.4	-1.40	0.0808	0.0525	-0.0359
9	-6.4	-1.21	0.1131	0.0369	-0.0202
10	-5.4	-1.02	0.1539	0.0128	0.0039
11	-4.7	-0.89	0.1867	-0.0034	0.0200
12	-4.7	-0.89	0.1867	0.0133	0.0034
13	-4.6	-0.87	0.1922	0.0245	-0.0078
14	-4.4	-0.83	0.2033	0.0300	-0.0134
15	-4.0	-0.75	0.2266	0.0234	-0.0067
16	-3.0	-0.57	0.2843	-0.0176	0.0343
17	-2.4	-0.45	0.3264	-0.0431	0.0597
18	-2.4	-0.45	0.3264	-0.0264	0.0431
19	-2.4	-0.45	0.3264	-0.0097	0.0264
20	-2.0	-0.38	0.3520	-0.0187	0.0353
21	-1.6	-0.30	0.3821	-0.0321	0.0488
22	-1.6	-0.30	0.3821	-0.0154	0.0321
23	-1.6	-0.30	0.3821	0.0012	0.0154
24	-1.6	-0.30	0.3821	0.0179	-0.0012
25	-1.0	-0.19	0.4247	-0.0080	0.0247
26	-0.7	-0.13	0.4483	-0.0150	0.0316
27	-0.7	-0.13	0.4483	0.0017	0.0150
28	-0.7	-0.13	0.4483	0.0184	-0.0017
29	-0.3	-0.06	0.4761	0.0072	0.0094
30	0.0	0.00	0.5000	0.0000	0.0167
31	0.6	0.11	0.5438	-0.0271	0.0438
32	0.6	0.11	0.5438	-0.0105	0.0271
33	0.6	0.11	0.5438	0.0062	0.0105
34	0.7	0.13	0.5517	0.0150	0.0017
35	1.3	0.25	0.5987	-0.0154	0.0320
36	1.3	0.25	0.5987	0.0013	0.0154
37	1.4	0.26	0.6026	0.0141	0.0026
38	2.0	0.38	0.6480	-0.0147	0.0313
39	2.3	0.43	0.6664	-0.0164	0.0331
40	2.3	0.43	0.6664	0.0003	0.0164
41	2.6	0.49	0.6879	-0.0046	0.0212
42	2.7	0.51	0.6950	0.0050	0.0117
43	3.4	0.64	0.7389	-0.0222	0.0389
44	3.6	0.68	0.7517	-0.0184	0.0350
45	4.0	0.75	0.7734	-0.0234	0.0401
46	4.3	0.81	0.7910	-0.0243	0.0410
47	4.4	0.83	0.7967	-0.0134	0.0300

TABLE B.9. EXAMPLE CALCULATION OF THE KOLMOGOROV "D" STATISTIC (CONTINUED)

i	$X^{(i)}$	z_i	p_i	D_i+	D_i-
48	4.6	0.87	0.8078	-0.0078	0.0245
49	4.6	0.87	0.8078	0.0089	0.0078
50	5.7	1.08	0.8599	-0.0266	0.0432
51	5.7	1.08	0.8599	-0.0099	0.0266
52	6.0	1.13	0.8708	-0.0041	0.0208
53	6.6	1.25	0.8944	-0.0111	0.0277
54	6.6	1.25	0.8944	0.0056	0.0111
55	7.0	1.32	0.9066	0.0101	0.0066
56	7.6	1.43	0.9236	0.0097	0.0069
57	8.6	1.62	0.9474	0.0026	0.0141
58	8.7	1.64	0.9495	0.0172	-0.0005
59	8.7	1.64	0.9495	0.0338	-0.0172
60	9.4	1.77	0.9616	0.0384	-0.0217

2.10.5 The next step is to standardize the ordered observations. Let z_i denote the standardized value of the ith ordered observation. Then,

$$z_i = \frac{X^{(i)}}{s} \quad and \quad s^2 = \frac{\Sigma[X^{(i)}]^2}{(n-1)}$$

For the example, s = 5.3, and the standardized observations are listed in Table B.9.

2.10.6 From Table B.10, obtain the value of the standard normal cumulative distribution function (standard normal CDF) at z_i. Denote this value as p_i. Note that negative z are not listed in Table B.10. The value of the standard normal CDF at a negative number is one minus the value of the standard normal CDF at the absolute value of that number. For example, since the value of the standard normal CDF at 3.21 is 0.9993, the value of the standard normal CDF at -3.21 is 1 - 0.9993 = 0.0007. The p_i values for the example data are listed in Table B.9.

TABLE B.10. P IS THE VALUE OF THE STANDARD NORMAL CUMULATIVE DISTRIBUTION AT Z

z	p	z	p	z	p	z	p
0.00	0.5000	0.41	0.6591	0.82	0.7939	1.23	0.8907
0.01	0.5040	0.42	0.6628	0.83	0.7967	1.24	0.8925
0.02	0.5080	0.43	0.6664	0.84	0.7995	1.25	0.8944
0.03	0.5120	0.44	0.6700	0.85	0.8023	1.26	0.8962
0.04	0.5160	0.45	0.6736	0.86	0.8051	1.27	0.8980
0.05	0.5199	0.46	0.6772	0.87	0.8078	1.28	0.8997
0.06	0.5239	0.47	0.6808	0.88	0.8106	1.29	0.9015
0.07	0.5279	0.48	0.6844	0.89	0.8133	1.30	0.9032
0.08	0.5319	0.49	0.6879	0.90	0.8159	1.31	0.9049
0.09	0.5359	0.50	0.6915	0.91	0.8186	1.32	0.9066
0.10	0.5398	0.51	0.6950	0.92	0.8212	1.33	0.9082
0.11	0.5438	0.52	0.6985	0.93	0.8238	1.34	0.9099
0.12	0.5478	0.53	0.7019	0.94	0.8264	1.35	0.9115
0.13	0.5517	0.54	0.7054	0.95	0.8289	1.36	0.9131
0.14	0.5557	0.55	0.7088	0.96	0.8315	1.37	0.9147
0.15	0.5596	0.56	0.7123	0.97	0.8340	1.38	0.9162
0.16	0.5636	0.57	0.7157	0.98	0.8365	1.39	0.9177
0.17	0.5675	0.58	0.7190	0.99	0.8389	1.40	0.9192
0.18	0.5714	0.59	0.7224	1.00	0.8413	1.41	0.9207
0.19	0.5753	0.60	0.7257	1.01	0.8438	1.42	0.9222
0.20	0.5793	0.61	0.7291	1.02	0.8461	1.43	0.9236
0.21	0.5832	0.62	0.7324	1.03	0.8485	1.44	0.9251
0.22	0.5871	0.63	0.7357	1.04	0.8508	1.45	0.9265
0.23	0.5910	0.64	0.7389	1.05	0.8531	1.46	0.9279
0.24	0.5948	0.65	0.7422	1.06	0.8554	1.47	0.9292
0.25	0.5987	0.66	0.7454	1.07	0.8577	1.48	0.9306
0.26	0.6026	0.67	0.7486	1.08	0.8599	1.49	0.9319
0.27	0.6064	0.68	0.7517	1.09	0.8621	1.50	0.9332
0.28	0.6103	0.69	0.7549	1.10	0.8643	1.51	0.9345
0.29	0.6141	0.70	0.7580	1.11	0.8665	1.52	0.9357
0.30	0.6179	0.71	0.7611	1.12	0.8686	1.53	0.9370
0.31	0.6217	0.72	0.7642	1.13	0.8708	1.54	0.9382
0.32	0.6255	0.73	0.7673	1.14	0.8729	1.55	0.9394
0.33	0.6293	0.74	0.7704	1.15	0.8749	1.56	0.9406
0.34	0.6331	0.75	0.7734	1.16	0.8770	1.57	0.9418
0.35	0.6368	0.76	0.7764	1.17	0.8790	1.58	0.9429
0.36	0.6406	0.77	0.7794	1.18	0.8810	1.59	0.9441
0.37	0.6443	0.78	0.7823	1.19	0.8830	1.60	0.9452
0.38	0.6480	0.79	0.7852	1.20	0.8849	1.61	0.9463
0.39	0.6517	0.80	0.7881	1.21	0.8869	1.62	0.9474
0.40	0.6554	0.81	0.7910	1.22	0.8888	1.63	0.9484

TABLE B.10. P IS THE VALUE OF THE STANDARD NORMAL CUMULATIVE DISTRIBUTION AT Z (CONTINUED)

z	p	z	p	z	p	z	p
1.64	0.9495	2.05	0.9798	2.46	0.9931	2.87	0.9979
1.65	0.9505	2.06	0.9803	2.47	0.9932	2.88	0.9980
1.66	0.9515	2.07	0.9808	2.48	0.9934	2.89	0.9981
1.67	0.9525	2.08	0.9812	2.49	0.9936	2.90	0.9981
1.68	0.9535	2.09	0.9817	2.50	0.9938	2.91	0.9982
1.69	0.9545	2.10	0.9821	2.51	0.9940	2.92	0.9982
1.70	0.9554	2.11	0.9826	2.52	0.9941	2.93	0.9983
1.71	0.9564	2.12	0.9830	2.53	0.9943	2.94	0.9984
1.72	0.9573	2.13	0.9834	2.54	0.9945	2.95	0.9984
1.73	0.9582	2.14	0.9838	2.55	0.9946	2.96	0.9985
1.74	0.9591	2.15	0.9842	2.56	0.9948	2.97	0.9985
1.75	0.9599	2.16	0.9846	2.57	0.9949	2.98	0.9986
1.76	0.9608	2.17	0.9850	2.58	0.9951	2.99	0.9986
1.77	0.9616	2.18	0.9854	2.59	0.9952	3.00	0.9987
1.78	0.9625	2.19	0.9857	2.60	0.9953	3.01	0.9987
1.79	0.9633	2.20	0.9861	2.61	0.9955	3.02	0.9987
1.80	0.9641	2.21	0.9864	2.62	0.9956	3.03	0.9988
1.81	0.9649	2.22	0.9868	2.63	0.9957	3.04	0.9988
1.82	0.9656	2.23	0.9871	2.64	0.9959	3.05	0.9989
1.83	0.9664	2.24	0.9875	2.65	0.9960	3.06	0.9989
1.84	0.9671	2.25	0.9878	2.66	0.9961	3.07	0.9989
1.85	0.9678	2.26	0.9881	2.67	0.9962	3.08	0.9990
1.86	0.9686	2.27	0.9884	2.68	0.9963	3.09	0.9990
1.87	0.9693	2.28	0.9887	2.69	0.9964	3.10	0.9990
1.88	0.9699	2.29	0.9890	2.70	0.9965	3.11	0.9991
1.89	0.9706	2.30	0.9893	2.71	0.9966	3.12	0.9991
1.90	0.9713	2.31	0.9896	2.72	0.9967	3.13	0.9991
1.91	0.9719	2.32	0.9898	2.73	0.9968	3.14	0.9992
1.92	0.9726	2.33	0.9901	2.74	0.9969	3.15	0.9992
1.93	0.9732	2.34	0.9904	2.75	0.9970	3.16	0.9992
1.94	0.9738	2.35	0.9906	2.76	0.9971	3.17	0.9992
1.95	0.9744	2.36	0.9909	2.77	0.9972	3.18	0.9993
1.96	0.9750	2.37	0.9911	2.78	0.9973	3.19	0.9993
1.97	0.9756	2.38	0.9913	2.79	0.9974	3.20	0.9993
1.98	0.9761	2.39	0.9916	2.80	0.9974	3.21	0.9993
1.99	0.9767	2.40	0.9918	2.81	0.9975	3.22	0.9994
2.00	0.9772	2.41	0.9920	2.82	0.9976	3.23	0.9994
2.01	0.9778	2.42	0.9922	2.83	0.9977	3.24	0.9994
2.02	0.9783	2.43	0.9925	2.84	0.9977	3.25	0.9994
2.03	0.9788	2.44	0.9927	2.85	0.9978	3.26	0.9994
2.04	0.9793	2.45	0.9929	2.86	0.9979	3.27	0.9995

TABLE B.10. P IS THE VALUE OF THE STANDARD NORMAL CUMULATIVE DISTRIBUTION AT Z (CONTINUED)

z	p	z	p	z	p	z	p
3.28	0.9995	3.46	0.9997	3.64	0.9999	3.82	0.9999
3.29	0.9995	3.47	0.9997	3.65	0.9999	3.83	0.9999
3.30	0.9995	3.48	0.0997	3.66	0.9999	3.84	0.9999
3.31	0.9995	3.49	0.9998	3.67	0.9999	3.85	0.9999
3.32	0.9995	3.50	0.9998	3.68	0.9999	3.86	0.9999
3.33	0.9996	3.51	0.9998	3.69	0.9999	3.87	0.9999
3.34	0.9996	3.52	0.9998	3.70	0.9999	3.88	0.9999
3.35	0.9996	3.53	0.9998	3.71	0.9999	3.89	0.9999
3.36	0.9996	3.54	0.9998	3.72	0.9999	3.90	1.0000
3.37	0.9996	3.55	0.9998	3.73	0.9999	3.91	1.0000
3.38	0.9996	3.56	0.9998	3.74	0.9999	3.92	1.0000
3.39	0.9997	3.57	0.9998	3.75	0.9999	3.93	1.0000
3.40	0.9997	3.58	0.9998	3.76	0.9999	3.94	1.0000
3.41	0.9997	3.59	0.9998	3.77	0.9999	3.95	1.0000
3.42	0.9997	3.60	0.9998	3.78	0.9999	3.96	1.0000
3.43	0.9997	3.61	0.9998	3.79	0.9999	3.97	1.0000
3.44	0.9997	3.62	0.9999	3.80	0.9999	3.98	1.0000
3.45	0.9997	3.63	0.9999	3.81	0.9999	3.99	1.0000

2.10.7 Next, calculate the following differences for each ordered observation:

$$D_i+ = (i/n) - p_i$$

$$D_i- = p_i - [(i-1)/n]$$

The differences for the example are listed in Table B.9.

2.10.8 Obtain the maximum of the D_i+, and denote it as D+. Obtain the maximum of the D_i-, and denote it as D-. For the example, D+ = 0.0525, and D- = 0.0597.

2.10.9 Next, obtain the maximum of D+ and D-, and denote it as D. For the example, D = 0.0597.

2.10.10 The test statistic, D*, is calculated as follows:

$$D^* = D(\sqrt{n} - 0.01 + \frac{0.85}{\sqrt{n}})$$

For the example, D* = 0.4684.

2.10.11 The decision rule for the two tailed test is to compare the critical value from Table B.11 to the computed D*. If the computed value is greater than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 is 1.035. The calculated value, 0.4684, is not greater than the critical value. Thus, the conclusion of the test is that the data are normally distributed.

2.10.12 In general, if the data fail the test for normality, a transformation such as the log transformation may normalize the data. After transforming the data, repeat the Kolmogorov "D" test for normality.

TABLE B.11. CRITICAL VALUES FOR THE KOLMOGOROV "D" TEST

Alpha Level	Critical Value
0.010	1.035
0.025	0.955
0.050	0.895
0.100	0.819
0.150	0.775

3. TEST FOR HOMOGENEITY OF VARIANCE

3.1 For Dunnett's Procedure and the t test with Bonferroni's adjustment, the variances of the data obtained from each toxicant concentration and the control are assumed to be equal. Bartlett's Test is a formal test of this assumption. In using this test, it is assumed that the data are normally distributed.

3.2 The data used in this example are growth data from a Fathead Minnow Larval Survival and Growth Test, and are the same data used in Appendices C and D. These data are listed in Table B.12, together with the calculated variance for the control and each toxicant concentration.

TABLE B.12. FATHEAD LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR BARTLETT'S TEST FOR HOMOGENEITY OF VARIANCE

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.711	0.646	0.669	0.629	0.650
B	0.662	0.626	0.669	0.680	0.558
C	0.718	0.723	0.694	0.513	0.606
D	0.767	0.700	0.676	0.672	0.508
Mean($\bar{Y}_i$)	0.714	0.674	0.677	0.624	0.580
S_i^2	0.0018	0.0020	0.0001	0.0059	0.0037
I	1	2	3	4	5

3.3 The test statistic for Bartlett's Test (Snedecor and Cochran, 1980) is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i) \ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each toxicant concentration and control

p = number of levels of toxicant concentration including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations

n_i = the number of replicates for concentration i.

$$\bar{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} \frac{1}{V_i} - (\sum_{i=1}^{P} V_i)^{-1}]$$

3.4 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value is obtained from a table of the chi-square distribution for p - 1 degrees of freedom and a significance level of 0.01. If B is less than the critical value then the variances are assumed to be equal.

3.5 For the data in this example, $V_i = 3$, p = 5, $\bar{S}^2 = 0.0027$, and C = 1.133. The calculated B value is:

$$B = \frac{(15)[\ln(0.0027)] - 3\sum_{i=1}^{P} \ln(S_i^2)}{1.133}$$

$$= \frac{15(-5.9145) - 3(-32.4771)}{1.133}$$

$$= 7.691$$

3.6 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value for the test is 13.277 for a significance level of 0.01. Since B = 7.691 is less than the critical value of 13.277, conclude that the variances are not different.

4. TRANSFORMATIONS OF THE DATA

4.1 When the assumptions of normality and/or homogeneity of variance are not met, transformations of the data may remedy the problem, so that the data can be analyzed by parametric procedures, rather than by nonparametric technique such as Steel's Many-one Rank Test or Wilcoxon's Rank Sum Test. Examples of transformations include log, square root, arc sine square root, and reciprocals. After the data have been transformed, Shapiro-Wilk's and Bartlett's tests should be performed on the transformed observations to determine whether the assumptions of normality and/or homogeneity of variance are met.

4.2 ARC SINE SQUARE ROOT TRANSFORMATION (USEPA, 1993)

4.2.1 For data consisting of proportions from a binomial (response/no response; live/dead) response variable, the variance within the ith treatment is proportional to $P_i (1 - P_i)$, where P_i is the expected proportion for the treatment. This clearly violates the homogeneity of variance assumption required by parametric procedures such as Dunnett's Procedure or the t test with Bonferroni's adjustment, since the existence of a treatment effect implies different values of P_i for different treatments, i. Also, when the observed proportions are based on small samples, or when P_i is

close to zero or one, the normality assumption may be invalid. The arc sine square root (arc sine $\sqrt{P}$) transformation is commonly used for such data to stabilize the variance and satisfy the normality requirement.

4.2.2 Arc sine transformation consists of determining the angle (in radians) represented by a sine value. In the case of arc sine square root transformation of mortality data, the proportion of dead (or affected) organisms is taken as the sine value, the square root of the sine value is calculated, and the angle (in radians) for the square root of the sine value is determined. Whenever the proportion dead is 0 or 1, a special modification of the arc sine square root transformation must be used (Bartlett, 1937). An explanation of the arc sine square root transformation and the modification is provided below.

4.2.3 Calculate the response proportion (RP) at each effluent concentration, where:

RP = (number of surviving or "unaffected" organisms)/(number exposed)

Example: If 12 of 20 animals in a given treatment replicate survive:

RP = 12/20

= 0.60

4.2.4 Transform each RP to its arc sine square root, as follows:

4.2.4.1 For RPs greater than zero or less than one:

Angle (radians) = arc sine $\sqrt{RP}$

Example: If RP = 0.60:

Angle = arc sine $\sqrt{0.60}$

= arc sine 0.7746

= 0.8861 radians

4.2.4.2 Modification of the arc sine square root when RP = 0:

Angle (in radians) = arc sine $\sqrt{1/4\,N}$

Where: N = Number of animals/treatment replicate

Example: If 20 animals are used:

Angle = arc sine $\sqrt{1/80}$

= arc sine 0.1118

= 0.1120 radians

4.2.4.3 Modification of the arc sine square root when RP = 1.0:

Angle = 1.5708 radians - (radians for RP = 0)

Example: Using above value:

Angle = 1.5708 - 0.1120

= 1.4588 radians

APPENDIX C

DUNNETT'S PROCEDURE

1. MANUAL CALCULATIONS

1.1 Dunnett's Procedure (Dunnett, 1955; Dunnett, 1964) is used to compare each concentration mean with the control mean to decide if any of the concentrations differ from the control. This test has an overall error rate of alpha, which accounts for the multiple comparisons with the control. It is based on the assumptions that the observations are independent and normally distributed and that the variance of the observations is homogeneous across all concentrations and control (see Appendix B for a discussion on validating the assumptions). Dunnett's Procedure uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance. Dunnett's Procedure can only be used when the same number of replicate test vessels have been used at each concentration and the control. When this condition is not met, a t test with Bonferroni's adjustment is used (see Appendix D).

1.2 The data used in this example are growth data from a Fathead Minnow Larval Survival and Growth Test, and are the same data used in Appendices B and D. These data are listed in Table C.1.

TABLE C.1. FATHEAD MINNOW, *PIMEPHALES PROMELAS,* LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR DUNNETT'S PROCEDURE

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.711	0.517	0.602	0.566	0.455
B	0.662	0.501	0.669	0.612	0.502
C	0.646	0.723	0.694	0.410	0.606
D	0.690	0.560	0.676	0.672	0.254
Mean($\bar{Y}_i$)	0.677	0.575	0.660	0.565	0.454
Total(T_i)	2.709	2.301	2.641	2.260	1.817

1.3 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, using the following formulas:

Where: p = number of effluent concentrations including:

$$SST = \sum_{ij} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSB = \sum_{i} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations; $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration i

Y_{ij} = the jth observation for concentration i

1.4 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 4$

$N = 20$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 2.709$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 2.301$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 2.641$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.260$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 1.817$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 11.728$

$SST = \sum_{ij} Y_{ij}^2 - G^2/N$

$= 7.146 - (11.728)^2/20$

$= 0.2687$

$SSB = \sum_i T_i^2/n_i - G^2/N$

$= ¼ (28.017 - 11.728)^2/20$

$= 0.1270$

$SSW = SST - SSB$

$= 0.2687 - 0.1270$

$= 0.1417$

1.5 Summarize these data in the ANOVA table (Table C.2).

TABLE C.2. ANOVA TABLE FOR DUNNETT'S PROCEDURE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

1.6 Summarize data for ANOVA (Table C.3).

TABLE C.3. COMPLETED ANOVA TABLE FOR DUNNETT'S PROCEDURE

Source	df	SS	Mean Square
Between	5 - 1 = 4	0.1270	0.0318
Within	20 - 5 = 15	0.1417	0.0094
Total	19	0.2687	

1.7 To perform the individual comparisons, calculate the t statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean for concentration i

$\bar{Y}_1$ = mean for the control

S_w = square root of the within mean square

n_1 = number of replicates in the control

n_i = number of replicates for concentration i.

1.8 Table C.4 includes the calculated t values for each concentration and control combination.

TABLE C.4. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.487
64	3	0.248
128	4	1.633
256	5	3.251

1.9 Since the purpose of the test is only to detect a decrease in growth from the control, a one-sided test is appropriate. The critical value for the one-sided comparison (2.36), with an overall alpha level of 0.05, 15 degrees of freedom and four concentrations excluding the control is read from the table of Dunnett's "T" values (Table C.5; this table assumes an equal number of replicates in all treatment concentrations and the control). The mean weight for concentration i is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Since T_5 is greater than 2.36, the 256 µg/L concentration has significantly lower growth than the control. Hence the NOEC and LOEC for growth are 128 µg/L and 256 µg/L, respectively.

TABLE C.5. DUNNETT'S "T" VALUES (Miller, 1981)

(One-tailed) d^{α}_{k}

k / ν	α = .05									α = 0.1								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
5	2.02	2.44	2.58	2.85	2.98	3.08	3.16	3.24	3.30	3.37	3.90	4.21	4.43	4.50	4.73	4.85	4.94	5.03
6	1.94	2.34	2.56	2.71	2.83	2.92	3.00	3.07	3.12	3.14	3.61	4.88	4.07	4.21	4.33	4.43	4.51	4.39
7	1.89	2.27	2.48	2.62	2.73	2.82	2.89	2.95	3.01	3.00	3.42	3.56	3.83	3.96	4.07	4.15	4.23	4.30
8	1.86	2.22	2.42	2.55	2.56	2.74	2.81	2.87	2.92	2.90	3.20	3.51	3.67	3.79	3.18	3.96	4.03	4.09
9	1.83	2.18	2.37	2.50	2.60	2.68	2.75	2.81	2.86	2.82	3.19	3.40	3.55	3.86	3.75	3.82	3.89	3.94
10	1.81	2.15	2.34	2.47	2.56	2.64	2.70	2.76	2.81	2.76	3.11	3.31	3.45	3.56	3.64	3.71	3.78	3.83
11	1.80	2.13	2.31	2.44	2.53	2.60	2.67	2.72	2.77	2.72	3.06	3.25	3.38	3.46	3.56	3.63	3.69	3.74
12	1.78	2.11	2.29	2.41	2.50	2.58	2.64	2.59	2.74	2.68	3.01	3.19	3.32	3.42	3.50	3.56	3.62	3.67
13	1.77	2.09	2.27	2.39	2.48	2.55	2.61	2.68	2.71	2.65	2.97	3.15	3.27	3.37	3.44	3.91	3.56	3.61
14	1.76	2.08	2.25	2.37	2.46	2.53	2.59	2.64	2.69	2.62	2.94	3.11	3.23	3.32	3.40	3.46	3.51	3.56
15	1.75	2.07	2.24	2.36	2.44	2.51	2.57	2.62	2.67	2.60	2.91	3.08	3.20	3.29	3.36	3.42	3.47	3.52
16	1.75	2.06	2.23	2.34	2.43	2.50	2.56	2.61	2.65	2.58	2.38	3.05	3.17	3.28	3.33	3.39	3.44	3.48
17	1.74	2.05	2.22	2.33	2.42	2.49	2.54	2.59	2.64	2.57	2.86	3.03	3.14	3.23	3.30	3.36	3.41	3.45
18	1.73	2.04	2.21	2.32	2.41	2.48	2.53	2.58	2.62	2.55	2.84	3.01	3.12	3.21	3.27	3.33	3.38	3.40
19	1.73	2.03	2.20	2.31	2.40	2.47	2.52	2.57	2.61	2.54	2.83	2.99	3.10	3.18	3.25	3.31	3.36	3.40
20	1.72	2.03	2.19	2.30	2.30	2.46	2.51	2.56	2.60	2.53	2.81	2.97	3.08	3.17	3.23	3.29	3.34	3.40
24	1.71	2.01	3.17	2.28	2.36	2.43	2.48	2.53	2.57	2.40	2.77	2.92	3.03	3.11	3.17	3.22	3.27	3.31
30	1.70	1.99	2.15	2.25	2.33	2.40	2.45	2.50	2.54	2.46	2.72	2.87	2.97	3.05	3.11	3.16	3.21	3.24
40	1.68	1.97	2.13	2.23	2.31	2.37	2.42	2.47	2.51	2.42	2.68	2.32	2.92	2.99	3.06	3.10	3.14	3.18
60	1.67	1.95	2.10	2.21	2.28	2.35	2.39	2.44	2.48	2.39	2.64	2.78	2.87	2.94	3.08	3.04	3.06	3.12
120	1.86	1.93	2.08	2.18	2.26	2.32	2.37	2.41	2.45	2.36	2.60	2.73	2.82	2.90	2.94	2.90	3.03	3.06
α	1.64	1.92	2.06	2.16	2.23	2.29	2.34	2.33	2.42	2.33	2.56	2.68	2.77	2.34	2.90	2.93	2.97	3.00

1.10 To quantify the sensitivity of the test, the minimum significant difference (MSD) may be calculated. The formula is as follows:

$$MSD = d\,S_w\sqrt{(1/n_1)+(1/n)}$$

Where: d = critical value for the Dunnett's Procedure

S_w = the square root of the within mean square

n = the number of replicates at each concentration, assuming an equal number of replicates at all treatment concentrations

n_1 = number of replicates in the control

For example:

$$MSD = 2.36(0.097)[3\sqrt{(1/4)+(1/4)}] = 2.36(0.097)(\sqrt{2/4})$$

$$= 2.36\ (0.097)(0.707)$$

$$= 0.162$$

1.11 For this set of data, the minimum difference between the control mean and a concentration mean that can be detected as statistically significant is 0.087 mg. This represents a decrease in growth of 24% from the control.

1.11.1 If the data have not been transformed, the MSD (and the percent decrease from the control mean that it represents) can be reported as is.

1.11.2 In the case where the data have been transformed, the MSD would be in transformed units. In this case carry out the following conversion to determine the MSD in untransformed units.

1.11.2.1 Subtract the MSD from the transformed control mean. Call this difference D. Next, obtain untransformed values for the control mean and the difference, D.

$MSD_u = control_u - D_u$

Where: MSD_u = the minimum significant difference for untransformed data

$Control_u$ = the untransformed control mean

D_u = the untransformed difference

1.11.2.2 Calculate the percent reduction from the control that MSD_u represents as:

$$\text{Percent Reduction} = \frac{MSD_u}{Control_u} \times 100$$

1.11.3 An example of a conversion of the MSD to untransformed units, when the arc sine square root transformation was used on the data, follows:

Step 1. Subtract the MSD from the transformed control mean. As an example, assume the data in Table C.1 were transformed by the arc sine square root transformation. Thus:

$0.677 - 0.162 = 0.515$

Step 2. Obtain untransformed values for the control mean (0.677) and the difference (0.515) obtained in Step 1 above.

$[\text{Sine } (0.677)]^2 = 0.392$
$[\text{Sine } (0.515)]^2 = 0.243$

Step 3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values obtained in Step 2.

$MSD_u = 0.392 - 0.243 = 0.149$

In this case, the MSD would represent a 38.0% decrease in survival from the control [(0.149/0.392)(100)].

2. COMPUTER CALCULATIONS

2.1 This computer program incorporates two analyses: an analysis of variance (ANOVA), and a multiple comparison of treatment means with the control mean (Dunnett's Procedure). The ANOVA is used to obtain the error value. Dunnett's Procedure indicates which toxicant concentration means (if any) are statistically different from the control mean at the 5% level of significance. The program also provides the minimum difference between the control and treatment means that could be detected as statistically significant, and tests the validity of the homogeneity of variance assumption by Bartlett's Test. The multiple comparison is performed based on procedures described by Dunnett (1955).

2.2 The source code for the Dunnett's program is structured into a series of subroutines, controlled by a driver routine. Each subroutine has a specific function in the Dunnett's Procedure, such as data input, transforming the data, testing for equality of variances, computing p values, and calculating the one-way analysis of variance.

2.3 The program compares up to seven toxicant concentrations against the control, and can accommodate up to 50 replicates per concentration.

2.4 If the number of replicates at each toxicant concentration and control are not equal, a t test with Bonferroni's adjustment is performed instead of Dunnett's Procedure (see Appendix D).

2.5 The program was written in IBM-PC FORTRAN by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled executable version of the program can be obtained from EMSL-Cincinnati by sending a written request to EMSL at 3411 Church Street, Cincinnati, OH 45244.

2.6 DATA INPUT AND OUTPUT

2.6.1 Reproduction data from a daphnid, *Ceriodaphnia dubia,* survival and reproduction test (Table C.6) are used to illustrate the data input and output for this program.

TABLE C.6. SAMPLE DATA FOR DUNNETT'S PROGRAM *CERIODAPHNIA DUBIA* REPRODUCTION DATA

		Effluent Concentration (%)			
Replicate	Control	1.56	3.12	6.25	12.5
1	27	32	39	27	10
2	30	35	30	34	13
3	29	32	33	36	7
4	31	26	33	34	7
5	16	18	36	31	7
6	15	29	33	27	10
7	18	27	33	33	10
8	17	16	27	31	16
9	14	35	38	33	12
10	27	13	44	31	2

2.6.2 Data Input

2.6.2.1 When the program is entered, the user is asked to select the type of data to be entered:

1. Response proportions, like survival or fertilization proportions.
2. Counts and measurements, like offspring counts, cystocarp counts or weights.

2.6.2.2 After the type of data is chosen, the user has the following options:

1. Create a data file
2. Edit a data file
3. Perform analysis on existing data set
4. Stop

2.6.2.3 When Option 1 (Create a data file) is selected for counts and measurements, the program prompts the user for the following information:

1. Number of concentrations, including control
2. For each concentration:
   - number of observations
   - data for each observation

2.6.2.4 After the data have been entered, the user may save the file on a disk, and the program returns to the menu (see below).

2.6.2.5 Sample data input is shown in Figure C.1.

```
                    EMSL Cincinnati Dunnett Software
                              Version 1.5

      1)    Create a data file
      2)    Edit a data file
      3)    Perform ANOVA on existing data
      4)    Stop

Your choice ? 1

Number of groups, including control ? 5

Number of observations for group  1 ? 10

Enter the data for group  1 one observation at a time.

NO.  1?  27

NO.  2?  30

NO.  3?  29

NO.  4?  31

NO.  5?  16

NO.  6?  15

NO.  7?  18

NO.  8?  17

NO.  9?  14

NO. 10?  27

Number of observations for group  2  ?  10

Do you wish to save the data on disk  ?y

Disk file for output  ?  cerio
```

Figure C.1. Sample Data Input for Dunnett's Program for Reproduction Data from Table C.6.

2.6.3 Program Output

2.6.3.1 When Option 3 (Perform analysis on existing data set) is selected from the menu, the user is asked to select the transformation desired, and indicate whether they expect the means of the test groups to be less or greater than the mean for the control group (see Figure C.2).

2.6.3.2 Summary statistics (Figure C.3) for the raw and transformed data, if applicable, the ANOVA table, results of Bartlett's Test, the results of the multiple comparison procedure and the minimum detectable difference are included in the program output.

EMSL Cincinnati Dunnett Software
Version 1.5

1) Create a data file
2) Edit a data file
3) Perform analysis on existing data set
4) Stop

Your choice ? 3

File name ? cerio

Available Transformations

1) no transform
2) square root
3) log10

Your choice ? 1

Dunnett's test as implemented in this program is
a one-sided test. You must specify the direction
the test is to be run; that is, do you expect the
means for the test groups to be less than or
greater than the mean for the control group mean.

Direction for Dunnett's test : L=less than, G=greater than ? L

Figure C.2. Example of Choosing Option 3 from the Menu of the Dunnett Program.

Ceriodaphnia Reproduction Data from Table C.6

Summary Statistics and ANOVA

Transformation = None

Group	n	Mean	s.d.	CV%
1 = control	10	22.4000	6.9314	30.9
2	10	26.3000	8.0007	30.4
3	10	34.6000	4.8351	14.0
4	10	31.7000	2.9458	9.3
5*	10	9.4000	3.8930	41.4

*) the mean for this group is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -5.628560
This difference corresponds to -25.13 percent of control

Between concentrations
Sum of squares = 3887.880000 with 4 degrees of freedom.

Error mean square = 31.853333 with 45 degrees of freedom.

Bartlett's test p-value for equality of variances = .029

Do you wish to restart the program ?

Figure C.3. Example of Program Output for the Dunnett's Program Using the Reproduction Data from Table C.6.

APPENDIX D

T TEST WITH BONFERRONI'S ADJUSTMENT

1. The t test with Bonferroni's adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus, Dunnett's Procedure is a more powerful test.

2. The t test with Bonferroni's adjustment is based on the same assumptions of normality and homogeneity of variance as Dunnett's Procedure (see Appendix B for testing these assumptions), and, like Dunnett's Procedure, uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance.

3. An example of the use of the t test with Bonferroni's adjustment is provided below. The data used in the example are the same as in Appendix C, except that the third replicate from the 256 µg/L concentration is presumed to have been lost. Thus, Dunnett's Procedure cannot be used. The weight data are presented in Table D.1.

TABLE D.1. FATHEAD MINNOW, *PIMEPHALES PROMELAS,* LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR THE T-TEST WITH BONFERRONI'S ADJUSTMENT

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.711	0.517	0.602	0.566	0.455
B	0.662	0.501	0.669	0.612	0.502
C	0.646	0.723	0.694	0.410	(LOST)
D	0.690	0.560	0.676	0.672	0.254
Mean($\bar{Y}$)	0.677	0.575	0.660	0.565	0.404
Total(T_i)	2.709	2.301	2.641	2.260	1.211

3.1 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, using the following formulas:

Where: p = number of effluent concentrations including the control

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration i

$SST = \sum_{ij} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSB = \sum_i T_i^2/n_i - G^2/N$ Between Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

Where: G = The grand total of all sample observations; $G = \sum_{i=1}^{P} T_i$

T_i = The total of the replicate measurements for concentration i

Y_{ij} = The jth observation for concentration i

3.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = 4$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 2.709$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 2.301$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 2.641$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.260$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 1.211$
$G = T_1 + T_2 + T_3 + T_4 + T_5 = 11.122$

$SSB = \sum_i T_i^2/n_i - G^2/N$

$= 6.668 - (11.122)^2/19$

$= 0.158$

$SST = \sum_{ij} Y_{ij}^2 - G^2/N$

$= 6.779 - (11.122)^2/19$

$= 0.269$

$SSW = SST - SSB$

= 0.269 - 0.158

= 0.111

3.3 Summarize these data in the ANOVA table (Table D.2):

TABLE D.2. ANOVA TABLE FOR BONFERRONI'S ADJUSTMENT

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

3.4 Summarize these data in the ANOVA table (Table D.3):

TABLE D.3. COMPLETED ANOVA TABLE FOR THE T-TEST WITH BONFERRONI'S ADJUSTMENT

Source	df	SS	Mean Square
Between	5 - 1 = 4	0.158	0.0395
Within	19 - 5 = 14	0.111	0.0029
Total	18	0.269	

3.5 To perform the individual comparisons, calculate the t statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean for each concentration

$\bar{Y}_1$ = mean for the control

S_w = square root of the within mean square

n_1 = number of replicates in the control.

n_i = number of replicates for concentration i.

3.6 Table D.4 includes the calculated t values for each concentration and control combination.

TABLE D.4. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.623
64	3	0.220
128	4	1.782
256	5	4.022

3.7 Since the purpose of the test is only to detect a decrease in growth from the control, a one-sided test is appropriate. The critical value for the one-sided comparison (2.510), with an overall alpha level of 0.05, fourteen degrees of freedom and four concentrations excluding the control, was obtained from Table D.5. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Since t_5 is greater than 2.510, the 256 µg/L concentration has significantly lower growth than the control. Hence the NOEC and LOEC for growth are 128 µg/L and 256 µg/L, respectively.

TABLE D.5. CRITICAL VALUES FOR "T" FOR THE T TEST WITH BONFERRONI'S ADJUSTMENT
P = 0.05 CRITICAL LEVEL, ONE TAILED

df	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
1	6.314	12.707	19.002	25.452	31.821	38.189	44.556	50.924	57.290	63.657
2	2.920	4.303	5.340	6.206	6.965	7.649	8.277	8.861	9.408	9.925
3	2.354	3.183	3.741	4.177	4.541	4.857	5.138	5.392	5.626	5.841
4	2.132	2.777	3.187	3.496	3.747	3.961	4.148	4.315	4.466	4.605
5	2.016	2.571	2.912	3.164	3.365	3.535	3.681	3.811	3.927	4.033
6	1.944	2.447	2.750	2.969	3.143	3.288	3.412	3.522	3.619	3.708
7	1.895	2.365	2.642	2.842	2.998	3.128	3.239	3.336	3.422	3.500
8	1.860	2.307	2.567	2.752	2.897	3.016	3.118	3.206	3.285	3.356
9	1.834	2.263	2.510	2.686	2.822	2.934	3.029	3.111	3.185	3.250
10	1.813	2.229	2.406	2.634	2.764	2.871	2.961	3.039	3.108	3.170
11	1.796	2.301	2.432	2.594	2.719	2.821	2.907	2.981	3.047	3.106
12	1.783	2.179	2.404	2.561	2.681	2.730	2.863	2.935	2.998	3.055
13	1.771	2.161	2.380	2.533	2.651	2.746	2.827	2.897	2.950	3.013
14	1.762	2.145	2.360	2.510	2.625	2.718	2.797	2.864	2.924	2.977
15	1.754	2.132	2.343	2.490	2.603	2.694	2.771	2.837	2.895	2.947
16	1.746	2.120	2.329	2.473	2.584	2.674	2.749	2.814	2.871	2.921
17	1.740	2.110	2.316	2.459	2.567	2.655	2.729	2.793	2.849	2.899
18	1.735	2.101	2.305	2.446	2.553	2.640	2.712	2.775	2.830	2.879
19	1.730	2.094	2.295	2.434	2.540	2.626	2.697	2.759	2.813	2.861
20	1.725	2.086	2.206	2.424	2.528	2.613	2.684	2.745	2.798	2.846
21	1.721	2.080	2.278	2.414	2.518	2.602	2.672	2.732	2.785	2.832
22	1.718	2.074	2.271	2.406	2.509	2.592	2.661	2.721	2.773	2.819
23	1.714	2.069	2.264	2.398	2.500	2.583	2.651	2.710	2.762	2.808
24	1.711	2.064	2.258	2.391	2.493	2.574	2.642	2.701	2.752	2.797
25	1.709	2.060	2.253	2.385	2.486	2.566	2.634	2.692	2.743	2.788
26	1.706	2.056	2.248	2.379	2.479	2.559	2.627	2.684	2.734	2.779
27	1.704	2.052	2.243	2.374	2.473	2.553	2.620	2.677	2.727	2.771
28	1.702	2.049	2.239	2.369	2.468	2.547	2.613	2.670	2.720	2.764

TABLE D.5. CRITICAL VALUES FOR "T" FOR THE T TEST WITH BONFERRONI'S ADJUSTMENT
P = 0.05 CRITICAL LEVEL, ONE TAILED (CONTINUED)

df	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
29	1.700	2.046	2.235	2.364	2.463	2.541	2.607	2.664	2.713	2.757
30	1.698	2.043	2.231	2.360	2.458	2.536	2.602	2.658	2.707	2.750
31	1.696	2.040	2.228	2.356	2.453	2.531	2.597	2.652	2.701	2.745
32	1.694	2.037	2.224	2.352	2.449	2.527	2.592	2.647	2.696	2.739
33	1.693	2.035	2.221	2.349	2.445	2.523	2.587	2.643	2.691	2.734
34	1.691	2.033	2.219	2.346	2.442	2.519	2.583	2.638	2.686	2.729
35	1.690	2.031	2.216	2.342	2.438	2.515	2.579	2.634	2.682	2.724
36	1.689	2.029	2.213	2.340	2.435	2.512	2.575	2.630	2.678	2.720
37	1.688	2.027	2.211	2.337	2.432	2.508	2.572	2.626	2.674	2.716
38	1.686	2.025	2.209	2.334	2.429	2.505	2.568	2.623	2.670	2.712
39	1.685	2.023	2.207	2.332	2.426	2.502	2.565	2.619	2.667	2.708
40	1.684	2.022	2.205	2.329	2.424	2.499	2.562	2.616	2.663	2.705
50	1.676	2.009	2.189	2.311	2.404	2.478	2.539	2.592	2.638	2.678
60	1.671	2.001	2.179	2.300	2.391	2.463	2.324	2.576	2.621	2.661
70	1.667	1.995	2.171	2.291	2.381	2.453	2.513	2.564	2.609	2.648
80	1.665	1.991	2.166	2.285	2.374	2.446	2.505	2.556	2.600	2.639
90	1.662	1.987	2.162	2.280	2.369	2.440	2.499	2.549	2.593	2.632
100	1.661	1.984	2.158	2.276	2.365	2.435	2.494	2.544	2.588	2.626
110	1.659	1.982	2.156	2.273	2.361	2.432	2.490	2.540	2.583	2.622
120	1.658	1.980	2.153	2.270	2.358	2.429	2.487	2.536	2.580	2.618
Infinite	1.645	1.960	2.129	2.242	2.327	2.394	2.450	2.498	2.540	2.576

d.f. = Degrees of freedom for MSE (Mean Square Error) from ANOVA.
K = Number of concentrations to be compared to the control.

APPENDIX E

STEEL'S MANY-ONE RANK TEST

1. Steel's Many-one Rank Test is a nonparametric test for comparing treatments with a control. This test is an alternative to Dunnett's Procedure, and may be applied to data when the normality assumption has not been met. Steel's Test requires equal variances across the treatments and the control, but it is thought to be fairly insensitive to deviations from this condition (Steel, 1959). The tables for Steel's Test require an equal number of replicates at each concentration. If this is not the case, use Wilcoxon's Rank Sum Test, with Bonferroni's adjustment (see Appendix F).

2. For an analysis using Steel's Test, for each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to the observation. (Extensive ties would invalidate this procedure). The sum of the ranks within each concentration and within the control is then calculated. To determine if the response in a concentration is significantly different from the response in the control, the minimum rank sum for each concentration and control combination is compared to the significant values of rank sums given later in this section. In this table, k equals the number of treatments excluding the control and n equals the number of replicates for each concentration and the control.

3. An example of the use of this test is provided below. The test employs reproduction data from a *Ceriodaphnia dubia* 7-day, chronic test. The data are listed in Table E.1. Significant mortality was detected via Fisher's Exact Test in the 50% effluent concentration. The data for this concentration is not included in the reproduction analysis.

TABLE E.1. EXAMPLE OF STEEL'S MANY-ONE RANK TEST: DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, 7-DAY CHRONIC TEST

Effluent Concentration	Replicate 1	2	3	4	5	6	7	8	9	10	No. Live Adults
Control	20	26	26	23	24	27	26	23	27	24	10
3%	13	15	14	13	23	26	0	25	26	27	9
6%	18	22	13	13	23	22	20	22	23	22	10
12%	14	22	20	23	20	23	25	24	25	21	10
25%	9	0	9	7	6	10	12	14	9	13	8
50%	0	0	0	0	0	0	0	0	0	0	0

4. For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign ranks (1, 2, 3,..., 16) to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to each tied observation.

5. An example of assigning ranks to the combined data for the control and 3% effluent concentration is given in Table E.2. This ranking procedure is repeated for each control and concentration combination. The complete set of rankings is listed in Table E.3. The ranks are then summed for each effluent concentration, as shown in Table E.4.

TABLE E.2. EXAMPLE OF STEEL'S MANY-ONE RANK TEST: ASSIGNING RANKS TO THE CONTROL AND 3% EFFLUENT CONCENTRATION

Rank	Number of Young Produced	Control or % Effluent
1	0	3
2.5	13	3
2.5	13	3
4	14	3
5	15	3
6	20	Control
8	23	Control
8	23	Control
8	23	3
10.5	24	Control
10.5	24	Control
12	25	3
15	26	Control
15	26	Control
15	26	Control
15	26	3
15	26	3
19	27	Control
19	27	Control
19	27	3

TABLE E.3. TABLE OF RANKS

Replicate (Organism)	Control[1]		Effluent Concentration (%)							
			3		6		12		25	
1	20	(6,4.5,3,11)	13	(2.5)	18	(3)	14	(1)	9	(5)
2	26	(15,17,17,17)	15	(5)	22	(7.5)	22	(6)	0	(1)
3	26	(15,17,17,17)	14	(4)	13	(1.5)	20	(3)	9	(5)
4	23	(8,11.5,8.5,12.5)	13	(2.5)	13	(1.5)	23	(8.5)	7	(3)
5	24	(10.5,14.5,12,14.5)	23	(8)	23	(11.5)	20	(3)	6	(2)
6	27	(19,19.5,19.5,19.5)	26	(15)	22	(7.5)	23	(8.5)	10	(7)
7	26	(15,17,17,17)	0	(1)	20	(4.5)	25	(14.5)	12	(8)
8	23	(8,11.5,8.5,12.5)	25	(12)	22	(7.5)	24	(12)	14	(10)
9	27	(19,19.5,19.5,19.5)	26	(15)	23	(11.5)	25	(14.5)	9	(5)
10	24	(10.5,14.5,12,14.5)	27	(19)	22	(7.5)	21	(5)	13	(9)

[1] Control ranks are given in the order of the concentration with which they were ranked.

TABLE E.4. RANK SUMS

Effluent Concentration (%)	Rank Sum
3	84
6	64
12	76
25	55

6. For this set of data, determine if the reproduction in any of the effluent concentrations is significantly lower than the reproduction by the control organisms. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for the reproduction of each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the reproduction would be considered to be significantly lower than the control. At a probability level of 0.05, the critical rank in a test with four concentrations and ten replicates is 76 (see Table E.5 , for R=4).

7. Comparing the rank sums in Table E.4 to the appropriate critical rank, the 6%, 12% and 25% effluent concentrations are found to be significantly different from the control. Thus the NOEC and LOEC for reproduction are 3% and 6%, respectively.

TABLE E.5. SIGNIFICANT VALUES OF RANK SUMS: JOINT CONFIDENCE COEFFICIENTS OF 0.95 (UPPER) and 0.99 (LOWER) FOR ONE-SIDED ALTERNATIVES (Steel, 1959)

	k = number of treatments (excluding control)							
n	2	3	4	5	6	7	8	9
4	11	10	10	10	10	--	--	--
	--	--	--	--	--	--	--	--
5	18	17	17	16	16	16	16	15
	15	--	--	--	--	--	--	--
6	27	26	25	25	24	24	24	23
	23	22	21	21	--	--	--	--
7	37	36	35	35	34	34	33	33
	32	31	30	30	29	29	29	29
8	49	48	47	46	46	45	45	44
	43	42	41	40	40	40	39	39
9	63	62	61	60	59	59	58	58
	56	55	54	53	52	52	51	51
10	79	77	76	75	74	74	73	72
	71	69	68	67	66	66	65	65
11	97	95	93	92	91	90	90	89
	87	85	84	83	82	81	81	80
12	116	114	112	111	110	109	108	108
	105	103	102	100	99	99	98	98
13	138	135	133	132	130	129	129	128
	125	123	121	120	119	118	117	117
14	161	158	155	154	153	152	151	150
	147	144	142	141	140	139	138	137
15	186	182	180	178	177	176	175	174
	170	167	165	164	162	161	160	160
16	213	209	206	204	203	201	200	199
	196	192	190	188	187	186	185	184
17	241	237	234	232	231	229	228	227
	223	219	217	215	213	212	211	210
18	272	267	264	262	260	259	257	256
	252	248	245	243	241	240	239	238
19	304	299	296	294	292	290	288	287
	282	278	275	273	272	270	268	267
20	339	333	330	327	325	323	322	320
	315	310	307	305	303	301	300	299

APPENDIX F

WILCOXON RANK SUM TEST

1. Wilcoxon's Rank Sum Test is a nonparametric test, to be used as an alternative to Steel's Many-one Rank Test when the number of replicates are not the same at each concentration. A Bonferroni's adjustment of the pairwise error rate for comparison of each concentration versus the control is used to set an upper bound of alpha on the overall error rate, in contrast to Steel's Many-one Rank Test, for which the overall error rate is fixed at alpha. Thus, Steel's Test is a more powerful test.

2. An example of the use of the Wilcoxon Rank Sum Test is provided in Table F.1. The data used in the example are the same as in Appendix E, except that two males are presumed to have occurred, one in the control and one in the 12% effluent concentration. Thus, there is unequal replication for the reproduction analysis.

3. For each concentration and control combination, combine the data and arrange the values in order of size, from smallest to largest. Assign ranks to the ordered observations (a rank of 1 to the smallest, 2 to the next smallest, etc.). If ties in rank occur, assign the average rank to each tied observation.

TABLE F.1. EXAMPLE OF WILCOXON'S RANK SUM TEST: DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, 7-DAY CHRONIC TEST

Effluent Concentration	Replicate 1	2	3	4	5	6	7	8	9	10	No. Live Adults
Cont	M	26	26	23	24	27	26	23	27	24	10
3%	13	15	14	13	23	26	0	25	26	27	9
6%	18	22	13	13	23	22	20	22	23	22	10
12%	14	22	20	23	M	23	25	24	25	21	10
25%	9	0	9	7	6	10	12	14	9	13	8
50%	0	0	0	0	0	0	0	0	0	0	0

4. An example of assigning ranks to the combined data for the control and 3% effluent concentration is given in Table F.2. This ranking procedure is repeated for each of the three remaining control versus test concentration combinations. The complete set of ranks is listed in Table F.3. The ranks are then summed for each effluent concentration, as shown in Table F.4.

5. For this set of data, determine if the reproduction in any of the effluent concentrations is significantly lower than the reproduction by the control organisms. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum for the control. Thus, compare the rank sums for the reproduction of each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the reproduction would be considered to be significantly lower than the control. At a probability level of 0.05, the critical rank in a test with four concentrations and nine replicates in the control is 72 for those concentrations with ten replicates, and 60 for those concentrations with nine replicates (see Table F.5, for K = 4).

6. Comparing the rank sums in Table F.4 to the appropriate critical rank, the 6%, 12% and 25% effluent concentrations are found to be significantly different from the control. Thus, the NOEC and LOEC for reproduction are 3% and 6%, respectively.

TABLE F.2. EXAMPLE OF WILCOXON'S RANK SUM TEST: ASSIGNING RANKS TO THE CONTROL AND EFFLUENT CONCENTRATIONS

Rank	Number of Young Produced	Control or % Effluent
1	0	3
2.5	13	3
2.5	13	3
4	14	3
5	15	3
7	23	Control
7	23	Control
7	23	3
9.5	24	Control
9.5	24	Control
11	25	3
14	26	Control
14	26	Control
14	26	Control
14	26	3
14	26	3
18	27	Control
18	27	Control
18	27	3

TABLE F.3. TABLE OF RANKS

Replicate (Organism)	Control[1]		Effluent Concentration (%) 3		6		12		25	
1	M		13	(2.5)	18	(3)	14	(1)	9	(5)
2	26	(14,16,15,16)	15	(5)	22	(6.5)	22	(4)	0	(1)
3	26	(14,16,15,16)	14	(4)	13	(1.5)	20	(2)	9	(5)
4	23	(7,10.5,6.5,11.5)	13	(2.5)	13	(1.5)	23	(6.5)	7	(3)
5	24	(9.5,13.5,10,13.5)	23	(7)	23	(10.5)	M		6	(2)
6	27	(18,18.5,17.5,18.5)	26	(14)	22	(6.5)	23	(6.5)	10	(7)
7	26	(14,16,15,16)	0	(1)	20	(4)	25	(12.5)	12	(8)
8	23	(7,10.5,6.5,11.5)	25	(11)	22	(6.5)	24	(10)	14	(10)
9	27	(18,18.5,17.5,18.5)	26	(14)	23	(10.5)	25	(12.5)	9	(5)
10	24	(9.5,13.5,10,13.5)	27	(18)	22	(6.5)	21	(3)	13	(9)

[1] Control ranks are given in the order of the concentration with which they were ranked.

TABLE F.4. RANK SUMS

Effluent Concentration	Rank Sum	No. of Replicates	Critical Rank Sum
3	79	10	72
6	57	10	72
12	58	9	60
25	55	10	72

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
1	3	6	10	16	23	30	39	49	59
	4	6	11	17	24	32	41	51	62
	5	7	12	19	26	34	44	54	66
	6	8	13	20	28	36	46	57	69
	7	8	14	21	29	39	49	60	72
	8	9	15	23	31	41	51	63	72
	9	10	16	24	33	43	54	66	79
	10	10	17	26	35	45	56	69	82
2	3	--	--	15	22	29	38	47	58
	4	--	10	16	23	31	40	49	60
	5	6	11	17	24	33	42	52	63
	6	7	12	18	26	34	44	55	66
	7	7	13	20	27	36	46	57	69
	8	8	14	21	29	38	49	60	72
	9	8	14	22	31	40	51	62	75
	10	9	15	23	32	42	53	65	78
3	3	--	--	--	21	29	37	46	57
	4	--	10	16	22	30	39	48	59
	5	--	11	17	24	32	41	51	62
	6	6	11	18	25	33	43	53	65
	7	7	12	19	26	35	45	56	68
	8	7	13	20	28	37	47	58	70
	9	7	13	21	29	39	49	61	73
	10	8	14	22	31	41	51	63	76

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
4	3	--	--	--	21	28	37	46	56
	4	--	--	15	22	30	38	48	59
	5	--	10	16	23	31	40	50	61
	6	6	11	17	24	33	42	52	64
	7	6	12	18	26	34	44	55	67
	8	7	12	19	27	36	46	57	69
	9	7	13	20	28	38	48	60	72
	10	7	14	21	30	40	50	62	75
5	3	--	--	--	--	28	36	46	56
	4	--	--	15	22	29	38	48	58
	5	--	10	16	23	31	40	50	61
	6	--	11	17	24	32	42	52	63
	7	6	11	18	25	34	43	54	66
	8	6	12	19	27	35	45	56	68
	9	7	13	20	28	37	47	59	71
	10	7	13	21	29	39	49	61	74
6	3	--	--	--	--	28	36	45	56
	4	--	--	15	21	29	38	47	58
	5	--	10	16	22	30	39	49	60
	6	--	11	16	24	32	41	51	63
	7	6	11	17	25	33	43	54	65
	8	6	12	18	26	35	45	56	68
	9	6	12	19	27	37	47	58	70
	10	7	13	20	29	38	49	60	73
7	3	--	--	--	--	--	36	45	56
	4	--	--	--	21	29	37	47	58
	5	--	--	15	22	30	39	49	60
	6	--	10	16	23	32	41	51	62
	7	--	11	17	25	33	43	53	65
	8	6	11	18	26	35	44	55	67
	9	6	12	19	27	36	46	58	70
	10	7	13	20	28	38	48	60	72

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicate Per Effluent Concentration							
		3	4	5	6	7	8	9	10
8	3	--	--	--	--	--	36	45	55
	4	--	--	--	21	29	37	47	57
	5	--	--	15	22	30	39	49	59
	6	--	10	16	23	31	40	51	62
	7	--	11	17	24	33	42	53	64
	8	6	11	18	25	34	44	55	67
	9	6	12	19	27	36	46	57	69
	10	6	12	19	28	37	48	59	72
9	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	30	39	48	59
	6	--	10	16	23	31	40	50	62
	7	--	10	17	24	33	42	52	64
	8	--	11	18	25	34	44	55	66
	9	6	11	18	26	35	46	57	69
	10	6	12	19	28	37	47	59	71
10	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	29	38	48	59
	6	--	10	16	23	31	40	50	61
	7	--	10	16	24	32	42	52	64
	8	--	11	17	25	34	43	54	66
	9	6	11	18	26	35	45	56	68
	10	6	12	19	27	37	47	58	71

APPENDIX G

FISHER'S EXACT TEST

1. Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) is a statistical method based on the hypergeometric probability distribution that can be used to test if the proportion of successes is the same in two Bernoulli (binomial) populations. When used with the *Ceriodaphnia dubia* data, it provides a conservative test of the equality of any two survival proportions assuming only the independence of responses from a Bernoulli population. Additionally, since it is a conservative test, a pair-wise comparison error rate of 0.05 is suggested rather that an experiment-wise error rate.

2. The basis for Fisher's Exact Test is a 2×2 contingency table. However, in order to use this table the contingency table must be arranged in the format shown in Table G.1. From the 2×2 table, set up for the control and the concentration you wish to compare, you can determine statistical significance by looking up a value in the table provided later in this section.

TABLE G.1. FORMAT FOR CONTINGENCY TABLE

	Number of		
	Successes	Failures	Number of Observations
Row 1	a	A - a	A
Row 2	b	B - b	B
Total	a + b	[(A + B) - a - b]	A + B

3. Arrange the table so that the total number of observations for row one is greater than or equal to the total for row two ($A \geq B$). Categorize a success such that the proportion of successes for row one is greater than or equal to the proportion of successes for row two ($a/A \geq b/B$). For the *Ceriodaphnia dubia* survival data, a success may be 'alive' or 'dead', whichever causes $a/A \geq b/B$. The test is then conducted by looking up a value in the table of significance levels of b and comparing it to the b value given in the contingency table. The table of significance levels of b is Table G.5. Enter Table G.5 in the section for A, subsection for B, and the line for a. If the b value of the contingency table is equal to or less than the integer in the column headed 0.05 in Table G.5, then the survival proportion for the effluent concentration is significantly different from the survival proportion for the control. A dash or absence of entry in Table G.5 indicates that no contingency table in that class is significant.

4. To illustrate Fisher's Exact Test, a set of survival data (Table G.2) from the daphnid, *Ceriodaphnia dubia*, survival and reproduction test will be used.

5. For each control and effluent concentration construct a 2x2 contingency table.

6. For the control and effluent concentration of 1% the appropriate contingency table for the test is given in Table G.3.

TABLE G.2. EXAMPLE OF FISHER'S EXACT TEST: *CERIODAPHNIA DUBIA* MORTALITY DATA

Effluent Concentration (%)	No. Dead	Total[1]
Control	1	9
1	0	10
3	0	10
6	0	10
12	0	10
25	10	10

[1] Total number of live adults at the beginning of the test.

7. Since 10/10 > 8/9, the category 'alive' is regarded as a success. For A = 10, B = 9 and, a = 10, under the column headed 0.05, the value from Table G.5 is b = 5. Since the value of b (b = 8) from the contingency table (Table G.3), is greater than the value of b (b = 5) from Table G.5, the test concludes that the proportion of survival is not significantly different for the control and 1% effluent.

8. The contingency tables for the combinations of control and effluent concentrations of 3%, 6%, 12% are identical to Table G.3. The conclusion of no significant difference in the proportion of survival for the control and the level of effluent would also remain the same.

9. For the combination of control and 25% effluent, the contingency table would be constructed as Table G.4. The category 'dead' is regarded as a success, since 10/10 > 1/9. The b value (b = 1) from the contingency table (Table G.4) is less than the b value (b = 5) from the table of significance levels of b (Table G.5). Thus, the percent mortality for 25% effluent is significantly greater than the percent mortality for the control. Thus, the NOEC and LOEC for survival are 12% and 25%, respectively.

TABLE G.3. 2×2 CONTINGENCY TABLE FOR CONTROL AND 1% EFFLUENT

	Number of		Number of Observations
	Alive	Dead	
1% Effluent	10	0	10
Control	8	1	9
Total	18	1	19

Table G.4. 2x2 CONTINGENCY TABLE FOR CONTROL AND 25% EFFLUENT

	Number of		Number of Observations
	Dead	Alive	
25% Effluent	10	0	10
Control	1	8	9
Total	11	8	19

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1]

	α	0-05	0-025	0-01	0-005		α	0-05	0-025	0-01	0-005
						A=8	8	4 -038	3 -013	2 -003	2 -003
A=3 B=3	3	0 -050			——		7	2 -020	2 -020	1 -005+	0 -001
							6	1 -020	1 -020	0 -003	0 -003
							5	0 -013	0 -013		
A=4 B=4	4	1 -014	1 -014	——	——		4	0 -038			
3	4	0 -029	——	——	——	7	8	3 -026	2 -007	2 -007	1 -001
							7	2 -035-	1 -009	1 -009	0 -001
A=5 B=5	5	1 -024	1 -024	0 -004	0 -004		6	1 -032	0 -006	0 -006	
	4	0 -024	1 -024	——	——		5	0 -019	0 -019		
4	5	1 -048	0 -008	0 -008	——	6	8	2 -015-	2 -015-	1 -003	1 -003
	4	0 -040	——	——	——		7	1 -016	1 -016	0 -002	0 -002
3	5	0 -018	0 -018	——	——		6	0 -009	0 -009	0 -009	
2	5	0 -048	——	——	——		5	0 -028	——	——	
						5	8	2 -035-	1 -007	1 -007	0 -001
							7	1 -032	0 -005-	0 -005-	0 -005-
A=6 B=6	6	2 -030	1 -008	1 -008	0 -001		6	0 -016	0 -016	——	
	5	1 -040	0 -008	0 -008	——		5	0 -044	——	——	
	4	0 -030	——	——	——	4	8	1 -018	1 -018	0 -002	0 -002
5	6	1 -015+	0 -015+	0 -002	0 -002		7	0 -010+	0 -010+	——	
	5	0 -013	0 -013	——	——		6	0 -030	——	——	
	4	0 -045+	——	——	——	3	8	0 -006	0 -006	0 -006	
4	6	1 -033	0 -005-	0 -005-	0 -005-		7	0 -024	0 -024	——	
	5	0 -024	0 -024	——	——	2	8	0 -022	0 -022	——	
3	6	0 -012	0 -012	——	——	A=9	9	5 -041	4 -015-	3 -005-	3 -005-
	5	0 -048	——	——	——		8	3 -025-	3 -025-	2 -008	1 -002
2	6	0 -036	——	——	——		7	2 -028	1 -008	1 -008	0 -001
							6	1 -025-	1 -025-	0 -005-	0 -005-
A=7 B=7	7	3 -035-	2 -010+	1 -002	1 -002		5	0 -015-	0 -015-	——	
	6	1 -015-	1 -015-	0 -002	0 -002		4	0 -041	——	——	
	5	1 -010+	0 -010+	——	——	8	9	4 -029	3 -009	3 -009	2 -002
	4	0 -035-	——	——	——		8	3 -043	2 -013	1 -003	1 -003
6	7	2 -021	2 -021	1 -005-	1 -005-		7	2 -044	1 -012	0 -002	0 -002
	6	1 -025+	0 -004	0 -004	0 -004		6	1 -036	0 -007	0 -007	
	5	0 -016	0 -016	——	——		5	0 -020	0 -020	——	
	4	0 -049	——	——	——	7	9	3 -019	3 -019	2 -005	2 -005-
5	7	2 -045+	1 -010+	0 -001	0 -001		8	2 -024	2 -024	1 -006	0 -001
	6	1 -045+	0 -008	0 -008	——		7	1 -020	1 -020	0 -003	0 -003
	5	0 -027	——	——	——		6	0 -010+	0 -010+	——	
	7	1 -024	1 -024	——	0 -003		5	0 -029	——	——	
	6	0 -015+	0 -015+	0 -003	——	6	9	3 -044	2 -011	1 -002	1 -002
	5	0 -045+	——	——	——		8	2 -047	1 -011	0 -001	0 -001
	7	0 -008	0 -008	0 -008	——		7	1 -035-	0 -006	0 -006	
	6	0 -033	——	——	——		6	0 -017	0 -017	——	
	7	0 -028	——	——	——		5	0 -042	——	——	

[1] The table shows:(1) In bold type, for given a, A and B, the value of b ([a) which is just significant at the probability level quoted (one-tailed test); and (2) In small type, for given A, B and r = a + b, the exact probability (if there is independence) that b is equal to or less than the integer shown in bold type. From Pearson and Hartley (1962).

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=9 B=5	9	2 -027	1 -005-	1 -005-	1 -005-	A=10 B=4	10	1 -011	1 -011	0 -001	0 -001
	8	1 -023	1 -023	0 -003	0 -003		9	1 -041	0 -005-	0 -005-	0 -005-
	7	0 -	0 -010+	———	———		8	0 -	0 -015-	———	———
	6	0 -028	———	———	———		7	0 -	———	———	———
4	9	1 -014	1 -014	0 -001	0 -001	3	10	1 -038	0 -003	0 -003	0 -003
	8	0 -007	0 -007	0 -007	———		9	0 -014	0 -014	———	———
	7	0 -021	0 -021	———	———		8	0 -	———	———	———
	6	0 -049	———	0 -005	———	2	10	0 -	0 -015+	———	———
3	9	1 -	0 -005-	0 -005-	0 -005-		9	0 -	———	———	———
	8	0 -018	0 -018	———	———						
	7	0 -	———	———	———						
2	9	0 -018	0 -018	———	———	A=11 B=11	11	7 -	6 -018	5 -006	4 -002
							10	5 -032	4 -012	3 -004	3 -004
							9	4 -040	3 -015-	2 -004	2 -004
A=10 B=10	10	6 -043	5 -016	4 -005+	3 -002		8	3 -043	2 -015-	1 -004	1 -004
	9	4 -029	3 -010-	3 -010	2 -003		7	2 -040	1 -012	0 -002	0 -002
	8	3 -	2 -012	1 -003	1 -003		6	1 -032	0 -006	0 -006	———
	7	2 -	1 -010-	1 -010-	0 -002		5	0 -018	0 -018	———	———
	6	1 -029	0 -005+	0 -005+	———		4	0 -	———	———	———
	5	0 -016	0 -016	———	———	10	11	6 -	5 -012	4 -004	4 -004
	4	0 -043	———	———	———		10	4 -021	4 -021	3 -007	2 -002
9	10	5 -033	4 -011	3 -003	3 -003		9	3 -024	3 -024	2 -007	1 -002
	9	4 -	3 -017	2 -005-	2 -005-		8	2 -023	2 -023	1 -006	0 -001
	8	2 -019	2 -019	1 -004	1 -004		7	1 -017	1 -017	0 -003	0 -003
	7	1 -	1 -015-	0 -002	0 -002		6	1 -043	0 -009	0 -009	———
	6	1 -040	0 -008	0 -008	———		5	0 -023	0 -023	———	———
	5	0 -022	0 -022	———	———	9	11	5 -026	4 -008	4 -008	3 -002
8	10	4 -023	4 -023	3 -007	2 -002		10	4 -038	3 -012	2 -003	2 -003
	9	3 -032	2 -009	2 -009	1 -002		9	3 -040	2 -012	1 -003	1 -003
	8	2 -031	1 -008	1 -008	0 -001		8	2 -	1 -009	1 -009	0 -001
	7	1 -023	1 -023	0 -004	0 -004		7	1 -	1 -025-	0 -004	0 -004
	6	0 -011	0 -011	———	———		6	0 -012	0 -012	———	———
	5	0 -029	———	———	———		5	0 -030	———	———	———
7	10	3 -	3 -015-	2 -003	2 -003	8	11	4 -018	4 -018	3 -005-	3 -005-
	9	2 -018	2 -018	1 -004	1 -004		10	3 -024	3 -024	2 -006	1 -001
	8	1 -013	1 -013	0 -002	0 -002		9	2 -022	2 -022	1 -005-	1 -005-
	7	1 -036	0 -006	0 -006	———		8	1 -	1 -015-	0 -002	0 -002
	6	0 -017	0 -017	———	———		7	1 -037	0 -007	0 -007	———
	5	0 -041	———	———	———		6	0 -017	0 -017	———	———
6	10	3 -036	2 -008	2 -008	1 -001		5	0 -040	———	———	———
	9	2 -036	1 -008	1 -008	0 -001	7	11	4 -043	3 -011	2 -002	2 -002
	8	1 -024	1 -024	0 -003	0 -003		10	3 -047	2 -013	1 002	1 -002
	7	0 -	0 -010+	———	———		9	2 -039	1 -009	1 -009	0 -001
	6	0 -026	———	———	———		8	1 -	1 -025-	0 -004	0 -004
5	10	2 -022	2 -022	1 -004	1 -004		7	0 -	0 -010+	———	———
	9	1 -017	1 -017	0 -002	0 -002		6	0 -	0 -025-	———	———
	8	1 -	0 -007	0 -007	———	6	11	3 -	2 006	2 -006	1 -001
	7	0 -	0 -019	———	———		10	2 -	1 -005+	1 -005+	0 -001
	6	0 -	———	———	———		9	1 -	1 -018	0 -002	0 -002

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=11 B=6	8	1 -043	0 -007	0 -007	——	A=12 B=9	7	1 -037	0 -007	0 -007	——
	7	0 -017	0 -017	——	——		6	0 -017	0 -017	——	——
	6	0 -037	——	——	——		5	0 -039	——	——	——
5	11	2 -018	2 -018	1 -003	1 -003	8	12	5 -049	4 -014	3 -004	3 -004
	10	1 -013	1 -013	0 -001	0 -001		11	3 -018	3 -018	2 -004	2 -004
	9	1 -036	0 -005⁻	0 -005⁻	0 -005⁻		10	2 -015⁺	2 -015⁺	1 -003	1 -003
	8	0 -013	0 -013	——	——		9	2 -040	1 -010⁻	1 -010⁻	0 -001
	7	0 -029	——	——	——		8	1 -025⁻	1 -025⁻	0 -004	0 -004
4	11	1 -009	1 -009	1 -009	0 -001		7	0 -010⁺	0 -010⁺	——	——
	10	1 -033	0 -004	0 -004	0 -004		6	0 -024	0 -024	——	——
	9	0 -011	0 -011	——	——	7	12	4 -036	3 -009	3 -009	2 -002
	8	0 -026	——	——	——		11	3 -038	2 -010⁻	2 -010⁻	1 -002
3	11	1 -033	0 -003	0 -003	0 -003		10	2 -029	1 -006	1 -006	0 -001
	10	0 -011	0 -011	——	——		9	1 -017	1 -017	0 -002	0 -002
	9	0 -027	——	——	——		8	1 -040	0 -007	0 -007	——
2	11	0 -013	0 -013	——	——		7	0 -016	0 -016	——	——
	10	0 -038	——	——	——		6	0 -034	——	——	——
						6	12	3 -025⁻	3 -025⁻	2 -005⁻	2 -005⁻
							11	2 -022	2 -022	1 -004	1 -004
A=12 B=12	12	8 -047	7 -019	6 -007	5 -002		10	1 -013	1 -013	0 -002	0 -002
	11	6 -034	5 -014	4 -005⁻	4 -005⁻		9	1 -032	0 -005⁻	0 -005⁻	0 -005⁻
	10	5 -045⁻	4 -018	3 -006	2 -002		8	0 -011	0 -011	——	——
	9	4 -050⁻	3 -020	2 -006	1 -001		7	0 -025⁻	0 -025⁻	——	——
	8	3 -050⁻	2 -018	1 -005⁻	1 -005⁻		6	0 -050⁻	——	——	——
	7	2 -045⁻	1 -014	0 -002	0 -002	5	12	2 -015⁻	2 -015	1 -002	1 -002
	6	1 -034	0 -007	0 -007	——		11	1 -010⁻	1 -010⁻	1 -010⁻	0 -001
	5	0 -019	0 -019	——	——		10	1 -028	0 -003	0 -003	0 -003
	4	0 -047	——	——	——		9	0 -009	0 -009	0 -009	——
11	12	7 -037	6 -014	5 -005⁻	5 -005⁻		8	0 -020	0 -020	——	——
	11	5 -024	5 -024	4 -008	3 -002		7	0 -041	——	——	——
	10	4 -029	3 -010⁺	2 -003	2 -003	4	12	2 -050	1 -007	1 -007	0 -001
	9	3 -030	2 -009	2 -009	1 -002		11	1 -027	0 -003	0 -003	0 -003
	8	2 -026	1 -007	1 -007	0 -001		10	0 -008	0 -008	0 -008	——
	7	1 -019	1 -019	0 -003	0 -003		9	0 -019	0 -019	——	——
	6	1 -045⁻	0 -009	0 -009	——		8	0 -038	——	——	——
	5	0 -024	0 -024	——	——	3	12	1 -029	0 -002	0 -002	0 -002
10	12	6 -029	5 -010	5 -010⁻	4 -003		11	0 -009	0 -009	0 -009	——
	11	5 -043	4 -015⁺	3 -005⁻	3 -005⁻		10	0 -022	0 -022	——	——
	10	4 -048	3 -017	2 -005⁻	2 -005⁻		9	0 -044	——	——	——
	9	3 -046	2 -015⁻	1 -004	1 -004	2	12	0 -011	0 -011	——	——
	8	2 -038	1 -010⁺	0 -002	0 -002		11	0 -033	——	——	——
	7	1 -026	0 -005⁻	0 -005⁻	0 -005-						
	6	0 -012	0 -012	——	——						
	5	0 -030	——	——	——	A=13 B=13	13	9 -048	8 -020	7 -007	6 -003
9	12	5 -021	5 -021	4 -006	3 -002		12	7 -037	6 -015⁺	5 -006	4 -002
	11	4 -029	3 -009	3 -009	2 -002		11	6 -048	5 -021	4 -008	3 -002
	10	3 -029	2 -008	2 -008	1 -002		10	4 -024	4 -024	3 -008	2 -002
	9	2 -024	2 -024	1 -006	0 -001		9	3 -024	3 -024	2 -008	1 -002
	8	1 -016	1 -016	0 -002	0 -002		8	2 -021	2 -021	1 -006	0 -001

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=13 B=13	7	2 -048	1 -015+	0 -003	0 -003	A=13 B=7	11	2 -022	2 -022	1 -004	1 -004
	6	1 -037	0 -007	0 -007	———		10	1 -012	1 -012	0 -002	0 -002
	5	0 -020	0 -020	———	———		9	1 -029	0 -004	0 -004	0 -004
	4	0 -048		———	———		8	0 -010+	0 -010+	———	———
12	1	8 -039	7 -015-	6 -005+	5 -002		7	0 -022	0 -022	———	———
	1	6 -027	5 -010-	5 -010-	4 -003		6	0 -044	———	———	———
	1	5 -033	4 -013	3 -004	3 -004	6	13	3 -021	3 -021	2 -004	2 -004
	1	4 -036	3 -013	2 -004	2 -004		12	2 -017	2 -017	1 -003	1 -003
	9	3 -034	2 -011	1 -003	1 -003		11	2 -046	1 -010-	1 -010-	0 -001
	8	2 -029	1 -008	1 -008	0 -001		10	1 -024	1 -024	0 -003	0 -003
	7	1 -020	1 -020	0 -004	0 -004		9	1 -050-	0 -008	0 -008	———
	6	1 -046	0 -010-	0 -010-	———		8	0 -017	0 -017	———	———
	5	0 -024	0 -024	———	———		7	0 -034	———	———	———
11	1	7 -031	6 -011	5 -003	5 -003	5	13	2 -012	2 -012	1 -002	1 -002
	1	6 -048	5 -018	4 -006	3 -002		12	2 -044	1 -008	1 -008	0 -001
	1	4 -021	4 -021	3 -007	2 -002		11	1 -022	1 -022	0 -002	0 -002
	1	3 -021	3 -021	2 -006	1 -001		10	1 -047	0 -007	0 -007	———
	9	3 -050-	2 -017	1 -004	1 -004		9	0 -015-	0 -015-	———	———
	8	2 -040	1 -011	0 -002	0 -002		8	0 -029	———	———	———
	7	1 -027	0 -005-	0 -005-	0 -005-	4	13	2 -044	1 -006	1 -006	0 -000
	6	0 -013	0 -013	———	———		12	1 -022	1 -022	0 -002	0 -002
		0 -030		———	———		11	0 -006	0 -006	0 -006	———
10	1	6 -024	6 -024	5 -007	4 -002		10	0 -015-	0 -015-	———	———
	1	5 -035-	4 -012	3 -003	3 -003		9	0 -029	———	———	———
	1	4 -037	3 -012	2 -003	2 -003	3	13	1 -025	1 -025	0 -002	0 -002
	1	3 -033	2 -010+	1 -002	1 -002		12	0 -007	0 -007	0 -007	———
	9	2 -026	1 -006	1 -006	0 -001		11	0 -018	0 -018	———	———
	8	1 -017	1 -017	0 -003	0 -003		10	0 -036	———	———	———
	7	1 -038	0 -007	0 -007	———	2	13	0 -010-	0 -010-	0 -010-	———
	6	0 -017	0 -017	———	———		12	0 -029	———	———	———
	5	0 -038		———	———						
9	1	5 -017	5 -017	4 -005-	4 -005-						
	1	4 -023	4 -023	3 -007	2 -001	A=14	14	10 -049	9 -020	8 -008	7 -003
	1	3 -022	3 -022	2 -006	1 -001		13	8 -038	7 -016	6 -006	5 -002
	1	2 -017	2 -017	1 -004	1 -004		12	6 -023	6 -023	5 -009	4 -003
	9	2 -040	1 -010+	0 -001	0 -001		11	5 -027	4 -011	3 -004	3 -004
	8	1 -025-	1 -025-	0 -004	0 -004		10	4 -028	3 -011	2 -003	2 -003
	7	0'-010+	0 -010+	———	———		9	3 -027	2 -009	2 -009	1 -002
	6	0 -023	0 -023	———	———		8	2 -023	2 -023	1 -006	0 -001
	5	0 -049		———	———		7	1 -016	1 -016	0 -003	0 -003
8	1	5 -042	4 -012	3 -003	3 -003		6	1 -038	0 -008	0 -008	———
	1	4 -047	3 -014	2 -003	2 -003		5	0 -020	0 -020	———	———
	1	3 -041	2 -011	1 -002	1 -002		4	0 -049	———	———	———
	1	2 -029	1 -007	1 -007	0 -001	13	14	9 -041	8 -016	7 -006	6 -002
	9	1 -017	1 -017	0 -002	0 -002		13	7 -029	6 -011	5 -004	5 -004
	8	1 -037	0 -006	0 -006	———		12	6 -037	5 -015+	4 -005+	3 -002
	7	0 -015-	0 -015-	———	———		11	5 -041	4 -017	3 -006	2 -001
	6	0 -032		———	———		10	4 -041	3 -016	2 -005-	2 -005-
7	1	4 -031	3 -007	3 -007	2 -001		9	3 -038	2 -013	1 -003	1 -003
	1	3 -031	2 -007	2 -007	1 -001		8	2 -031	1 -009	1 -009	0 -001

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=14	7	1 -021	1 -021	0 -004	0 -004	A=14 B=7	14	4 -026	3 -006	3 -006	2 -001
	6	1 -048	0 -010+	———	———		13	3 -025	2 -006	2 -006	1 -001
	5	0 -025-	0 -025-	———	———		12	2 -017	2 -017	1 -003	1 -003
12	1	8 -033	7 -012	6 -004	6 -004		11	2 -041	1 -009	1 -009	0 -001
	1	6 -021	6 -021	5 -007	4 -002		10	1 -021	1 -021	0 -003	0 -003
	1	5 -025+	4 -009	4 -009	3 -003		9	1 -043	0 -007	0 -007	———
	1	4 -026	3 -009	3 -009	2 -002		8	0 -015-	0 -015-	———	———
	1	3 -024	3 -024	2 -007	1 -002		7	0 -030	———	———	———
	9	2 -019	2 -019	1 -005-	1 -005-	6	14	3 -018	3 -018	2 -003	2 -003
	8	2 -042	1 -012	0 -002	0 -002		13	2 -014	2 -014	1 -002	1 -002
	7	1 -028	0 -005+	0 -005+	———		12	2 -037	1 -007	1 -007	0 -001
	6	0 -013	0 -013	———	———		11	1 -018	1 -018	0 -002	0 -002
	5	0 -030	———	———	———		10	1 -038	0 -005+	0 -005+	———
11	1	7 -026	6 -009	6 -009	5 -003		9	0 -012	0 -012	———	———
	1	6 -039	5 -014	4 -004	4 -004		8	0 -024	0 -024	———	———
	1	5 -043	4 -016	3 -005-	3 -005-		7	0 -044	———	———	———
	1	4 -042	3 -015-	2 -004	2 -004	5	14	2 -010+	2 -010+	1 -001	1 -001
	1	3 -036	2 -011	1 -003	1 -003		13	2 -037	1 -006	1 -006	0 -001
	9	2 -027	1 -007	1 -007	0 -001		12	1 -017	1 -017	0 -002	0 -002
	8	1 -017	1 -017	0 -003	0 -003		11	1 -038	0 -005-	0 -005-	0 -005-
	7	1 -038	0 -007	0 -007	———		10	0 -011	0 -011	———	———
	6	0 -017	0 -017	———	———		9	0 -022	0 -022	———	———
	5	0 -038	———	———	———		8	0 -040	———	———	———
10	1	6 -020	6 -020	5 -006	4 -002	4	14	2 -039	1 -005-	1 -005-	1 -005-
	1	5 -028	4 -009	4 -009	3 -002		13	1 -019	1 -019	0 -002	0 -002
	1	4 -028	3 -009	3 -009	2 -002		12	1 -044	0 -005-	0 -005-	0 -005-
	1	3 -024	3 -024	2 -007	1 -001		11	0 -011	0 -011	———	———
	1	2 -018	2 -018	1 -004	1 -004		10	0 -023	0 -023	———	———
	9	2 -040	1 -011	0 -002	0 -002		9	0 -041	———	———	———
	8	1 -024	1 -024	0 -004	0 -004	3	14	1 -022	1 -022	0 -001	0 -001
	7	0 -010-	0 -010-	0 -010-	———		13	0 -006	0 -006	0 -006	———
	6	0 -022	0 -022	———	———		12	0 -015-	0 -015-	———	———
	5	0 -047	———	———	———		11	0 -029	———	———	———
9	1	6 -047	5 -014	4 -004	4 -004	2	14	0 -008	0 -008	0 -008	———
	1	4 -018	4 -018	3 -005-	3 -005-		13	0 -025	0 -025	———	———
	1	3 -017	3 -017	2 -004	2 -004		12	0 -050	———	———	———
	1	3 -042	2 -012	1 -002	1 -002						
	1	2 -029	1 -007	1 -007	0 -001						
	9	1 -017	1 -017	0 -002	0 -002						
	8	1 -036	0 -006	0 -006	———	A=15 B=15	15	11 -050-	10 -021	9 -008	8 -003
	7	0 -014	0 -014	———	———		14	9 -040	8 -018	7 -007	6 -003
	6	0 -030	———	———	———		13	7 -025+	6 -010+	5 -004	5 -004
8	1	5 -036	4 -010-	4 -010-	3 -002		12	6 -030	5 -013	4 -005-	4 -005-
	1	4 -039	3 -011	2 -002	2 -002		11	5 -033	4 -013	3 -005-	3 -005-
	1	3 -032	2 -008	2 -008	1 -001		10	4 -033	3 -013	2 -004	2 -004
	1	2 -022	2 -022	1 -005-	1 -005-		9	3 -030	2 -010+	1 -003	1 -003
	1	2 -048	1 -012	0 -002	0 -002		8	2 -025+	1 -007	1 -007	0 -001
	9	1 -026	0 -004	0 -004	0 -004		7	1 -018	1 -018	0 -003	0 -003
	8	0 -009	0 -009	0 -009	———		6	1 -040	0 -008	0 -008	———
	7	0 -020	0 -020	———	———		5	0 -021	0 -012	———	———
	6	0 -040	———	———	———		4	0 -050-	———	———	———

TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=15 B=14	15	10 -042	9 -017	8 -006	7 -002	A=15 B=9	13	4 -042	3 -013	2 -003	2 -003
	14	8 -031	7 -013	6 -005-	6 -005-		12	3 -032	2 -009	2 -009	1 -002
	13	7 -041	6 -017	5 -007	4 -002		11	2 -021	2 -021	1 -005-	1 -005-
	12	6 -046	5 -020	4 -007	3 -002		10	2 -045-	1 -011	0 -002	0 -002
	11	5 -048	4 -020	3 -007	2 -002		9	1 -024	1 -024	0 -004	0 -004
	10	4 -046	3 -018	2 -006	1 -001		8	1 -048	0 -009	0 -009	——
	9	3 -041	2 -014	1 -004	1 -004		7	0 -019	0 -019	——	——
	8	2 -033	1 -009	1 -009	0 -001		6	0 -037	——	——	——
	7	1 -022	1 -022	0 -004	0 -004	8	15	5 -032	4 -008	4 -008	3 -002
	6	1 -049	0 -011	——	——		14	4 -033	3 -009	3 -009	2 -002
	5	0 -025+	——	——	——		13	3 -026	2 -006	2 -006	1 -001
13	15	9 -035-	8 -013	7 -005-	7 -005-		12	2 -017	2 -017	1 -003	1 -003
	14	7 -023	7 -023	6 -009	5 -003		11	2 -037	1 -008	1 -008	0 -001
	13	6 -029	5 -011	4 -004	4 -004		10	1 -019	1 -019	0 -003	0 -003
	12	5 -031	4 -012	3 -004	3 -004		9	1 -038	0 -006	0 -006	——
	11	4 -030	3 -011	2 -003	2 -003		8	0 -013	0 -013	——	——
	10	3 -026	2 -008	2 -008	1 -002		7	0 -026	——	——	——
	9	2 -020	2 -020	1 -005+	0 -001		6	0 -050-	——	——	——
	8	2 -043	1 -013	0 -002	0 -002	7	15	4 -023	4 -023	3 -005-	3 -005-
	7	1 -029	0 -005+	0 -005+	——		14	3 -021	3 -021	2 -004	2 -004
	6	0 -013	0 -013	——	——		13	2 -014	2 -014	1 -002	1 -002
	5	0 -031	——	——	——		12	2 -032	1 -007	1 -007	0 -001
12	15	8 -028	7 -010-	7 -010-	6 -003		11	1 -015+	1 -015+	0 -002	0 -002
	14	7 -043	6 -016	5 -006	4 -002		10	1 -032	0 -005-	0 -005-	0 -005-
	13	6 -049	5 -019	4 -007	3 -002		9	0 -010+	0 -010+	——	——
	12	5 -049	4 -019	3 -006	2 -002		8	0 -020	0 -020	——	——
	11	4 -045+	3 -017	2 -005-	2 -005-		7	0 -038	——	——	——
	10	3 -038	2 -012	1 -003	1 -003	6	15	3 -015+	3 -015+	2 -003	2 -003
	9	2 -028	1 -007	1 -007	0 -001		14	2 -011	2 -011	1 -002	1 -002
	8	1 -018	1 -018	0 -003	0 -003		13	2 -031	1 -006	1 -006	0 -001
	7	1 -038	0 -007	0 -007	——		12	1 -014	1 -014	0 -002	0 -002
	6	0 -017	0 -017	——	——		11	1 -029	0 -004	0 -004	0 -004
	5	0 -037	——	——	——		10	0 -009	0 -009	0 -009	——
11	15	7 -022	7 -022	6 -007	5 -002		9	0 -017	0 -017	——	——
	14	6 -032	5 -011	4 -003	4 -003		8	0 -032	——	——	——
	13	5 -034	4 -012	3 -003	3 -003	5	15	2 -009	2 -009	2 -009	1 -001
	12	4 -032	3 -010+	2 -003	2 -003		14	2 -032	1 -005-	1 -005-	1 -005-
	11	3 -026	2 -008	2 -008	1 002		13	1 -014	1 -014	0 -001	0 -001
	10	2 -019	2 -019	1 -004	1 -004		12	1 -031	0 -004	0 -004	0 -004
	9	2 -040	1 -011	0 -002	0 -002		11	0 -008	0 -008	0 -008	——
	8	1 -024	1 -024	0 -004	0 -004		10	0 -016	0 -016	——	——
	7	1 -049	0 -010-	0 -010-	——		9	0 -030	——	——	——
	6	0 -022	0 -022	——	——	4	15	2 -035+	1 -004	1 -004	1 -004
	5	0 -046	——	——	——		14	1 -016	1 -016	0 -001	0 -001
10	15	6 -017	6 -017	5 -005-	5 -005-		13	1 -037	0 -004	0 -004	0 -004
	14	5 -023	5 -023	4 -007	3 -002		12	0 -009	0 -009	0 -009	——
	13	4 -022	4 -022	3 -007	2 -001		11	0 -018	0 -018	——	——
	12	3 -018	3 -018	2 -005-	2 -005-		10	0 -033	——	——	——
	11	3 -042	2 -013	1 -003	1 -003	3	15	1 -020	1 -020	0 -001	0 -001
	10	2 -029	1 -007	1 -007	0 -001		14	0 -005-	0 -005-	0 -005-	0 -005-
	9	1 -016	0 -016	0 -002	0 -002		13	0 -012	0 -012	——	——
	8	1 -034	0 -006	0 -006	——		12	0 -025-	0 -025-	——	——
	7	0 -013	1 -013	——	——		11	0 -043	——	——	——
	6	0 -028	——	——	——	2	15	0 -007	0 -007	0 -007	——
9	15	6 -042	5 -012	4 -003	4 -003		14	0 -022	0 -022	——	——
	14	5 -047	4 -015-	3 -004	3 -004		13	0 -044	——	——	——

APPENDIX H

SINGLE CONCENTRATION TOXICITY TEST - COMPARISON OF CONTROL WITH 100% EFFLUENT OR RECEIVING WATER

1. To statistically compare a control with one concentration, such as 100% effluent or the instream waste concentration, a t-test is the recommended analysis. The t-test is based on the assumptions that the observations are independent and normally distributed and that the variances of the observations are equal between the two groups.

2. Shapiro Wilk's test may be used to test the normality assumption (see Appendix B for details). If the data do not meet the normality assumption, the nonparametric test, Wilcoxon's Rank Sum Test, may be used to analyze the data. An example of this test is given in Appendix F. Since a control and one concentration are being compared, the K = 1 section of Table F.5 contains the needed critical values.

3. The F test for equality of variances is used to test the homogeneity of variance assumption. When conducting the F test, the alternative hypothesis of interest is that the variances are not equal.

4. To make the two-tailed F test at the 0.01 level of significance, put the larger of the two variances in the numerator of F.

$$F = \frac{S_1^2}{S_2^2} \quad where\ S_1^2 > S_2^2$$

5. Compare F with the 0.005 level of a tabled F value with n_1 - 1 and n_2 - 1 degrees of freedom, where n_1 and n_2 are the number of replicates for each of the two groups.

6. A set of *Ceriodaphnia dubia* reproduction data from an effluent screening test will be used to illustrate the F test. The raw data, mean and variance for the control and 100% effluent are given in Table H.1.

TABLE H.1. *CERIODAPHNIA DUBIA* REPRODUCTION DATA FROM AN EFFLUENT SCREENING TEST

	Replicate											
	1	2	3	4	5	6	7	8	9	10	$\overline{X}$	S^2
Control	36	38	35	35	28	41	37	33	.	.	35.4	14.5
100% Effluent	23	14	21	7	12	17	23	8	18	.	15.9	36.6

7. Since the variability of the 100% effluent is greater than the variability of the control, S^2 for the 100% effluent concentration is placed in the numerator of the F statistic and S^2 for the control is placed in the denominator.

$$F = \frac{36.61}{14.55}$$

8. There are 9 replicates for the effluent concentration and 8 replicates for the control. Thus, the numerator degrees of freedom is 8 and the denominator degrees of freedom is 7. For a two-tailed test at the 0.01 level of

significance, the critical F value is obtained from a table of the F distribution (Snedecor and Cochran, 1980). The critical F value for this test is 8.68. Since 2.52 is not greater than 8.68, the conclusion is that the variances of the control and 100% effluent are homogeneous.

9. **EQUAL VARIANCE T-TEST**

9.1 To perform the t-test, calculate the following test statistic:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{S_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where: $\bar{Y}_1$ = Mean for the control

$\bar{Y}_2$ = Mean for the effluent concentration

$$S_p = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}$$

S_1^2 = Estimate of the variance for the control

S_2^2 = Estimate of the variance for the effluent concentration

n_1 = Number of replicates for the control

n_2 = Number of replicates for the effluent concentration

9.2 Since we are usually concerned with a decreased response from the control, such as a decrease in survival or a decrease in reproduction, a one-tailed test is appropriate. Thus, compare the calculated t with a critical t, where the critical t is at the 5% level of significance with $n_1 + n_2 - 2$ degrees of freedom. If the calculated t exceeds the critical t, the mean responses are declared different.

9.3 Using the data from Table H.1 to illustrate the t-test, the calculation of t is as follows:

$$t = \frac{35.4 - 15.9}{5.13\sqrt{\frac{1}{8} + \frac{1}{9}}} = 7.82$$

Where:

$$S_p = \sqrt{\frac{(8-1)14.5 + (9-1)36.6}{(8+9-2)}} = 5.13$$

9.4 For an 0.05 level of significance test with 15 degrees of freedom the critical t is 1.754 (Note: Table D.5 for K = 1 includes the critical t values for comparing two groups). Since 7.82 is greater than 1.754, the conclusion is that the reproduction in the 100% effluent concentration is significantly lower than the control reproduction.

10. UNEQUAL VARIANCE T-TEST

10.1 If the F test for equality of variance fails, the t-test is still a valid test. However, the denominator of the t statistic is adjusted as follows:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where: $\bar{Y}_1$ = Mean for the control

$\bar{Y}_2$ = Mean for the effluent concentration

S_1^2 = Estimate of the variance for the control

S_2^2 = Estimate of the variance for the effluent concentration

n_1 = Number of replicates for the control

n_2 = Number of replicates for the effluent concentration

10.2 Additionally, the degrees of freedom for the test are adjusted using the following formula:

$$df' = \frac{(n_1 - 1)(n_2 - 1)}{(n_2 - 1)C^2 + (1 - C)^2(n_1 - 1)}$$

Where:

$$C = \frac{\frac{S_1^2}{n_1}}{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

10.3 The modified degrees of freedom is usually not an integer. Common practice is to round down to the nearest integer.

10.4 The t-test is then conducted as the equal variance t-test. The calculated t is compared to the critical t at the 0.05 significance level with the modified degrees of freedom. If the calculated t exceeds the critical t, the mean responses are found to be statistically different.

APPENDIX I

PROBIT ANALYSIS

1. This program calculates the EC1 and EC50 (or LC1 and LC50), and the associated 95% confidence intervals.

2. The program is written in IBM PC Basic for the IBM compatible PC by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled, executable version of the program can be obtained from EMSL-Cincinnati by sending a written request to EMSL at 3411 Church Street, Cincinnati, OH 45244.

2.1 Data input is illustrated by a set of total mortality data (Figure I.1) from a fathead minnow embryo-larval survival and teratogenicity test. The program requests the following input:

1. Desired output of abbreviated (A) or full (F) output? (Note: only abbreviated output is shown below.)
2. Output designation (P = printer, D = disk file).
3. Title for the output.
4. The number of exposure concentrations.
5. Toxicant concentration data.

2.2 The program output for the abbreviated output includes the following:

1. A table of the observed proportion responding and the proportion responding adjusted for the controls (see Figure I.2).
2. The calculated chi-square statistic for heterogeneity and the tabular value. This test is one indicator of how well the data fit the model. The program will issue a warning when the test indicates that the data do not fit the model.
3. Estimated LC1 and LC50 values and associated 95% confidence intervals (see Figure I.2).

USEPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

Do you wish abbreviated (A) or full (F) input/output? A
Output to printer (P) or disk file (D)? P
Title ? Example of Probit Analysis

Number responding in the control group = ? 2
Number of animals exposed in the concurrent control group = ? 20
Number of exposure concentrations, exclusive of controls ? 5

Input data starting with the lowest exposure concentration

Concentration = ? 0.5
Number responding = ? 2
Number exposed = ? 20

Concentration = ? 1.0
Number responding = ? 1
Number exposed = ? 20

Concentration = ? 2.0
Number responding = ? 4
Number exposed = ? 20

Concentration = ? 4.0
Number responding = ? 16
Number exposed = ? 20

Concentration = ? 8.0
Number responding = ? 20
Number exposed = ? 20

Number	Number Conc.	Number Resp.	Exposed
1	0.5000	2	20
2	1.0000	1	20
3	2.0000	4	20
4	4.0000	16	20
5	8.0000	20	20

Do you wish to modify your data ? N

The number of control animals which responded = 2
The number of control animals exposed = 20
Do you wish to modify these values ? N

Figure I.1. Sample Data Input for USEPA Probit Analysis program, Version 1.5.

Example of Probit Analysis

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	20	2	0.1000	0.0000
0.5000	20	2	0.1000	0.0174
1.0000	20	1	0.0500	-.0372
2.0000	20	4	0.2000	0.1265
4.0000	20	16	0.8000	0.7816
8.0000	20	20	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 0.441
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 7.815

Example of Probit Analysis

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	Lower 95% Confidence	Upper Limits
LC/EC 1.00	1.346	0.453	1.922
LC/EC 50.00	3.018	2.268	3.672

Figure I.2. USEPA Probit Analysis Program Used for Calculating LC/EC Values, Version 1.5.

APPENDIX J

SPEARMAN-KARBER METHOD

1. The Spearman-Karber Method is a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978). The Spearman-Karber Method estimates the mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Spearman-Karber Method is recommended when partial mortalities occur in the test solutions, but the data do not fit the Probit model.

4. To calculate the LC50 using the Spearman-Karber Method, the following must be true: 1) the smoothed adjusted proportion mortality for the lowest effluent concentration (not including the control) must be zero, and 2) the smoothed adjusted proportion mortality for the highest effluent concentration must be one.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed adjusted proportion mortalities must be between zero and one.

6. The Spearman-Karber Method is illustrated below using a set of mortality data from a Fathead Minnow Larval Survival and Growth test. These data are listed in Table J.1.

TABLE J.1. EXAMPLE OF SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25%	2	0.05
12.5%	0	0.00
25.0%	0	0.00
50.0%	26	0.65
100.0%	40	1.00

7. Let $p_0, p_1, ..., p_k$ denote the observed response proportion mortalities for the control and k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \le p_1 \le ... \le p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \le p_1 \le ... \le p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i-1}^{s} = p_i^{s} = \frac{(p_i + p_{i-1})}{2}$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

7.1 For the data in this example, because the observed mortality proportions for the control and the 6.25% effluent concentration are greater than the observed response proportions for the 12.5% and 25.0% effluent concentrations, the responses for these four groups must be averaged:

$$p_0^{s} = p_1^{s} = p_2^{s} = p_3^{s} = \frac{0.05+0.05+0.00+0.00}{4} = \frac{0.10}{4} = 0.025$$

7.2 Since $p_4 = 0.65$ is larger than p_3^s, set $p_4^s = 0.65$. Similarly, $p_5 = 1.00$ is larger than p_4^s, so set $p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table J.2.

8. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

Where: $p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$

p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

8.1 For the data in this example, the data for each effluent concentration must be adjusted for control mortality using Abbott's formula, as follows:

$$p_0^{a} = p_1^{a} = p_2^{a} = p_3^{a} = \frac{p_1^{s} - p_0^{s}}{1 - p_0^{s}} = \frac{0.025 - 0.025}{1 - 0.025} = \frac{0.0}{0.975} = 0.0$$

$$p_4^{a} = \frac{p_4^{s} - p_0^{s}}{1 - p_0^{s}} = \frac{0.650 - 0.025}{1 - 0.025} = \frac{0.0625}{0.975} = 0.641$$

$$p_5^{a} = \frac{p_5^{s} - p_0^{s}}{1 - p_0^{s}} = \frac{1.000 - 0.025}{1 - 0.025} = \frac{0.975}{0.975} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table J.2. A plot of the smoothed, adjusted data is shown in Figure J.1.

9. Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k-1} \frac{(p_{i+1}^{a})(x_i + x_{i+1})}{2}$$

Where: p_i^a = the smoothed adjusted proportion mortality at concentration i

X_i = the $\log_{10}$ of concentration i

k = the number of effluent concentrations tested, not including the control.

9.1 For this example, the $\log_{10}$ of the estimated LC50, m, is calculated as follows:

m = [(0.000 - 0.000) (0.7959 + 1.0969)]/2 +
[(0.000 - 0.000) (1.0969 + 1.3979)]/2 +
[(0.641 - 0.000) (1.3979 + 1.6990)]/2 +
[(1.000 - 0.641) (1.6990 + 2.0000)]/2

= 1.656527

TABLE J.2. EXAMPLE OF SPEARMAN-KARBER METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.05	0.025	0.000
6.25%	0.05	0.025	0.000
12.5%	0.00	0.025	0.000
25.0%	0.00	0.025	0.000
50.0%	0.65	0.650	0.641
100.0%	1.00	1.000	1.000

10. Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k-1} \frac{p_i^a(1-p_i^a)(X_{i+1}+X_{i-1})^2}{4(n_i-1)}$$

Where: X_i = the $\log_{10}$ of concentration i

n_i = the number of organisms tested at effluent concentration i

p_i^a = the smoothed adjusted observed proportion mortality at effluent concentration i

k = the number of effluent concentrations tested, not including the control.

10.1 For this example, the estimated variance of m, V(m), is calculated as follows:

V(m) = $(0.000)(1.000)(1.3979 - 0.7959)^2/4(39)$ +
$(0.000)(1.000)(1.6990 - 1.0969)^2/4(39)$ +
$(0.641)(0.359)(2.0000 - 1.3979)^2/4(39)$

= 0.00053477

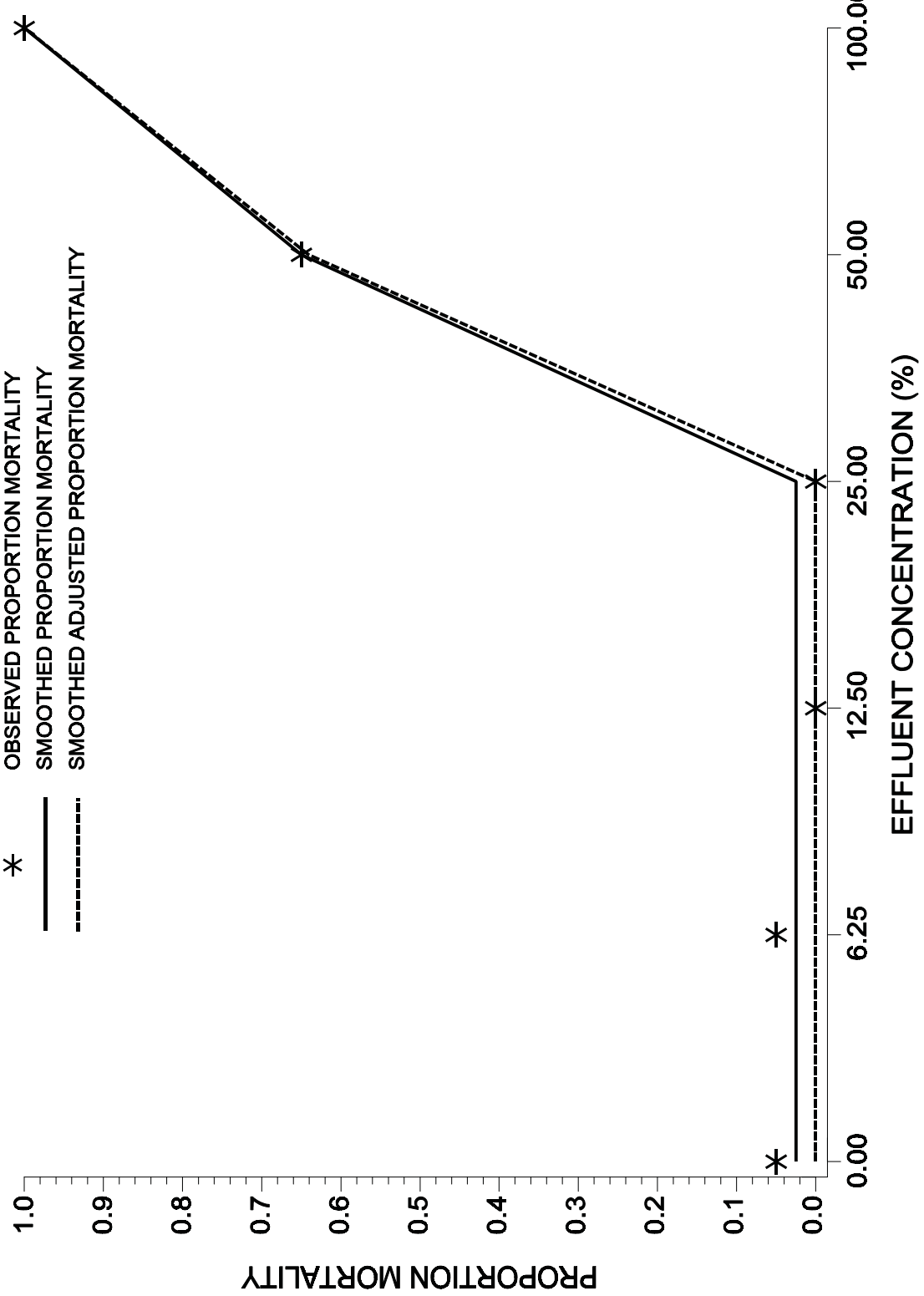

Figure J.1. Plot of the smoothed, adjusted data for the fathead minnow larval survival and growth test.

11. Calculate the 95% confidence interval for m: $m \pm 2.0\sqrt{V(m)}$

11.1 For this example, the 95% confidence interval for m is calculated as follows:

$$1.656527 \pm 2\sqrt{0.00053477} = (1.610277,\ 1.702777)$$

12. The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking base$_{10}$ antilogs of the above values.

12.1 For this example, the estimated LC50 is calculated as follows:

LC50 = antilog(m) = antilog(1.656527) = 45.3%.

12.2 The limits of the 95% confidence interval for the estimated LC50 are calculated by taking the antilogs of the upper and lower limits of the 95% confidence interval for m as follows:

lower limit: antilog(1.610277) = 40.8%

upper limit: antilog(1.702777) = 50.4%

APPENDIX K

TRIMMED SPEARMAN-KARBER METHOD

1. The Trimmed Spearman-Karber Method is a modification of the Spearman-Karber Method, a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Hamilton et al, 1977). Appendix The Trimmed Spearman-Karber Method estimates the trimmed mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the trimmed mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Trimmed Spearman-Karber Analysis is recommended only when the requirements for the Probit Method and the Spearman-Karber Method are not met.

4. To calculate the LC50 using the Trimmed Spearman-Karber Method, the smoothed, adjusted, observed proportion mortalities must bracket 0.5.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed, adjusted, observed proportion mortalities must be between zero and one.

6. Let $p_0, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \le p_1 \le ... \le p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \le p_1 \le ... \le p_k$, with their average. For example, if p_i is less than p_{i-1} then:

Where: $p^s_{i-1} = p^s_i = (p_i + p_{i-1})/2$

p^s_i = the smoothed observed proportion mortality for effluent concentration i.

7. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

Where: $p^a_i = (p^s_i - p^s_0) / (1 - p^s_0)$

p^s_0 = the smoothed observed proportion mortality for the control

p^s_i = the smoothed observed proportion mortality for effluent concentration i.

8. Calculate the amount of trim to use in the estimation of the LC50 as follows:

Where: $Trim = \max(p^a_1, 1-p^a_k)$

p^a_1 = the smoothed, adjusted proportion mortality for the lowest effluent concentration, exclusive of the control

p^a_k = the smoothed, adjusted proportion mortality for the highest effluent concentration

k = the number of effluent concentrations, exclusive of the control.

The minimum trim should be calculated for each data set rather than using a fixed amount of trim for each data set.

9. Due to the intensive nature of the calculation for the estimated LC50 and the calculation of the associated 95% confidence interval using the Trimmed Spearman-Karber Method, it is recommended that the data be analyzed by computer.

10. A computer program which estimates the LC50 and associated 95% confidence interval using the Trimmed Spearman-Karber Method, can be obtained from EMSL-Cincinnati by sending a written request to EMSL, 3411 Church Street, Cincinnati, OH 45244.

11. The Trimmed Spearman-Karber program automatically performs the following functions:

a. Smoothing.
b. Adjustment for mortality in the control.
c. Calculation of the necessary trim.
d. Calculation of the LC50.
e. Calculation of the associated 95% confidence interval.

12. To illustrate the Trimmed Spearman-Karber method using the Trimmed Spearman-Karber computer program, a set of data from a Fathead Minnow Larval Survival and Growth test will be used. The data are listed in Table K.1.

TABLE K.1. EXAMPLE OF TRIMMED SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25	0	0.00
12.5	2	0.05
25.0	0	0.00
50.0	0	0.00
100.0	32	0.80

12.1 The program requests the following input (Figure K.1):

a. Output destination (D = disk file, P = printer).
b. Control data.
c. Data for each toxicant concentration.

12.2 The program output includes the following (Figure K.2):

a. A table of the concentrations tested, number of organisms exposed, and mortalities.
b. The amount of trim used in the calculation.
c. The estimated LC50 and the associated 95% confidence interval.

```
A:>spearman

 TRIMMED SPEARMAN-KARBER METHOD.  VERSION 1.5

ENTER DATE OF TEST:
1
ENTER TEST NUMBER:
2
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
 L
ENTER TEST SPECIES NAME:
Fathead minnow
ENTER TOXICANT  NAME:
Effluent
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT:
%
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
 40
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
2
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL;  MAX = 10):
5
ENTER THE  5 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
6.25  12.5  25  50  100
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:        40

ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
Days
ENTER DURATION OF TEST:
7
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:        0 2 0 0 32

WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
 y
```

Figure K.1. Example input for Trimmed Spearman-Karber Method.

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 1 TEST NUMBER: 2 DURATION: 7 Days
TOXICANT: effluent
SPECIES: fathead minnow

RAW DATA: Concentration (%)	Number Exposed	Mortalities
.00	40	2
6.25	40	0
12.50	40	2
25.00	40	0
50.00	40	0
100.00	40	32

SPEARMAN-KARBER TRIM: 20.41%

SPEARMAN-KARBER ESTIMATES: LC50: 77.28
95% CONFIDENCE LIMITS
ARE NOT RELIABLE.

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

Figure K.2. Example output for Trimmed Spearman-Karber Method.

APPENDIX L

GRAPHICAL METHOD

1. The Graphical Method is used to calculate the LC50. It is a mathematical procedure which estimates the LC50 by linearly interpolating between points of a plot of observed percent mortality versus the base 10 logarithm ($\log_{10}$) of percent effluent concentration. This method does not provide a confidence interval for the LC50 estimate and its use is only recommended when there are no partial mortalities. The only requirement for the Graphical Method is that the observed percent mortalities bracket 50%.

2. For an analysis using the Graphical Method the data must first be smoothed and adjusted for mortality in the control replicates. The procedure for smoothing and adjusting the data is detailed in the following steps.

3. The Graphical Method is illustrated below using a set of mortality data from an Fathead Minnow Larval Survival and Growth test. These data are listed in Table L.1.

TABLE L.1. EXAMPLE OF GRAPHICAL METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25	0	0.00
12.5	0	0.00
25.0	0	0.00
50.0	40	1.00
100.0	40	1.00

4. Let $p_0, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \le p_1 \le ... \le p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \le p_1 \le ... \le p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i-1}^s = p_i^s = (p_i + p_{i-1})/2$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

4.1 For the data in this example, because the observed mortality proportions for the 6.25%, 12.5%, and 25.0% effluent concentrations are less than the observed response proportion for the control, the values for these four groups must be averaged:

$$p_0^s = p_1^s = p_2^s = p_3^s = \frac{0.05+0.00+0.00+0.00}{4} = \frac{0.05}{4} = 0.0125$$

4.2 Since $p_4 = p_5 = 1.00$ are larger then 0.0125, set $p_4^s = p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table L.2.

TABLE L.2. EXAMPLE OF GRAPHICAL METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration %	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.05	0.0125	0.00
6.25	0.00	0.0125	0.00
12.5	0.00	0.0125	0.00
25.0	0.00	0.0125	0.00
50.0	1.00	1.0000	1.00
100.0	1.00	1.0000	1.00

5. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

5.1 Because the smoothed observed proportion mortality for the control group is greater than zero, the responses must be adjusted using Abbott's formula, as follows:

$$p_0^a = p_1^a = p_2^a = p_3^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.0125 - 0.0125}{1 - 0.0125} = \frac{0.0}{0.9875} = 0.0$$

$$p_4^a = p_5^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{1.00 - 0.0125}{1 - 0.0125} = \frac{0.9875}{0.9875} = 1.00$$

A table of the smoothed, adjusted response proportions for the effluent concentrations are shown in Table L.2.

5.2 Plot the smoothed, adjusted data on 2-cycle semi-log graph paper with the logarithmic axis (the y axis) used for percent effluent concentration and the linear axis (the x axis) used for observed percent mortality. A plot of the smoothed, adjusted data is shown in Figure L.1.

6. Locate the two points on the graph which bracket 50% mortality and connect them with a straight line.

7. On the scale for percent effluent concentration, read the value for the point where the plotted line and the 50% mortality line intersect. This value is the estimated LC50 expressed as a percent effluent concentration.

7.1 For this example, the two points on the graph which bracket the 50% mortality line (0% mortality at 25% effluent, and 100% mortality at 50% effluent) are connected with a straight line. The point at which the plotted line intersects the 50% mortality line is the estimated LC50. The estimated LC50 = 35% effluent.

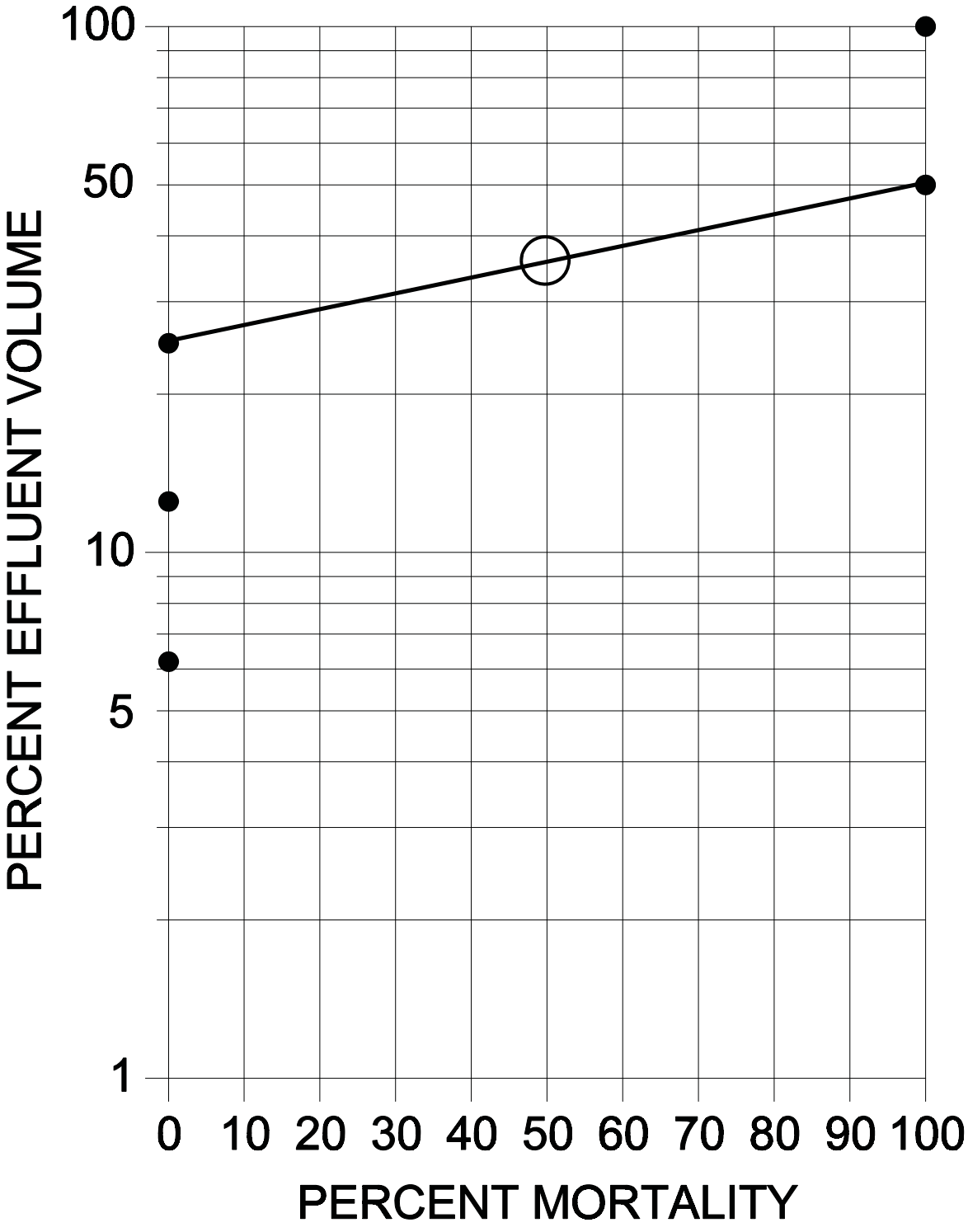

Figure L.1 Plot of the smoothed adjusted response proportions for fathead minnow, *Pimephales promelas*, survival data.

APPENDIX M

LINEAR INTERPOLATION METHOD

1. GENERAL PROCEDURE

1.1 The Linear Interpolation Method is used to calculate a point estimate of the effluent or other toxicant concentration that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms (Inhibition Concentration, or IC). The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests, and the generation of an endpoint from a continuous model that allows a traditional quantitative assessment of the precision of the endpoint, such as confidence limits for the endpoint of a single test, and a mean and coefficient of variation for the endpoints of multiple tests.

1.2 The Linear Interpolation Method assumes that the responses (1) are monotonically non-increasing, where the mean response for each higher concentration is less than or equal to the mean response for the previous concentration, (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. If the data are not monotonically nonincreasing, they are adjusted by smoothing (averaging). In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean. Also, no assumption is made about the distribution of the data except that the data within a group being resampled are independent and identically distributed.

2. DATA SUMMARY AND PLOTS

2.1 Calculate the mean responses for the control and each toxicant concentration, construct a summary table, and plot the data.

3. MONOTONICITY

3.1 If the assumption of monotonicity of test results is met, the observed response means ($\bar{Y}_i$) should stay the same or decrease as the toxicant concentration increases. If the means do not decrease monotonically, the responses are "smoothed" by averaging (pooling) adjacent means.

3.2 Observed means at each concentration are considered in order of increasing concentration, starting with the control mean ($\bar{Y}_1$). If the mean observed response at the lowest toxicant concentration ($\bar{Y}_2$) is equal to or smaller than the control mean ($\bar{Y}_1$), it is used as the response. If it is larger than the control mean, it is averaged with the control, and this average is used for both the control response (M_1) and the lowest toxicant concentration response (M_2). This mean is then compared to the mean observed response for the next higher toxicant concentration ($\bar{Y}_3$). Again, if the mean observed response for the next higher toxicant concentration is smaller than the mean of the control and the lowest toxicant concentration, it is used as the response. If it is higher than the mean of the first two, it is averaged with the first two, and the mean is used as the response for the control and two lowest concentrations of toxicant. This process is continued for data from the remaining toxicant concentrations. A numerical example of smoothing the data is provided below. (Note: Unusual patterns in the deviations from monotonicity may require an additional step of smoothing). Where $\bar{Y}_i$ decrease monotonically, the $\bar{Y}_i$ become M_i without smoothing.

4. LINEAR INTERPOLATION METHOD

4.1 The method assumes a linear response from one concentration to the next. Thus, the ICp is estimated by linear interpolation between two concentrations whose responses bracket the response of interest, the (p) percent reduction from the control.

4.2 To obtain the estimate, determine the concentrations C_J and C_{J+1} which bracket the response M_1 (1 - p/100), where M_1 is the smoothed control mean response and p is the percent reduction in response relative to the control

response. These calculations can easily be done by hand or with a computer program as described below. The linear interpolation estimate is calculated as follows:

$$ICp = C_J + [\ M_1\ (1 - p/100) - M_J\ ]\ \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

Where: C_J = tested concentration whose observed mean response is greater than $M_1(1 - p/100)$.

C_{J+1} = tested concentration whose observed mean response is less than $M_1(1 - p/100)$.

M_1 = smoothed mean response for the control.

M_J = smoothed mean response for concentration J.

M_{J+1} = smoothed mean response for concentration J + 1.

p = percent reduction in response relative to the control response.

ICp = estimated concentration at which there is a percent reduction from the smoothed mean control response. The ICp is reported for the test, together with the 95% confidence interval calculated by the ICPIN.EXE program described below.

4.3 If the C_J is the highest concentration tested, the ICp would be specified as *greater than* C_J. If the response at the lowest concentration tested is used to extrapolate the ICp value, the ICp should be expressed as a *less than the lowest test concentration.*

5. CONFIDENCE INTERVALS

5.1 Due to the use of a linear interpolation technique to calculate an estimate of the ICp, standard statistical methods for calculating confidence intervals are not applicable for the ICp. This limitation is avoided by use a technique known as the bootstrap method as proposed by Efron (1982) for deriving point estimates and confidence intervals.

5.2 In the Linear Interpolation Method, the smoothed response means are used to obtain the ICp estimate reported for the test. The bootstrap method is used to obtain the 95% confidence interval for the true mean. In the bootstrap method, the test data Y_{ji} is randomly resampled with replacement to produce a new set of data Y_{ji}*, that is statistically equivalent to the original data, but a new and slightly different estimate of the ICp (ICp*) is obtained. This process is repeated at least 80 times (Marcus and Holtzman, 1988) resulting in multiple "data" sets, each with an associate ICp* estimate. The distribution of the ICp* estimates derived from the sets of resampled data approximates the sampling distribution of the ICp estimate. The standard error of the ICp is estimated by the standard deviation of the individual ICp* estimates. Empirical confidence intervals are derived from the quantiles of the ICp* empirical distribution. For example, if the test data are resampled a minimum of 80 time, the empirical 2.5% and the 97.5% confidence limits are approximately the second smallest and second largest ICp* estimates (Marcus and Holtzman, 1988).

5.3 The width of the confidence intervals calculated by the bootstrap method is related to the variability of the data. When confidence intervals are wide, the reliability of the IC estimate is in question. However, narrow intervals do not necessarily indicate that the estimate is highly reliable, because of undetected violations of assumptions and the fact that the confidence limits based on the empirical quantiles of a bootstrap distribution of 80 samples may be unstable.

5.4 The bootstrapping method of calculating confidence intervals is computationally intensive. For this reason, all of the calculations associated with determining the confidence intervals for the ICp estimate have been incorporated into a computer program. Computations are most easily done with a computer program such as the revision of the

BOOTSTRP program (USEPA, 1988; USEPA, 1989) which is now called "ICPIN" which is described below in subsection 7.

6. MANUAL CALCULATIONS

6.1 DATA SUMMARY AND PLOTS

6.1.1 The data used in this example are the *Ceriodaphnia dubia* reproduction data used in the example in Section 13. Table M.1 includes the raw data and the mean reproduction for each concentration. Data are included for all animals tested regardless of death of the organism. If an animal died during the test without producing young, a zero is entered. If death occurred after producing young, the number of young produced prior to death is entered. A plot of the data is provided in Figure M.1.

TABLE M.1. *CERIODAPHNIA DUBIA* REPRODUCTION DATA

		Effluent Concentration (%)				
Replicate	Control	1.56	3.12	6.25	12.5	25.0
1	27	32	39	27	10	0
2	30	35	30	34	13	0
3	29	32	33	36	7	0
4	31	26	33	34	7	0
5	16	18	36	31	7	0
6	15	29	33	27	10	0
7	18	27	33	33	10	0
8	17	16	27	31	16	0
9	14	35	38	33	12	0
10	27	13	44	31	2	0
Mean ($\overline{Y}_i$)	22.4	26.3	34.6	31.7	9.4	0
i	1	2	3	4	5	6

6.2 MONOTONICITY

6.2.1 As can be seen from the plot in Figure M.1, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

6.2.2 Starting with the control mean $\overline{Y}_1 = 22.4$ and $\overline{Y}_2 = 26.3$, we see that $\overline{Y}_1 < \overline{Y}_2$. Calculate the smoothed means:

$$M_1 = M_2 = (\overline{Y}_1 + \overline{Y}_2)/2 = 24.35$$

6.2.3 Since $\overline{Y}_3 = 34.6$ is larger than M_2, average $\overline{Y}_3$ with the previous concentrations:

6.2.4 Additionally, $\overline{Y}_4 = 31.7$ is larger than M_3, and is pooled with the first three means. Thus,

$$M_1 = M_2 = M_3 = M_4 = (M_1 + M_2 + M_3 + \overline{Y}_4)/4 = 28.7$$

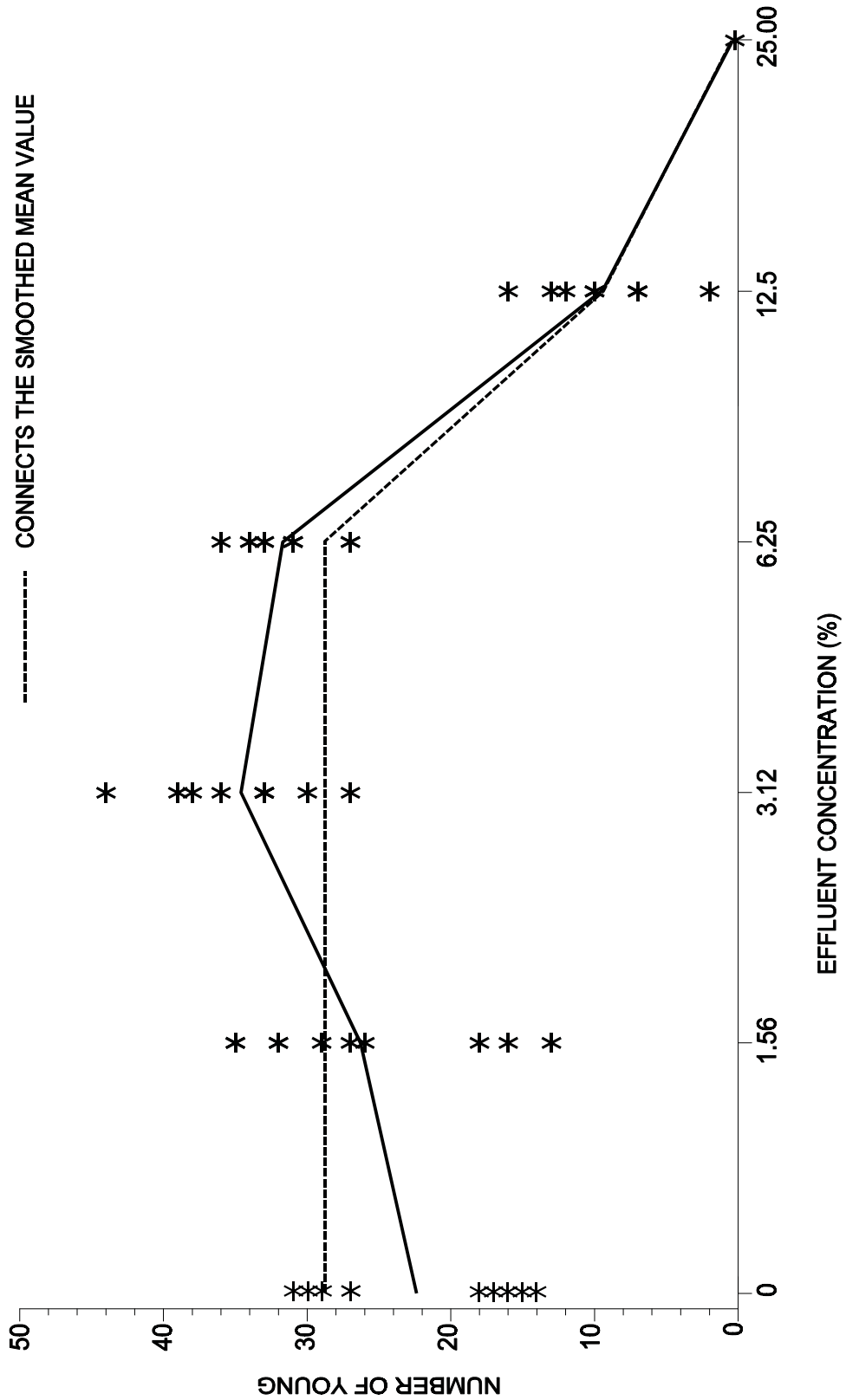

Figure M.1. Plot of raw data, observed means, and smoothed means for the daphnid, *Ceriodaphnia dubia*, reproductive data.

TABLE M.2. *CERIODAPHNIA DUBIA* REPRODUCTION MEAN RESPONSE AFTER SMOOTHING

Effluent Concentration %	i	Response Mean (Y_i) (Young/female)	Smoothed Mean (M_i) (Young/female)
Control	1	22.4	28.75
1.56	2	26.3	28.75
3.12	3	34.6	28.75
6.25	4	31.7	28.75
12.5	5	9.4	9.40
25.0	6	0.0	0.00

6.2.5 Since $M_4 > \overline{Y}_5 = 9.4$, set $M_5 = 9.4$. Likewise, $M_5 > \overline{Y}_6 = 0$ and M_6 becomes 0. Table M.2 contains the smoothed means and Figure M.1 gives a plot of the smoothed response curve.

6.3 LINEAR INTERPOLATION

6.3.1 Estimates of the IC25 and IC50 are calculated using the Linear Interpolation Method. A 25% reduction in reproduction, compared to the controls, would result in a mean reproduction of 21.56 young per adult, where $M_1(1-p/100) = 28.75(1-25/100)$. A 50% reduction in reproduction, compared to the controls, would result in a mean reproduction of 14.38 young per adult, where $M_1(1-p/100) = 28.75(1-50/100)$. Examining the smoothed means and their associated concentrations (Table M.2), the two effluent concentrations bracketing the reproduction of 21.56 young per adult are $C_4 = 6.25\%$ effluent and $C_5 = 12.5\%$ effluent. The two effluent concentrations bracketing a response of 14.38 young per adult are also $C_4 = 6.25\%$ effluent and $C_5 = 12.5\%$ effluent.

6.3.2 Using Equation 1 from 4.2, the estimate of the IC25 is calculated as follows:

$$ICp = C_J + [\, M_1\,(1 - p/100) - M_J\,] \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

$$IC25 = 6.25 + [28.75\,(1 - 25/100) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

$$= 8.57\% \text{ effluent}$$

6.3.3 Using the equation from section 4.2, the estimate of the IC50 is calculated as follows:

$$ICp = C_J + [\, M_1\,(1 - p/100) - M_J\,] \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

$$IC50 = 6.25 + [28.75\,(1 - 50/100) - 28.75] \frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

$$= 10.89\% \text{ effluent}$$

6.4 CONFIDENCE INTERVALS

6.4.1 Confidence intervals for the ICp are derived using the bootstrap method. As described above, this method involves randomly resampling the individual observations and recalculating the ICp at least 80 times, and determining the mean ICp, standard deviation, and empirical 95% confidence intervals. For this reason, the confidence intervals are calculated using a computer program called ICPIN. This program is described below and is available to carry out all the calculations of both the interpolation estimate (ICp) and the confidence intervals.

7. COMPUTER CALCULATIONS

7.1 The computer program, ICPIN, prepared for the Linear Interpolation Method was written in TURBO PASCAL for IBM compatible PCs. The program (version 2.0) has been modified by Computer Science Corporation, Duluth, MN with funding provided by the Environmental Research Laboratory, Duluth, MN (Norberg-King, 1993). The program was originally developed by Battelle Laboratories, Columbus, OH through a government contract supported by the Environmental Research Laboratory, Duluth, MN (USEPA, 1988). To obtain the program and supporting documentation, send a written request to EMSL-Cincinnati at 3411 Church Street, Cincinnati, OH 45244.

7.2 The ICPIN.EXE program performs the following functions: 1) it calculates the observed response means (Y_i) (response means); 2) it calculates the standard deviations; 3) checks the responses for monotonicity; 4) calculates smoothed means (M_i) (pooled response means) if necessary; 5) uses the means, M_i, to calculate the initial ICp of choice by linear interpolation; 6) performs a user-specified number of bootstrap resamples between 80 and 1000 (as multiples of 40); 7) calculates the mean and standard deviation of the bootstrapped ICp estimates; and 8) provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven (Norberg-King, 1993).

7.3 For the IC*p* calculation, up to twelve treatments can be used (which includes the control). There can be up to 40 replicates per concentration, and the program does not require an equal number of replicates per concentration. The value of p can range from 1% to 99%.

7.4 DATA INPUT

7.4.1 Data is entered directly into the program onscreen. A sample data entry screen in shown in Figure M.2. The program documentation provides guidance on the entering and analysis of data for the Linear Interpolation Method (Norberg-King, 1993).

7.4.2 The user selects the ICp estimate desired (e.g., IC25 or IC50) and the number of resamples to be taken for the bootstrap method of calculating the confidence intervals. The program has the capability of performing any number of resamples from 80 to 1000 as multiples of 40. However, Marcus and Holtzman (1988) recommend a minimum of 80 resamples for the bootstrap method be used and at least 250 resamples are better (Norberg-King, 1993).

7.5 DATA OUTPUT.

7.5.1 The program output includes the following (Figures M.3 and M.4):

1. A table of the concentration identification, the concentration tested and raw data response for each replicate and concentration.
2. A table of test concentrations, number of replicates, concentration (units), response means ($\bar{Y}_i$), standard deviations for each response mean, and the pooled response means (smoothed means; M_i).
3. The linear interpolation estimate of the ICp using the means (M_i). *Use this value for the ICp estimate.*
4. The mean ICp and standard deviation from the bootstrap resampling.
5. The confidence intervals calculated by the bootstrap method for the ICp. Provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is

over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven.

7.6 ICPIN program output for the analysis of the *Ceriodaphnia dubia* reproduction data in Table M.1 is provided in Figures M.3 and M.4.

7.6.1 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 8.57% effluent. The empirical 95% confidence intervals for the true mean were 8.30% to 8.85% effluent.

7.6.2 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 10.89% effluent. The empirical 95% confidence intervals for the true mean were 10.36% to 11.62% effluent.

ICp Data Entry/Edit Screen Current File:

Conc. ID	1	2	3	4	5	6
Conc. Tested						
Conc. Tested						
Response 1						
Response 2						
Response 3						
Response 4						
Response 5						
Response 6						
Response 7						
Response 8						
Response 9						
Response 10						
Response 11						
Response 12						
Response 13						
Response 14						
Response 15						
Response 16						
Response 17						
Response 18						
Response 19						
Response 20						

F10 for Command Menu Use arrow Keys to Switch Fields

Figure M.2. ICp data entry/edit screen. Twelve concentrating identifications can be used. Data for concentrations are entered in columns 1 through 6. For concentrations 7 through 12 and responses 21-40 the data is entered in additional fields of the same screen.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent:
Test Start Date: app M Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration: 7-d
DATA FILE: cerioman.icp
OUTPUT FILE: cerioman.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 8.5715 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 8.6014 Standard Deviation: 0.1467
Original Confidence Limits: Lower: 8.3040 Upper: 8.8496
Resampling time in Seconds: 2.53 Random Seed: -1652543090

Figure M.3. Example of ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent:
Test Start Date: app M Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration: 7-d
DATA FILE: cerioman.icp
OUTPUT FILE: cerioman.i50

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 10.8931 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 10.9108 Standard Deviation: 0.3267
Original Confidence Limits: Lower: 10.3618 Upper: 11.6201
Resampling time in Seconds: 2.58 Random Seed: 340510286

Figure M.4. Example of ICPIN program output for the IC50.

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United States Environmental Protection Agency (EPA)
National Pollutant Discharge Elimination System (NPDES)

MULTI-SECTOR GENERAL PERMIT FOR STORMWATER DISCHARGES ASSOCIATED WITH INDUSTRIAL ACTIVITY (MSGP)

AUTHORIZATION TO DISCHARGE UNDER THE
NATIONAL POLLUTANT DISCHARGE ELIMINATION SYSTEM

In compliance with the provisions of the Clean Water Act (CWA), as amended (33 U.S.C. 1251 *et seq.*), operators of stormwater discharges associated with industrial activity located in an area identified in Appendix C where EPA is the permitting authority are authorized to discharge to waters of the United States in accordance with the eligibility and Notice of Intent (NOI) requirements, effluent limitations, inspection requirements, and other conditions set forth in this permit. This permit is structured as follows:

- general requirements that apply to all facilities are found in Parts 1 through 7;
- industry sector-specific requirements are found in Part 8; and
- specific requirements that apply in individual States and Indian Country Lands are found in Part 9.

The Appendices (A through K) contain additional permit conditions that apply to all operators covered under this permit.

This permit becomes effective on September 29, 2008.

This permit and the authorization to discharge expire at midnight, September 29, 2013.

Robert W. Varney, Regional Administrator
EPA Region 1

Carl-Axel P. Soderberg, Division Director, Caribbean Environmental Protection Division
EPA Region 2

Jon M. Capacasa, Director, Water Protection Division
EPA Region 3

Timothy C. Henry, Acting Director, Water Division
EPA Region 5

Miguel I. Flores, Director, Water Quality Protection Division
EPA Region 6

Alexis Strauss, Director, Water Division
EPA Region 9

Michael Gearheard, Director, Office of Water and Watersheds
EPA Region 10

NPDES MULTI-SECTOR GENERAL PERMITS FOR STORMWATER DISCHARGES ASSOCIATED WITH INDUSTRIAL ACTIVITY
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1. Coverage under this Permit.

1.1 Eligibility.

1.1.1 Facilities Covered.

To be eligible to discharge under this permit, you must (1) have a stormwater discharge associated with industrial activity from your primary industrial activity, as defined in Appendix A, provided your primary industrial activity is included in Appendix D, or (2) be notified by EPA that you are eligible for coverage under Sector AD of this permit.

1.1.2 Allowable Stormwater Discharges.

Unless otherwise made ineligible under Part 1.1.4, the following discharges are eligible for coverage under this permit:

1.1.2.1 Stormwater discharges associated with industrial activity for any primary industrial activities and co-located industrial activities, as defined in Appendix A;

1.1.2.2 Discharges designated by EPA as needing a stormwater permit as provided in Sector AD;

1.1.2.3 Discharges that are not otherwise required to obtain NPDES permit authorization but are commingled with discharges that are authorized under this permit;

1.1.2.4 Discharges subject to any of the national stormwater-specific effluent limitations guidelines listed in Table 1-1; and

Table 1-1. Stormwater-specific Effluent Limitations Guidelines

Regulated Discharge	40 CFR Section	MSGP Sector	New Source Performance Standard (NSPS)	New Source Date
Discharges resulting from spray down or intentional wetting of logs at wet deck storage areas	Part 429, Subpart I	A	Yes	1/26/81
Runoff from phosphate fertilizer manufacturing facilities that comes into contact with any raw materials, finished product, by-products or waste products (SIC 2874)	Part 418, Subpart A	C	Yes	4/8/74
Runoff from asphalt emulsion facilities	Part 443, Subpart A	D	Yes	7/28/75
Runoff from material storage piles at cement manufacturing facilities	Part 411, Subpart C	E	Yes	2/20/74

Mine dewatering discharges at crushed stone, construction sand and gravel, or industrial sand mining facilities	Part 436, Subparts B, C, and D	J	No	N/A
Runoff from hazardous waste and non-hazardous waste landfills	Part 445, Subparts A and B	K, L	Yes	2/2/00
Runoff from coal storage piles at steam electric generating facilities	Part 423	O	Yes	11/19/82 (10/8/74)[1]

1.1.2.5 Discharges subject to any New Source Performance Standards (NSPS) identified in Table 1-1 (i.e., where facilities were constructed after the promulgation of that industry's NSPS), provided that you obtain and retain the following EPA documentation with your SWPPP, prior to submitting your NOI, and that you comply with any limits pursuant to Part 2.4:

- Determination of "No Significant Impact" under the National Environmental Policy Act (NEPA); or
- A completed Environmental Impact Statement in accordance with an environmental review conducted by EPA pursuant to 40 CFR 6.102(a)(6)[2].

1.1.3 Allowable Non-Stormwater Discharges.

The following are the non-stormwater discharges authorized under this permit, provided the non-stormwater component of your discharge is in compliance with Part 2.1.2.10:

- Discharges from fire-fighting activities;
- Fire hydrant flushings;
- Potable water, including water line flushings;
- Uncontaminated condensate from air conditioners, coolers, and other compressors and from the outside storage of refrigerated gases or liquids;
- Irrigation drainage;
- Landscape watering provided all pesticides, herbicides, and fertilizer have been applied in accordance with the approved labeling;
- Pavement wash waters where no detergents are used and no spills or leaks of toxic or hazardous materials have occurred (unless all spilled material has been removed);
- Routine external building washdown that does not use detergents;
- Uncontaminated ground water or spring water;

[1] NSPS promulgated in 1974 were not removed via the 1982 regulation; therefore wastewaters generated by Part 423-applicable sources that were New Sources under the 1974 regulations are subject to the 1974 NSPS.

[2] Note that if you have previously completed an Environmental Impact Statement or obtained a "No Significant Impact" statement for discharges subject to NSPS, you have met your obligation under this provision and you only need to retain this documentation for your files.

- Foundation or footing drains where flows are not contaminated with process materials; and
- Incidental windblown mist from cooling towers that collects on rooftops or adjacent portions of your facility, but not intentional discharges from the cooling tower (e.g., "piped" cooling tower blowdown or drains).

1.1.4 Limitations on Coverage.

1.1.4.1 Discharges Mixed with Non-Stormwater. Stormwater discharges that are mixed with non-stormwater, other than those non-stormwater discharges listed in Part 1.1.3, are not eligible for coverage under this permit.

1.1.4.2 Stormwater Discharges Associated with Construction Activity. Stormwater discharges associated with construction activity disturbing one acre or more are not eligible for coverage under this permit, unless in conjunction with mining activities or certain oil and gas extraction activities as specified in Sectors G, H, I, and J of this permit.

1.1.4.3 Discharges Currently or Previously Covered by Another Permit. Unless you received written notification from EPA specifically allowing these discharges to be covered under this permit, you are not eligible for coverage under this permit for any of the following:

- Stormwater discharges associated with industrial activity that are currently covered under an individual NPDES permit or an alternative NPDES general permit;
- Discharges covered within five years prior to the effective date of this permit by an individual permit or alternative general permit where that permit established site-specific numeric water quality-based limitations developed for the stormwater component of the discharge; or
- Discharges from facilities where any NPDES permit has been or is in the process of being denied, terminated, or revoked by EPA (this does not apply to the routine reissuance of permits every five years).

1.1.4.4 Stormwater Discharges Subject to Effluent Limitations Guidelines. For discharges subject to stormwater effluent limitation guidelines under 40 CFR, Subchapter N, only those stormwater discharges identified in Table 1-1 are eligible for coverage under this permit.

1.1.4.5 Endangered and Threatened Species and Critical Habitat Protection. Coverage under this permit is available only if your stormwater discharges, allowable non-stormwater discharges, and stormwater discharge-related activities will not adversely affect any species that are federally-listed as endangered or threatened ("listed") under the Endangered Species Act (ESA) and will not result in the adverse modification or destruction of habitat that is federally-designated as "critical habitat" under the ESA. You must meet one of the criteria below, following the procedures in Appendix E:

Criterion A. No federally-listed threatened or endangered species or their designated critical habitat are likely to occur in the "action area" as defined in Appendix A; or

Criterion B. Consultation between a Federal agency and the U.S. Fish and Wildlife Service and/or the National Marine Fisheries Service (together, the "Services") under section 7 of the ESA has been concluded. Consultations can be either formal or informal, and would have occurred only as a result of a separate federal action (e.g., during application for an individual wastewater discharge permit or the issuance of a wetlands dredge and fill permit).

The consultation must have addressed the effects of your facility's stormwater discharges, allowable non-stormwater discharges, and stormwater discharge-related activities on federally-listed threatened or endangered species and federally-designated critical habitat, and must have resulted in either:

i. a biological opinion finding no jeopardy to federally-listed species or destruction/adverse modification of federally-designated critical habitat; or

ii. written concurrence from the Service(s) with a finding that the facility's stormwater discharges associated with industrial activity, discharge-related activities and allowable non-stormwater discharges are not likely to adversely affect federally-listed species or federally-designated critical habitat; or

Criterion C. Your industrial activities are authorized through the issuance of a permit under section 10 of the ESA, and authorization addresses the effects of the stormwater discharges associated with industrial activity, discharge-related activities, and allowable non-stormwater discharges on federally-listed species and federally-designated critical habitat; or

Criterion D. Coordination between you and the U.S. Fish and Wildlife Service and/or the National Marine Fisheries Service has been concluded. The coordination must have addressed the effects of the facility's stormwater discharges associated with industrial activity, discharge-related activities, and allowable non-stormwater discharges on federally-listed threatened or endangered species and federally-designated critical habitat. The result of the coordination must be a written statement from the Service concluding that authorizing your stormwater discharges, discharge-related activities, and allowable non-stormwater discharges is consistent with the determination that the issuance of the MSGP is not likely to adversely affect federally-listed threatened or endangered species and federally-designated critical habitat. Any conditions or prerequisites deemed necessary to achieve consistency with the "not likely to adversely effect" determination become eligibility conditions for MSGP coverage, and permit requirements under Part 2.3; or

Criterion E. Authorizing your stormwater discharges associated with industrial activity, discharge-related activities, and allowable non-stormwater discharges is

consistent with the determination that the issuance of the MSGP is not likely to adversely affect any federally-listed endangered and threatened ("listed") species or designated critical habitat ("critical habitat"). To support your determination that you meet Criterion E, you must provide supporting documentation for your determination.

i. If you are an existing discharger, you must provide the following information with your completed Notice of Intent (NOI) form: (1) a list of the federally-listed threatened or endangered species or their designated critical habitat that are likely to occur in the "action area"; (2) a list of the pollutant parameters for which you have ever exceeded an applicable benchmark or effluent limitations guideline, or for which your discharge has ever been found to cause or contribute to an exceedance of an applicable water quality standard, or to violate State or Tribal water quality requirements (Part 9); and (3) your rationale supporting your determination that you meet Criterion E, including appropriate measures to be undertaken to avoid or eliminate the likelihood of adverse effects.

ii. If you are a new discharger, you must provide the following information with your completed NOI form: (1) a list of the federally-listed threatened or endangered species or their designated critical habitat that are likely to occur in the "action area"; (2) a list of the potential pollutants in your discharge; and (3) your rationale supporting your determination that you meet Criterion E, including appropriate measures to be undertaken to avoid or eliminate the likelihood of adverse effects; or

Criterion F. The facility's stormwater discharges associated with industrial activity, discharge-related activities, and allowable non-stormwater discharges were already addressed in another operator's valid certification of eligibility that included these discharges and activities and there is no reason to believe that federally-listed species or federally-designated critical habitat not considered in the prior certification may be present or located in the "action area". To certify eligibility under this criterion there must be no lapse of coverage in the other operator's certification. By certifying eligibility under this criterion, you agree to comply with any measures or controls upon which the other operator's certification was based. You must comply with any applicable terms, conditions, or other requirements developed in the process of meeting the eligibility requirements of the criteria in this section to remain eligible for coverage under this permit. If your certification is based on another operator's certification under Criterion E, that certification is valid only if you have documentation showing that the other operator had certified under Criterion E, and you provide EPA with the supporting information required of existing dischargers in Criterion E (above, under subparagraph (i)) in your NOI form.

1.1.4.6 Historic Properties Preservation. Coverage under this permit is available only if your stormwater discharges, allowable non-stormwater discharges, and stormwater discharge-

related activities meet one of the eligibility criteria below, following the procedures in Appendix F:

Criterion A. Your stormwater discharges and allowable non-stormwater discharges do not have the potential to have an effect on historic properties and you are not constructing or installing new stormwater control measures on your site that cause subsurface disturbance; or

Criterion B. Your discharge-related activities (i.e., construction and/or installation of stormwater control measures that involve subsurface disturbance) will not affect historic properties; or

Criterion C. Your stormwater discharges, allowable non-stormwater discharges, and discharge-related activities have the potential to have an effect on historic properties, and you have consulted with the State Historic Preservation Officer (SHPO), Tribal Historic Preservation Officer (THPO), or other tribal representative regarding measures to mitigate or prevent any adverse effects on historic properties, and you have either (1) obtained and are in compliance with a written agreement that outlines all such measures, or (2) been unable to reach agreement on such measures; or

Criterion D. You have contacted the State Historic Preservation Officer, Tribal Historic Preservation Officer, or other tribal representative and EPA in writing informing them that you have the potential to have an effect on historic properties and you did not receive a response from the SHPO, THPO, or tribal representative within 30 days of receiving your letter.

If you have been unable to reach agreement with a SHPO, THPO, or other tribal representative regarding appropriate measures to mitigate or prevent adverse effects, EPA may notify you of additional measures you must implement to be eligible for coverage under this permit.

1.1.4.7 New Discharges to Water Quality Impaired Waters. If you are a new discharger you are not eligible for coverage under this permit to discharge to an "impaired water", as defined in Appendix A unless you:

a. prevent all exposure to stormwater of the pollutant(s) for which the waterbody is impaired, and retain documentation of procedures taken to prevent exposure onsite with your SWPPP; or

b. document that the pollutant(s) for which the waterbody is impaired is not present at your site, and retain documentation of this finding with your SWPPP; or

c. in advance of submitting your NOI, provide to the appropriate EPA Regional Office data to support a showing that the discharge is not expected to cause or contribute to an exceedance of a water quality standard, and retain such data

onsite with your SWPPP. To do this, you must provide data and other technical information to the Regional Office sufficient to demonstrate:

i. For discharges to waters without an EPA approved or established TMDL, that the discharge of the pollutant for which the water is impaired will meet in-stream water quality criteria at the point of discharge to the waterbody; or

ii. For discharges to waters with an EPA approved or established TMDL, that there are sufficient remaining wasteload allocations in an EPA approved or established TMDL to allow your discharge and that existing dischargers to the waterbody are subject to compliance schedules designed to bring the waterbody into attainment with water quality standards.

You are eligible under Part 1.1.4.7.c if you receive an affirmative determination from the Regional Office that your discharge will not contribute to the existing impairment, in which case you must maintain such determination onsite with your SWPPP, or if the Regional Office fails to respond within 30 days of submission of data to the Regional Office.

1.1.4.8 New Discharges to Waters Designated as Tier 3 for Antidegradation Purposes. If you are a new discharger, you are not eligible for coverage under this permit for discharges to waters designated by a State or Tribe as Tier 3 (outstanding natural resource waters) for antidegradation purposes under 40 CFR 131.13(a)(3) (see list of Tier 3 waters on EPA's website at http://www.epa.gov/npdes/stormwater/msgp).

1.2 Permit Compliance.

Any noncompliance with any of the requirements of this permit constitutes a violation of the Clean Water Act. As detailed in Part 3 (Corrective Actions) of this permit, failure to take any required corrective actions constitute an independent, additional violation of this permit and the Clean Water Act. As such, any actions and time periods specified for remedying noncompliance do not absolve parties of the initial underlying noncompliance. However, where corrective action is triggered by an event that does not itself constitute permit noncompliance, such as an exceedance of an applicable benchmark, there is no permit violation provided you take the required corrective action within the relevant deadlines established in Part 3.3.

1.3 Authorization under this Permit.

1.3.1 How to Obtain Authorization.

To obtain authorization under this permit, you must:

- Be located in a State, territory, or Indian Country, or be a Federal Facility identified in Appendix C where EPA is the permitting authority;
- Meet the Part 1.1 eligibility requirements;

- Select, design, install, and implement control measures in accordance with Part 2.1 to meet numeric and non-numeric effluent limits;
- Submit a complete and accurate Notice of Intent (NOI) either using EPA's electronic Notice of Intent (eNOI) system (accessible at www.epa.gov/npdes/eNOI) or using a paper form (included in Appendix G of this permit) and then submitting that paper form to the address listed in Part 7.6.1; and
- Develop a SWPPP according to the requirements in Part 5 of this permit.

EPA will post on the Internet, at www.epa.gov/npdes/noisearch, all NOIs received. Late NOIs will be accepted but authorization to discharge will not be retroactive.

Timeframes for discharge authorization are contained in Table 1-2. Some authorization dates in Table 1-2 are dependent on you posting a copy of your SWPPP on the Internet. Posting requires that (1) your NOI identifies the Uniform Resource Locator (URL) that provides direct access to your SWPPP, (2) you post a complete copy of your SWPPP at that URL, and (3) the SWPPP is available from that URL at least for the period starting the day you submit your NOI until you are authorized to discharge. You are not required to post any confidential business information (CBI) at this URL, but you must clearly identify those portions of the SWPPP that are being withheld from public access as a result of your determination of CBI.

Table 1-2. NOI Submittal Deadlines/Discharge Authorization Dates		
Category	**NOI Submission Deadline**	**Discharge Authorization Date[1]**
Existing Dischargers – in operation as of October 30, 2005 and authorized for coverage under MSGP 2000.	No later than January 5, 2009.	30 days after EPA posts your NOI. Your authorization under the MSGP 2000 is automatically continued until you have been granted coverage under this permit or an alternative permit, or coverage is otherwise terminated.
New Dischargers or New Sources - have commenced discharging between October 30, 2005 and January 5, 2009.	As soon as possible but no later than January 5, 2009.	30 days after EPA posts your NOI.
New Dischargers or New Sources - commence discharging after January 5, 2009.	A minimum of 60 days prior to commencing discharge, or a minimum of 30 days if your SWPPP is posted on the Internet during this period and the Internet address (i.e., URL) to your SWPPP is provided on the NOI form.	If you post your SWPPP on the Internet, 30 days after EPA posts your NOI. Otherwise, 60 days after EPA posts your NOI.
New Owner/Operator of Existing Discharger - transfer of ownership and/or operation of a facility whose discharge is authorized under this permit	A minimum of 30 days prior to date that the transfer will take place to the new owner/operator.	30 days after EPA posts your NOI.
Other Eligible Dischargers - in operation prior to October 30, 2005, but not covered under the MSGP 2000 or another NPDES permit.	Immediately, to minimize the time discharges from the facility will continue to be unauthorized.	If you post your SWPPP on the Internet, 30 days after EPA posts your NOI. Otherwise, 60 days after EPA posts your NOI.

[1] Based on a review of your NOI or other information, EPA may delay your authorization for further review, notify you that additional effluent limitations are necessary, or may deny coverage under this permit and require submission of an application for an individual NPDES permit, as detailed in Part 1.6. In these instances, EPA will notify you in writing of the delay, of the need for additional effluent limits, or of the request for submission of an individual NPDES permit application.

1.3.2 Continuation of this Permit.

If this permit is not reissued or replaced prior to the expiration date, it will be administratively continued in accordance with 40 CFR 122.6 and remain in force and effect. If

you were authorized to discharge under this permit prior to the expiration date, any discharges authorized under this permit will automatically remain covered by this permit until the earliest of:

- Your authorization for coverage under a reissued permit or a replacement of this permit following your timely and appropriate submittal of a complete NOI requesting authorization to discharge under the new permit and compliance with the requirements of the new permit; or
- Your submittal of a Notice of Termination; or
- Issuance or denial of an individual permit for the facility's discharges; or
- A formal permit decision by EPA not to reissue this general permit, at which time EPA will identify a reasonable time period for covered dischargers to seek coverage under an alternative general permit or an individual permit. Coverage under this permit will cease at the end of this time period.

1.4 Terminating Coverage.

1.4.1 Submitting a Notice of Termination.

To terminate permit coverage, you must submit a complete and accurate Notice of Termination either electronically (strongly encouraged) at www.epa.gov/npdes/eNOI or using the paper Notice of Termination form included in Appendix H of this permit, to the address listed in Part 7.6.1. Your authorization to discharge under this permit terminates at midnight of the day that a complete Notice of Termination is processed and posted on EPA's website (www.epa.gov/npdes/noisearch). If you submit a Notice of Termination without meeting one or more of the conditions identified in Part 1.4.2, then your Notice of Termination is not valid. You are responsible for meeting the terms of this permit until your authorization is terminated.

1.4.2 When to Submit a Notice of Termination.

You must submit a Notice of Termination within 30 days after one or more of the following conditions have been met:

- A new owner or operator has taken over responsibility for the facility; or
- You have ceased operations at the facility, there are not or no longer will be discharges of stormwater associated with industrial activity from the facility, and you have already implemented necessary sediment and erosion controls as required by Part 2.1.2.5;
- You are a Sector G, H, or J facility and you have met the applicable termination requirements; or
- You have obtained coverage under an individual or alternative general permit for all discharges required to be covered by an NPDES permit, unless EPA has required that you obtain such coverage under authority of Part 1.6.1, in which case coverage under this permit will terminate automatically.

1.5 Conditional Exclusion for No Exposure.

If you are covered by this permit, and become eligible for a no exposure exclusion from permitting under 40 CFR 122.26(g), you may file a No Exposure Certification. You are no longer required to have a permit upon submission of a complete and accurate no exposure certification to EPA. If you are no longer required to have permit coverage because of a no exposure exclusion and have submitted a No Exposure Certification form to EPA, you are not required to submit a Notice of Termination. You must submit a No Exposure Certification to EPA once every five years. File your No Exposure Certification using the eNOI system at www.epa.gov/npdes/eNOI.

1.6 Alternative Permits.

1.6.1 EPA Requiring Coverage under an Alternative Permit.

EPA may require you to apply for and/or obtain authorization to discharge under either an individual NPDES permit or an alternative NPDES general permit in accordance with 40 CFR 122.64 and 124.5. Any interested person may petition EPA to take action under this paragraph. If EPA requires you to apply for an individual NPDES permit, EPA will notify you in writing that a permit application is required. This notification will include a brief statement of the reasons for this decision and will provide application information. In addition, if you are an existing discharger authorized to discharge under this permit, the notice will set a deadline to file the permit application, and will include a statement that on the effective date of the individual NPDES permit, or the alternative general permit as it applies to you, coverage under this general permit will terminate. EPA may grant additional time to submit the application if you request it. If you are covered under this permit and fail to submit an individual NPDES permit application as required by EPA, then the applicability of this permit to you is terminated at the end of the day specified by EPA as the deadline for application submittal. EPA may take appropriate enforcement action for any unpermitted discharge.

1.6.2 Permittee Requesting Coverage under an Alternative Permit.

You may request to be excluded from coverage under this general permit by applying for an individual permit. In such a case, you must submit an individual permit application in accordance with the requirements of 40 CFR 122.26(c)(1)(ii), with reasons supporting the request, to EPA at the applicable EPA Regional Office listed in Part 7.6.2 of this permit. The request may be granted by issuance of an individual permit or authorization of coverage under an alternative general permit if your reasons are adequate to support the request.

When an individual NPDES permit is issued to you or you are authorized to discharge under an alternative NPDES general permit, your authorization to discharge under this permit is terminated on the effective date of the individual permit or the date of authorization of coverage under the alternative general permit.

1.7 Severability.

Invalidation of a portion of this permit does not necessarily render the whole permit invalid. EPA's intent is that the permit is to remain in effect to the extent possible; in the event that any part of this permit is invalidated, EPA will advise the regulated community as to the effect of such invalidation.

2. Control Measures and Effluent Limits.

In the technology-based limits included in Part 2.1 and in Part 8, the term "minimize" means reduce and/or eliminate to the extent achievable using control measures (including best management practices) that are technologically available and economically practicable and achievable in light of best industry practice.

2.1 Control Measures.

You must select, design, install, and implement control measures (including best management practices) to address the selection and design considerations in Part 2.1.1, meet the non-numeric effluent limits in Part 2.1.2, and meet limits contained in applicable effluent limitations guidelines in Part 2.1.3. The selection, design, installation, and implementation of these control measures must be in accordance with good engineering practices and manufacturer's specifications. Note that you may deviate from such manufacturer's specifications where you provide justification for such deviation and include documentation of your rationale in the part of your SWPPP that describes your control measures, consistent with Part 5.1.4. If you find that your control measures are not achieving their intended effect of minimizing pollutant discharges, you must modify these control measures as expeditiously as practicable. Regulated stormwater discharges from your facility include stormwater run-on that commingles with stormwater discharges associated with industrial activity at your facility.

2.1.1 Control Measure Selection and Design Considerations

You must consider the following when selecting and designing control measures:

- preventing stormwater from coming into contact with polluting materials is generally more effective, and less costly, than trying to remove pollutants from stormwater;
- using control measures in combination is more effective than using control measures in isolation for minimizing pollutants in your stormwater discharge;
- assessing the type and quantity of pollutants, including their potential to impact receiving water quality, is critical to designing effective control measures that will achieve the limits in this permit;
- minimizing impervious areas at your facility and infiltrating runoff onsite (including bioretention cells, green roofs, and pervious pavement, among other approaches) can reduce runoff and improve groundwater recharge and stream base flows in local streams, although care must be taken to avoid ground water contamination;

- attenuating flow using open vegetated swales and natural depressions can reduce in-stream impacts of erosive flows;
- conserving and/or restoring of riparian buffers will help protect streams from stormwater runoff and improve water quality; and
- using treatment interceptors (e.g., swirl separators and sand filters) may be appropriate in some instances to minimize the discharge of pollutants.

2.1.2 Non-Numeric Technology-Based Effluent Limits (BPT/BAT/BCT).

2.1.2.1 Minimize Exposure. You must minimize the exposure of manufacturing, processing, and material storage areas (including loading and unloading, storage, disposal, cleaning, maintenance, and fueling operations) to rain, snow, snowmelt, and runoff by either locating these industrial materials and activities inside or protecting them with storm resistant coverings (although significant enlargement of impervious surface area is not recommended). In minimizing exposure, you should pay particular attention to the following:

- use grading, berming, or curbing to prevent runoff of contaminated flows and divert run-on away from these areas;
- locate materials, equipment, and activities so that leaks are contained in existing containment and diversion systems (confine the storage of leaky or leak-prone vehicles and equipment awaiting maintenance to protected areas);
- clean up spills and leaks promptly using dry methods (e.g., absorbents) to prevent the discharge of pollutants;
- use drip pans and absorbents under or around leaky vehicles and equipment or store indoors where feasible;
- use spill/overflow protection equipment;
- drain fluids from equipment and vehicles prior to on-site storage or disposal;
- perform all cleaning operations indoors, under cover, or in bermed areas that prevent runoff and run-on and also that capture any overspray; and
- ensure that all washwater drains to a proper collection system (i.e., not the stormwater drainage system).

The discharge of vehicle and equipment washwater, including tank cleaning operations, is not authorized by this permit. These wastewaters must be covered under a separate NPDES permit, discharged to a sanitary sewer in accordance with applicable industrial pretreatment requirements, or disposed of otherwise in accordance with applicable law.

Note: Industrial materials do not need to be enclosed or covered if stormwater runoff from affected areas will not be discharged to receiving waters or if discharges are authorized under another NPDES permit.

2.1.2.2 Good Housekeeping. You must keep clean all exposed areas that are potential sources of pollutants, using such measures as sweeping at regular intervals, keeping materials orderly and labeled, and storing materials in appropriate containers.

2.1.2.3 Maintenance. You must regularly inspect, test, maintain, and repair all industrial equipment and systems to avoid situations that may result in leaks, spills, and other releases of pollutants in stormwater discharged to receiving waters. You must maintain all control measures that are used to achieve the effluent limits required by this permit in effective operating condition. Nonstructural control measures must also be diligently maintained (e.g., spill response supplies available, personnel appropriately trained). If you find that your control measures need to be replaced or repaired, you must make the necessary repairs or modifications as expeditiously as practicable.

2.1.2.4 Spill Prevention and Response Procedures. You must minimize the potential for leaks, spills and other releases that may be exposed to stormwater and develop plans for effective response to such spills if or when they occur. At a minimum, you must implement:

- Procedures for plainly labeling containers (e.g., "Used Oil," "Spent Solvents," "Fertilizers and Pesticides," etc.) that could be susceptible to spillage or leakage to encourage proper handling and facilitate rapid response if spills or leaks occur;
- Preventative measures such as barriers between material storage and traffic areas, secondary containment provisions, and procedures for material storage and handling;
- Procedures for expeditiously stopping, containing, and cleaning up leaks, spills, and other releases. Employees who may cause, detect, or respond to a spill or leak must be trained in these procedures and have necessary spill response equipment available. If possible, one of these individuals should be a member of your stormwater pollution prevention team (see Part 5.1.1); and
- Procedures for notification of appropriate facility personnel, emergency response agencies, and regulatory agencies. Where a leak, spill, or other release containing a hazardous substance or oil in an amount equal to or in excess of a reportable quantity established under either 40 CFR Part 110, 40 CFR Part 117, or 40 CFR Part 302, occurs during a 24-hour period, you must notify the National Response Center (NRC) at (800) 424-8802 or, in the Washington, DC, metropolitan area, call (202) 267-2675 in accordance with the requirements of 40 CFR Part 110, 40 CFR Part 117, and 40 CFR Part 302 as soon as you have knowledge of the discharge. State or local requirements may necessitate reporting spills or discharges to local emergency response, public health, or drinking water supply agencies. Contact information must be in locations that are readily accessible and available.

2.1.2.5 Erosion and Sediment Controls. You must stabilize exposed areas and contain runoff using structural and/or non-structural control measures to minimize onsite erosion and sedimentation, and the resulting discharge of pollutants. Among other actions you must take to meet this limit, you must place flow velocity dissipation devices at discharge locations and within outfall channels where necessary to reduce erosion and/or settle out pollutants. In selecting, designing, installing, and implementing appropriate control measures, you are encouraged to consult with EPA's internet-based resources relating to BMPs for erosion and sedimentation, including the sector-specific *Industrial Stormwater*

Fact Sheet Series, (www.epa.gov/npdes/stormwater/msgp), *National Menu of Stormwater BMPs* (www.epa.gov/npdes/stormwater/menuofbmps), and *National Management Measures to Control Nonpoint Source Pollution from Urban Areas* (www.epa.gov/owow/nps/urbanmm/index.html), and any similar State or Tribal publications.

2.1.2.6 Management of Runoff. You must divert, infiltrate, reuse, contain, or otherwise reduce stormwater runoff, to minimize pollutants in your discharges. In selecting, designing, installing, and implementing appropriate control measures, you are encouraged to consult with EPA's internet-based resources relating to runoff management, including the sector-specific *Industrial Stormwater Fact Sheet Series*, (www.epa.gov/npdes/stormwater/msgp), *National Menu of Stormwater BMPs* (www.epa.gov/npdes/stormwater/menuofbmps), and *National Management Measures to Control Nonpoint Source Pollution from Urban Areas* (www.epa.gov/owow/nps/urbanmm/index.html), and any similar State or Tribal publications.

2.1.2.7 Salt Storage Piles or Piles Containing Salt. You must enclose or cover storage piles of salt, or piles containing salt, used for deicing or other commercial or industrial purposes, including maintenance of paved surfaces. You must implement appropriate measures (e.g., good housekeeping, diversions, containment) to minimize exposure resulting from adding to or removing materials from the pile. Piles do not need to be enclosed or covered if stormwater runoff from the piles is not discharged or if discharges from the piles are authorized under another NPDES permit.

2.1.2.8 Sector Specific Non-Numeric Effluent Limits. You must achieve any additional non-numeric limits stipulated in the relevant sector-specific section(s) of Part 8.

2.1.2.9 Employee Training. You must train all employees who work in areas where industrial materials or activities are exposed to stormwater, or who are responsible for implementing activities necessary to meet the conditions of this permit (e.g., inspectors, maintenance personnel), including all members of your Pollution Prevention Team. Training must cover both the specific control measures used to achieve the effluent limits in this Part, and monitoring, inspection, planning, reporting, and documentation requirements in other parts of this permit. EPA recommends training be conducted at least annually (or more often if employee turnover is high).

2.1.2.10 Non-Stormwater Discharges. You must eliminate non-stormwater discharges not authorized by an NPDES permit. See Part 1.2.3 for a list of non-stormwater discharges authorized by this permit.

2.1.2.11 Waste, Garbage and Floatable Debris. You must ensure that waste, garbage, and floatable debris are not discharged to receiving waters by keeping exposed areas free of such materials or by intercepting them before they are discharged.

2.1.2.12 Dust Generation and Vehicle Tracking of Industrial Materials. You must minimize generation of dust and off-site tracking of raw, final, or waste materials.

2.1.3 Numeric Effluent Limitations Based on Effluent Limitations Guidelines

If you are in an industrial category subject to one of the effluent limitations guidelines identified in Table 6-1 (see Part 6.2.2.1), you must meet the effluent limits referenced in Table 2-1 below:

Table 2-1. Applicable Effluent Limitations Guidelines

Regulated Activity	40 CFR Part/Subpart	Effluent Limit
Discharges resulting from spray down or intentional wetting of logs at wet deck storage areas	Part 429, Subpart I	See Part 8.A.7
Runoff from phosphate fertilizer manufacturing facilities that comes into contact with any raw materials, finished product, by-products or waste products (SIC 2874)	Part 418, Subpart A	See Part 8.C.4
Runoff from asphalt emulsion facilities	Part 443, Subpart A	See Part 8.D.4
Runoff from material storage piles at cement manufacturing facilities	Part 411, Subpart C	See Part 8.E.5
Mine dewatering discharges at crushed stone, construction sand and gravel, or industrial sand mining facilities	Part 436, Subparts B, C, or D	See Part 8.J.9
Runoff from hazardous waste landfills	Part 445, Subpart A	See Part 8.K.6
Runoff from non-hazardous waste landfills	Part 445, Subpart B	See Part 8.L.10
Runoff from coal storage piles at steam electric generating facilities	Part 423	See Part 8.O.8

2.2 Water Quality-Based Effluent Limitations.

2.2.1 Water Quality Standards

Your discharge must be controlled as necessary to meet applicable water quality standards.

EPA expects that compliance with the other conditions in this permit will control discharges as necessary to meet applicable water quality standards. If at any time you become aware, or EPA determines, that your discharge causes or contributes to an exceedance of applicable water quality standards, you must take corrective action as required in Part 3.1, document the corrective actions as required in Parts 3.4 and 5.4, and report the corrective actions to EPA as required in Part 7.2.

Additionally, EPA may impose additional water quality-based limitations on a site-specific basis, or require you to obtain coverage under an individual permit, if information in your NOI, required reports, or from other sources indicates that your discharges are not controlled as necessary to meet applicable water quality standards.

2.2.2 Discharges to Water Quality Impaired Waters.

2.2.2.1 Existing Discharge to an Impaired Water with an EPA Approved or Established TMDL. If you discharge to an impaired water with an EPA approved or established TMDL, EPA will inform you if any additional limits or controls are necessary for your discharge to be consistent with the assumptions of any available wasteload allocation in the TMDL, or if coverage under an individual permit is necessary in accordance with Part 1.6.1.

2.2.2.2 Existing Discharge to an Impaired Water without an EPA Approved or Established TMDL. If you discharge to an impaired water without an EPA approved or established TMDL, you are required to comply with Part 2.2.1 and the monitoring requirement of Part 6.2.4. Note that this provision also applies to situations where EPA determines that your discharge is not controlled as necessary to meet water quality standards in a downstream water segment, even if your discharge is to a receiving water that is not specifically identified on a Section 303(d) list.

2.2.2.3 New Discharge to an Impaired Water. If your authorization to discharge under this permit relied on Part 1.1.4.7 for a new discharge to an impaired water, you must implement and maintain any control measures or conditions on your site that enabled you to become eligible under Part 1.1.4.7, and modify such measures or conditions as necessary pursuant to any Part 3 corrective actions. You are also required to comply with Part 2.2.1 and the monitoring requirements of Parts 6.2.4.

2.2.3 Tier 2 Antidegradation Requirements for New or Increased Dischargers

If you are a new discharger, or an existing discharger required to notify EPA of an increased discharge consistent with Part 7.4 (i.e., a "planned changes" report), and you discharge directly to waters designated by a State or Tribe as Tier 2 or Tier 2.5 for antidegradation purposes under 40 CFR 131.12(a) (see list of Tier 2 and 2.5 waters on EPA's website at http://www.epa.gov/npdes/stormwater/msgp), EPA may notify you that additional analyses, control measures, or other permit conditions are necessary to comply with the applicable antidegradation requirements, or notify you that an individual permit application is necessary in accordance with Part 1.6.1.

2.3 Requirements Relating to Endangered Species and Historic Properties

If your eligibility under either Part 1.1.4.5 or Part 1.1.4.6 was made possible through your, or another operator's, agreement to include certain measures or prerequisite actions, or implement certain terms and conditions, you must comply with all such agreed-upon requirements to maintain eligibility under the MSGP.

2.4 Requirements Relating to the National Environmental Policy Act (NEPA) Review

If your eligibility under Part 1.1.2.5 was made possible through your agreement to implement any mitigation measures as a result of the NEPA review process, you must comply with all such agreed-upon measures to maintain eligibility under the MSGP.

3. Corrective Actions

3.1 Conditions Requiring Review and Revision to Eliminate Problem

If any of the following conditions occur, you must review and revise the selection, design, installation, and implementation of your control measures to ensure that the condition is eliminated and will not be repeated in the future:

- an unauthorized release or discharge (e.g., spill, leak, or discharge of non-stormwater not authorized by this or another NPDES permit) occurs at your facility;
- a discharge violates a numeric effluent limit;
- you become aware, or EPA determines, that your control measures are not stringent enough for the discharge to meet applicable water quality standards;
- an inspection or evaluation of your facility by an EPA official, or local, State, or Tribal entity, determines that modifications to the control measures are necessary to meet the non-numeric effluent limits in this permit; or
- you find in your routine facility inspection, quarterly visual assessment, or comprehensive site inspection that your control measures are not being properly operated and maintained.

3.2 Conditions Requiring Review to Determine if Modifications Are Necessary

If any of the following conditions occur, you must review the selection, design, installation, and implementation of your control measures to determine if modifications are necessary to meet the effluent limits in this permit:

- construction or a change in design, operation, or maintenance at your facility significantly changes the nature of pollutants discharged in stormwater from your facility, or significantly increases the quantity of pollutants discharged; or
- the average of 4 quarterly sampling results exceeds an applicable benchmark. If less than 4 benchmark samples have been taken, but the results are such that an exceedence of the 4 quarter average is mathematically certain (i.e., if the sum of quarterly sample results to date is more than 4 times the benchmark level) this is considered a benchmark exceedence, triggering this review.

3.3 Corrective Action Deadlines

You must document your discovery of any of the conditions listed in Parts 3.1 and 3.2 within 24 hours of making such discovery. Subsequently, within 14 days of such discovery, you

must document any corrective action(s) to be taken to eliminate or further investigate the deficiency, or if no corrective action is needed, the basis for that determination. Specific documentation required within 24 hours and 14 days is detailed in Part 3.4. If you determine that changes are necessary following your review, any modifications to your control measures must be made before the next storm event if possible, or as soon as practicable following that storm event. These time intervals are not grace periods, but are schedules considered reasonable for documenting your findings and for making repairs and improvements. They are included in this permit to ensure that the conditions prompting the need for these repairs and improvements are not allowed to persist indefinitely.

3.4 Corrective Action Report

Within 24 hours of discovery of any condition listed in Parts 3.1 and 3.2, you must document the following information (i.e., questions 3-5 of the Corrective Actions section in the Annual Reporting Form, provided in Appendix I):

- Identification of the condition triggering the need for corrective action review;
- Description of the problem identified; and
- Date the problem was identified.

Within 14 days of discovery of any condition listed in Parts 3.1 and 3.2, you must document the following information (i.e., questions 7-11 of the Corrective Actions section in the Annual Reporting Form, provided in Appendix I):

- Summary of corrective action taken or to be taken (or, for triggering events identified in Part 3.2 where you determine that corrective action is not necessary, the basis for this determination);
- Notice of whether SWPPP modifications are required as a result of this discovery or corrective action;
- Date corrective action initiated; and
- Date corrective action completed or expected to be completed.

You must submit this documentation in an annual report as required in Part 7.2 and retain a copy onsite with your SWPPP as required in Part 5.4.

3.5 Effect of Corrective Action

If the event triggering the review is a permit violation (e.g., non-compliance with an effluent limit), correcting it does not remove the original violation. Additionally, failing to take corrective action in accordance with this section is an additional permit violation. EPA will consider the appropriateness and promptness of corrective action in determining enforcement responses to permit violations.

3.6 Substantially Identical Outfalls

If the event triggering corrective action is linked to an outfall that represents other substantially identical outfalls, your review must assess the need for corrective action for each outfall represented by the outfall that triggered the review. Any necessary changes to control measures that affect these other outfalls must also be made before the next storm event if possible, or as soon as practicable following that storm event.

4. Inspections

You must conduct the inspections in Parts 4.1, 4.2, and 4.3 at your facility.

4.1 Routine Facility Inspections.

4.1.1 Routine Facility Inspection Procedures.

Conduct routine facility inspections of all areas of the facility where industrial materials or activities are exposed to stormwater, and of all stormwater control measures used to comply with the effluent limits contained in this permit. Routine facility inspections must be conducted at least quarterly (i.e., once each calendar quarter) although in many instances, more frequent inspection (e.g., monthly) may be appropriate for some types of equipment, processes, and control measures or areas of the facility with significant activities and materials exposed to stormwater. Perform these inspections during periods when the facility is in operation. You must specify the relevant inspection schedules in your SWPPP document as required in Part 5.1.5. These routine inspections must be performed by qualified personnel (for definition see Appendix A) with at least one member of your stormwater pollution prevention team participating. At least once each calendar year, the routine facility inspection must be conducted during a period when a stormwater discharge is occurring.

4.1.2 Routine Facility Inspection Documentation.

You must document the findings of each routine facility inspection performed and maintain this documentation onsite with your SWPPP as required in Part 5.4. You are not required to submit your routine facility inspection findings to EPA, unless specifically requested to do so. At a minimum, your documentation of each routine facility inspection must include:

- The inspection date and time;
- The name(s) and signature(s) of the inspector(s);
- Weather information and a description of any discharges occurring at the time of the inspection;
- Any previously unidentified discharges of pollutants from the site;
- Any control measures needing maintenance or repairs;
- Any failed control measures that need replacement;
- Any incidents of noncompliance observed; and
- Any additional control measures needed to comply with the permit requirements.

Any corrective action required as a result of a routine facility inspection must be performed consistent with Part 3 of this permit.

4.1.3 Exceptions to Routine Facility Inspections.

Inactive and Unstaffed Sites: The requirement to conduct routine facility inspections on a quarterly basis does not apply at a facility that is inactive and unstaffed, as long as there are no industrial materials or activities exposed to stormwater. Such a facility is only required to conduct an annual comprehensive site inspection in accordance with the requirements of Part 4.3. To invoke this exception, you must maintain a statement in your SWPPP pursuant to Part 5.1.5.2 indicating that the site is inactive and unstaffed, and that there are no industrial materials or activities exposed to precipitation, in accordance with the substantive requirements in 40 CFR 122.26(g)(4)(iii). The statement must be signed and certified in accordance with Appendix B, Subsection 11. If circumstances change and industrial materials or activities become exposed to stormwater or your facility becomes active and/or staffed, this exception no longer applies and you must immediately resume quarterly facility inspections. If you are not qualified for this exception at the time you are authorized under this permit, but during the permit term you become qualified because your facility is inactive and unstaffed, and there are no industrial materials or activities that are exposed to stormwater, then you must include the same signed and certified statement as above and retain it with your records pursuant to Part 5.4.

Inactive and unstaffed facilities covered under Sectors G (Metal Mining), H (Coal Mines and Coal Mining-Related Facilities), and J (Non-Metallic Mineral Mining and Dressing), are not required to meet the "no industrial materials or activities exposed to stormwater" standard to be eligible for this exception from routine inspections, consistent with the requirements established in Parts 8.G.8.4, 8.H.8.1, and 8.J.8.1.

4.2 Quarterly Visual Assessment of Stormwater Discharges.

4.2.1 Quarterly Visual Assessment Procedures.

Once each quarter for the entire permit term, you must collect a stormwater sample from each outfall (except as noted in Part 4.2.3) and conduct a visual assessment of each of these samples. These samples are not required to be collected consistent with 40 CFR Part 136 procedures but should be collected in such a manner that the samples are representative of the stormwater discharge.

The visual assessment must be made:

- Of a sample in a clean, clear glass, or plastic container, and examined in a well-lit area;
- On samples collected within the first 30 minutes of an actual discharge from a storm event. If it is not possible to collect the sample within the first 30 minutes of discharge, the sample must be collected as soon as practicable after the first 30

minutes and you must document why it was not possible to take samples within the first 30 minutes. In the case of snowmelt, samples must be taken during a period with a measurable discharge from your site; and

- For storm events, on discharges that occur at least 72 hours (3 days) from the previous discharge. The 72-hour (3-day) storm interval does not apply if you document that less than a 72-hour (3-day) interval is representative for local storm events during the sampling period.

You must visually inspect the sample for the following water quality characteristics:

- Color;
- Odor;
- Clarity;
- Floating solids;
- Settled solids;
- Suspended solids;
- Foam;
- Oil sheen; and
- Other obvious indicators of stormwater pollution.

4.2.2 Quarterly Visual Assessment Documentation.

You must document the results of your visual assessments and maintain this documentation onsite with your SWPPP as required in Part 5.4. You are not required to submit your visual assessment findings to EPA, unless specifically requested to do so. At a minimum, your documentation of the visual assessment must include:

- Sample location(s)
- Sample collection date and time, and visual assessment date and time for each sample;
- Personnel collecting the sample and performing visual assessment, and their signatures;
- Nature of the discharge (i.e., runoff or snowmelt);
- Results of observations of the stormwater discharge;
- Probable sources of any observed stormwater contamination,
- If applicable, why it was not possible to take samples within the first 30 minutes.

Any corrective action required as a result of a quarterly visual assessment must be performed consistent with Part 3 of this permit.

4.2.3 Exceptions to Quarterly Visual Assessments.

Adverse Weather Conditions: When adverse weather conditions prevent the collection of samples during the quarter, you must take a substitute sample during the next qualifying storm event. Documentation of the rationale for no visual assessment for the quarter must be included with your SWPPP records as described in Part 5.4. Adverse conditions are

those that are dangerous or create inaccessibility for personnel, such as local flooding, high winds, or electrical storms, or situations that otherwise make sampling impractical, such as drought or extended frozen conditions.

Climates with Irregular Stormwater Runoff: If your facility is located in an area where limited rainfall occurs during many parts of the year (e.g., arid or semi-arid climate) or in an area where freezing conditions exist that prevent runoff from occurring for extended periods, then your samples for the quarterly visual assessments may be distributed during seasons when precipitation runoff occurs.

Areas Subject to Snow: In areas subject to snow, at least one quarterly visual assessment must capture snowmelt discharge, as described in Part 6.1.3, taking into account the exception described above for climates with irregular stormwater runoff.

Inactive and unstaffed sites: The requirement for a quarterly visual assessment does not apply at a facility that is inactive and unstaffed, as long as there are no industrial materials or activities exposed to stormwater. To invoke this exception, you must maintain a statement in your SWPPP as required in Part 5.1.5.2 indicating that the site is inactive and unstaffed, and that there are no industrial materials or activities exposed to precipitation, in accordance with the substantive requirements in 40 CFR 122.26(g)(4)(iii). The statement must be signed and certified in accordance with Appendix B, Subsection 11. If circumstances change and industrial materials or activities become exposed to stormwater or your facility becomes active and/or staffed, this exception no longer applies and you must immediately resume quarterly visual assessments. If you are not qualified for this exception at the time you are authorized under this permit, but during the permit term you become qualified because your facility is inactive and unstaffed, and there are no industrial materials or activities that are exposed to stormwater, then you must include the same signed and certified statement as above and retain it with your records pursuant to Part 5.4.

Inactive and unstaffed facilities covered under Sectors G (Metal Mining), H (Coal Mines and Coal Mining-Related Facilities), and J (Non-Metallic Mineral Mining and Dressing), are not required to meet the “no industrial materials or activities exposed to stormwater” standard to be eligible for this exception from quarterly visual assessment, consistent with the requirements established in Parts 8.G.8.4, 8.H.8.1, and 8.J.8.1.

Substantially identical outfalls: If your facility has two or more outfalls that you believe discharge substantially identical effluents, as documented in Part 5.1.5.2, you may conduct quarterly visual assessments of the discharge at just one of the outfalls and report that the results also apply to the substantially identical outfall(s) provided that you perform visual assessments on a rotating basis of each substantially identical outfall throughout the period of your coverage under this permit.

If stormwater contamination is identified through visual assessment performed at a substantially identical outfall, you must assess and modify your control measures as appropriate for each outfall represented by the monitored outfall.

4.3 Comprehensive Site Inspections.

4.3.1 Comprehensive Site Inspection Procedures.

You must conduct annual comprehensive site inspections while you are covered under this permit. Annual, as defined in this Part, means once during each of the following inspection periods beginning with the period you are authorized to discharge under this permit:

Year 1:	September 29, 2008 – September 29, 2009
Year 2:	September 29, 2009 – September 29, 2010
Year 3:	September 29, 2010 – September 29, 2011
Year 4:	September 29, 2011 – September 29, 2012
Year 5:	September 29, 2012 – September 29, 2013

You are waived from having to perform a comprehensive site inspection for an inspection period, as defined above, if you obtain authorization to discharge less than three months before the end of that inspection period.

Should your coverage be administratively continued after the expiration date of this permit, you must continue to perform these inspections annually until you are no longer covered.

Comprehensive site inspections must be conducted by qualified personnel with at least one member of your stormwater pollution prevention team participating in the comprehensive site inspections.

Your comprehensive site inspections must cover all areas of the facility affected by the requirements in this permit, including the areas identified in the SWPPP as potential pollutant sources (see Part 5.1.3) where industrial materials or activities are exposed to stormwater, any areas where control measures are used to comply with the effluent limits in Part 2, and areas where spills and leaks have occurred in the past 3 years. The inspections must also include a review of monitoring data collected in accordance with Part 6.2. Inspectors must consider the results of the past year's visual and analytical monitoring when planning and conducting inspections. Inspectors must examine the following:

- Industrial materials, residue, or trash that may have or could come into contact with stormwater;
- Leaks or spills from industrial equipment, drums, tanks, and other containers;
- Offsite tracking of industrial or waste materials, or sediment where vehicles enter or exit the site;
- Tracking or blowing of raw, final, or waste materials from areas of no exposure to exposed areas; and
- Control measures needing replacement, maintenance, or repair.

Stormwater control measures required by this permit must be observed to ensure that they are functioning correctly. If discharge locations are inaccessible, nearby downstream locations must be inspected.

Your annual comprehensive site inspection may also be used as one of the routine inspections, as long as all components of both types of inspections are included.

4.3.2 Comprehensive Site Inspection Documentation.

You must document the findings of each comprehensive site inspection and maintain this documentation onsite with your SWPPP as required in Part 5.4. In addition, you must submit this documentation in an annual report as required in Part 7.2. At a minimum, your documentation of the comprehensive site inspection must include (see the Annual Reporting Form included as Appendix I):

- The date of the inspection;
- The name(s) and title(s) of the personnel making the inspection;
- Findings from the examination of areas of your facility identified in Part 4.3.1;
- All observations relating to the implementation of your control measures including:
  - previously unidentified discharges from the site,
  - previously unidentified pollutants in existing discharges,
  - evidence of, or the potential for, pollutants entering the drainage system;
  - evidence of pollutants discharging to receiving waters at all facility outfall(s), and the condition of and around the outfall, including flow dissipation measures to prevent scouring, and
  - additional control measures needed to address any conditions requiring corrective action identified during the inspection.
- Any required revisions to the SWPPP resulting from the inspection;
- Any incidents of noncompliance observed or a certification stating the facility is in compliance with this permit (if there is no noncompliance); and
- A statement, signed and certified in accordance with Appendix B, Subsection 11 of the permit.

Any corrective action required as a result of the comprehensive site inspection must be performed consistent with Part 3 of this permit.

5. Stormwater Pollution Prevention Plan (SWPPP).

You must prepare a SWPPP for your facility before submitting your Notice of Intent (NOI) for permit coverage. If you prepared a SWPPP for coverage under a previous NPDES permit, you must review and update the SWPPP to implement all provisions of this permit prior to submitting your NOI. The SWPPP does not contain effluent limitations; the limitations are contained in Part 2 of the permit, and for some sectors, Parts 8 and 9 of the permit. The SWPPP is intended to document the selection, design, and installation of control measures. As distinct from the SWPPP, the additional documentation requirements (see Part 5.4) are intended to

document the implementation (including inspection, maintenance, monitoring, and corrective action) of the permit requirements.

5.1 Contents of Your SWPPP.

For coverage under this permit, your SWPPP must contain all of the following elements:

- Stormwater pollution prevention team (see Part 5.1.1);
- Site description (see Part 5.1.2);
- Summary of potential pollutant sources (see Part 5.1.3);
- Description of control measures (see Part 5.1.4);
- Schedules and procedures (see Part 5.1.5);
- Documentation to support eligibility considerations under other federal laws (see Part 5.1.6); and
- Signature requirements (see Part 5.1.7).

Where your SWPPP refers to procedures in other facility documents, such as a Spill Prevention, Control and Countermeasure (SPCC) Plan or an Environmental Management System (EMS) developed for a National Environmental Performance Track facility, copies of the relevant portions of those documents must be kept with your SWPPP.

5.1.1 Stormwater Pollution Prevention Team.

You must identify the staff members (by name or title) that comprise the facility's stormwater pollution prevention team as well as their individual responsibilities. Your stormwater pollution prevention team is responsible for assisting the facility manager in developing and revising the facility's SWPPP as well as maintaining control measures and taking corrective actions where required. Each member of the stormwater pollution prevention team must have ready access to either an electronic or paper copy of applicable portions of this permit and your SWPPP.

5.1.2 Site Description.

Your SWPPP must include the following:

- *Activities at the Facility.* Provide a description of the nature of the industrial activities at your facility.
- *General location map.* Provide a general location map (e.g., U.S. Geological Survey (USGS) quadrangle map) with enough detail to identify the location of your facility and all receiving waters for your stormwater discharges.
- *Site map.* Provide a map showing:
  - the size of the property in acres;
  - the location and extent of significant structures and impervious surfaces;
  - directions of stormwater flow (use arrows);
  - locations of all existing structural control measures;

- locations of all receiving waters in the immediate vicinity of your facility, indicating if any of the waters are impaired and, if so, whether the waters have TMDLs established for them;
- locations of all stormwater conveyances including ditches, pipes, and swales;
- locations of potential pollutant sources identified under Part 5.1.3.2;
- locations where significant spills or leaks identified under Part 5.1.3.3 have occurred;
- locations of all stormwater monitoring points;
- locations of stormwater inlets and outfalls, with a unique identification code for each outfall (e.g., Outfall No. 1, No. 2, etc), indicating if you are treating one or more outfalls as "substantially identical" under Parts 4.2.3, 5.1.5.2, and 6.1.1, and an approximate outline of the areas draining to each outfall;
- municipal separate storm sewer systems, where your stormwater discharges to them;
- locations and descriptions of all non-stormwater discharges identified under Part 2.1.2.10;
- locations of the following activities where such activities are exposed to precipitation:
  - fueling stations;
  - vehicle and equipment maintenance and/or cleaning areas;
  - loading/unloading areas;
  - locations used for the treatment, storage, or disposal of wastes;
  - liquid storage tanks;
  - processing and storage areas;
  - immediate access roads and rail lines used or traveled by carriers of raw materials, manufactured products, waste material, or by-products used or created by the facility;
  - transfer areas for substances in bulk; and
  - machinery; and
- locations and sources of run-on to your site from adjacent property that contains significant quantities of pollutants.

5.1.3 Summary of Potential Pollutant Sources.

You must document areas at your facility where industrial materials or activities are exposed to stormwater and from which allowable non-stormwater discharges are released. *Industrial materials or activities* include, but are not limited to: material handling equipment or activities; industrial machinery; raw materials; industrial production and processes; and intermediate products, by-products, final products, and waste products. *Material handling activities* include, but are not limited to: the storage, loading and unloading, transportation, disposal, or conveyance of any raw material, intermediate product, final product or waste product. For each area identified, the description must include:

5.1.3.1 Activities in the area. A list of the industrial activities exposed to stormwater (e.g., material storage; equipment fueling, maintenance, and cleaning; cutting steel beams).

5.1.3.2 Pollutants. A list of the pollutant(s) or pollutant constituents (e.g., crankcase oil, zinc, sulfuric acid, and cleaning solvents) associated with each identified activity. The pollutant list must include all significant materials that have been handled, treated, stored, or disposed, and that have been exposed to stormwater in the 3 years prior to the date you prepare or amend your SWPPP.

5.1.3.3 Spills and Leaks. You must document where potential spills and leaks could occur that could contribute pollutants to stormwater discharges, and the corresponding outfall(s) that would be affected by such spills and leaks. You must document all significant spills and leaks of oil or toxic or hazardous pollutants that actually occurred at exposed areas, or that drained to a stormwater conveyance, in the 3 years prior to the date you prepare or amend your SWPPP.

Note: Significant spills and leaks include, but are not limited to, releases of oil or hazardous substances in excess of quantities that are reportable under CWA Section 311 (see 40 CFR 110.6 and 40 CFR 117.21) or Section 102 of the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA), 42 USC §9602. This permit does not relieve you of the reporting requirements of 40 CFR 110, 40 CFR 117, and 40 CFR 302 relating to spills or other releases of oils or hazardous substances.

5.1.3.4 Non-Stormwater Discharges. You must document that you have evaluated for the presence of non-stormwater discharges and that all unauthorized discharges have been eliminated. Documentation of your evaluation must include:

- The date of any evaluation;
- A description of the evaluation criteria used;
- A list of the outfalls or onsite drainage points that were directly observed during the evaluation;
- The different types of non-stormwater discharge(s) and source locations; and
- The action(s) taken, such as a list of control measures used to eliminate unauthorized discharge(s), if any were identified. For example, a floor drain was sealed, a sink drain was re-routed to sanitary, or an NPDES permit application was submitted for an unauthorized cooling water discharge.

5.1.3.5 Salt Storage. You must document the location of any storage piles containing salt used for deicing or other commercial or industrial purposes.

5.1.3.6 Sampling Data. You must summarize all stormwater discharge sampling data collected at your facility during the previous permit term.

5.1.4 Description of Control Measures.

5.1.4.1 Control Measures to Meet Technology-Based and Water Quality-Based Effluent Limits. You must document the location and type of control measures you have installed and implemented at your site to achieve the non-numeric effluent limits in Part 2.1.2, and where applicable in Part 8, the effluent limitations guidelines-based limits in Part 2.1.3,

the water quality-based effluent limits in Part 2.2, and any agreed-upon endangered species or NEPA-related requirements in Parts 2.3 and 2.4, and describe how you addressed the control measure selection and design considerations in Part 2.1.1. This documentation must describe how the control measures at your site address both the pollutant sources identified in Part 5.1.3, and any stormwater run-on that commingles with any discharges covered under this permit.

5.1.5 Schedules and Procedures

5.1.5.1 Pertaining to Control Measures Used to Comply with the Effluent Limits in Part 2. The following must be documented in your SWPPP:

- Good Housekeeping (See Part 2.1.2.2) – A schedule for regular pickup and disposal of waste materials, along with routine inspections for leaks and conditions of drums, tanks and containers;
- Maintenance (See Part 2.1.2.3) – Preventative maintenance procedures, including regular inspections, testing, maintenance, and repair of all industrial equipment and systems, and control measures, to avoid situations that may result in leaks, spills, and other releases, and any back-up practices in place should a runoff event occur while a control measure is off-line;
- Spill Prevention and Response Procedures (See Part 2.1.2.4) – Procedures for preventing and responding to spills and leaks. You may reference the existence of other plans for Spill Prevention Control and Countermeasure (SPCC) developed for the facility under Section 311 of the CWA or BMP programs otherwise required by an NPDES permit for the facility, provided that you keep a copy of that other plan onsite and make it available for review consistent with Part 5.3; and
- Employee Training (Part 2.1.2.9) – A schedule for all types of necessary training.

5.1.5.2 Pertaining to Monitoring and Inspection. You must document in your SWPPP your procedures for conducting the five types of analytical monitoring specified by this permit, where applicable to your facility, including:

- Benchmark monitoring (see Part 6.2.1);
- Effluent limitations guidelines monitoring (see Part 6.2.2);
- State- or Tribal-specific monitoring (see Part 6.2.3);
- Impaired waters monitoring (see Part 6.2.4); and
- Other monitoring as required by EPA (see Part 6.2.5).

For each type of monitoring, your SWPPP must document:

- Locations where samples are collected, including any determination that two or more outfalls are substantially identical;
- Parameters for sampling and the frequency of sampling for each parameter;
- Schedules for monitoring at your facility, including schedule for alternate monitoring periods for climates with irregular stormwater runoff (see Part 6.1.6);

- Any numeric control values (benchmarks, effluent limitations guidelines, TMDL-related requirements, or other requirements) applicable to discharges from each outfall; and
- Procedures (e.g., responsible staff, logistics, laboratory to be used, etc.) for gathering storm event data, as specified in Part 6.1.

If you are invoking the exception for inactive and unstaffed sites for benchmark monitoring, you must include in your SWPPP the information to support this claim as required by Part 6.2.1.3.

You must document the following in your SWPPP if you plan to use the substantially identical outfall exception for your quarterly visual assessment requirements in Part 4.2 or your benchmark monitoring requirements in Part 6.2.1:

- Location of each of the substantially identical outfalls;
- Description of the general industrial activities conducted in the drainage area of each outfall;
- Description of the control measures implemented in the drainage area of each outfall;
- Description of the exposed materials located in the drainage area of each outfall that are likely to be significant contributors of pollutants to stormwater discharges;
- An estimate of the runoff coefficient of the drainage areas (low = under 40%; medium = 40 to 65%; high = above 65%); and
- Why the outfalls are expected to discharge substantially identical effluents.

You must document in your SWPPP your procedures for performing, as appropriate, the three types of inspections specified by this permit, including:

- Routine facility inspections (see Part 4.1);
- Quarterly visual assessment of stormwater discharges (see Part 4.2); and
- Comprehensive site inspections (see Part 4.3).

For each type of inspection performed, your SWPPP must identify:

- Person(s) or positions of person(s) responsible for inspection;
- Schedules for conducting inspections, including tentative schedule for facilities in climates with irregular stormwater runoff discharges (see Part 4.2.3); and
- Specific items to be covered by the inspection, including schedules for specific outfalls.

If you are invoking the exception for inactive and unstaffed sites relating to routine facility inspections and quarterly visual assessments, you must include in your SWPPP the information to support this claim as required by Parts 4.1.3 and 4.2.3.

5.1.6 Documentation to Support Eligibility Considerations Under Other Federal Laws.

5.1.6.1 Documentation Regarding Endangered Species. You must keep with your SWPPP the documentation supporting your determination with regard to Part 1.1.4.5 (Endangered and Threatened Species and Critical Habitat Protection).

5.1.6.2 Documentation Regarding Historic Properties. You must keep with your SWPPP the documentation supporting your determination with regard to Part 1.1.4.6 (Historic Properties Preservation).

5.1.6.3 Documentation Regarding NEPA Review. You must keep with your SWPPP the documentation supporting your certification of eligibility under Part 1.1.2.5 (Discharges Subject to Any New Source Performance Standards).

5.1.7 Signature Requirements.

You must sign and date your SWPPP in accordance with Appendix B, Subsection 11, including the date of signature.

5.2 Required SWPPP Modifications.

You must modify your SWPPP whenever necessary to address any of the triggering conditions for corrective action in Part 3.1 and to ensure that they do not reoccur, or to reflect changes implemented when a review following the triggering conditions in Part 3.2 indicates that changes to your control measures are necessary to meet the effluent limits in this permit. Changes to your SWPPP document must be made in accordance with the corrective action deadlines in Parts 3.3 and 3.4, and must be signed and dated in accordance with Appendix B, Subsection 11.

5.3 SWPPP Availability.

You must retain a copy of the current SWPPP required by this permit at the facility, and it must be immediately available to EPA; a State, Tribal, or local agency approving stormwater management plans; the operator of an MS4 receiving discharges from the site; and representatives of the U.S. Fish and Wildlife Service (USFWS) or the National Marine Fisheries Service (NMFS) at the time of an onsite inspection or upon request. EPA may provide access to portions of your SWPPP to a member of the public upon request. Confidential Business Information (CBI) may be withheld from the public, but may not be withheld from those staff cleared for CBI review within EPA, USFWS, or NMFS.

EPA encourages you to post your SWPPP online and provide the website address on your NOI.

5.4 Additional Documentation Requirements.

You are required to keep the following inspection, monitoring, and certification records with your SWPPP that together keep your records complete and up-to-date, and demonstrate your full compliance with the conditions of this permit:

- A copy of the NOI submitted to EPA along with any correspondence exchanged between you and EPA specific to coverage under this permit;
- A copy of the acknowledgment letter you receive from the NOI Processing Center or eNOI system assigning your permit tracking number;
- A copy of this permit (an electronic copy easily available to SWPPP personnel is also acceptable);
- Descriptions and dates of any incidences of significant spills, leaks, or other releases that resulted in discharges of pollutants to waters of the U.S., through stormwater or otherwise; the circumstances leading to the release and actions taken in response to the release; and measures taken to prevent the recurrence of such releases (see Part 2.1.2.4);
- Records of employee training, including date training received (see Part 2.1.2.9);
- Documentation of maintenance and repairs of control measures, including the date(s) of regular maintenance, date(s) of discovery of areas in need of repair/replacement, and for repairs, date(s) that the control measure(s) returned to full function, and the justification for any extended maintenance/repair schedules (see Part 2.1.2.3);
- All inspection reports, including the Routine Facility Inspection Reports (see Part 4.1), the Quarterly Visual Assessment Reports (see Part 4.2), and the Comprehensive Site Inspection Reports (see Part 4.3);
- Description of any deviations from the schedule for visual assessments and/or monitoring, and the reason for the deviations (e.g., adverse weather or it was impracticable to collect samples within the first 30 minutes of a measurable storm event) (see Parts 4.2.1, 6.1.4, and 6.2.1.2);
- Description of any corrective action taken at your site, including triggering event and dates when problems were discovered and modifications occurred;
- Documentation of any benchmark exceedances and how they were responded to, including either (1) corrective action taken, (2) a finding that the exceedence was due to natural background pollutant levels, or (3) a finding that no further pollutant reductions were technologically available and economically practicable and achievable in light of best industry practice consistent with Part 6.2.1.2;
- Documentation to support any determination that pollutants of concern are not expected to be present above natural background levels if you discharge directly to impaired waters, and that such pollutants were not detected in your discharge or were solely attributable to natural background sources (see Part 6.2.4.2); and
- Documentation to support your claim that your facility has changed its status from active to inactive and unstaffed with respect to the requirements to conduct routine facility inspections (see Part 4.1.3), quarterly visual assessments (see Part 4.2.3), and/or benchmark monitoring (see Part 6.2.1.3).

6. Monitoring.

You must collect and analyze stormwater samples and document monitoring activities consistent with the procedures described in Part 6 and Appendix B, Subsections 10 – 12, and any additional sector-specific or State/Tribal-specific requirements in Parts 8 and 9, respectively. Refer to Part 7 for reporting and recordkeeping requirements.

6.1 Monitoring Procedures

6.1.1 Monitored Outfalls.

Applicable monitoring requirements apply to each outfall authorized by this permit, except as otherwise exempt from monitoring as a "substantially identical outfall." If your facility has two or more outfalls that you believe discharge substantially identical effluents, based on the similarities of the general industrial activities and control measures, exposed materials that may significantly contribute pollutants to stormwater, and runoff coefficients of their drainage areas, you may monitor the effluent of just one of the outfalls and report that the results also apply to the substantially identical outfall(s). As required in Part 5.1.5.2, your SWPPP must identify each outfall authorized by this permit and describe the rationale for any substantially identical outfall determinations. The allowance for monitoring only one of the substantially identical outfalls is not applicable to any outfalls with numeric effluent limitations. You are required to monitor each outfall covered by a numeric effluent limit as identified in Part 6.2.2.

6.1.2 Commingled Discharges.

If discharges authorized by this permit commingle with discharges not authorized under this permit, any required sampling of the authorized discharges must be performed at a point before they mix with other waste streams, to the extent practicable.

6.1.3 Measurable Storm Events.

All required monitoring must be performed on a storm event that results in an actual discharge from your site ("measurable storm event") that follows the preceding measurable storm event by at least 72 hours (3 days). The 72-hour (3-day) storm interval does not apply if you are able to document that less than a 72-hour (3-day) interval is representative for local storm events during the sampling period. In the case of snowmelt, the monitoring must be performed at a time when a measurable discharge occurs at your site.

For each monitoring event, except snowmelt monitoring, you must identify the date and duration (in hours) of the rainfall event, rainfall total (in inches) for that rainfall event, and time (in days) since the previous measurable storm event. For snowmelt monitoring, you must identify the date of the sampling event.

6.1.4 Sample Type.

You must take a minimum of one grab sample from a discharge resulting from a measurable storm event as described in Part 6.1.3. Samples must be collected within the first 30 minutes of a measurable storm event. If it is not possible to collect the sample within the first 30 minutes of a measurable storm event, the sample must be collected as soon as practicable after the first 30 minutes and documentation must be kept with the SWPPP explaining why it was not possible to take samples within the first 30 minutes. In the case of snowmelt, samples must be taken during a period with a measurable discharge.

6.1.5 Adverse Weather Conditions.

When adverse weather conditions as described in Part 4.2.3 prevent the collection of samples according to the relevant monitoring schedule, you must take a substitute sample during the next qualifying storm event. Adverse weather does not exempt you from having to file a benchmark monitoring report in accordance with your sampling schedule. You must report any failure to monitor as specified in Part 7.1 indicating the basis for not sampling during the usual reporting period.

6.1.6 Climates with Irregular Stormwater Runoff.

If your facility is located in areas where limited rainfall occurs during parts of the year (e.g., arid or semi-arid climates) or in areas where freezing conditions exist that prevent runoff from occurring for extended periods, required monitoring events may be distributed during seasons when precipitation occurs, or when snowmelt results in a measurable discharge from your site. You must still collect the required number of samples.

6.1.7 Monitoring Periods.

Monitoring requirements in this permit begin in the first full quarter following either April 1, 2009 or your date of discharge authorization, whichever date comes later. If your monitoring is required on a quarterly basis (e.g., benchmark monitoring), you must monitor at least once in each of the following 3-month intervals:

- January 1 – March 31;
- April 1 – June 30;
- July 1 – September 30; and
- October 1 – December 31.

For example, if you obtain permit coverage on June 2, 2009, then your first monitoring quarter is July 1 - September 30, 2009. This monitoring schedule may be modified in accordance with Part 6.1.6 if the revised schedule is documented with your SWPPP and provided to EPA with your first monitoring report.

6.1.8 Monitoring for Allowable Non-Stormwater Discharges

You are only required to monitor allowable non-stormwater discharges (as delineated in Part 1.1.3) when they are commingled with stormwater discharges associated with industrial activity.

6.2 Required Monitoring.

This permit includes five types of required analytical monitoring, one or more of which may apply to your discharge:

- Quarterly benchmark monitoring (see Part 6.2.1)
- Annual effluent limitations guidelines monitoring (see Part 6.2.2);
- State- or Tribal-specific monitoring (see Part 6.2.3);
- Impaired waters monitoring (see Part 6.2.4); and
- Other monitoring as required by EPA (see Part 6.2.5).

When more than one type of monitoring for the same parameter at the same outfall applies (e.g., total suspended solids once per year for an effluent limit and once per quarter for benchmark monitoring at a given outfall), you may use a single sample to satisfy both monitoring requirements (i.e., one sample satisfying both the annual effluent limit sample and one of the 4 quarterly benchmark monitoring samples).

All required monitoring must be conducted in accordance with the procedures described in Appendix B, Subsection 10.D.

6.2.1 Benchmark Monitoring.

This permit stipulates pollutant benchmark concentrations that may be applicable to your discharge. The benchmark concentrations are not effluent limitations; a benchmark exceedance, therefore, is not a permit violation. Benchmark monitoring data are primarily for your use to determine the overall effectiveness of your control measures and to assist you in knowing when additional corrective action(s) may be necessary to comply with the effluent limitations in Part 2.

6.2.1.1 Applicability of Benchmark Monitoring. You must monitor for any benchmark parameters specified for the industrial sector(s), both primary industrial activity and any co-located industrial activities, applicable to your discharge. Your industry-specific benchmark concentrations are listed in the sector-specific sections of Part 8. If your facility is in one of the industrial sectors subject to benchmark concentrations that are hardness-dependent, you are required to submit to EPA with your first benchmark report a hardness value, established consistent with the procedures in Appendix J, which is representative of your receiving water.

Samples must be analyzed consistent with 40 CFR Part 136 analytical methods and using test procedures with quantitation limits at or below benchmark values for all benchmark parameters for which you are required to sample.

6.2.1.2 Benchmark Monitoring Schedule. Benchmark monitoring must be conducted quarterly, as identified in Part 6.1.7, for your first 4 full quarters of permit coverage commencing no earlier than April 1, 2009. Facilities in climates with irregular stormwater runoff, as described in Part 6.1.6, may modify this quarterly schedule provided that this revised schedule is reported to EPA when the first benchmark sample is collected and reported, and that this revised schedule is kept with the facility's SWPPP as specified in Part 5.4.

Data not exceeding benchmarks: After collection of 4 quarterly samples, if the average of the 4 monitoring values for any parameter does not exceed the benchmark, you have fulfilled your monitoring requirements for that parameter for the permit term. For averaging purposes, use a value of zero for any individual sample parameter, analyzed using procedures consistent with Part 6.2.1.1, which is determined to be less than the method detection limit. For sample values that fall between the method detection level and the quantitation limit (i.e., a confirmed detection but below the level that can be reliably quantified), use a value halfway between zero and the quantitation limit.

Data exceeding benchmarks: After collection of 4 quarterly samples, if the average of the 4 monitoring values for any parameter exceeds the benchmark, you must, in accordance with Part 3.2, review the selection, design, installation, and implementation of your control measures to determine if modifications are necessary to meet the effluent limits in this permit, and either:

- Make the necessary modifications and continue quarterly monitoring until you have completed 4 additional quarters of monitoring for which the average does not exceed the benchmark; or

- Make a determination that no further pollutant reductions are technologically available and economically practicable and achievable in light of best industry practice to meet the technology-based effluent limits or are necessary to meet the water-quality-based effluent limitations in Parts 2 of this permit, in which case you must continue monitoring once per year. You must also document your rationale for concluding that no further pollutant reductions are achievable, and retain all records related to this documentation with your SWPPP. You must also notify EPA of this determination in your next benchmark monitoring report.

In accordance with Part 3.2, you must review your control measures and perform any required corrective action immediately (or document why no corrective action is required), without waiting for the full 4 quarters of monitoring data, if an exceedance of the 4 quarter average is mathematically certain. If after modifying your control measures and conducting 4 additional quarters of monitoring, your average still exceeds the benchmark (or if an exceedance of the benchmark by the 4 quarter average is mathematically certain prior to conducting the full 4 additional quarters of monitoring), you must again review your control measures and take one of the two actions above.

***Natural background pollutant levels*:** Following the first 4 quarters of benchmark monitoring (or sooner if the exceedance is triggered by less than 4 quarters of data, see above), if the average concentration of a pollutant exceeds a benchmark value, and you determine that exceedance of the benchmark is attributable solely to the presence of that pollutant in the natural background, you are not required to perform corrective action or additional benchmark monitoring provided that:

- The average concentration of your benchmark monitoring results is less than or equal to the concentration of that pollutant in the natural background;
- You document and maintain with your SWPPP, as required in Part 5.4, your supporting rationale for concluding that benchmark exceedances are in fact attributable solely to natural background pollutant levels. You must include in your supporting rationale any data previously collected by you or others (including literature studies) that describe the levels of natural background pollutants in your stormwater discharge; and
- You notify EPA on your final quarterly benchmark monitoring report that the benchmark exceedances are attributable solely to natural background pollutant levels.

Natural background pollutants include those substances that are naturally occurring in soils or groundwater. Natural background pollutants do not include legacy pollutants from earlier activity on your site, or pollutants in run-on from neighboring sources which are not naturally occurring.

6.2.1.3 Exception for Inactive and Unstaffed Sites. The requirement for benchmark monitoring does not apply at a facility that is inactive and unstaffed, as long as there are no industrial materials or activities exposed to stormwater. To invoke this exception, you must do the following:

- Maintain a statement onsite with your SWPPP stating that the site is inactive and unstaffed, and that there are no industrial materials or activities exposed to stormwater in accordance with the substantive requirements in 40 CFR 122.26(g) and sign and certify the statement in accordance with Appendix B, Subsection 11; and
- If circumstances change and industrial materials or activities become exposed to stormwater or your facility becomes active and/or staffed, this exception no longer applies and you must immediately begin complying with the applicable benchmark monitoring requirements under Part 6.2 as if you were in your first year of permit coverage. You must indicate in your first benchmark monitoring report that your facility has materials or activities exposed to stormwater or has become active and/or staffed.
- If you are not qualified for this exception at the time you are authorized under this permit, but during the permit term you become qualified because your facility is inactive and unstaffed, and there are no industrial materials or activities that are exposed to stormwater, then you must notify EPA of this change in your next benchmark monitoring report. You may discontinue benchmark monitoring once

you have notified EPA, and prepared and signed the certification statement described above concerning your facility's qualification for this special exception.

Note: This exception has different requirements for Sectors G, H, and J (see Part 8).

6.2.2 Effluent Limitations Monitoring.

6.2.2.1 Monitoring Based on Effluent Limitations Guidelines. Table 6-1 identifies the stormwater discharges subject to effluent limitation guidelines that are authorized for coverage under this permit. Beginning in the first full quarter following April 1, 2009 or your date of discharge authorization, whichever date comes later, you must monitor once per year at each outfall containing the discharges identified in Table 6-1 for the parameters specified in the sector-specific section of Part 8.

Table 6-1. Required Monitoring for Effluent Limits Based on Effluent Limitations Guidelines

Regulated Activity	Effluent Limit	Monitoring Frequency	Sample Type
Discharges resulting from spray down or intentional wetting of logs at wet deck storage areas	See Part 8.A.7	1/year	Grab
Runoff from phosphate fertilizer manufacturing facilities that comes into contact with any raw materials, finished product, by-products or waste products (SIC 2874)	See Part 8.C.4	1/year	Grab
Runoff from asphalt emulsion facilities	See Part 8.D.4	1/year	Grab
Runoff from material storage piles at cement manufacturing facilities	See Part 8.E.5	1/year	Grab
Mine dewatering discharges at crushed stone, construction sand and gravel, or industrial sand mining facilities	See Part 8.J.9	1/year	Grab
Runoff from hazardous waste landfills	See Part 8.K.6	1/year	Grab
Runoff from non-hazardous waste landfills	See Part 8.L.10	1/year	Grab
Runoff from coal storage piles at steam electric generating facilities	See Part 8.O.8	1/year	Grab

6.2.2.2 Substantially Identical Outfalls. You must monitor each outfall discharging runoff from any regulated activity identified in Table 6-1. The substantially identical outfall monitoring provisions are not available for numeric effluent limits monitoring.

6.2.3 State or Tribal Provisions Monitoring

6.2.3.1 Sectors Required to Conduct State or Tribal Monitoring. You must comply with any State or Tribal monitoring requirements (see Part 9) applicable to your facility's location.

6.2.3.2 State or Tribal Monitoring Schedule. If a monitoring frequency is not specified for an applicable requirement in Part 9, you must monitor once per year for the entire permit term.

6.2.4 Discharges to Impaired Waters Monitoring.

6.2.4.1 Permittees Required to Monitor Discharges to Impaired Waters. If you discharge to an impaired water, you must monitor for all pollutants for which the waterbody is impaired and for which a standard analytical method exists (see 40 CFR Part 136).

If the pollutant for which the waterbody is impaired is suspended solids, turbidity or sediment/sedimentation, you must monitor for Total Suspended Solids (TSS). If the pollutant for which the waterbody is impaired is expressed in the form of an indicator or surrogate pollutant, you must monitor for that indicator or surrogate pollutant. No monitoring is required when a waterbody's biological communities are impaired but no pollutant, including indicator or surrogate pollutants, is specified as causing the impairment, or when a waterbody's impairment is related to hydrologic modifications, impaired hydrology, or temperature.

6.2.4.2 Impaired Waters Monitoring Schedule.

Discharges to impaired waters without an EPA approved or established TMDL:
Beginning in the first full quarter following April 1, 2009 or your date of discharge authorization, whichever date comes later, you must monitor once per year at each outfall (except substantially identical outfalls) discharging stormwater to impaired waters without an EPA approved or established TMDL. This monitoring requirement does not apply after one year if the pollutant for which the waterbody is impaired is not detected above natural background levels in your stormwater discharge, and you document, as required in Part 5.4 (Additional Documentation Requirements), that this pollutant is not expected to be present above natural background levels in your discharge.

If the pollutant for which the water is impaired is not present and not expected to be present in your discharge, or it is present but you have determined that its presence is caused solely by natural background sources, you should include a notification to this effect in your first monitoring report, after which you may discontinue annual monitoring. To support a determination that the pollutant's presence is caused solely by natural background sources, you must keep the following documentation with your SWPPP records:

- An explanation of why you believe that the presence of the pollutant causing the impairment in your discharge is not related to the activities at your facility; and
- Data and/or studies that tie the presence of the pollutant causing the impairment in your discharge to natural background sources in the watershed.

Natural background pollutants include those substances that are naturally occurring in soils or groundwater. Natural background pollutants do not include legacy pollutants from earlier activity on your site, or pollutants in run-on from neighboring sources which are not naturally occurring.

Discharges to impaired waters with an EPA approved or established TMDL: For stormwater discharges to waters for which there is an EPA approved or established TMDL, you are not required to monitor for the pollutant for which the TMDL was written unless EPA informs you, upon examination of the applicable TMDL and/or WLA, that you are subject to such a requirement consistent with the assumptions of the applicable TMDL and/or WLA. EPA's notice will include specifications on which pollutant to monitor and the required monitoring frequency during the first year of permit coverage. Following the first year of monitoring:

- If the TMDL pollutant is not detected in any of your first year samples, you may discontinue further sampling, unless the TMDL has specific instructions to the contrary, in which case you must follow those instructions. You must keep records of this finding onsite with your SWPPP.

- If you detect the presence of the pollutant causing the impairment in your stormwater discharge for any of the samples collected in your first year, you must continue monitoring annually throughout the term of this permit, unless the TMDL specifies more frequent monitoring, in which case you must follow the TMDL requirements.

6.2.5 Additional Monitoring Required by EPA.

EPA may notify you of additional discharge monitoring requirements. Any such notice will briefly state the reasons for the monitoring, locations, and parameters to be monitored, frequency and period of monitoring, sample types, and reporting requirements.

6.3 Follow-up Actions if Discharge Exceeds Numeric Effluent Limit.

You must conduct follow-up monitoring within 30 calendar days (or during the next qualifying runoff event, should none occur within 30 days) of implementing corrective action(s) taken pursuant to Part 3 in response to an exceedance of a numeric effluent limit contained in this permit. See Part 9 for specific monitoring requirements applicable to individual States or Tribes. Monitoring must be performed for any pollutant(s) that exceeds the effluent limit. If this follow-up monitoring exceeds the applicable effluent limitation, you must comply with both Parts 6.3.1 and 6.3.2.

6.3.1 Submit an Exceedance Report.

You must submit an Exceedance Report consistent with Part 7.3.

6.3.2 Continue to Monitor.

You must continue to monitor, at least quarterly, until your discharge is in compliance with the effluent limit or until EPA waives the requirement for additional monitoring.

7. Reporting and Recordkeeping

7.1 Reporting Monitoring Data to EPA.

All monitoring data collected pursuant to Parts 6.2 and 6.3 must be submitted to EPA using EPA's online eNOI system (www.epa.gov/npdes/eNOI) no later than 30 days (email date or postmark date) after you have received your complete laboratory results for all monitored outfalls for the reporting period. If you cannot access eNOI, paper reporting forms must be submitted by the same deadline to the appropriate address identified in Part 7.6.1. If you are using paper reporting forms, EPA strongly recommends that you use the MSGP discharge monitoring report (MDMR) available at www.epa.gov/npdes/stormwater/msgp. See Part 9 for specific reporting requirements applicable to individual States or Tribes.

For benchmark monitoring, note that you are required to submit sampling results to EPA no later than 30 days after receiving laboratory results for each quarter that you are required to collect benchmark samples, in accordance with Part 6.2.1.2. If you collect multiple samples in a single quarter (e.g., due to adverse weather conditions, climates with irregular stormwater runoff, or areas subject to snow), you are required to submit all sampling results to EPA within 30 days of receiving the laboratory results.

7.2 Annual Report

You must submit an annual report to EPA that includes the findings from your Part 4.3 comprehensive site inspection and any corrective action documentation as required in Part 3.4. If corrective action is not yet completed at the time of submission of this annual report, you must describe the status of any outstanding corrective action(s). In addition to the information required in Parts 3.4 (Corrective Action Report) and 4.3.2 (Comprehensive Site Inspection Documentation), you must include the following information with your annual report:

- Facility name
- NPDES permit tracking number
- Facility physical address
- Contact person name, title, and phone number

EPA strongly recommends that you submit this report using the Annual Reporting Form provided as Appendix I. You must submit the annual report to EPA within 45 days (postmark date) after conducting the comprehensive site inspection to the address identified in Part 7.6.1.

7.3 Exceedance Report for Numeric Effluent Limits

If follow-up monitoring pursuant to Part 6.3 exceeds a numeric effluent limit, you must submit an Exceedance Report to EPA no later than 30 days after you have received your lab results. Your report must include the following:

- NPDES permit tracking number;

- Facility name, physical address and location;
- Name of receiving water;
- Monitoring data from this and the preceding monitoring event(s);
- An explanation of the situation; what you have done and intend to do (should your corrective actions not yet be complete) to correct the violation; and
- An appropriate contact name and phone number.

7.4 Additional Reporting.

In addition to the reporting requirements stipulated in Part 7, you are also subject to the standard permit reporting provisions of Appendix B, Subsection 12.

Where applicable, you must submit the following reports to the appropriate EPA Regional Office listed in Part 7.6.2, as applicable. If you discharge through an MS4, you must also submit these reports to the MS4 operator (identified pursuant to Part 5.1.2).

- 24-hour reporting (see Appendix B, Subsection 12.F) - You must report any noncompliance which may endanger health or the environment. Any information must be provided orally within 24 hours from the time you become aware of the circumstances;
- 5-day follow-up reporting to the 24 hour reporting (see Appendix B, Subsection 12.F) - A written submission must also be provided within five days of the time you become aware of the circumstances;
- Reportable quantity spills (see Part 2.1.2.4) - You must provide notification, as required under Part 2.1.2.4, as soon as you have knowledge of a leak, spill, or other release containing a hazardous substance or oil in an amount equal to or in excess of a reportable quantity.

Where applicable, you must submit the following reports to EPA Headquarters at the appropriate address in Part 7.6.1:

- Planned changes (see Appendix B, Subsection 12.A) – You must give notice to EPA as soon as possible of any planned physical alterations or additions to the permitted facility that qualify the facility as a new source or that could significantly change the nature or significantly increase the quantity of pollutants discharged;
- Anticipated noncompliance (see Appendix B, Subsection 12.B) – You must give advance notice to EPA of any planned changes in the permitted facility or activity which you anticipate will result in noncompliance with permit requirements;
- Transfer of ownership and/or operation – You must submit a complete and accurate NOI in accordance with the requirements of Appendix G of this permit and by the deadlines specified in Table 1-2;
- Compliance schedules (see Appendix B, Subsection 12.F) - Reports of compliance or noncompliance with, or any progress reports on, interim and final requirements contained in any compliance schedule of this permit must be submitted no later than 14 days following each schedule date;

- Other noncompliance (see Appendix B, Subsection 12.G) - You must report all instances of noncompliance not reported in your monitoring report (pursuant to Part 7.1), compliance schedule report, or 24-hour report at the time monitoring reports are submitted; and
- Other information (see Appendix B, Subsection 12.H) – You must promptly submit facts or information if you become aware that you failed to submit relevant facts in your NOI, or that you submitted incorrect information in your NOI or in any report.

7.5 Recordkeeping.

You must retain copies of your SWPPP (including any modifications made during the term of this permit), additional documentation requirements pursuant to Part 5.4 (including documentation related to corrective actions taken pursuant to Part 3), all reports and certifications required by this permit, monitoring data, and records of all data used to complete the NOI to be covered by this permit, for a period of at least 3 years from the date that your coverage under this permit expires or is terminated.

7.6 Addresses for Reports

7.6.1 EPA Addresses

Paper copies of any reports required in Part 6 and 7, not otherwise submitted electronically via EPA's eNOI system (www.epa.gov/npdes/eNOI) must be sent to one of the following addresses:

Via U.S. mail:

U.S. Environmental Protection Agency
Office of Water, Water Permits Division
Mail Code 4203M, ATTN: MSGP Reports
1200 Pennsylvania Avenue, NW
Washington, D.C. 20460

Or Via Overnight/Express Delivery:

U.S. Environmental Protection Agency
Office of Water, Water Permits Division
Room 7420, ATTN: MSGP Reports
1201 Constitution Avenue, NW
Washington, D.C. 20004
Phone number: 202-564-9545

Notices of Intent and Notices of Termination should be submitted using EPA's eNOI system (www.epa.gov/npdes/eNOI) or sent to EPA's NOI Center (see Appendix G for the address).

All other written correspondence concerning discharges in any State, Indian Country land, Territory, or from any Federal facility covered under this permit and directed to the EPA, including individual permit applications, must be sent to the address of the appropriate EPA Regional Office listed below:

7.6.2 Regional Addresses

7.6.2.1 Region 1: Connecticut, Massachusetts, and New Hampshire, Rhode Island, Vermont.

U.S. EPA Region 1
Office of Ecosystem Protection
One Congress Street - CIP
Boston, MA 02114

7.6.2.2 Region 2: New Jersey, New York, Puerto Rico, and Virgin Islands.

For Puerto Rico and the Virgin Islands

U.S. EPA Region 2
Caribbean Environmental Protection Division
Environmental Management Branch
Centro Europa Building
1492 Ponce de Leon Avenue, Suite 417
San Juan, PR 00907-4127

For New Jersey and New York:

(Coverage not available under this permit.)

U.S. EPA Region 2
Division of Environmental Planning and Protection
290 Broadway
New York, NY 10007-1866

7.6.2.3 Region 3: Delaware, District of Columbia, Maryland, Pennsylvania, Virginia, West Virginia.

U.S. EPA Region 3
Water Protection Division (3WP40)
Stormwater Coordinator
1650 Arch Street
Philadelphia, PA 19103

7.6.2.4 Region 4: Alabama, Florida, Georgia, Kentucky, Mississippi, North Carolina, South Carolina, Tennessee.

(Coverage not available under this permit.)

U.S. EPA Region 4
Clean Water Act Enforcement Section
Water Programs Enforcement Branch
Water Management Division
Atlanta Federal Center
61 Forsyth Street SW
Atlanta, GA 30303

7.6.2.5 Region 5: Illinois, Indiana, Michigan, Minnesota, Ohio, Wisconsin.

U.S. EPA Region 5
Water Division
NPDES Programs Branch
77 W. Jackson Blvd.
Mail Code WN16J
Chicago, IL 60604

7.6.2.6 Region 6: Arkansas, Louisiana, Oklahoma, Texas, and New Mexico (except see Region 9 for Navajo lands, and see Region 8 for Ute Mountain Reservation lands).

U.S. EPA Region 6
Stormwater Coordinator
Compliance Assurance and Enforcement Division (6EN-WC)
EPA SW MSGP
P.O. Box 50625
Dallas, TX 75205

7.6.2.7 Region 7: Iowa, Kansas, Missouri, Nebraska.

(Coverage not available under this permit.)

U.S. EPA - Region 7
901 N. 5th Street
Kansas City, KS 66101

7.6.2.8 Region 8: Colorado, Montana, North Dakota, South Dakota, Wyoming, Utah (except see Region 9 for Goshute Reservation and Navajo Reservation lands), the Ute Mountain Reservation in New Mexico, and the Pine Ridge Reservation in Nebraska.

(Coverage not available under this permit.)

U.S. EPA Region 8
Stormwater Coordinator (8P-W-P)
999 18th Street, Suite 300
Denver, CO 80202-2466

7.6.2.9 Region 9: Arizona, California, Hawaii, Nevada, Guam, American Samoa, the Commonwealth of the Northern Mariana Islands, the Goshute Reservation in Utah and Nevada, the Navajo Reservation in Utah, New Mexico, and Arizona, the Duck Valley Reservation in Idaho, Fort McDermitt Reservation in Oregon.

U.S. EPA Region 9
Water Management Division, WTR-5
Stormwater Coordinator
75 Hawthorne Street
San Francisco, CA 94105

7.6.2.10 Region 10: Alaska, Idaho, Oregon (except see Region 9 for Fort McDermitt Reservation), Washington.

U.S. EPA Region 10
Office of Water and Watersheds OWW-130
Stormwater Coordinator
1200 6th Avenue
Seattle, WA 98101

7.6.3 State and Tribal Addresses.

See Part 9 (States and Tribes) for the addresses of applicable States or Tribes that require submission of information to their agencies.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart A – Sector A – Timber Products.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.A.1 Covered Stormwater Discharges.

The requirements in Subpart A apply to stormwater discharges associated with industrial activity from Timber Products facilities as identified by the SIC Codes specified under Sector A in Table D-1 of Appendix D of the permit.

8.A.2 Limitation on Coverage

8.A.2.1 *Prohibition of Discharges.* (See also Part 1.1.4) Not covered by this permit: stormwater discharges from areas where there may be contact with the chemical formulations sprayed to provide surface protection. These discharges must be covered by a separate NPDES permit.

8.A.2.2 *Authorized Non-Stormwater Discharges.* (See also Part 1.1.3) Also authorized by this permit, provided the non-stormwater component of the discharge is in compliance with the requirements in Part 2.1.2 (Non-Numeric Effluent Limits): discharges from the spray down of lumber and wood product storage yards where no chemical additives are used in the spray-down waters and no chemicals are applied to the wood during storage.

8.A.3 Additional Technology-Based Effluent Limits.

8.A.3.1 *Good Housekeeping.* (See also Part 2.1.2.2) In areas where storage, loading and unloading, and material handling occur, perform good housekeeping to limit the discharge of wood debris, minimize the leachate generated from decaying wood materials, and minimize the generation of dust.

8.A.4 Additional SWPPP Requirements.

8.A.4.1 *Drainage Area Site Map.* (See also Part 5.1.2) Document in your SWPPP where any of the following may be exposed to precipitation or surface runoff: processing areas, treatment chemical storage areas, treated wood and residue storage areas, wet decking areas, dry decking areas, untreated wood and residue storage areas, and treatment equipment storage areas.

8.A.4.2 *Inventory of Exposed Materials.* (See also Part 5.1.3.2) Where such information exists, if your facility has used chlorophenolic, creosote, or chromium-copper-arsenic formulations for wood surface protection or preserving, document in your SWPPP the following: areas where contaminated soils, treatment equipment, and stored materials still remain and the management practices employed to minimize the contact of these materials with stormwater runoff.

8.A.4.3 *Description of Stormwater Management Controls.* (See also Part 5.1.4) Document measures implemented to address the following activities and sources: log, lumber, and wood product storage areas; residue storage areas; loading and unloading areas; material handling areas; chemical storage areas; and equipment and vehicle maintenance, storage, and repair areas. If your facility performs wood surface protection and preservation activities, address the specific control measures, including any BMPs, for these activities.

8.A.5 Additional Inspection Requirements.

See also Part 4.1. If your facility performs wood surface protection and preservation activities, inspect processing areas, transport areas, and treated wood storage areas monthly to assess the usefulness of practices to minimize the deposit of treatment chemicals on unprotected soils and in areas that will come in contact with stormwater discharges.

8.A.6 Sector-Specific Benchmarks

Table 8.A-1 identifies benchmarks that apply to the specific subsectors of Sector A. These benchmarks apply to both your primary industrial activity and any co-located industrial activities, which describe your site activities.

Table 8.A-1

Subsector (You may be subject to requirements for more than one sector/subsector)	Parameter	Benchmark Monitoring Concentration
Subsector A1. General Sawmills and Planing Mills (SIC 2421)	Chemical Oxygen Demand (COD)	120.0 mg/L
	Total Suspended Solids (TSS)	100 mg/L
	Total Zinc[1]	Hardness Dependent
Subsector A2. Wood Preserving (SIC 2491)	Total Arsenic	0.15 mg/L
	Total Copper[1]	Hardness Dependent
Subsector A3. Log Storage and Handling (SIC 2411)	Total Suspended Solids (TSS)	100 mg/L
Subsector A4. Hardwood Dimension and Flooring Mills; Special Products Sawmills, not elsewhere classified; Millwork, Veneer, Plywood, and Structural Wood; Wood Pallets and Skids; Wood Containers, not elsewhere classified; Wood Buildings and Mobile Homes; Reconstituted Wood Products; and Wood Products Facilities not elsewhere classified (SIC 2426, 2429, 2431-2439 (except 2434), 2441, 2448, 2449, 2451, 2452, 2493, and 2499)	Chemical Oxygen Demand (COD)	120.0 mg/L
	Total Suspended Solids (TSS)	100.0 mg/L

[1] The benchmark values of some metals are dependent on water hardness. For these parameters, permittees must determine the hardness of the receiving water (see Appendix J, "Calculating Hardness in Receiving Waters for Hardness Dependent Metals," for methodology), in accordance with Part 6.2.1.1, to identify the applicable 'hardness range' for determining their benchmark value applicable to their facility. The ranges occur in 25 mg/L increments. Hardness Dependent Benchmarks follow in the table below:

Water Hardness Range	Copper (mg/L)	Zinc (mg/L)
0-25 mg/L	0.0038	0.04
25-50 mg/L	0.0056	0.05
50-75 mg/L	0.0090	0.08
75-100 mg/L	0.0123	0.11
100-125 mg/L	0.0156	0.13
125-150 mg/L	0.0189	0.16
150-175 mg/L	0.0221	0.18
175-200 mg/L	0.0253	0.20
200-225 mg/L	0.0285	0.23
225-250 mg/L	0.0316	0.25
250+ mg/L	0.0332	0.26

8.A.7 Effluent Limitations Based on Effluent Limitations Guidelines (See also Part 6.2.2.1 of the permit.)

Table 8.A-2 identifies effluent limits that apply to the industrial activities described below. Compliance with these effluent limits is to be determined based on discharges from these industrial activities independent of commingling with any other wastestreams that may be covered under this permit.

Table 8.A-2[1]		
Industrial Activity		
Discharges resulting from spray down or intentional wetting of logs at wet deck storage areas	pH	6.0 - 9.0 s.u
	Debris (woody material such as bark, twigs, branches, heartwood, or sapwood)	No discharge of debris that will not pass through a 2.54-cm (1-in.) diameter round opening

[1] Monitor annually.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart B – Sector B – Paper and Allied Products.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.B.1 Covered Stormwater Discharges.

The requirements in Subpart B apply to stormwater discharges associated with industrial activity from Paper and Allied Products Manufacturing facilities, as identified by the SIC Codes specified under Sector B in Table D-1 of Appendix D of the permit.

8.B.2 Sector-Specific Benchmarks. (See also Part 6 of the permit.)

Table 8.B-1.

Subsector (You may be subject to requirements for more than one sector/subsector)	Parameter	Benchmark Monitoring Concentration
Subsector B1. Paperboard Mills (SIC Code 2631)	Chemical Oxygen Demand (COD)	120 mg/L

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart C – Sector C – Chemical and Allied Products Manufacturing, and Refining.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.C.1 Covered Stormwater Discharges.

The requirements in Subpart C apply to stormwater discharges associated with industrial activity from Chemical and Allied Products Manufacturing, and Refining facilities, as identified by the SIC Codes specified under Sector C in Table D-1 of Appendix D of the permit.

8.C.2 Limitations on Coverage.

8.C.2.1 *Prohibition of Non-Stormwater Discharges.* (See also Part 1.1.4) The following are not covered by this permit: non-stormwater discharges containing inks, paints, or substances (hazardous, nonhazardous, etc.) resulting from an onsite spill, including materials collected in drip pans; washwater from material handling and processing areas; and washwater from drum, tank, or container rinsing and cleaning.

8.C.3 Sector-Specific Benchmarks

Table 8.C-1 identifies benchmarks that apply to the specific subsectors of Sector C. These benchmarks apply to both your primary industrial activity and any co-located industrial activities.

Table 8.C-1.		
Subsector (You may be subject to requirements for more than one sector/subsector)	**Parameter**	**Benchmark Monitoring Concentration**
Subsector C1. Agricultural Chemicals (SIC 2873-2879)	Nitrate plus Nitrite Nitrogen	0.68 mg/L
	Total Lead[1]	Hardness Dependent
	Total Iron	1.0 mg/L
	Total Zinc[1]	Hardness Dependent
	Phosphorus	2.0 mg/L
Subsector C2. Industrial Inorganic Chemicals (SIC 2812-2819)	Total Aluminum	0.75 mg/ L
	Total Iron	1.0 mg/L
	Nitrate plus Nitrite Nitrogen	0.68 mg/L
Subsector C3. Soaps, Detergents, Cosmetics, and Perfumes (SIC 2841-2844)	Nitrate plus Nitrite Nitrogen	0.68 mg/L
	Total Zinc[1]	Hardness Dependent
Subsector C4. Plastics, Synthetics, and Resins (SIC 2821-2824)	Total Zinc[1]	Hardness Dependent

[1] The benchmark values of some metals are dependent on water hardness. For these parameters, permittees must determine the hardness of the receiving water (see Appendix J, "Calculating Hardness in Receiving Waters for Hardness Dependent Metals," for methodology), in accordance with Part 6.2.1.1, to identify the applicable 'hardness range' for determining their benchmark value applicable to their facility. The ranges occur in 25 mg/L increments. Hardness Dependent Benchmarks follow in the table below:

Water Hardness Range	**Lead** (mg/L)	**Zinc** (mg/L)
0-25 mg/L	0.014	0.04
25-50 mg/L	0.023	0.05
50-75 mg/L	0.045	0.08
75-100 mg/L	0.069	0.11
100-125 mg/L	0.095	0.13
125-150 mg/L	0.122	0.16
150-175 mg/L	0.151	0.18
175-200 mg/L	0.182	0.20
200-225 mg/L	0.213	0.23
225-250 mg/L	0.246	0.25
250+ mg/L	0.262	0.26

8.C.4 Effluent Limitations Based on Effluent Limitations Guidelines (See also Part 6.2.2.1 of the permit.)

Table 8.C-2 identifies effluent limits that apply to the industrial activities described below. Compliance with these effluent limits is to be determined based on discharges from these industrial activities independent of commingling with any other wastestreams that may be covered under this permit.

Table 8.C-2[1]		
Industrial Activity	**Parameter**	**Effluent Limit**
Runoff from phosphate fertilizer manufacturing facilities that comes into contact with any raw materials, finished product, by-products or waste products (SIC 2874)	Total Phosphorus (as P)	105.0 mg/L, daily maximum
		35 mg/L, 30-day avg.
	Fluoride	75.0 mg/L, daily maximum
		25.0 mg/L, 30-day avg.

[1] Monitor annually.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart D – Sector D – Asphalt Paving and Roofing Materials and Lubricant Manufacturing.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.D.1 Covered Stormwater Discharges.

The requirements in Subpart D apply to stormwater discharges associated with industrial activity from Asphalt Paving and Roofing Materials and Lubricant Manufacturing facilities, as identified by the SIC Codes specified under Sector D in Table D-1 of Appendix D of the permit.

8.D.2 Limitations on Coverage.

The following stormwater discharges associated with industrial activity are not authorized by this permit (See also Part 1.1.4)

8.D.2.1 Discharges from petroleum refining facilities, including those that manufacture asphalt or asphalt products, that are subject to nationally established effluent limitation guidelines found in 40 CFR Part 419 (Petroleum Refining); or

8.D.2.2 Discharges from oil recycling facilities; or

8.D.2.3 Discharges associated with fats and oils rendering.

8.D.3 Sector-Specific Benchmarks

Table 8.D-1 identifies benchmarks that apply to the specific subsectors of Sector D. These benchmarks apply to both your primary industrial activity and any co-located industrial activities, which describe your site activities.

Table 8.D-1.

Subsector	Parameter	Benchmark Monitoring Concentration
Subsector D1. Asphalt Paving and Roofing Materials (SIC 2951, 2952)	Total Suspended Solids (TSS)	100 mg/L

8.D.4 Effluent Limitations Based on Effluent Limitations Guidelines (See also Part 6.2.2.1 of the permit.)

Table 8.D-2 identifies effluent limits that apply to the industrial activities described below. Compliance with these effluent limits is to be determined based on discharges from these industrial activities independent of commingling with any other wastestreams that may be covered under this permit.

Table 8.D-2[1]

Industrial Activity	Parameter	Effluent Limit
Discharges from asphalt emulsion facilities.	Total Suspended Solids (TSS)	23.0 mg/L, daily maximum 15.0 mg/L, 30-day avg.
	pH	6.0 - 9.0 s.u.
	Oil and Grease	15.0 mg/L, daily maximum
		10 mg/L, 30-day avg.

[1]Monitor annually.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart E – Sector E – Glass, Clay, Cement, Concrete, and Gypsum Products.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.E.1 Covered Stormwater Discharges.

The requirements in Subpart E apply to stormwater discharges associated with industrial activity from Glass, Clay, Cement, Concrete, and Gypsum Products facilities, as identified by the SIC Codes specified under Sector E in Table D-1 of Appendix D of the permit.

8.E.2 Additional Technology-Based Effluent Limits.

8.E.2.1 *Good Housekeeping Measures.* (See also Part 2.1.2.2) With good housekeeping, prevent or minimize the discharge of spilled cement, aggregate (including sand or gravel), kiln dust, fly ash, settled dust, or other significant material in stormwater from paved portions of the site that are exposed to stormwater. Consider sweeping regularly or using other equivalent measures to minimize the presence of these materials. Indicate in your SWPPP the frequency of sweeping or equivalent measures. Determine the frequency based on the amount of industrial activity occurring in the area and the frequency of precipitation, but it must be performed at least once a week if cement, aggregate, kiln dust, fly ash, or settled dust are being handled or processed. You must also prevent the exposure of fine granular solids (cement, fly ash, kiln dust, etc.) to stormwater, where practicable, by storing these materials in enclosed silos, hoppers, or buildings, or under other covering.

8.E.3 Additional SWPPP Requirements.

8.E.3.1 *Drainage Area Site Map*. (See also Part 5.1.2) Document in the SWPPP the locations of the following, as applicable: bag house or other dust control device; recycle/sedimentation pond, clarifier, or other device used for the treatment of process wastewater; and the areas that drain to the treatment device.

8.E.3.2 *Certification.* (See also Part 5.1.3.4) For facilities producing ready-mix concrete, concrete block, brick, or similar products, include in the non-stormwater discharge certification a description of measures that ensure that process waste waters resulting from washing trucks, mixers, transport buckets, forms, or other equipment are discharged in accordance with NPDES requirements or are recycled.

8.E.4 Sector-Specific Benchmarks.

Table 8.E-1 identifies benchmarks that apply to the specific subsectors of Sector E. These benchmarks apply to both your primary industrial activity and any co-located industrial activities, which describe your site activities.

Table 8.E-1.		
Subsector (You may be subject to requirements for more than one sector/subsector)	**Parameter**	**Benchmark Monitoring Cutoff Concentration**
Subsector E1. Clay Product Manufacturers (SIC 3251-3259, 3261-3269)	Total Aluminum	0.75 mg/L
Subsector E2. Concrete and Gypsum Product Manufacturers (SIC 3271-3275)	Total Suspended Solids (TSS)	100 mg/L
	Total Iron	1.0 mg/L

8.E.5 Effluent Limitations Based on Effluent Limitations Guidelines (See also Part 6.2.2.1 of the permit.)

Table 8.E-2 identifies effluent limits that apply to the industrial activities described below. Compliance with these limits is to be determined based on discharges from these industrial activities independent of commingling with any other wastestreams that may be covered under this permit.

Table 8.E-2[1]		
Industrial Activity	**Parameter**	**Effluent Limit**
Discharges from material storage piles at cement manufacturing facilities	Total Suspended Solids (TSS)	50 mg/L, daily maximum
	pH	6.0 - 9.0 s.u.

[1]Monitor annually.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart F – Sector F – Primary Metals.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.F.1 Covered Stormwater Discharges.

The requirements in Subpart F apply to stormwater discharges associated with industrial activity from Primary Metals facilities, as identified by the SIC Codes specified under Sector F in Table D-1 of Appendix D of the permit.

8.F.2 Additional Technology-Based Effluent Limits

8.F.2.1 *Good Housekeeping Measures.* (See also Part 2.1.2.2) As part of your good housekeeping program, include a cleaning and maintenance program for all impervious areas of the facility where particulate matter, dust, or debris may accumulate, especially areas where material loading and unloading, storage, handling, and processing occur; and, where practicable, the paving of areas where vehicle traffic or material storage occur but where vegetative or other stabilization methods are not practicable (institute a sweeping program in these areas too). For unstabilized areas where sweeping is not practicable, consider using stormwater management devices such as sediment traps, vegetative buffer strips, filter fabric fence, sediment filtering boom, gravel outlet protection, or other equivalent measures that effectively trap or remove sediment.

8.F.3 Additional SWPPP Requirements.

8.F.3.1 *Drainage Area Site Map.* (See also Part 5.1.2) Identify in the SWPPP where any of the following activities may be exposed to precipitation or surface runoff: storage or disposal of wastes such as spent solvents and baths, sand, slag and dross; liquid storage tanks and drums; processing areas including pollution control equipment (e.g., baghouses); and storage areas of raw material such as coal, coke, scrap, sand, fluxes, refractories, or metal in any form. In addition, indicate where an accumulation of significant amounts of particulate matter could occur from such sources as furnace or oven emissions, losses from coal and coke handling operations, etc., and could result in a discharge of pollutants to waters of the United States.

8.F.3.2 *Inventory of Exposed Material.* (See also Part 5.1.3.2) Include in the inventory of materials handled at the site that potentially may be exposed to precipitation or runoff, areas where deposition of particulate matter from process air emissions or losses during material-handling activities are possible

8.F.4 Additional Inspection Requirements. (See also Part 4.1) As part of conducting your quarterly routine facility inspections (Part 4.1), address all potential sources of pollutants, including (if applicable) air pollution control equipment (e.g., baghouses, electrostatic precipitators, scrubbers, and cyclones), for any signs of degradation (e.g., leaks,

corrosion, or improper operation) that could limit their efficiency and lead to excessive emissions. Consider monitoring air flow at inlets and outlets (or use equivalent measures) to check for leaks (e.g., particulate deposition) or blockage in ducts. Also inspect all process and material handling equipment (e.g., conveyors, cranes, and vehicles) for leaks, drips, or the potential loss of material; and material storage areas (e.g., piles, bins, or hoppers for storing coke, coal, scrap, or slag, as well as chemicals stored in tanks and drums) for signs of material losses due to wind or stormwater runoff.

8.F.5 Sector-Specific Benchmarks. (See also Part 6 of the permit.)

Table 8.F-1.

Subsector (You may be subject to requirements for more than one sector/subsector)	Parameter	Benchmark Monitoring Cutoff Concentration
Subsector F1. Steel Works, Blast Furnaces, and Rolling and Finishing Mills (SIC 3312-3317)	Total Aluminum	0.75 mg/L
	Total Zinc[1]	Hardness Dependent
Subsector F2. Iron and Steel Foundries (SIC 3321-3325)	Total Aluminum	0.75 mg/L
	Total Suspended Solids (TSS)	100 mg/L
	Total Copper[1]	Hardness Dependent
	Total Iron	1.0 mg/L
	Total Zinc[1]	Hardness Dependent
Subsector F3. Rolling, Drawing, and Extruding of Nonferrous Metals (SIC 3351-3357)	Total Copper[1]	Hardness Dependent
	Total Zinc[1]	Hardness Dependent
Subsector F4. Nonferrous Foundries (SIC 3363-3369)	Total Copper[1]	Hardness Dependent
	Total Zinc[1]	Hardness Dependent

[1] The benchmark values of some metals are dependent on water hardness. For these parameters, permittees must determine the hardness of the receiving water (see Appendix J, "Calculating Hardness in Receiving Waters for Hardness Dependent Metals," for methodology), in accordance with Part 6.2.1.1, to identify the applicable 'hardness range' for determining their benchmark value applicable to their facility. The ranges occur in 25 mg/L increments. Hardness Dependent Benchmarks follow in the table below:

Water Hardness Range	Copper (mg/L)	Zinc (mg/L)
0-25 mg/L	0.0038	0.04
25-50 mg/L	0.0056	0.05
50-75 mg/L	0.0090	0.08
75-100 mg/L	0.0123	0.11
100-125 mg/L	0.0156	0.13
125-150 mg/L	0.0189	0.16
150-175 mg/L	0.0221	0.18
175-200 mg/L	0.0253	0.20
200-225 mg/L	0.0285	0.23
225-250 mg/L	0.0316	0.25
250+ mg/L	0.0332	0.26

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart G – Sector G – Metal Mining.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.G.1 Covered Stormwater Discharges.

The requirements in Subpart G apply to stormwater discharges associated with industrial activity from Metal Mining facilities, including mines abandoned on Federal lands, as identified by the SIC Codes specified under Sector G in Table D-1 of Appendix D. Coverage is required for metal mining facilities that discharge stormwater contaminated by contact with, or that has come into contact with, any overburden, raw material, intermediate product, finished product, byproduct, or waste product located on the site of the operation.

8.G.1.1 Covered Discharges from Inactive Facilities. All stormwater discharges.

8.G.1.2 Covered Discharges from Active and Temporarily Inactive Facilities. Only the stormwater discharges from the following areas are covered: waste rock and overburden piles if composed entirely of stormwater and not combining with mine drainage; topsoil piles; offsite haul and access roads; onsite haul and access roads constructed of waste rock, overburden, or spent ore if composed entirely of stormwater and not combining with mine drainage; onsite haul and access roads not constructed of waste rock, overburden, or spent ore except if mine drainage is used for dust control; runoff from tailings dams or dikes when not constructed of waste rock or tailings and no process fluids are present; runoff from tailings dams or dikes when constructed of waste rock or tailings and no process fluids are present, if composed entirely of stormwater and not combining with mine drainage; concentration building if no contact with material piles; mill site if no contact with material piles; office or administrative building and housing if mixed with stormwater from industrial area; chemical storage area; docking facility if no excessive contact with waste product that would otherwise constitute mine drainage; explosive storage; fuel storage; vehicle and equipment maintenance area and building; parking areas (if necessary); power plant; truck wash areas if no excessive contact with waste product that would otherwise constitute mine drainage; unreclaimed, disturbed areas outside of active mining area; reclaimed areas released from reclamation requirements prior to December 17, 1990; and partially or inadequately reclaimed areas or areas not released from reclamation requirements.

8.G.1.3 Covered Discharges from Exploration and Construction of Metal Mining and/or Ore Dressing Facilities. All stormwater discharges.

8.G.1.4 Covered Discharges from Facilities Undergoing Reclamation. All stormwater discharges.

8.G.2 Limitations on Coverage.

8.G.2.1 *Prohibition of Stormwater Discharges.* Stormwater discharges not authorized by this permit: discharges from active metal mining facilities that are subject to effluent limitation guidelines for the Ore Mining and Dressing Point Source Category (40 CFR Part 440).

NOTE: Stormwater runoff from these sources are subject to 40 CFR Part 440 if they are mixed with other discharges subject to Part 440. In this case, they are not eligible for coverage under this permit. Discharges from overburden/waste rock and overburden/waste rock-related areas are not subject to 40 CFR Part 440 unless they: (1) drain naturally (or are intentionally diverted) to a point source; and (2) combine with "mine drainage" that is otherwise regulated under the Part 440 regulations. For such sources, coverage under this permit would be available if the discharge composed entirely of stormwater does not combine with other sources of mine drainage that are not subject to 40 CFR Part 440, and meets the other eligibility criteria contained in Part 1.2 of the permit. Permit applicants bear the initial responsibility for determining if they are eligible for coverage under this permit, or must seek coverage under another NPDES permit. EPA recommends that permit applicants contact the relevant NPDES permit issuance authority for assistance to determine the nature and scope of the "active mining area" on a mine-by-mine basis, as well as to determine the appropriate permitting mechanism for authorizing such discharges.

8.G.2.2 *Prohibition of Non-Stormwater* Discharges. Not authorized by this permit: adit drainage, and contaminated springs or seeps discharging from waste rock dumps that do not directly result from precipitation events (see also the standard Limitations on Coverage in Part 1.1.4).

8.G.3 Definitions.

The following definitions are not intended to supersede the definitions of active and inactive mining facilities established by 40 CFR 122.26(b)(14)(iii).

8.G.3.1 *Mining operation* - Consists of the active and temporarily inactive phases, and the reclamation phase, but excludes the exploration and construction phases.

8.G.3.2 *Exploration phase* - Entails exploration and land disturbance activities to determine the viability of a site. The exploration phase is not considered part of “mining operations.”

8.G.3.3 *Construction phase* - Includes the building of site access roads and removal of overburden and waste rock to expose mineable minerals. The construction phase is not considered part of “mining operations.”

8.G.3.4 *Active phase* - Activities including the extraction, removal or recovery of metal ore. For surface mines, this definition does not include any land where grading has returned the earth to a desired contour and reclamation has begun. This definition is derived from the definition of “active mining area” found at 40 CFR 440.132(a). The active phase is considered part of “mining operations.”

8.G.3.5 *Reclamation phase* - Activities undertaken, in compliance with applicable mined land reclamation requirements, following the cessation of the "active phase", intended to return the land to an appropriate post-mining land use in order to meet applicable Federal and State reclamation requirements. The reclamation phase is considered part of "mining operations."

8.G.3.6 *Active metal mining facility* - A place where work or other activity related to the extraction, removal, or recovery of metal ore is being conducted. For surface mines, this definition does not include any land where grading has returned the earth to a desired contour and reclamation has begun. This definition is derived from the definition of "active mining area" found at 40 CFR 440.132(a).

8.G.3.7 *Inactive metal mining facility* - A site or portion of a site where metal mining and/or milling occurred in the past but is not an active facility as defined above, and where the inactive portion is not covered by an active mining permit issued by the applicable State or Federal agency. An inactive metal mining facility has an identifiable owner / operator. Sites where mining claims are being maintained prior to disturbances associated with the extraction, beneficiation, or processing of mined materials and sites where minimal activities are undertaken for the sole purpose of maintaining a mining claim are not considered either active or inactive mining facilities and do not require an NPDES industrial stormwater permit.

8.G.3.8 *Temporarily inactive metal mining facility* - A site or portion of a site where metal mining and/or milling occurred in the past but currently are not being actively undertaken, and the facility is covered by an active mining permit issued by the applicable State or Federal agency.

8.G.3.9 *Final Stabilization* - A site or portion of a site is "finally stabilized" when it has implemented all applicable Federal and State reclamation requirements.

8.G.4 Technology-Based Effluent Limits for Clearing, Grading, and Excavation Activities.

Clearing, grading, and excavation activities being conducted as part of the exploration and construction phase of mining activities are covered under this permit.

8.G.4.1 Management Practices for Clearing, Grading, and Excavation Activities.

8.G.4.1.1 *Selecting and installing control measures*. For all areas affected by clearing, grading, and excavation activities, you must select, design, install, and implement control measures that meet applicable Part 2 effluent limits.

8.G.4.1.2 *Good Housekeeping*. Litter, debris, and chemicals must be prevented from becoming a pollutant source in stormwater discharges.

8.G.4.1.3 *Retention and Detention of Stormwater Runoff*. For drainage locations serving more than one acre, sediment basins and/or temporary sediment traps should be used. At a minimum, silt fences, vegetative buffer strips, or equivalent sediment controls are required for all down slope boundaries (and for side slope boundaries as necessary based on individual site conditions) of the development area unless a sediment basin providing storage for a calculated

volume of runoff from a 2-year, 24-hour storm or 3,600 cubic feet of storage per acre drained is provided. You are required to remove sediment from sediment traps or sedimentation ponds when design capacity has been reduced by 50 percent. Due to high sediment discharges from some Sector G facilities, permittees may need to implement a combination of structural BMP approaches to sufficiently decrease discharge of sediment from their facilities.

8.G.4.2 Inspection of Clearing, Grading, and Excavation Activities.

8.G.4.2.1 *Inspection Frequency*. Inspections must be conducted either at least once every 7 calendar days, or at least once every 14 calendar days and within 24 hours of the end of a storm event of 0.5 inches or greater. Inspection frequency may be reduced to at least once every month if the entire site is temporarily stabilized (pursuant to Part 8.G.4.3.2), if runoff is unlikely due to winter (e.g., site is covered with snow or ice) or frozen conditions, or construction is occurring during seasonal dry periods in arid areas and semi-arid areas.

8.G.4.2.2 *Location of Inspections*. Inspections must include all areas of the site disturbed by clearing, grading, and/or excavation activities and areas used for storage of materials that are exposed to precipitation. Sedimentation and erosion control measures must be observed to ensure proper operation. Discharge locations must be inspected to ascertain whether erosion control measures are effective in preventing significant impacts to waters of the United States, where accessible. Where discharge locations are inaccessible, nearby downstream locations must be inspected to the extent that such inspections are practicable. Locations where vehicles enter or exit the site must be inspected for evidence of significant off-site sediment tracking.

8.G.4.2.3 *Inspection Reports*. For each inspection required above, you must complete an inspection report. At a minimum, the inspection report must include the information required in Part 4.1.

8.G.4.3 Requirements for Cessation of Clearing, Grading, and Excavation Activities.

8.G.4.3.1 *Inspections and Maintenance.* Inspections and maintenance of control measures, including BMPs, associated with clearing, grading, and excavation activities being conducted as part of the exploration and construction phase of a mining operation must continue until final stabilization has been achieved on all portions of the disturbed area, or until the commencement of the active mining phase for those areas that have been temporarily stabilized as a precursor to mining.

8.G.4.3.2 *Temporary Stabilization of Disturbed Areas*. Stabilization measures should be initiated immediately in portions of the site where clearing, grading and/or excavation activities have temporarily ceased, but in no case more than 14 days after the clearing, grading and/or excavation activities in that portion of the site have temporarily ceased. In arid, semiarid, and drought-stricken areas, or in areas subject to snow or freezing conditions, where initiating perennial

vegetative stabilization measures is not possible within 14 days after mining, exploration, and/or construction activity has temporarily ceased, temporary vegetative stabilization measures must be initiated as soon as practicable. Until temporary vegetative stabilization is achieved, interim measures such as erosion control blankets with an appropriate seed base and tackifiers must be employed. In areas of the site, where exploration and/or construction has permanently ceased prior to active mining, temporary stabilization measures must be implemented to minimize mobilization of sediment or other pollutants until such time as the active mining phase commences.

8.G.4.3.3 *Final Stabilization of Disturbed Areas.* Stabilization measures should be initiated immediately in portions of the site where exploration and/or construction activities have permanently ceased, but in no case more than 14 days after the exploration and/or construction activity in that portion of the site has permanently ceased. In arid, semiarid, and drought-stricken areas, or in areas subject to snow or freezing conditions, where initiating perennial vegetative stabilization measures is not possible within 14 days after mining, exploration, and/or construction activity has permanently ceased, final vegetative stabilization measures must be initiated as soon as possible. Until final stabilization is achieved temporary stabilization measures, such as erosion control blankets with an appropriate seed base and tackifiers, must be used.

8.G.5 Additional Technology-Based Effluent Limits.

8.G.5.1 *Employee Training.* (See also Part 2.1.2.9) Conduct employee training at least annually at active and temporarily inactive sites.

8.G.5.2 *Stormwater Controls.* Apart from the control measures you implement to meet your Part 2 effluent limits, consider implementing the following control measures at your site. The potential pollutants identified in Part 8.G.6.3 shall determine the priority and appropriateness of the control measures selected.

8.G.5.2.1 *Stormwater Diversions*: Consider diverting stormwater away from potential pollutant sources. Following are some options: interceptor or diversion controls (e.g., dikes, swales, curbs, or berms); pipe slope drains; subsurface drains; conveyance systems (e.g., channels or gutters, open-top box culverts, and waterbars; rolling dips and road sloping; roadway surface water deflector and culverts); or their equivalents.

8.G.5.2.2 *Capping*: When capping is necessary to minimize pollutant discharges in stormwater, identify the source being capped and the material used to construct the cap.

8.G.5.2.3 *Treatment*: If treatment of stormwater (e.g., chemical or physical systems, oil and water separators, artificial wetlands) is necessary to protect water quality, describe the type and location of treatment used. Passive and/or active treatment of stormwater runoff is encouraged where practicable. Treated runoff may be discharged as a stormwater source regulated under this permit

provided the discharge is not combined with discharges subject to effluent limitation guidelines for the Ore Mining and Dressing Point Source Category (40 CFR Part 440).

8.G.5.3 *Certification of Discharge Testing.* (See also Part 5.1.3.4) Test or evaluate all outfalls covered under this permit for the presence of specific mining-related non-stormwater discharges such as seeps or adit discharges, or discharges subject to effluent limitations guidelines (e.g., 40 CFR Part 440), such as mine drainage or process water. Alternatively (if applicable), you may keep a certification with your SWPPP consistent with Part 8.G.6.6.

8.G.6 Additional SWPPP Requirements.

8.G.6.1 *Nature of Industrial Activities*. (See also Part 5.1.2) Briefly document in your SWPPP the mining and associated activities that can potentially affect the stormwater discharges covered by this permit, including a general description of the location of the site relative to major transportation routes and communities**.**

8.G.6.2 *Site Map*. (See also Part 5.1.2) Document in your SWPPP the locations of the following (as appropriate): mining or milling site boundaries; access and haul roads; outline of the drainage areas of each stormwater outfall within the facility with indications of the types of discharges from the drainage areas; location(s) of all permitted discharges covered under an individual NPDES permit, outdoor equipment storage, fueling, and maintenance areas; materials handling areas; outdoor manufacturing, outdoor storage, and material disposal areas; outdoor chemicals and explosives storage areas; overburden, materials, soils, or waste storage areas; location of mine drainage (where water leaves mine) or other process water; tailings piles and ponds (including proposed ones); heap leach pads; off-site points of discharge for mine drainage and process water; surface waters; boundary of tributary areas that are subject to effluent limitations guidelines; and location(s) of reclaimed areas.

8.G.6.3 *Potential Pollutant Sources.* (See also Part 5.1.3) For each area of the mine or mill site where stormwater discharges associated with industrial activities occur, identify the types of pollutants (e.g., heavy metals, sediment) likely to be present in significant amounts. Consider these factors: the mineralogy of the ore and waste rock (e.g., acid forming); toxicity and quantity of chemicals used, produced, or discharged; the likelihood of contact with stormwater; vegetation of site (if any); and history of significant leaks or spills of toxic or hazardous pollutants. Also include a summary of any existing ore or waste rock or overburden characterization data and test results for potential generation of acid rock. If any new data is acquired due to changes in ore type being mined, update your SWPPP with this information.

8.G.6.4 *Documentation of Control Measures.* Document all control measures that you implement consistent with Part 8.G.5.2. If control measures are implemented or planned but are not listed in Part 8.G.5.2 (e.g., substituting a less toxic chemical for a more toxic one), include descriptions of them in your SWPPP.

8.G.6.5 *Employee Training.* All employee training(s) must be documented in the SWPPP.

8.G.6.6 *Certification of Permit Coverage for Commingled Non-Stormwater Discharges:* If you are able, consistent with Part 8.G.5.3 above, to certify that a particular discharge composed of commingled stormwater and non-stormwater is covered under a separate NPDES permit, and that permit subjects the non-stormwater portion to effluent limitations prior to any commingling, retain such certification with your SWPPP. This certification must identify the non-stormwater discharges, the applicable NPDES permit(s), the effluent limitations placed on the non-stormwater discharge by the permit(s), and the points at which the limitations are applied.

8.G.7 Additional Inspection Requirements.

(See also Part 4.1 and 8.G.4.2.) Except for areas of the site subject to clearing, grading, and/or excavation activities conducted as part of the exploration and construction phase, which are subject to Part 8.G.4.2.1, inspect sites at least quarterly unless adverse weather conditions make the site inaccessible. Sites which discharge to waters designated as outstanding waters or waters which are impaired for sediment or nitrogen must be inspected monthly. See Part 8.G.8.4 for inspection requirements for inactive and unstaffed sites.

8.G.8 Monitoring and Reporting Requirements. (See also Part 6 of the permit.)

Note: There are no Part 8.G.8 monitoring and reporting requirements for inactive and unstaffed sites.

8.G.8.1 *Benchmark Monitoring for Active Copper Ore Mining and Dressing Facilities.* Active copper ore mining and dressing facilities, must sample and analyze stormwater discharges for the pollutants listed in Table 8.G-1.

Table 8.G-1

Subsector (You may be subject to requirements for more than one sector/subsector)	**Parameter**	**Benchmark Monitoring Concentration**
Subsector G1. Active Copper Ore Mining and Dressing Facilities (SIC 1021)	Total Suspended Solids (TSS)	100 mg/L
	Nitrate plus Nitrite Nitrogen	0.68 mg/L
	Chemical Oxygen Demand (COD)	120 mg/L

8.G.8.2 *Benchmark Monitoring Requirements for Discharges From Waste Rock and Overburden Piles at Active Metal Mining Facilities.* For discharges from waste rock and overburden piles, perform benchmark monitoring once in the first year for the parameters listed in Table 8.G-2, and twice annually in all subsequent years of coverage under this permit for any parameters for which the benchmark has been exceeded. You are also required to conduct analytic monitoring for the parameters listed in Table 8.G-3 in accordance with the requirements in Part 8.G.6.3. The Director may also notify you that you must perform additional monitoring to accurately characterize the quality and quantity of pollutants discharged from your waste rock and overburden piles.

Table 8.G-2.		
Subsector (Discharges may be subject to requirements for more than one sector/subsector)	**Parameter**	**Benchmark Monitoring Cutoff Concentration**
Subsector G2. Iron Ores; Copper Ores; Lead and Zinc Ores; Gold and Silver Ores; Ferroalloy Ores, Except Vanadium; and Miscellaneous Metal Ores (SIC Codes 1011, 1021, 1031, 1041, 1044, 1061, 1081, 1094, 1099) (Note: when analyzing hardness for a suite of metals, it is more cost effective to add analysis of calcium and magnesium, and have hardness calculated than to require hardness analysis separately)	Total Suspended Solids (TSS)	100 mg/L
	Turbidity	50 NTU
	pH	6.0-9.0 s.u.
	Hardness (as $CaCO_3$; calc. from Ca, Mg)[1]	no benchmark value
	Total Antimony	0.64 mg/L
	Total Arsenic	0.15 mg/ L
	Total Beryllium	0.13 mg/L
	Total Cadmium[1]	Hardness Dependent
	Total Copper[1]	Hardness Dependent
	Total Iron	1.0 mg/L
	Total Lead[1]	Hardness Dependent
	Total Mercury	0.0014 mg/L
	Total Nickel[1]	Hardness Dependent
	Total Selenium	0.005 mg/L
	Total Silver[1]	Hardness Dependent
	Total Zinc[1]	Hardness Dependent

[1] The benchmark values of some metals are dependent on water hardness. For these parameters, permittees must determine the hardness of the receiving water (see Appendix J, "Calculating Hardness in Receiving Waters for Hardness Dependent Metals," for methodology), in accordance with Part 6.2.1.1, to identify the applicable 'hardness range' for determining their benchmark value applicable to their facility. The ranges occur in 25 mg/L increments. Hardness Dependent Benchmarks follow in the table below:

Water Hardness Range	**Cadmium** (mg/L)	**Copper** (mg/L)	**Lead** (mg/L)	**Nickel** (mg/L)	**Silver** (mg/L)	**Zinc** (mg/L)
0-25 mg/L	0.0005	0.0038	0.014	0.15	0.0007	0.04
25-50 mg/L	0.0008	0.0056	0.023	0.20	0.0007	0.05
50-75 mg/L	0.0013	0.0090	0.045	0.32	0.0017	0.08
75-100 mg/L	0.0018	0.0123	0.069	0.42	0.0030	0.11
100-125 mg/L	0.0023	0.0156	0.095	0.52	0.0046	0.13
125-150 mg/L	0.0029	0.0189	0.122	0.61	0.0065	0.16
150-175 mg/L	0.0034	0.0221	0.151	0.71	0.0087	0.18
175-200 mg/L	0.0039	0.0253	0.182	0.80	0.0112	0.20
200-225 mg/L	0.0045	0.0285	0.213	0.89	0.0138	0.23
225-250 mg/L	0.0050	0.0316	0.246	0.98	0.0168	0.25
250+ mg/L	0.0053	0.0332	0.262	1.02	0.0183	0.26

8.G.8.3 Additional Analytic Monitoring Requirements for Discharges From Waste Rock and Overburden Piles at Active Metal Mining Facilities. In addition to the monitoring required in Part 8.G.6.2 for discharges from waste rock and overburden piles, you must also conduct monitoring for additional parameters based on the type of ore you mine at your site. Where a parameter in Table 8.G-3 is the same as a pollutant you are required

to monitor for in Table 8.G-2 (i.e., for all of the metals, you must use the corresponding benchmark in Table 8.G-2 and you may use any monitoring results conducted for Part 8.G.6.2 to satisfy the monitoring requirement for that parameter for Part 8.G.6.3. For radium and uranium, which do not have corresponding benchmarks in Table 8.G-2, there are no applicable benchmarks.) The frequency and schedule for monitoring for these additional parameters is the same as that specified in Part 6.2.1.2.

Table 8.G-3. Additional Monitoring Requirements for Discharges from Waste Rock and Overburden Piles

Supplemental Requirements			
Type of Ore Mined	**Pollutants of Concern**		
	Total Suspended Solids (TSS)	**pH**	**Metals, Total**
Tungsten Ore	X	X	Arsenic, Cadmium (H), Copper (H), Lead (H), Zinc (H)
Nickel Ore	X	X	Arsenic, Cadmium (H), Copper (H), Lead (H), Zinc (H)
Aluminum Ore	X	X	Iron
Mercury Ore	X	X	Nickel (H)
Iron Ore	X	X	Iron (Dissolved)
Platinum Ore			Cadmium (H), Copper (H), Mercury, Lead (H), Zinc (H)
Titanium Ore	X	X	Iron, Nickel (H), Zinc (H)
Vanadium Ore	X	X	Arsenic, Cadmium (H), Copper (H), Lead (H), Zinc (H)
Molybdenum	X	X	Arsenic, Cadmium (H), Copper (H), Lead (H), Mercury, Zinc (H)
Uranium, Radium, and Vanadium Ore	X	X	Chemical Oxygen Demand, Arsenic, Radium (Dissolved and Total), Uranium, Zinc (H)

Note: An "X" indicated for TSS and/or pH means that you are required to monitor for those parameters. (H) indicates that hardness must also be measured when this pollutant is measured.

8.G.8.4 Inactive and Unstaffed Sites – Conditional Exemption from No Exposure Requirements for Quarterly Visual Assessments and Routine Facility Inspections. As a Sector G facility, if you are seeking to exercise a waiver from the quarterly visual assessment and routine facility inspection requirements for inactive and unstaffed sites (including temporarily inactive sites), you are conditionally exempt from the requirement to certify that "there are no industrial materials or activities exposed to stormwater" in Part 4.2.3. This exemption is conditioned on the following:

- If circumstances change and your facility becomes active and/or staffed, this exception no longer applies and you must immediately begin complying with the quarterly visual assessment requirements; and
- EPA retains the authority to revoke this exemption and/or the monitoring waiver where it is determined that the discharge causes, has a reasonable potential to cause,

or contributes to an instream excursion above an applicable water quality standard, including designated uses.

Subject to the two conditions above, if your facility is inactive and unstaffed, you are waived from the requirement to conduct quarterly visual assessments and routine facility inspections. You are not waived from conducting the Part 4.3 comprehensive site inspection. You are encouraged to inspect your site more frequently where you have reason to believe that severe weather or natural disasters may have damaged control measures or increased discharges.

Table 8.G-4. Applicability of the Multi-Sector General Permit to Stormwater Runoff From Active Mining and Dressing Sites, Temporarily Inactive Sites, and Sites Undergoing Reclamation

Discharge/Source of Discharge	Note/Comment
Piles	
Waste rock/overburden	If composed entirely of stormwater and not combining with mine drainage. See note below.
Topsoil	--
Roads constructed of waste rock or spent ore	
Onsite haul roads	If composed entirely of stormwater and not combining with mine drainage. See note below.
Offsite haul and access roads	--
Roads not constructed of waste rock or spent ore	
Onsite haul roads	Except if mine drainage is used for dust control
Offsite haul and access roads	--
Milling/concentrating	
Runoff from tailings dams and dikes when constructed of waste rock/tailings	Except if process fluids are present and only if composed entirely of stormwater and not combining with mine drainage. See Note below.
Runoff from tailings dams/dikes when not constructed of waste rock and tailings	Except if process fluids are present
Concentration building	If stormwater only and no contact with piles
Mill site	If stormwater only and no contact with piles
Ancillary areas	
Office and administrative building and housing	If mixed with stormwater from the industrial area
Chemical storage area	--
Docking facility	Except if excessive contact with waste product that would otherwise constitute mine drainage
Explosive storage	--
Fuel storage (oil tanks/coal piles)	--
Vehicle and equipment maintenance area/building	--
Parking areas	But coverage unnecessary if only employee and visitor-type parking
Power plant	
Truck wash area	Except when excessive contact with waste product that would otherwise constitute mine drainage

Table 8.G-4. Applicability of the Multi-Sector General Permit to Stormwater Runoff From Active Mining and Dressing Sites, Temporarily Inactive Sites, and Sites Undergoing Reclamation	
Reclamation-related areas	
Any disturbed area (unreclaimed)	Only if not in active mining area
Reclaimed areas released from reclamation requirements prior to Dec. 17, 1990	--
Partially/inadequately reclaimed areas or areas not released from reclamation requirements	--

Note: Stormwater runoff from these sources are subject to the NPDES program for stormwater unless mixed with discharges subject to 40 CFR Part 440 that are regulated by another permit prior to mixing. Non-stormwater discharges from these sources are subject to NPDES permitting and may be subject to the effluent limitation guidelines under 40 CFR Part 440. Discharges from overburden/waste rock and overburden/waste rock-related areas are not subject to 40 CFR Part 440 unless: (1) it drains naturally (or is intentionally diverted) to a point source; and (2) combines with "mine drainage" that is otherwise regulated under the Part 440 regulations. For such sources, coverage under this permit would be available if the discharge composed entirely of stormwater does not combine with other sources of mine drainage that are not subject to 40 CFR Part 440, as well as meeting other eligibility criteria contained in Part 1.1 of the permit. Permit applicants bear the initial responsibility for determining the applicable technology-based standard for such discharges. EPA recommends that permit applicants contact the relevant NPDES permit issuance authority for assistance to determine the nature and scope of the "active mining area" on a mine-by-mine basis, as well as to determine the appropriate permitting mechanism for authorizing such discharges.

8.G.9. Termination of Permit Coverage

8.G.9.1 *Termination of Permit Coverage for Sites Reclaimed After December 17, 1990.* A site or a portion of a site that has been released from applicable state or federal reclamation requirements after December 17, 1990, is no longer required to maintain coverage under this permit. If the site or portion of a site reclaimed after December 17, 1990, was not subject to reclamation requirements, the site or portion of the site is no longer required to maintain coverage under this permit if the site or portion of the site has been reclaimed as defined in Part 8.G.7.2.

8.G.9.2 *Termination of Permit Coverage for Sites Reclaimed Before December 17, 1990.* A site or portion of a site that was released from applicable state or federal reclamation requirements before December 17, 1990, or that was otherwise reclaimed before December 17, 1990, is no longer required to maintain coverage under this permit if the site or portion of the site has been reclaimed. A site or portion of a site is considered to have been reclaimed if: (1) stormwater runoff that comes into contact with raw materials, intermediate byproducts, finished products, and waste products does not have the potential to cause or contribute to violations of state water quality standards, (2) soil disturbing activities related to mining at the sites or portion of the site have been completed, (3) the site or portion of the site has been stabilized to minimize soil erosion, and (4) as appropriate depending on location, size, and the potential to contribute pollutants to stormwater discharges, the site or portion of the site has been revegetated, will be amenable to natural revegetation, or will be left in a condition consistent with the post-mining land use.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart H – Sector H – Coal Mines and Coal Mining-Related Facilities.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.H.1 Covered Stormwater Discharges.

The requirements in Subpart H apply to stormwater discharges associated with industrial activity from Coal Mines and Coal Mining-Related facilities as identified by the SIC Codes specified under Sector H in Table D-1 of Appendix D.

8.H.2 Limitations on Coverage.

8.H.2.1 *Prohibition of Non-Stormwater Discharges.* (See also Part 1.1.4) Not covered by this permit: discharges from pollutant seeps or underground drainage from inactive coal mines and refuse disposal areas that do not result from precipitation events, and discharges from floor drains in maintenance buildings and other similar drains in mining and preparation plant areas.

8.H.2.2 *Discharges Subject to Stormwater Effluent Guidelines.* (See also Part 1.1.4.4) Not authorized by this permit: stormwater discharges subject to an existing effluent limitation guideline at 40 CFR Part 434.

8.H.3 Definitions

The following definitions are not intended to supersede the definitions of active and inactive mining facilities established by 40 CFR 122.26(b)(14)(iii).

8.H.3.1 *Mining operation* - Consists of the active and temporarily inactive phases, and the reclamation phase, but excludes the exploration and construction phases.

8.H.3.2 *Exploration phase* - Entails exploration and land disturbance activities to determine the financial viability of a site. The exploration phase is not considered part of "mining operations."

8.H.3.3 *Construction phase* - Includes the building of site access roads and removal of overburden and waste rock to expose mineable coal. The construction phase is not considered part of "mining operations."

8.H.3.4 *Active phase* - Activities including the extraction, removal or recovery of coal. For surface mines, this definition does not include any land where grading has returned the earth to a desired contour and reclamation has begun. This definition is derived from the definition of "active mining area" found at 40 CFR 434.11(b). The active phase is considered part of "mining operations."

8.H.3.5 *Reclamation phase* - Activities undertaken, in compliance with applicable mined land reclamation requirements, following the cessation of the "active phase", intended to return the land to an appropriate post-mining land use. The reclamation phase is considered part of "mining operations."

8.H.3.6 *Active coal mining facility* - A place where work or other activity related to the extraction, removal, or recovery of coal is being conducted. For surface mines, this definition does not include any land where grading has returned the earth to a desired contour and reclamation has begun. This definition is derived from the definition of "active mining area" found at 40 CFR 434.11(b).

8.H.3.7 *Inactive coal mining facility* - A site or portion of a site where coal mining and/or milling occurred in the past but is not an active facility as defined above, and where the inactive portion is not covered by an active mining permit issued by the applicable State or Federal agency. An inactive coal mining facility has an identifiable owner / operator. Sites where mining claims are being maintained prior to disturbances associated with the extraction, beneficiation, or processing of mined materials and sites where minimal activities are undertaken for the sole purpose of maintaining a mining claim are not considered either active or inactive mining facilities and do not require an NPDES industrial stormwater permit.

8.H.3.8 *Temporarily inactive coal mining facility* - A site or portion of a site where coal mining and/or milling occurred in the past but currently are not being actively undertaken, and the facility is covered by an active mining permit issued by the applicable State or Federal agency.

8.H.3.9 *Final Stabilization* - A site or portion of a site is "finally stabilized" when it has implemented all applicable Federal and State reclamation requirements.

8.H.4 Technology-Based Effluent Limits for Clearing, Grading, and Excavation Activities.

Clearing, grading, and excavation activities being conducted as part of the exploration and construction phase of mining activities are covered under this permit.

8.H.4.1 Management Practices for Clearing, Grading, and Excavation Activities.

8.H.4.1.1 *Selecting and installing control measures.* For all areas affected by clearing, grading, and excavation activities, you must select, design, install, and implement control measures that meet applicable Part 2 effluent limits.

8.H.4.1.2 *Good Housekeeping.* Litter, debris, and chemicals must be prevented from becoming a pollutant source in stormwater discharges.

8.H.4.1.3 *Retention and Detention of Stormwater Runoff.* For drainage locations serving more than one acre, sediment basins and/or temporary sediment traps should be used. At a minimum, silt fences, vegetative buffer strips, or equivalent sediment controls are required for all down slope boundaries (and side slope boundaries as necessary based on individual site conditions) of the development area unless a sediment basin providing storage for a calculated volume of runoff from a 2-year, 24-hour storm or 3,600 cubic feet of storage

per acre drained is provided. You are required to remove sediment from sediment traps or sedimentation ponds when design capacity has been reduced by 50 percent. Due to high sediment discharges from some Sector H facilities, permittees may need to implement a combination of structural BMP approaches to sufficiently decrease discharge of sediment from their facilities.

8.H.4.2 *Inspection of Clearing, Grading, and Excavation Activities.*

8.H.4.2.1 *Inspection Frequency.* Inspections must be conducted either at least once every 7 calendar days, or at least once every 14 calendar days and within 24 hours of the end of a storm event of 0.5 inches or greater. Inspection frequency may be reduced to at least once every month if the entire site is temporarily stabilized (pursuant to Part 8.H.4.3.2), if runoff is unlikely due to winter (e.g., site is covered with snow or ice) or frozen conditions, or construction is occurring during seasonal dry periods in arid areas and semi-arid areas.

8.H.4.2.2 *Location of Inspections.* Inspections must include all areas of the site disturbed by clearing, grading, and/or excavation activities and areas used for storage of materials that are exposed to precipitation. Sedimentation and erosion control measures must be observed to ensure proper operation. Discharge locations must be inspected to ascertain whether erosion control measures are effective in preventing significant impacts to waters of the United States, where accessible. Where discharge locations are inaccessible, nearby downstream locations must be inspected to the extent that such inspections are practicable. Locations where vehicles enter or exit the site must be inspected for evidence of significant off-site sediment tracking.

8.H.4.2.3 *Inspection Reports.* For each inspection required above, you must complete an inspection report. At a minimum, the inspection report must include the information required in Part 4.1.

8.H.4.3 *Requirements for Cessation of Clearing, Grading, and Excavation Activities.*

8.H.4.3.1 *Inspections and Maintenance.* Inspections and maintenance of control measures, including BMPs, associated with clearing, grading, and/or excavation activities being conducted as part of the exploration and construction phase of a mining operation must continue until final stabilization has been achieved on all portions of the disturbed area.

8.H.4.3.2 *Temporary Stabilization of Disturbed Areas.* Stabilization measures should be initiated immediately in portions of the site where clearing, grading and/or excavation activities have temporarily ceased, but in no case more than 14 days after the clearing, grading and/or excavation activities in that portion of the site have temporarily ceased. In arid, semiarid, and drought-stricken areas, or in areas subject to snow or freezing conditions, where initiating perennial vegetative stabilization measures is not possible within 14 days after mining, exploration, and/or construction activity has temporarily ceased, temporary vegetative stabilization measures must be initiated as soon as practicable.

Until temporary vegetative stabilization is achieved, interim measures such as erosion control blankets with an appropriate seed base and tackifiers must be employed. In areas of the site, where exploration and/or construction has permanently ceased prior to active mining, temporary stabilization measures must be implemented to minimize mobilization of sediment or other pollutants until such time as the active mining phase commences.

8.H.4.3.2 *Final Stabilization of Disturbed Areas.* Stabilization measures should be initiated immediately in portions of the site where exploration and/or construction activities have permanently ceased, but in no case more than 14 days after the exploration and/or construction activity in that portion of the site has permanently ceased. In arid, semiarid, and drought-stricken areas, or in areas subject to snow or freezing conditions, or in areas subject to snow or freezing conditions, where initiating perennial vegetative stabilization measures is not possible within 14 days after mining, exploration, and/or construction activity has permanently ceased, temporary vegetative stabilization measures must be initiated as soon as possible. Until final stabilization is achieved temporary stabilization measures, such as erosion control blankets with an appropriate seed base and tackifiers, must be used.

8.H.5 Additional Technology-Based Effluent Limits.

8.H.5.1 *Good Housekeeping Measures.* (See also Part 2.1.2.2) As part of your good housekeeping program, consider using sweepers and covered storage, watering haul roads to minimize dust generation, and conserving vegetation (where possible) to minimize erosion.

8.H.5.2 *Preventive Maintenance.* (See also Part 2.1.2.3) Perform inspections or other equivalent measures of storage tanks and pressure lines of fuels, lubricants, hydraulic fluid, and slurry to prevent leaks due to deterioration or faulty connections.

8.H.6 Additional SWPPP Requirements.

8.H.6.1 *Other Applicable Regulations.* Most active coal mining-related areas (SIC Codes 1221-1241) are subject to sediment and erosion control regulations of the U.S. Office of Surface Mining (OSM) that enforces the Surface Mining Control and Reclamation Act (SMCRA). OSM has granted authority to most coal-producing states to implement SMCRA through State SMCRA regulations. All SMCRA requirements regarding control of stormwater-related pollutant discharges must be addressed and then documented with the SWPPP (directly or by reference).

8.H.6.2 *Site Map.* (See also Part 5.1.2) Document in your SWPPP where any of the following may be exposed to precipitation or surface runoff: haul and access roads; railroad spurs, sliding, and internal hauling lines; conveyor belts, chutes, and aerial tramways; equipment storage and maintenance yards; coal handling buildings and structures; and inactive mines and related areas; acidic spoil, refuse, or unreclaimed disturbed areas; and liquid storage tanks containing pollutants such as caustics, hydraulic fluids, and lubricants.

8.H.6.3 *Potential Pollutant Sources.* (See also Part 5.1.3) Document in your SWPPP the following sources and activities that have potential pollutants associated with them: truck traffic on haul roads and resulting generation of sediment subject to runoff and dust generation; fuel or other liquid storage; pressure lines containing slurry, hydraulic fluid, or other potential harmful liquids; and loading or temporary storage of acidic refuse or spoil.

8.H.7 Additional Inspection Requirements.

8.H.7.1 *Inspections of Active Mining-Related Areas.* (See also Part 4) Except for areas of the site subject to clearing, grading, and/or excavation activities conducted as part of the exploration and construction phase, which are subject to Part 8.H.4.2.1, perform quarterly inspections of active mining areas covered by this permit, corresponding with the inspections as performed by SMCRA inspectors, of all mining-related areas required by SMCRA. Also maintain the records of the SMCRA authority representative. See Part 8.H.8.1 for inspection requirements for inactive and unstaffed sties.

8.H.7.2 *Sediment and Erosion Control.* (See also Part 2.1.2.5) As indicated in Part 8.H.6.1, SMCRA requirements regarding sediment and erosion control measures must be complied with for those areas subject to SMCRA authority, including inspection requirements.

8.H.7.3 *Comprehensive Site Inspections.* (See also Part 4.3) Your inspection program must include inspections for pollutants entering the drainage system from activities located on or near coal mining-related areas. Among the areas to be inspected are haul and access roads; railroad spurs, sliding, and internal hauling lines; conveyor belts, chutes, and aerial tramways; equipment storage and maintenance yards; coal handling buildings and structures; and inactive mines and related areas.

8.H.8 Sector-Specific Benchmarks. (See also Part 6 of the permit.)

Table 8.H-1.

Subsector (You may be subject to requirements for more than one sector/subsector)	Parameter	Benchmark Monitoring Concentration
Subsector H1. Coal Mines and Related Areas (SIC 1221-1241)	Total Aluminum	0.75 mg/L
	Total Iron	1.0 mg/L
	Total Suspended Solids (TSS)	100 mg/L

8.H.8.1 *Inactive and Unstaffed Sites – Conditional Exemption from No Exposure Requirement for Routine Inspections, Quarterly Visual Assessments, and Benchmark Monitoring.* As a Sector H facility, if you are seeking to exercise a waiver from either the quarterly visual assessment or the benchmark monitoring requirements for inactive and unstaffed sites (including temporarily inactive sites), you are conditionally exempt from the requirement to certify that "there are no industrial materials or activities exposed to

stormwater" in Parts 4.2.3 and 6.2.1.3, respectively. Additionally, if you are seeking to reduce your required quarterly routine inspection frequency to a once annual comprehensive inspection, as is allowed under Part 4.1.3, you are also conditionally exempt from the requirement to certify that "there are no industrial materials or activities exposed to stormwater." These conditional exemptions are based on the following requirements:

- If circumstances change and your facility becomes active and/or staffed, this exception no longer applies and you must immediately begin complying with the applicable benchmark monitoring requirements as if you were in your first year of permit coverage, and the quarterly visual assessment requirements; and
- EPA retains the authority to revoke this exemption and/or the monitoring waiver where it is determined that the discharge causes, has a reasonable potential to cause or contribute to an instream excursion above an applicable water quality standard, including designated uses.

Subject to the two conditions above, if your facility is inactive and unstaffed, you are waived from the requirement to conduct quarterly visual assessments and routine facility inspections. You are not waived from conducting the Part 4.3 comprehensive site inspection. You are encouraged to inspect your site more frequently where you have reason to believe that severe weather or natural disasters may have damaged control measures or increased discharges.

8.H.9 Termination of Permit Coverage

8.H.9.1 *Termination of Permit Coverage for Sites Reclaimed After December 17, 1990.* A site or a portion of a site that has been released from applicable state or federal reclamation requirements after December 17, 1990, is no longer required to maintain coverage under this permit. If the site or portion of a site reclaimed after December 17, 1990, was not subject to reclamation requirements, the site or portion of the site is no longer required to maintain coverage under this permit if the site or portion of the site has been reclaimed as defined in Part 8.H.7.2.

8.H.9.2 *Termination of Permit Coverage for Sites Reclaimed Before December 17, 1990.* A site or portion of a site that was released from applicable state or federal reclamation requirements before December 17, 1990, or that was otherwise reclaimed before December 17, 1990, is no longer required to maintain coverage under this permit if the site or portion of the site has been reclaimed. A site or portion of a site is considered to have been reclaimed if: (1) stormwater runoff that comes into contact with raw materials, intermediate byproducts, finished products, and waste products does not have the potential to cause or contribute to violations of state water quality standards, (2) soil disturbing activities related to mining at the sites or portion of the site have been completed, (3) the site or portion of the site has been stabilized to minimize soil erosion, and (4) as appropriate depending on location, size, and the potential to contribute pollutants to stormwater discharges, the site or portion of the site has been revegetated, will be amenable to natural revegetation, or will be left in a condition consistent with the post-mining land use.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart I – Sector I – Oil and Gas Extraction.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.I.1 Covered Stormwater Discharges.

The requirements in Subpart I apply to stormwater discharges associated with industrial activity from Oil and Gas Extraction facilities as identified by the SIC Codes specified under Sector I in Table D-1 of Appendix D of the permit.

Discharges of stormwater runoff from field activities or operations associated with oil and gas exploration, production, processing, or treatment operations or transmission facilities are exempt from NPDES permit coverage unless, in accordance with 40 CFR 122.26(c)(1)(iii), the facility:

- Has had a discharge of stormwater resulting in the discharge of a reportable quantity for which notification is or was required pursuant to 40 CFR 117.21 or 40 CFR 302.6 at anytime since November 16, 1987; or
- Has had a discharge of stormwater resulting in the discharge of a reportable quantity for which notification is or was required pursuant to 40 CFR 110.6 at any time since November 16, 1987; or
- Contributes to a violation of a water quality standard.

Any stormwater discharges that require permit coverage as a result of meeting one of the conditions of 122.26(c)(1)(iii) may be covered under this permit unless otherwise required to obtain coverage under an alternative NPDES general permit or an individual NPDES permit as specified in Part 1.6.1.

8.I.2 Limitations on Coverage.

8.I.2.1 *Stormwater Discharges Subject to Effluent Limitation Guidelines.* (See also Part 1.1.4.4) This permit does not authorize stormwater discharges from petroleum drilling operations that are subject to nationally established effluent limitation guidelines found at 40 CFR Part 435, respectively.

8.I.2.2 *Non-Stormwater Discharges.* Discharges of vehicle and equipment washwater, including tank cleaning operations, are not authorized by this permit. Alternatively, washwater discharges must be authorized under a separate NPDES permit, or be discharged to a sanitary sewer in accordance with applicable industrial pretreatment requirements.

8.I.3 Additional Technology-Based Effluent Limits.

8.I.3.1 *Vegetative Controls.* Implement vegetative practices designed to preserve existing vegetation, where attainable, and revegetate open areas as soon as practicable after grade drilling. Consider the following (or equivalent measures): temporary or permanent seeding, mulching, sod stabilization, vegetative buffer strips, and tree protection practices. Begin implementing appropriate vegetative practices on all disturbed areas within 14 days following the last activity in that area.

8.I.4 Additional SWPPP Requirements.

8.I.4.1 *Drainage Area Site Map.* (See also Part 5.1.2) Document in your SWPPP where any of the following may be exposed to precipitation or surface runoff: Reportable Quantity (RQ) releases; locations used for the treatment, storage, or disposal of wastes; processing areas and storage areas; chemical mixing areas; construction and drilling areas; all areas subject to the effluent guidelines requirements for "No Discharge" in accordance with 40 CFR 435.32; and the structural controls to achieve compliance with the "No Discharge" requirements.

8.I.4.2 *Potential Pollutant Sources.* (See also Part 5.1.3) Also document in your SWPPP the following sources and activities that have potential pollutants associated with them: chemical, cement, mud, or gel mixing activities; drilling or mining activities; and equipment cleaning and rehabilitation activities. In addition, include information about the reportable quantity (RQ) release that triggered the permit application requirements: the nature of the release (e.g., spill of oil from a drum storage area), amount of oil or hazardous substance released, amount of substance recovered, date of the release, cause of the release (e.g., poor handling techniques and lack of containment in the area), areas affected by the release (i.e., land and water), procedure to clean up release, actions or procedures implemented to prevent or improve response to a release, and remaining potential contamination of stormwater from release (taking into account human health risks, the control of drinking water intakes, and the designated uses of the receiving water).

8.I.4.3 *Erosion and Sedimentation Control.* (See also Part 2.1.2.5) Unless covered by the current Construction General Permit (CGP), the additional documentation requirements for sediment and erosion controls for well drillings and sand/shale mining areas include the following:

8.I.4.3.1 *Site Description.* Also include a description in your SWPPP of the nature of the exploration activity, estimates of the total area of site and area disturbed due to exploration activity, an estimate of runoff coefficient of the site, a site drainage map, including approximate slopes, and the names of all receiving waters.

8.I.4.3.2 *Vegetative Controls.* Document vegetative practices used consistent with Part 8.I.3.1 in the SWPPP.

8.I.5 Additional Inspection Requirements.

All erosion and sedimentation control measures must be inspected every 7 days.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart J – Sector J – Non-Metallic Mineral Mining and Dressing.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.J.1 Covered Stormwater Discharges.

The requirements in Subpart J apply to stormwater discharges associated with industrial activity from Active and Inactive Non-Metallic Mineral Mining and Dressing facilities as identified by the SIC Codes specified under Sector J in Table D-1 of Appendix D of the permit.

8.J.1.1 *Covered Discharges from Inactive Facilities.* All stormwater discharges.

8.J.1.2 *Covered Discharges from Active and Temporarily Inactive Facilities.* All stormwater discharges, except for most stormwater discharges subject to the existing effluent limitation guideline at 40 CFR Part 436. Mine dewatering discharges composed entirely of stormwater or uncontaminated ground water seepage from: construction sand and gravel, industrial sand, and crushed stone mining facilities in Regions 1, 2, 3, 6, 9, and 10 are covered by this permit.

8.J.1.3 *Covered Discharges from Exploration and Construction of Non-Metallic Mineral Mining Facilities.* All stormwater discharges.

8.J.1.4 Covered Discharges from Sites Undergoing Reclamation. All stormwater discharges.

8.J.2 Limitations on Coverage.

Most stormwater discharges subject to an existing effluent limitation guideline at 40 CFR Part 436 are not authorized by this permit. The exceptions to this limitation, which are covered by this permit, are mine dewatering discharges composed entirely of stormwater or uncontaminated ground water seepage from construction sand and gravel, industrial sand, and crushed stone mining facilities in Regions 1, 2, 3, 6, 9, and 10.

8.J.3 Definitions.

The following definitions are not intended to supersede the definitions of active and inactive mining facilities established by 40 CFR 122.26(b)(14)(iii).

8.J.3.1 *Mining operations* - Consists of the active and temporarily inactive phases, and the reclamation phase, but excludes the exploration and construction phases.

8.J.3.2 *Exploration phase* - Entails exploration and land disturbance activities to determine the financial viability of a site. The exploration phase is not considered part of "mining operations."

8.J.3.3 *Construction phase* - Includes the building of site access roads and removal of overburden and waste rock to expose mineable minerals. The construction phase is not considered part of "mining operations".

8.J.3.4 *Active phase* - Activities including the extraction, removal or recovery of minerals. For surface mines, this definition does not include any land where grading has returned the earth to a desired contour and reclamation has begun. This definition is derived from the definition of "active mining area" found at 40 CFR 440.132(a). The active phase is considered part of "mining operations."

8.J.3.5 *Reclamation phase* - Activities undertaken, in compliance with applicable mined land reclamation requirements, following the cessation of the "active phase", intended to return the land to an appropriate post-mining land use. The reclamation phase is considered part of "mining operations".

NOTE: The following definitions are not intended to supersede the definitions of active and inactive mining facilities established by 40 CFR 122.26(b)(14)(iii).

8.J.3.6 *Active Mineral Mining Facility* - A place where work or other activity related to the extraction, removal, or recovery of minerals is being conducted. For surface mines, this definition does not include any land where grading has returned the earth to a desired contour and reclamation has begun. This definition is derived from the definition of "active mining area" found at 40 CFR 440.132(a).

8.J.3.7 *Inactive Mineral Mining Facility* - A site or portion of a site where mineral mining and/or milling occurred in the past but is not an active facility as defined above, and where the inactive portion is not covered by an active mining permit issued by the applicable State or Federal agency. An inactive mineral mining facility has an identifiable owner / operator. Sites where mining claims are being maintained prior to disturbances associated with the extraction, beneficiation, or processing of mined materials, and sites where minimal activities are undertaken for the sole purpose of maintaining a mining claim are not considered either active or inactive mining facilities and do not require an NPDES industrial stormwater permit.

8.J.3.8 *Temporarily Inactive Mineral Mining Facility* - A site or portion of a site where metal mining and/or milling occurred in the past but currently are not being actively undertaken, and the facility is covered by an active mining permit issued by the applicable State or Federal agency.

8.J.3.9 *Final Stabilization* - a site or portion of a site is "finally stabilized" when it has implemented all applicable Federal and State reclamation requirements.

8.J.3.10 *Uncontaminated* - Free from the presence of pollutants attributable to industrial activity.

8.J.4 Technology-Based Effluent Limits for Clearing, Grading, and Excavation Activities.

Clearing, grading, and excavation activities being conducted as part of the exploration and construction phase of mining activities are covered under this permit.

8.J.4.1 *Management Practices for Clearing, Grading, and Excavation Activities.*

8.J.4.1.1 *Selecting and installing control measures.* For all areas affected by clearing, grading, and excavation activities, you must select, design, install, and implement control measures that meet applicable Part 2 effluent limits.

8.J.4.1.2 *Good Housekeeping.* (See also Part 2.1.2.2) Litter, debris, and chemicals must be prevented from becoming a pollutant source in stormwater discharges.

8.J.4.1.3 *Retention and Detention of Stormwater Runoff.* For drainage locations serving more than one acre, sediment basins and/or temporary sediment traps should be used. At a minimum, silt fences, vegetative buffer strips, or equivalent sediment controls are required for all down slope boundaries (and for those side slope boundaries deemed appropriate as dictated by individual site conditions) of the development area unless a sediment basin providing storage for a calculated volume of runoff from a 2-year, 24-hour storm or 3,600 cubic feet of storage per acre drained is provided.

8.J.4.2 *Inspection of Clearing, Grading, and Excavation Activities.* (See also Part 4)

8.J.4.2.1 *Inspection Frequency.* Inspections must be conducted either at least once every 7 calendar days or at least once every 14 calendar days and within 24 hours of the end of a storm event of 0.5 inches or greater. Inspection frequency may be reduced to at least once every month if the entire site is temporarily stabilized (pursuant to Part 8.J.4.3.2), if runoff is unlikely due to winter conditions (e.g., site is covered with snow, ice, or the ground is frozen), or construction is occurring during seasonal arid periods in arid areas and semi-arid areas.

8.J.4.2.2 *Location of Inspections.* Inspections must include all areas of the site disturbed by clearing, grading, and/or excavation activities and areas used for storage of materials that are exposed to precipitation. Sedimentation and erosion control measures implemented must be observed to ensure proper operation. Discharge locations must be inspected to ascertain whether erosion control measures are effective in preventing significant impacts to waters of the United States, where accessible. Where discharge locations are inaccessible, nearby downstream locations must be inspected to the extent that such inspections are practicable. Locations where vehicles enter or exit the site must be inspected for evidence of significant off-site sediment tracking.

8.J.4.2.3 *Inspection Reports.* (See also Part 4.1) For each inspection required above, you must complete an inspection report. At a minimum, the inspection report must include the information required in Part 4.1.

8.J.4.3 *Requirements for Cessation of Clearing, Grading, and Excavation Activities.*

8.J.4.3.1 *Inspections and Maintenance.* Inspections and maintenance of control measures, including any BMPs, associated with clearing, grading, and/or excavation activities being conducted as part of the exploration and construction phase of a mining operation must continue until final stabilization has been achieved on all portions of the disturbed area or until the

commencement of the active mining phase for those areas that have been temporarily stabilized as a precursor to mining

8.J.4.3.2 *Temporary Stabilization of Disturbed Areas.* Stabilization measures should be initiated immediately in portions of the site where clearing, grading and/or excavation activities have temporarily ceased, but in no case more than 14 days after the clearing, grading and/or excavation activities in that portion of the site have temporarily ceased. In arid, semiarid, and drought-stricken areas, or in areas subject to snow or freezing conditions, where initiating perennial vegetative stabilization measures is not possible within 14 days after mining, exploration, and/or construction activity has temporarily ceased, temporary vegetative stabilization measures must be initiated as soon as practicable. Until temporary vegetative stabilization is achieved, interim measures such as erosion control blankets with an appropriate seed base and tackifiers must be employed. In areas of the site, where exploration and/or construction has permanently ceased prior to active mining, temporary stabilization measures must be implemented to minimize mobilization of sediment or other pollutants until such time as the active mining phase commences.

8.J.4.3.3 *Final Stabilization of Disturbed Areas.* Stabilization measures should be initiated immediately in portions of the site where mining, exploration, and/or construction activities have permanently ceased, but in no case more than 14 days after the exploration and/or construction activity in that portion of the site has permanently ceased. In arid, semiarid, and drought-stricken areas, or in areas subject to snow or freezing conditions, where initiating perennial vegetative stabilization measures is not possible within 14 days after mining, exploration, and/or construction activity has permanently ceased, final vegetative stabilization measures must be initiated as soon as possible. Until final stabilization is achieved temporary stabilization measures, such as erosion control blankets with an appropriate seed base and tackifiers must be used.

8.J.5 Additional Technology-Based Effluent Limits.

8.J.5.1 *Employee Training.* Conduct employee training at least annually at active and temporarily inactive sites. (See also Part 2.1.2.9)

8.J.5.2 *Stormwater Controls.* Apart from the control measures you implement to meet your Part 2 effluent limits, where necessary to minimize pollutant discharges, implement the following control measures at your site. The potential pollutants identified in Part 8.J.5.3 shall determine the priority and appropriateness of the control measures selected.

8.J.5.2.1 *Stormwater Diversions:* Consider diverting stormwater away from potential pollutant sources. Following are some control measure options: interceptor or diversion controls (e.g., dikes, swales, curbs, or berms); pipe slope drains; subsurface drains; conveyance systems (e.g., channels or gutters, open-top box culverts, and waterbars; rolling dips and road sloping; roadway surface water deflector and culverts); or their equivalents.

8.J.5.2.2 *Capping:* When capping is necessary to minimize pollutant discharges in stormwater, identify the source being capped and the material used to construct the cap.

8.J.5.2.3 *Treatment:* If treatment of stormwater (e.g., chemical or physical systems, oil and water separators, artificial wetlands) is necessary to protect water quality, describe the type and location of treatment used. Passive and/or active treatment of stormwater runoff is encouraged. Treated runoff may be discharged as a stormwater source regulated under this permit provided the discharge is not combined with discharges subject to effluent limitation guidelines for the Mineral Mining and Processing Point Source Category (40 CFR Part 436).

8.J.5.3 *Certification of Discharge Testing:* (See also Part 5.1.4.4) Test or evaluate all outfalls covered under this permit for the presence of specific mining-related non-stormwater discharges such as discharges subject to effluent limitations guidelines (e.g., 40 CFR Part 436). Alternatively (if applicable), you may keep a certification with your SWPPP.

8.J.6 Additional SWPPP Requirements.

The requirements in Part 8.J.6 are applicable for sites undergoing exploration and construction, active mineral mining facilities, temporarily inactive mineral mining facilities, and sites undergoing reclamation. The requirements in Part 8.J.6 are not applicable to inactive mineral mining facilities.

8.J.6.1 *Nature of Industrial Activities.* (See also Part 5.1.2) Document in your SWPPP the mining and associated activities that can potentially affect the stormwater discharges covered by this permit, including a general description of the location of the site relative to major transportation routes and communities**.**

8.J.6.2 *Site Map.* (See also Part 5.1.2) Document in your SWPPP the locations of the following (as appropriate): mining or milling site boundaries; access and haul roads; outline of the drainage areas of each stormwater outfall within the facility with indications of the types of discharges from the drainage areas; location(s) of all permitted discharges covered under an individual NPDES permit, outdoor equipment storage, fueling, and maintenance areas; materials handling areas; outdoor manufacturing, outdoor storage, and material disposal areas; outdoor chemicals and explosives storage areas; overburden, materials, soils, or waste storage areas; location of mine drainage dewatering or other process water; heap leach pads; off-site points of discharge for mine dewatering and process water; surface waters; boundary of tributary areas that are subject to effluent limitations guidelines; and location(s) of reclaimed areas.

8.J.6.3 *Potential Pollutant Sources.* (See also Part 5.1.3) For each area of the mine or mill site where stormwater discharges associated with industrial activities occur, document in your SWPPP the types of pollutants (e.g., heavy metals, sediment) likely to be present in significant amounts. For example, phosphate mining facilities will likely need to document pollutants such as selenium, which can be present in significant amounts in their discharges. Consider these factors: the mineralogy of the waste rock (e.g., acid forming); toxicity and quantity of chemicals used, produced, or discharged; the

likelihood of contact with stormwater; vegetation of site (if any); and history of significant leaks or spills of toxic or hazardous pollutants. Also include a summary of any existing waste rock or overburden characterization data and test results for potential generation of acid rock drainage.

8.J.6.4 *Stormwater Controls.* To the extent that you use any of the control measures in Part 8.J.5.2, document them in your SWPPP pursuant to Part 5.1.4. If control measures are implemented or planned but are not listed here (e.g., substituting a less toxic chemical for a more toxic one), include descriptions of them in your SWPPP.

8.J.6.4 *Employee Training.* All employee training(s) conducted in accordance with Part 8.J.5.1 must be documented with the SWPPP.

8.J.6.5 *Certification of Permit Coverage for Commingled Non-Stormwater Discharges.* If you determine that you are able to certify, consistent with Part 8.J.5.3, that a particular discharge composed of commingled stormwater and non-stormwater is covered under a separate NPDES permit, and that permit subjects the non-stormwater portion to effluent limitations prior to any commingling, you must retain such certification with your SWPPP. This certification must identify the non-stormwater discharges, the applicable NPDES permit(s), the effluent limitations placed on the non-stormwater discharge by the permit(s), and the points at which the limitations are applied.

8.J.7 Additional Inspection Requirements.

Except for areas of the site subject to clearing, grading, and/or excavation activities conducted as part of the exploration and construction phase, which are subject to Part 8.J.4.2.1, you must inspect sites at least quarterly unless adverse weather conditions make the site inaccessible. Sites which discharge to waters which are designated as outstanding waters or waters which are impaired for sediment or nitrogen must be inspected monthly. See Part 8.J.8.1 for inspection requirements for inactive and unstaffed sites. (See also Part 4.1 and 8.J.4.2.)

8.J.8 Sector-Specific Benchmarks

Table 8.J-1 identifies benchmarks that apply to the specific subsectors of Sector J. These benchmarks apply to both your primary industrial activity and any co-located industrial activities, which describe your site activities.

Table 8.J-1.

Subsector (You may be subject to requirements for more than one sector/subsector)	Parameter	Benchmark Monitoring Concentration
Subsector J1. Sand and Gravel Mining (SIC 1442, 1446)	Nitrate plus Nitrite Nitrogen	0.68 mg/L
	Total Suspended Solids (TSS)	100 mg/L
Subsector J2. Dimension and Crushed Stone and Nonmetallic Minerals (except fuels) (SIC 1411, 1422-1429, 1481, 1499)	Total Suspended Solids (TSS)	100 mg/L

8.J.8.1 *Inactive and Unstaffed Sites – Conditional Exemption from No Exposure Requirement for Routine Inspections, Quarterly Visual Assessments, and Benchmark Monitoring.* As a Sector J facility, if you are seeking to exercise a waiver from either the routine inspection, quarterly visual assessment or the benchmark monitoring requirements for inactive and unstaffed sites (including temporarily inactive sites), you are conditionally exempt from the requirement to certify that "there are no industrial materials or activities exposed to stormwater" in Parts 4.2.3 and 6.2.1.3, respectively. This exemption is conditioned on the following:

- If circumstances change and your facility becomes active and/or staffed, this exception no longer applies and you must immediately begin complying with the applicable benchmark monitoring requirements as if you were in your first year of permit coverage, and the quarterly visual assessment requirements; and
- EPA retains the authority to revoke this exemption and/or the monitoring waiver where it is determined that the discharge causes, has a reasonable potential to cause, or contributes to an instream excursion above an applicable water quality standard, including designated uses.

Subject to the two conditions above, if your facility is inactive and unstaffed, you are waived from the requirement to conduct quarterly visual assessments and routine facility inspections. You are not waived from conducting the Part 4.3 comprehensive site inspection. You are encouraged to inspect your site more frequently where you have reason to believe that severe weather or natural disasters may have damaged control measures or increased discharges.

8.J.9 Effluent Limitations Based on Effluent Limitations Guidelines (See also Part 6.2.2.1 of the permit)

Table 8.J-2 identifies effluent limits that apply to the industrial activities described below. Compliance with these effluent limits is to be determined based on discharges from these industrial activities independent of commingling with any other wastestreams that may be covered under this permit.

Table 8.J-2

Industrial Activity	Parameter	Effluent Limit[1]
Mine dewatering discharges at crushed stone mining facilities (SIC 1422 - 1429)	pH	6.0 - 9.0
Mine dewatering discharges at construction sand and gravel mining facilities (SIC 1442)	pH	6.0 - 9.0
Mine dewatering discharges at industrial sand mining facilities (SIC 1446)	Total Suspended Solids (TSS)	25 mg/L, monthly avg.
		45 mg/L, daily maximum
	pH	6.0 - 9.0

[1]Monitor annually.

8.J.10 Termination of Permit Coverage

8.J.10.1 *Termination of Permit Coverage for Sites Reclaimed After December 17, 1990.* A site or a portion of a site that has been released from applicable state or federal reclamation requirements after December 17, 1990, is no longer required to maintain coverage under this permit. If the site or portion of a site reclaimed after December 17, 1990, was not subject to reclamation requirements, the site or portion of the site is no longer required to maintain coverage under this permit if the site or portion of the site has been reclaimed as defined in Part 8.J.7.2.

8.J.10.2 *Termination of Permit Coverage for Sites Reclaimed Before December 17, 1990.* A site or portion of a site that was released from applicable state or federal reclamation requirements before December 17, 1990, or that was otherwise reclaimed before December 17, 1990, is no longer required to maintain coverage under this permit if the site or portion of the site has been reclaimed. A site or portion of a site is considered to have been reclaimed if: (1) stormwater runoff that comes into contact with raw materials, intermediate byproducts, finished products, and waste products does not have the potential to cause or contribute to violations of state water quality standards, (2) soil disturbing activities related to mining at the sites or portion of the site have been completed, (3) the site or portion of the site has been stabilized to minimize soil erosion, and (4) as appropriate depending on location, size, and the potential to contribute pollutants to stormwater discharges, the site or portion of the site has been revegetated, will be amenable to natural revegetation, or will be left in a condition consistent with the post-mining land use.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart K – Sector K – Hazardous Waste Treatment, Storage, or Disposal Facilities.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.K.1 Covered Stormwater Discharges.

The requirements in Subpart K apply to stormwater discharges associated with industrial activity from Hazardous Waste Treatment, Storage, or Disposal facilities (TSDFs) as identified by the Activity Code specified under Sector K in Table D-1 of Appendix D of the permit.

8.K.2 Industrial Activities Covered by Sector K.

This permit authorizes stormwater discharges associated with industrial activity from facilities that treat, store, or dispose of hazardous wastes, including those that are operating under interim status or a permit under subtitle C of RCRA.

Disposal facilities that have been properly closed and capped, and have no significant materials exposed to stormwater, are considered inactive and do not require permits.

8.K.3 Limitations on Coverage.

8.K.3.1 *Prohibition of Non-Stormwater Discharges.* (See also Part 1.1.4) The following are not authorized by this permit: leachate, gas collection condensate, drained free liquids, contaminated ground water, laboratory-derived wastewater, and contact washwater from washing truck and railcar exteriors and surface areas that have come in direct contact with solid waste at the landfill facility.

8.K.3.2 *Limitations on Coverage for Facilities Providing Commercial TSDF Services.* For facilities located in Region 6 (see Appendix C) coverage is limited to hazardous waste TSDFs that are self-generating (including occasionally accepting wastes from community household hazardous waste collection events as public service), handle only residential wastes, and/or only store hazardous wastes and do not treat or dispose of them. Coverage under this permit is not available to commercial waste disposal and treatment facilities located in Region 6 that dispose and treat on a commercial basis any produced hazardous wastes (i.e., not their own) as a service to commercial or industrial generators.

8.K.4 Definitions.

8.K.4.1 *Contaminated stormwater* - stormwater that comes into direct contact with landfill wastes, the waste handling and treatment areas, or landfill wastewater as defined in Part 8.K.4.5. Some specific areas of a landfill that may produce contaminated stormwater include (but are not limited to) the open face of an active landfill with exposed waste (no cover added); the areas around wastewater treatment operations; trucks, equipment, or machinery that has been in direct contact with the waste; and waste dumping areas.

8.K.4.2 *Drained free liquids* - aqueous wastes drained from waste containers (e.g., drums) prior to landfilling.

8.K.4.3 *Landfill* - an area of land or an excavation in which wastes are placed for permanent disposal, but that is not a land application or land treatment unit, surface impoundment, underground injection well, waste pile, salt dome formation, salt bed formation, underground mine, or cave as these terms are defined in 40 CFR 257.2, 258.2, and 260.10.

8.K.4.4 *Landfill wastewater* - as defined in 40 CFR Part 445 (Landfills Point Source Category), all wastewater associated with, or produced by, landfilling activities except for sanitary wastewater, non-contaminated stormwater, contaminated groundwater, and wastewater from recovery pumping wells. Landfill wastewater includes, but is not limited to, leachate, gas collection condensate, drained free liquids, laboratory derived wastewater, contaminated stormwater, and contact washwater from washing truck, equipment, and railcar exteriors and surface areas that have come in direct contact with solid waste at the landfill facility.

8.K.4.5 *Leachate* - liquid that has passed through or emerged from solid waste and contains soluble, suspended, or miscible materials removed from such waste.

8.K.4.6 *Non-contaminated stormwater* - stormwater that does not come into direct contact with landfill wastes, the waste handling and treatment areas, or landfill wastewater as defined in Part 8.K.4.4. Non-contaminated stormwater includes stormwater that flows off the cap, cover, intermediate cover, daily cover, and/or final cover of the landfill.

8.K.5 Sector-Specific Benchmarks

Table 8.K-1 identifies benchmarks that apply to the specific subsectors of Sector K. These benchmarks apply to both your primary industrial activity and any co-located industrial activities, which describe your site activities.

Table 8.K-1.		
Subsector (You may be subject to requirements for more than one sector/subsector)	**Parameter**	**Benchmark Monitoring Concentration**
Subsector K1. ALL - Industrial Activity Code "HZ" (Note: permit coverage limited in some States). Benchmarks only applicable to discharges not subject to effluent limitations in 40 CFR Part 445 Subpart A (see below).	Ammonia	2.14 mg/L
	Total Magnesium	0.064 mg/L
	Chemical Oxygen Demand (COD)	120 mg/L
	Total Arsenic	0.15 mg/L
	Total Cadmium[1]	Hardness Dependent
	Total Cyanide	0.022 mg/ L
	Total Lead[1]	Hardness Dependent
	Total Mercury	0.0014 mg/ L
	Total Selenium	0.005 mg/L
	Total Silver[1]	Hardness Dependent

[1] The benchmark values of some metals are dependent on water hardness. For these parameters, permittees must determine the hardness of the receiving water (see Appendix J, "Calculating Hardness in Receiving Waters for Hardness Dependent Metals," for methodology), in accordance with Part 6.2.1.1, to identify the applicable 'hardness range' for determining their benchmark value applicable to their facility. The ranges occur in 25 mg/L increments. Hardness Dependent Benchmarks follow in the table below:

Water Hardness Range	**Cadmium** (mg/L)	**Lead** (mg/L)	**Silver** (mg/L)
0-25 mg/L	0.0005	0.014	0.0007
25-50 mg/L	0.0008	0.023	0.0007
50-75 mg/L	0.0013	0.045	0.0017
75-100 mg/L	0.0018	0.069	0.0030
100-125 mg/L	0.0023	0.095	0.0046
125-150 mg/L	0.0029	0.122	0.0065
150-175 mg/L	0.0034	0.151	0.0087
175-200 mg/L	0.0039	0.182	0.0112
200-225 mg/L	0.0045	0.213	0.0138
225-250 mg/L	0.0050	0.246	0.0168
250+ mg/L	0.0053	0.262	0.0183

8.K.6 Effluent Limitations Based on Effluent Limitations Guidelines (See also Part 6.2.2.1 of the permit.)

Table 8.K-2 identifies effluent limits that apply to the industrial activities described below. Compliance with these effluent limits is to be determined based on discharges from these industrial activities independent of commingling with any other wastestreams that may be covered under this permit.

Table 8.K-2[1]		
Industrial Activity	**Parameter**	**Effluent Limit**
Discharges from hazardous waste landfills subject to effluent limitations in 40 CFR Part 445 Subpart A (see footnote).	Biochemical Oxygen Demand (BOD_5)	220 mg/L, daily maximum
		56 mg/L, monthly avg. maximum
	Total Suspended Solids (TSS)	88 mg/L, daily maximum
		27 mg/L, monthly avg. maximum
	Ammonia	10 mg/L, daily maximum
		4.9 mg/L, monthly avg. maximum
	Alpha Terpineol	0.042 mg/L, daily maximum
		0.019 mg/L, monthly avg. maximum
	Aniline	0.024 mg/L, daily maximum
		0.015 mg/L, monthly avg. maximum
	Benzoic Acid	0.119 mg/L, daily maximum
		0.073 mg/L, monthly avg. maximum
	Naphthalene	0.059 mg/L, daily maximum
		0.022 mg/L, monthly avg. maximum
	p-Cresol	0.024 mg/L, daily maximum
		0.015 mg/L, monthly avg. maximum
	Phenol	0.048 mg/L, daily maximum
		0.029 mg/L, monthly avg. maximum
	Pyridine	0.072 mg/L, daily maximum
		0.025 mg/L, monthly avg. maximum
	Total Arsenic	1.1 mg/L, daily maximum
		0.54 mg/L, monthly avg. maximum
	Total Chromium	1.1 mg/L, daily maximum
		0.46 mg/L, monthly avg. maximum
	Total Zinc	0.535 mg/L, daily maximum
		0.296 mg/L, monthly avg. maximum
	pH	Within the range of 6-9 standard pH units (s.u.)

[1] Monitor annually. As set forth at 40 CFR Part 445 Subpart A, these numeric limitations apply to contaminated stormwater discharges from hazardous waste landfills subject to the provisions of RCRA Subtitle C at 40 CFR Parts 264 (Subpart N) and 265 (Subpart N) except for any of the following facilities:

(a) landfills operated in conjunction with other industrial or commercial operations when the landfill receives only wastes generated by the industrial or commercial operation directly associated with the landfill;

(b) landfills operated in conjunction with other industrial or commercial operations when the landfill receives wastes generated by the industrial or commercial operation directly associated with the landfill and also receives other wastes, provided that the other wastes received for disposal are generated by a facility that is subject to the same provisions in 40 CFR Subchapter N as the industrial or commercial operation or that the other wastes received are of similar nature to the wastes generated by the industrial or commercial operation;

(c) landfills operated in conjunction with Centralized Waste Treatment (CWT) facilities subject to 40 CFR Part 437, so long as the CWT facility commingles the landfill wastewater with other non-landfill wastewater for discharge. A landfill directly associated with a CWT facility is subject to this part if the CWT facility discharges landfill wastewater separately from other CWT wastewater or commingles the wastewater from its landfill only with wastewater from other landfills; or

(d) landfills operated in conjunction with other industrial or commercial operations when the landfill receives wastes from public service activities, so long as the company owning the landfill does not receive a fee or other remuneration for the disposal service.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart L – Sector L – Landfills, Land Application Sites, and Open Dumps.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.L.1 Covered Stormwater Discharges.

The requirements in Subpart L apply to stormwater discharges associated with industrial activity from Landfills and Land Application Sites and Open Dumps as identified by the Activity Code specified under Sector L in Table D-1 of Appendix D of the permit.

8.L.2 Industrial Activities Covered by Sector L.

This permit may authorize stormwater discharges for Sector L facilities associated with waste disposal at landfills, land application sites, and open dumps that receive or have received industrial waste, including sites subject to regulation under Subtitle D of RCRA. This permit does not cover discharges from landfills that receive only municipal wastes.

8.L.3 Limitations on Coverage.

8.L.3.1 *Prohibition of Non-Stormwater Discharges.* (See also Part 1.1.4) The following discharges are not authorized by this permit: leachate, gas collection condensate, drained free liquids, contaminated ground water, laboratory wastewater, and contact washwater from washing truck and railcar exteriors and surface areas that have come in direct contact with solid waste at the landfill facility.

8.L.4 Definitions.

8.L.4.1 *Contaminated stormwater* - stormwater that comes into direct contact with landfill wastes, the waste handling and treatment areas, or landfill wastewater. Some areas of a landfill that may produce contaminated stormwater include (but are not limited to) the open face of an active landfill with exposed waste (no cover added); the areas around wastewater treatment operations; trucks, equipment, or machinery that has been in direct contact with the waste; and waste dumping areas.

8.L.4.2 *Drained free liquids* - aqueous wastes drained from waste containers (e.g., drums) prior to landfilling.

8.L.4.3 *Landfill wastewater* - as defined in 40 CFR Part 445 (Landfills Point Source Category) all wastewater associated with, or produced by, landfilling activities except for sanitary wastewater, non-contaminated stormwater, contaminated groundwater, and wastewater from recovery pumping wells. Landfill process wastewater includes, but is not limited to, leachate; gas collection condensate; drained free liquids; laboratory-derived wastewater; contaminated stormwater; and contact washwater from washing truck,

equipment, and railcar exteriors and surface areas that have come in direct contact with solid waste at the landfill facility.

8.L.4.4 *Leachate* - liquid that has passed through or emerged from solid waste and contains soluble, suspended, or miscible materials removed from such waste.

8.L.4.5 *Non-contaminated stormwater* - stormwater that does not come into direct contact with landfill wastes, the waste handling and treatment areas, or landfill wastewater. Non-contaminated stormwater includes stormwater that flows off the cap, cover, intermediate cover, daily cover, and/or final cover of the landfill.

8.L.5 Additional Technology-Based Effluent Limits.

8.L.5.1 *Preventive Maintenance Program.* (See also Part 2.1.2.3) As part of your preventive maintenance program, maintain the following: all elements of leachate collection and treatment systems, to prevent commingling of leachate with stormwater; the integrity and effectiveness of any intermediate or final cover (including repairing the cover as necessary), to minimize the effects of settlement, sinking, and erosion.

8.L.5.2 *Erosion and Sedimentation Control.* (See also Part 2.1.2.5) Provide temporary stabilization (e.g., temporary seeding, mulching, and placing geotextiles on the inactive portions of stockpiles) for the following: materials stockpiled for daily, intermediate, and final cover; inactive areas of the landfill or open dump; landfills or open dump areas that have gotten final covers but where vegetation has yet to establish itself; and land application sites where waste application has been completed but final vegetation has not yet been established.

8.L.5.3 *Unauthorized Discharge Test Certification.* (See also Part 5.1.3.4) The discharge test and certification must also be conducted for the presence of leachate and vehicle washwater.

8.L.6 Additional SWPPP Requirements.

8.L.5.1 *Drainage Area Site Map.* (See also Part 5.1.2) Document in your SWPPP where any of the following may be exposed to precipitation or surface runoff: active and closed landfill cells or trenches, active and closed land application areas, locations where open dumping is occurring or has occurred, locations of any known leachate springs or other areas where uncontrolled leachate may commingle with runoff, and leachate collection and handling systems.

8.L.5.2 *Summary of Potential Pollutant Sources.* (See also Part 5.1.3) Document in your SWPPP the following sources and activities that have potential pollutants associated with them: fertilizer, herbicide, and pesticide application; earth and soil moving; waste hauling and loading or unloading; outdoor storage of significant materials, including daily, interim, and final cover material stockpiles as well as temporary waste storage areas; exposure of active and inactive landfill and land application areas; uncontrolled leachate flows; and failure or leaks from leachate collection and treatment systems.

8.L.7 Additional Inspection Requirements. (See also Part 4)

8.L.7.1 *Inspections of Active Sites.* Except in arid and semi-arid climates, inspect operating landfills, open dumps, and land application sites at least once every 7 days. Focus on areas of landfills that have not yet been finally stabilized; active land application areas, areas used for storage of material and wastes that are exposed to precipitation, stabilization, and structural control measures; leachate collection and treatment systems; and locations where equipment and waste trucks enter and exit the site. Ensure that sediment and erosion control measures are operating properly. For stabilized sites and areas where land application has been completed, or where the climate is arid or semi-arid, conduct inspections at least once every month.

8.L.7.2 *Inspections of Inactive Sites.* Inspect inactive landfills, open dumps, and land application sites at least quarterly. Qualified personnel must inspect landfill (or open dump) stabilization and structural erosion control measures, leachate collection and treatment systems, and all closed land application areas.

8.L.8 Additional Post-Authorization Documentation Requirements.

8.L.8.1 *Recordkeeping and Internal Reporting.* Keep records with your SWPPP of the types of wastes disposed of in each cell or trench of a landfill or open dump. For land application sites, track the types and quantities of wastes applied in specific areas.

8.L.9 Sector-Specific Benchmarks

Table 8.L-1 identifies benchmarks that apply to the specific subsectors of Sector L. These benchmarks apply to both your primary industrial activity and any co-located industrial activities, which describe your site activities.

Table 8.L-1.

Subsector (You may be subject to requirements for more than one sector/subsector)	Parameter	Benchmark Monitoring Concentration[1]
Subsector L1. All Landfill, Land Application Sites and Open Dumps (Industrial Activity Code "LF")	Total Suspended Solids (TSS)	100 mg/L
Subsector L2. All Landfill, Land Application Sites and Open Dumps, except Municipal Solid Waste Landfill (MSWLF) Areas Closed in Accordance with 40 CFR 258.60 (Industrial Activity Code "LF")	Total Iron	1.0 mg/L

[1]Benchmark monitoring required only for discharges not subject to effluent limitations in 40 CFR Part 445 Subpart B (see Table L-2 above).

8.L.10. Effluent Limitations Based on Effluent Limitations Guidelines (See also Part 6.2.2.1 of the permit.)

Table 8.L-2 identifies effluent limits that apply to the industrial activities described below. Compliance with these effluent limits is to be determined based on discharges from these industrial activities independent of commingling with any other wastestreams that may be covered under this permit.

Table 8.L-2[1]

Industrial Activity	Parameter	Effluent Limit
Discharges from non-hazardous waste landfills subject to effluent limitations in 40 CFR Part 445 Subpart B.	Biochemical Oxygen Demand (BOD_5)	140 mg/L, daily maximum
		37 mg/L, monthly avg. maximum
	Total Suspended Solids (TSS)	88 mg/L, daily maximum
		27 mg/L, monthly avg. maximum
	Ammonia	10 mg/L, daily maximum
		4.9 mg/L, monthly avg. maximum
	Alpha Terpineol	0.033 mg/L, daily maximum
		0.016 mg/L monthly avg. maximum
	Benzoic Acid	0.12 mg/L, daily maximum
		0.071 mg/L, monthly avg. maximum
	p-Cresol	0.025 mg/L, daily maximum
		0.014 mg/L, monthly avg. maximum
	Phenol	0.026 mg/L, daily maximum
		0.015 mg/L, monthly avg. maximum
	Total Zinc	0.20 mg/L, daily maximum
		0.11 mg/L, monthly avg. maximum
	pH	Within the range of 6-9 standard pH units (s.u.)

[1] Monitor annually. As set forth at 40 CFR Part 445 Subpart B, these numeric limitations apply to contaminated stormwater discharges from MSWLFs that have not been closed in accordance with 40 CFR 258.60, and to contaminated stormwater discharges from those landfills that are subject to the provisions of 40 CFR Part 257 except for discharges from any of the following facilities:

(a) landfills operated in conjunction with other industrial or commercial operations, when the landfill receives only wastes generated by the industrial or commercial operation directly associated with the landfill;

(b) landfills operated in conjunction with other industrial or commercial operations, when the landfill receives wastes generated by the industrial or commercial operation directly associated with the landfill and also receives other wastes, provided that the other wastes received for disposal are generated by a facility that is subject to the same provisions in 40 CFR Subchapter N as the industrial or commercial operation, or that the other wastes received are of similar nature to the wastes generated by the industrial or commercial operation;

(c) landfills operated in conjunction with CWT facilities subject to 40 CFR Part 437, so long as the CWT facility commingles the landfill wastewater with other non-landfill wastewater for discharge. A landfill directly associated with a CWT facility is subject to this part if the CWT facility discharges landfill wastewater separately from other CWT wastewater or commingles the wastewater from its landfill only with wastewater from other landfills; or

(d) landfills operated in conjunction with other industrial or commercial operations when the landfill receives wastes from public service activities, so long as the company owning the landfill does not receive a fee or other remuneration for the disposal service.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart M – Sector M – Automobile Salvage Yards.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.M.1 Covered Stormwater Discharges.

The requirements in Subpart M apply to stormwater discharges associated with industrial activity from Automobile Salvage Yards as identified by the SIC Code specified under Sector M in Table D-1 of Appendix D of this permit.

8.M.2 Additional Technology-Based Effluent Limits.

8.M.2.1 *Spill and Leak Prevention Procedures.* (See also Part 2.1.2.4) Drain vehicles intended to be dismantled of all fluids upon arrival at the site (or as soon thereafter as feasible), or employ some other equivalent means to prevent spills and leaks.

8.M.2.2 *Employee Training.* (See also Part 2.1.2.9) If applicable to your facility, address the following areas (at a minimum) in your employee training program: proper handling (collection, storage, and disposal) of oil, used mineral spirits, anti-freeze, mercury switches, and solvents.

8.M.2.3 *Management of Runoff.* (See also Part 2.1.2.6) Consider the following management practices: berms or drainage ditches on the property line (to help prevent run-on from neighboring properties); berms for uncovered outdoor storage of oily parts, engine blocks, and above-ground liquid storage; installation of detention ponds; and installation of filtering devices and oil and water separators.

8.M.3 Additional SWPPP Requirements.

8.M.3.1 *Drainage Area Site Map.* (See also Part 5.1.2) Identify locations used for dismantling, storage, and maintenance of used motor vehicle parts. Also identify where any of the following may be exposed to precipitation or surface runoff: dismantling areas, parts (e.g., engine blocks, tires, hub caps, batteries, hoods, mufflers) storage areas, and liquid storage tanks and drums for fuel and other fluids.

8.M.3.2 *Potential Pollutant Sources.* (See also Part 5.1.3) Assess the potential for the following to contribute pollutants to stormwater discharges: vehicle storage areas, dismantling areas, parts storage areas (e.g., engine blocks, tires, hub caps, batteries, hoods, mufflers), and fueling stations.

8.M.4 Additional Inspection Requirements. (See also Part 4.1) Immediately (or as soon thereafter as feasible) inspect vehicles arriving at the site for leaks. Inspect quarterly for signs of leakage all equipment containing oily parts, hydraulic fluids, any other types of fluids, or mercury switches. Also, inspect quarterly for signs of leakage all vessels and

areas where hazardous materials and general automotive fluids are stored, including, but not limited to, mercury switches, brake fluid, transmission fluid, radiator water, and antifreeze.

8.M.5 Sector-Specific Benchmarks. (See also Part 6 of the permit.)

Table 8.M-1.

Subsector (You may be subject to requirements for more than one sector/subsector)	Parameter	Benchmark Monitoring Concentration
Subsector M1. Automobile Salvage Yards (SIC 5015)	Total Suspended Solids (TSS)	100 mg/L
	Total Aluminum	0.75 mg/L
	Total Iron	1.0 mg/L
	Total Lead[1]	Hardness Dependent

[1] The benchmark values of some metals are dependent on water hardness. For these parameters, permittees must determine the hardness of the receiving water (see Appendix J, "Calculating Hardness in Receiving Waters for Hardness Dependent Metals," for methodology), in accordance with Part 6.2.1.1, to identify the applicable 'hardness range' for determining their benchmark value applicable to their facility. The ranges occur in 25 mg/L increments. Hardness Dependent Benchmarks follow in the table below:

Water Hardness Range	Lead (mg/L)
0-25 mg/L	0.014
25-50 mg/L	0.023
50-75 mg/L	0.045
75-100 mg/L	0.069
100-125 mg/L	0.095
125-150 mg/L	0.122
150-175 mg/L	0.151
175-200 mg/L	0.182
200-225 mg/L	0.213
225-250 mg/L	0.246
250+ mg/L	0.262

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart N – Sector N – Scrap Recycling and Waste Recycling Facilities.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.N.1 Covered Stormwater Discharges.

The requirements in Subpart N apply to stormwater discharges associated with industrial activity from Scrap Recycling and Waste Recycling facilities as identified by the SIC Code specified under Sector N in Table D-1 of Appendix D of the permit.

8.N.2 Limitation on Coverage.

Separate permit requirements have been established for recycling facilities that only receive source-separated recyclable materials primarily from non-industrial and residential sources (i.e., common consumer products including paper, newspaper, glass, cardboard, plastic containers, and aluminum and tin cans). This includes recycling facilities commonly referred to as material recovery facilities (MRF).

8.N.2.1 *Prohibition of Non-Stormwater Discharges.* (See also Part 1.1.4) Non-stormwater discharges from turnings containment areas are not covered by this permit (see also Part 8.N.3.2.3). Discharges from containment areas in the absence of a storm event are prohibited unless covered by a separate NPDES permit.

8.N.3 Additional Technology-Based Effluent Limits.

8.N.3.1 *Scrap and Waste Recycling Facilities (Non-Source Separated, Nonliquid Recyclable Materials).* Requirements for facilities that receive, process, and do wholesale distribution of nonliquid recyclable wastes (e.g., ferrous and nonferrous metals, plastics, glass, cardboard, and paper). These facilities may receive both nonrecyclable and recyclable materials. This section is not intended for those facilities that accept recyclables only from primarily non-industrial and residential sources.

8.N.3.1.1 *Inbound Recyclable and Waste Material Control Program.* Minimize the chance of accepting materials that could be significant sources of pollutants by conducting inspections of inbound recyclables and waste materials. Following are some control measure options: (a) provide information and education to suppliers of scrap and recyclable waste materials on draining and properly disposing of residual fluids (e.g., from vehicles and equipment engines, radiators and transmissions, oil filled transformers, and individual containers or drums) and removal of mercury switches from vehicles before delivery to your facility; (b) establish procedures to minimize the potential of any residual fluids from coming into contact with precipitation or runoff; (c) establish procedures for accepting scrap lead-acid batteries (additional requirements for

the handling, storage, and disposal or recycling of batteries are contained in the scrap lead-acid battery program provisions in Part 8.N.3.2.6); (d) provide training targeted for those personnel engaged in the inspection and acceptance of inbound recyclable materials; and (e) establish procedures to ensure that liquid wastes, including used oil, are stored in materially compatible and non-leaking containers and are disposed of or recycled in accordance with the Resource Conservation and Recovery Act (RCRA).

8.N.3.1.2 *Scrap and Waste Material Stockpiles and Storage (Outdoor).* Minimize contact of stormwater runoff with stockpiled materials, processed materials, and nonrecyclable wastes. Following are some control measure options: (a) permanent or semi-permanent covers; (b) sediment traps, vegetated swales and strips, catch basin filters, and sand filters to facilitate settling or filtering of pollutants; (c) dikes, berms, containment trenches, culverts, and surface grading to divert runoff from storage areas; (d) silt fencing; and (e) oil and water separators, sumps, and dry absorbents for areas where potential sources of residual fluids are stockpiled (e.g., automobile engine storage areas).

8.N.3.1.3 *Stockpiling of Turnings Exposed to Cutting Fluids (Outdoor Storage).* Minimize contact of surface runoff with residual cutting fluids by: (a) storing all turnings exposed to cutting fluids under some form of permanent or semi-permanent cover, or (b) establishing dedicated containment areas for all turnings that have been exposed to cutting fluids. Any containment areas must be constructed of concrete, asphalt, or other equivalent types of impermeable material and include a barrier (e.g., berms, curbing, elevated pads) to prevent contact with stormwater run-on. Stormwater runoff from these areas can be discharged, provided that any runoff is first collected and treated by an oil and water separator or its equivalent. You must regularly maintain the oil and water separator (or its equivalent) and properly dispose of or recycle collected residual fluids.

8.N.3.1.4 *Scrap and Waste Material Stockpiles and Storage (Covered or Indoor Storage).* Minimize contact of residual liquids and particulate matter from materials stored indoors or under cover with surface runoff. Following are some control measure options: (a) good housekeeping measures, including the use of dry absorbents or wet vacuuming to contain, dispose of, or recycle residual liquids originating from recyclable containers, or mercury spill kits for spills from storage of mercury switches; (b) not allowing washwater from tipping floors or other processing areas to discharge to the storm sewer system; and (c) disconnecting or sealing off all floor drains connected to the storm sewer system.

8.N.3.1.5 *Scrap and Recyclable Waste Processing Areas.* Minimize surface runoff from coming in contact with scrap processing equipment. Pay attention to operations that generate visible amounts of particulate residue (e.g., shredding) to minimize the contact of accumulated particulate matter and residual fluids with runoff (i.e., through good housekeeping, preventive maintenance, etc.). Following are some control measure options: (a) regularly

inspect equipment for spills or leaks and malfunctioning, worn, or corroded parts or equipment; (b) establish a preventive maintenance program for processing equipment; (c) use dry-absorbents or other cleanup practices to collect and dispose of or recycle spilled or leaking fluids or use mercury spill kits for spills from storage of mercury switches; (d) on unattended hydraulic reservoirs over 150 gallons in capacity, install protection devices such as low-level alarms or equivalent devices, or secondary containment that can hold the entire volume of the reservoir; (e) containment or diversion structures such as dikes, berms, culverts, trenches, elevated concrete pads, and grading to minimize contact of stormwater runoff with outdoor processing equipment or stored materials; (f) oil and water separators or sumps; (g) permanent or semi-permanent covers in processing areas where there are residual fluids and grease; (h) retention or detention ponds or basins; sediment traps, and vegetated swales or strips (for pollutant settling and filtration); (i) catch basin filters or sand filters.

8.N.3.1.6 *Scrap Lead-Acid Battery Program.* Properly handle, store, and dispose of scrap lead-acid batteries. Following are some control measure options (a) segregate scrap lead-acid batteries from other scrap materials; (b) properly handle, store, and dispose of cracked or broken batteries; (c) collect and dispose of leaking lead-acid battery fluid; (d) minimize or eliminate (if possible) exposure of scrap lead-acid batteries to precipitation or runoff; and (e) provide employee training for the management of scrap batteries.

8.N.3.1.7 *Spill Prevention and Response Procedures.* (See also Part 2.1.2.4)Install alarms and/or pump shutoff systems on outdoor equipment with hydraulic reservoirs exceeding 150 gallons in the event of a line break. Alternatively, a secondary containment system capable of holding the entire contents of the reservoir plus room for precipitation can be used. Use a mercury spill kit for any release of mercury from switches, anti-lock brake systems, and switch storage areas.

8.N.3.1.8 *Supplier Notification Program.* As appropriate, notify major suppliers which scrap materials will not be accepted at the facility or will be accepted only under certain conditions.

8.N.3.2 Waste Recycling Facilities (Liquid Recyclable Materials).

8.N.3.2.1 *Waste Material Storage (Indoor).* Minimize or eliminate contact between residual liquids from waste materials stored indoors and from surface runoff. The plan may refer to applicable portions of other existing plans, such as Spill Prevention, Control, and Countermeasure (SPCC) plans required under 40 CFR Part 112. Following are some control measure options (a) procedures for material handling (including labeling and marking); (b) clean up spills and leaks with dry absorbent materials, a wet vacuum system; (c) appropriate containment structures (trenching, curbing, gutters, etc.); and (d) a drainage system, including appurtenances (e.g., pumps or ejectors, manually operated valves), to handle discharges from diked or bermed areas. Drainage should be

discharged to an appropriate treatment facility or sanitary sewer system, or otherwise disposed of properly. These discharges may require coverage under a separate NPDES wastewater permit or industrial user permit under the pretreatment program.

8.N.3.2.2 *Waste Material Storage (Outdoor).* Minimize contact between stored residual liquids and precipitation or runoff. The plan may refer to applicable portions of other existing plans, such as SPCC plans required under 40 CFR Part 112. Discharges of precipitation from containment areas containing used oil must also be in accordance with applicable sections of 40 CFR Part 112. Following are some control measure options (a) appropriate containment structures (e.g., dikes, berms, curbing, pits) to store the volume of the largest tank, with sufficient extra capacity for precipitation; (b) drainage control and other diversionary structures; (c) corrosion protection and/or leak detection systems for storage tanks; and (d) dry-absorbent materials or a wet vacuum system to collect spills.

8.N.3.2.3 *Trucks and Rail Car Waste Transfer Areas.* Minimize pollutants in discharges from truck and rail car loading and unloading areas. Include measures to clean up minor spills and leaks resulting from the transfer of liquid wastes. Following are two control measure options: (a) containment and diversionary structures to minimize contact with precipitation or runoff, and (b) dry clean-up methods, wet vacuuming, roof coverings, or runoff controls.

8.N.3.3 *Recycling Facilities (Source-Separated Materials).* The following identifies considerations for facilities that receive only source-separated recyclables, primarily from non-industrial and residential sources.

8.N.3.3.1 *Inbound Recyclable Material Control.* Minimize the chance of accepting nonrecyclables (e.g., hazardous materials) that could be a significant source of pollutants by conducting inspections of inbound materials. Following are some control measure options: (a) providing information and education measures to inform suppliers of recyclables about acceptable and non-acceptable materials, (b) training drivers responsible for pickup of recycled material, (c) clearly marking public drop-off containers regarding which materials can be accepted, (d) rejecting nonrecyclable wastes or household hazardous wastes at the source, and (e) establishing procedures for handling and disposal of nonrecyclable material.

8.N.3.3.2 *Outdoor Storage.* Minimize exposure of recyclables to precipitation and runoff. Use good housekeeping measures to prevent accumulation of particulate matter and fluids, particularly in high traffic areas. Following are some control measure options (a) provide totally enclosed drop-off containers for the public; (b) install a sump and pump with each container pit and treat or discharge collected fluids to a sanitary sewer system; (c) provide dikes and curbs for secondary containment (e.g., around bales of recyclable waste paper); (d) divert surface water runoff away from outside material storage areas; (e) provide covers over containment bins, dumpsters, and roll-off boxes;

and (f) store the equivalent of one day's volume of recyclable material indoors.

8.N.3.3.3 *Indoor Storage and Material Processing.* Minimize the release of pollutants from indoor storage and processing areas. Following are some control measure options (a) schedule routine good housekeeping measures for all storage and processing areas, (b) prohibit tipping floor washwater from draining to the storm sewer system, and (c) provide employee training on pollution prevention practices.

8.N.3.3.4 *Vehicle and Equipment Maintenance.* Following are some control measure options for areas where vehicle and equipment maintenance occur outdoors (a) prohibit vehicle and equipment washwater from discharging to the storm sewer system, (b) minimize or eliminate outdoor maintenance areas whenever possible, (c) establish spill prevention and clean-up procedures in fueling areas, (d) avoid topping off fuel tanks, (e) divert runoff from fueling areas, (f) store lubricants and hydraulic fluids indoors, and (g) provide employee training on proper handling and storage of hydraulic fluids and lubricants.

8.N.4 Additional SWPPP Requirements.

8.N.4.1 *Drainage Area Site Map.* (See also Part 5.1.2) Document in your SWPPP the locations of any of the following activities or sources that may be exposed to precipitation or surface runoff: scrap and waste material storage, outdoor scrap and waste processing equipment; and containment areas for turnings exposed to cutting fluids.

8.N.4.2 *Maintenance Schedules/Procedures for Collection, Handling, and Disposal or Recycling of Residual Fluids at Scrap and Waste Recycling Facilities.* If you are subject to Part 8.N.3.1.3, your SWPPP must identify any applicable maintenance schedule and the procedures to collect, handle, and dispose of or recycle residual fluids.

8.N.5 Additional Inspection Requirements.

8.N.5.1 *Inspections for Waste Recycling Facilities.* The inspections must be performed quarterly, pursuant to Part 4.1, and include, at a minimum, all areas where waste is generated, received, stored, treated, or disposed of and that are exposed to either precipitation or stormwater runoff.

8.N.6 Sector-Specific Benchmarks. (See also Part 6 of the permit.)

Table 8.N-1.		
Subsector (You may be subject to requirements for more than one sector/subsector)	**Parameter**	**Benchmark Monitoring Concentration**
Subsector N1. Scrap Recycling and Waste Recycling Facilities except Source-Separated Recycling (SIC 5093)	Chemical Oxygen Demand (COD)	120 mg/L
	Total Suspended Solids (TSS)	100 mg/L
	Total Recoverable Aluminum	0.75 mg/L
	Total Recoverable Copper[1]	Hardness Dependent
	Total Recoverable Iron	1.0 mg/L
	Total Recoverable Lead[1]	Hardness Dependent
	Total Recoverable Zinc[1]	Hardness Dependent

[1] The benchmark values of some metals are dependent on water hardness. For these parameters, permittees must determine the hardness of the receiving water (see Appendix J, "Calculating Hardness in Receiving Waters for Hardness Dependent Metals," for methodology), in accordance with Part 6.2.1.1, to identify the applicable 'hardness range' for determining their benchmark value applicable to their facility. The ranges occur in 25 mg/L increments. Hardness Dependent Benchmarks follow in the table below:

Water Hardness Range	**Copper** (mg/L)	**Lead** (mg/L)	**Zinc** (mg/L)
0-25 mg/L	0.0038	0.014	0.04
25-50 mg/L	0.0056	0.023	0.05
50-75 mg/L	0.0090	0.045	0.08
75-100 mg/L	0.0123	0.069	0.11
100-125 mg/L	0.0156	0.095	0.13
125-150 mg/L	0.0189	0.122	0.16
150-175 mg/L	0.0221	0.151	0.18
175-200 mg/L	0.0253	0.182	0.20
200-225 mg/L	0.0285	0.213	0.23
225-250 mg/L	0.0316	0.246	0.25
250+ mg/L	0.0332	0.262	0.26

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart O – Sector O – Steam Electric Generating Facilities.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.O.1 Covered Stormwater Discharges.

The requirements in Subpart O apply to stormwater discharges associated with industrial activity from Steam Electric Power Generating Facilities as identified by the Activity Code specified under Sector O in Table D-1 of Appendix D.

8.O.2 Industrial Activities Covered by Sector O.

This permit authorizes stormwater discharges from the following industrial activities at Sector O facilities:

8.O.2.1 steam electric power generation using coal, natural gas, oil, nuclear energy, etc., to produce a steam source, including coal handling areas;

8.O.2.2 coal pile runoff, including effluent limitations established by 40 CFR Part 423; and

8.O.2.3 dual fuel facilities that could employ a steam boiler.

8.O.3 Limitations on Coverage.

8.O.3.1 *Prohibition of Non-Stormwater Discharges.* Non-stormwater discharges subject to effluent limitations guidelines are not covered by this permit.

8.O.3.2 *Prohibition of Stormwater Discharges.* Stormwater discharges from the following are not covered by this permit:

8.O.3.2.1 ancillary facilities (e.g., fleet centers and substations) that are not contiguous to a stream electric power generating facility;

8.O.3.2.2 gas turbine facilities (providing the facility is not a dual-fuel facility that includes a steam boiler), and combined-cycle facilities where no supplemental fuel oil is burned (and the facility is not a dual-fuel facility that includes a steam boiler); and

8.O.3.2.3 cogeneration (combined heat and power) facilities utilizing a gas turbine.

8.O.4 Additional Technology-Based Effluent Limits. The following good housekeeping measures are required in addition to Part 2.1.2.2:

8.O.4.1 *Fugitive Dust Emissions.* Minimize fugitive dust emissions from coal handling areas. To minimize the tracking of coal dust offsite, consider procedures such as installing

specially designed tires or washing vehicles in a designated area before they leave the site and controlling the wash water.

8.O.4.2 *Delivery Vehicles.* Minimize contamination of stormwater runoff from delivery vehicles arriving at the plant site. Consider procedures to inspect delivery vehicles arriving at the plant site and ensure overall integrity of the body or container and procedures to deal with leakage or spillage from vehicles or containers.

8.O.4.3 *Fuel Oil Unloading Areas.* Minimize contamination of precipitation or surface runoff from fuel oil unloading areas. Consider using containment curbs in unloading areas, having personnel familiar with spill prevention and response procedures present during deliveries to ensure that any leaks or spills are immediately contained and cleaned up, and using spill and overflow protection devices (e.g., drip pans, drip diapers, or other containment devices placed beneath fuel oil connectors to contain potential spillage during deliveries or from leaks at the connectors).

8.O.4.4 *Chemical Loading and Unloading.* Minimize contamination of precipitation or surface runoff from chemical loading and unloading areas. Consider using containment curbs at chemical loading and unloading areas to contain spills, having personnel familiar with spill prevention and response procedures present during deliveries to ensure that any leaks or spills are immediately contained and cleaned up, and loading and unloading in covered areas and storing chemicals indoors.

8.O.4.5 *Miscellaneous Loading and Unloading Areas.* Minimize contamination of precipitation or surface runoff from loading and unloading areas. Consider covering the loading area; grading, berming, or curbing around the loading area to divert run-on; locating the loading and unloading equipment and vehicles so that leaks are contained in existing containment and flow diversion systems; or equivalent procedures.

8.O.4.6 *Liquid Storage Tanks.* Minimize contamination of surface runoff from above-ground liquid storage tanks. Consider protective guards around tanks, containment curbs, spill and overflow protection, dry cleanup methods, or equivalent measures.

8.O.4.7 *Large Bulk Fuel Storage Tanks.* Minimize contamination of surface runoff from large bulk fuel storage tanks. Consider containment berms (or their equivalent). You must also comply with applicable State and Federal laws, including Spill Prevention, Control and Countermeasure (SPCC) Plan requirements.

8.O.4.8 *Spill Reduction Measures.* Minimize the potential for an oil or chemical spill, or reference the appropriate part of your SPCC plan. Visually inspect as part of your routine facility inspection the structural integrity of all above-ground tanks, pipelines, pumps, and related equipment that may be exposed to stormwater, and make any necessary repairs immediately.

8.O.4.9 *Oil-Bearing Equipment in Switchyards.* Minimize contamination of surface runoff from oil-bearing equipment in switchyard areas. Consider using level grades and gravel surfaces to retard flows and limit the spread of spills, or collecting runoff in perimeter ditches.

8.O.4.10 *Residue-Hauling Vehicles.* Inspect all residue-hauling vehicles for proper covering over the load, adequate gate sealing, and overall integrity of the container body. Repair

vehicles without load covering or adequate gate sealing, or with leaking containers or beds.

8.O.4.11 *Ash Loading Areas.* Reduce or control the tracking of ash and residue from ash loading areas. Clear the ash building floor and immediately adjacent roadways of spillage, debris, and excess water before departure of each loaded vehicle.

8.O.4.12 *Areas Adjacent to Disposal Ponds or Landfills.* Minimize contamination of surface runoff from areas adjacent to disposal ponds or landfills. Reduce ash residue that may be tracked on to access roads traveled by residue handling vehicles, and reduce ash residue on exit roads leading into and out of residue handling areas.

8.O.4.13 *Landfills, Scrap yards, Surface Impoundments, Open Dumps, General Refuse Sites.* Minimize the potential for contamination of runoff from these areas.

8.O.5 Additional SWPPP Requirements.

8.O.5.1 *Drainage Area Site Map.* (See also Part 5.1.2) Document in your SWPPP the locations of any of the following activities or sources that may be exposed to precipitation or surface runoff: storage tanks, scrap yards, and general refuse areas; short- and long-term storage of general materials (including but not limited to supplies, construction materials, paint equipment, oils, fuels, used and unused solvents, cleaning materials, paint, water treatment chemicals, fertilizer, and pesticides); landfills and construction sites; and stock pile areas (e.g., coal or limestone piles).

8.O.5.2 *Documentation of Good Housekeeping Measures.* You must document in your SWPPP the good housekeeping measures implemented to meet the effluent limits in Part 8.O.4.

8.O.6 Additional Inspection Requirements.

8.O.6.1 *Comprehensive Site Compliance Inspection.* (See also Part 4.3) As part of your inspection, inspect the following areas monthly: coal handling areas, loading or unloading areas, switchyards, fueling areas, bulk storage areas, ash handling areas, areas adjacent to disposal ponds and landfills, maintenance areas, liquid storage tanks, and long term and short term material storage areas.

8.O.7 Sector-Specific Benchmarks

Table 8.O-1 identifies benchmarks that apply to the specific subsectors of Sector O. These benchmarks apply to both your primary industrial activity and any co-located industrial activities, which describe your site activities.

Table 8.O-1.

Subsector (You may be subject to requirements for more than one sector/subsector)	Parameter	Benchmark Monitoring Concentration
Subsector O1. Steam Electric Generating Facilities (Industrial Activity Code "SE")	Total Iron	1.0 mg/L

8.O.8 Effluent Limitations Based on Effluent Limitations Guidelines (See also Part 6.2.2.1 of the permit.)

Table 8.O-2 identifies effluent limits that apply to the industrial activities described below. Compliance with these effluent limits is to be determined based on discharges from these industrial activities independent of commingling with any other wastestreams that may be covered under this permit.

Table 8.O-2[1]

Industrial Activity	Parameter	Effluent Limit
Discharges from coal storage piles at Steam Electric Generating Facilities	TSS	50 mg/l[2]
	pH	6.0 min - 9.0 max

[1] Monitor annually.

[2] If your facility is designed, constructed, and operated to treat the volume of coal pile runoff that is associated with a 10-year, 24-hour rainfall event, any untreated overflow of coal pile runoff from the treatment unit is not subject to the 50 mg/L limitation for total suspended solids.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart P – Sector P – Land Transportation and Warehousing.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.P.1 Covered Stormwater Discharges.

The requirements in Subpart P apply to stormwater discharges associated with industrial activity from Land Transportation and Warehousing facilities as identified by the SIC Codes specified under Sector P in Table D-1 of Appendix D of the permit.

8.P.2 Limitation on Coverage

8.P.2.1 *Prohibited Discharges* (see also Parts 1.1.4 and 8.P.3.6) This permit does not authorize the discharge of vehicle/equipment/surface washwater, including tank cleaning operations. Such discharges must be authorized under a separate NPDES permit, discharged to a sanitary sewer in accordance with applicable industrial pretreatment requirements, or recycled on-site.

8.P.3 Additional Technology-Based Effluent Limits.

8.P.3.1 *Good Housekeeping Measures.* (See also Part 2.1.2.2) In addition to the Good Housekeeping requirements in Part 2.1.2.2, you must do the following. Recommended control measures are discussed as indicated:

8.P.3.1.1 *Vehicle and Equipment Storage Areas.* Minimize the potential for stormwater exposure to leaky or leak-prone vehicles/equipment awaiting maintenance. Consider the following (or other equivalent measures): use of drip pans under vehicles/equipment, indoor storage of vehicles and equipment, installation of berms or dikes, use of absorbents, roofing or covering storage areas, and cleaning pavement surfaces to remove oil and grease.

8.P.3.1.2 *Fueling Areas.* Minimize contamination of stormwater runoff from fueling areas. Consider the following (or other equivalent measures): Covering the fueling area; using spill/overflow protection and cleanup equipment; minimizing stormwater run-on/runoff to the fueling area; using dry cleanup methods; and treating and/or recycling collected stormwater runoff.

8.P.3.1.3 *Material Storage Areas.* Maintain all material storage vessels (e.g., for used oil/oil filters, spent solvents, paint wastes, hydraulic fluids) to prevent contamination of stormwater and plainly label them (e.g., “Used Oil,” “Spent Solvents,” etc.). Consider the following (or other equivalent measures): storing the materials indoors; installing berms/dikes around the areas; minimizing runoff of stormwater to the areas; using dry cleanup methods; and treating and/or recycling collected stormwater runoff.

8.P.3.1.4 *Vehicle and Equipment Cleaning Areas.* Minimize contamination of stormwater runoff from all areas used for vehicle/equipment cleaning. Consider the following (or other equivalent measures): performing all cleaning operations indoors; covering the cleaning operation, ensuring that all washwater drains to a proper collection system (i.e., not the stormwater drainage system); treating and/or recycling collected washwater, or other equivalent measures.

8.P.3.1.5 *Vehicle and Equipment Maintenance Areas.* Minimize contamination of stormwater runoff from all areas used for vehicle/equipment maintenance. Consider the following (or other equivalent measures): performing maintenance activities indoors; using drip pans; keeping an organized inventory of materials used in the shop; draining all parts of fluid prior to disposal; prohibiting wet clean up practices if these practices would result in the discharge of pollutants to stormwater drainage systems; using dry cleanup methods; treating and/or recycling collected stormwater runoff, minimizing run on/runoff of stormwater to maintenance areas.

8.P.3.1.6 *Locomotive Sanding (Loading Sand for Traction) Areas.* Consider the following (or other equivalent measures): covering sanding areas; minimizing stormwater run on/runoff; or appropriate sediment removal practices to minimize the offsite transport of sanding material by stormwater.

8.P.3.2 *Employee Training.* (See also Part 2.1.2.9) Train personnel at least once a year and address the following activities, as applicable: used oil and spent solvent management; fueling procedures; general good housekeeping practices; proper painting procedures; and used battery management.

8.P.4 Additional SWPPP Requirements.

8.P.4.1 *Drainage Area Site Map.* (See also Part 5.1.2) Identify in the SWPPP the following areas of the facility and indicate whether activities occurring there may be exposed to precipitation/surface runoff: Fueling stations; vehicle/equipment maintenance or cleaning areas; storage areas for vehicle/equipment with actual or potential fluid leaks; loading/unloading areas; areas where treatment, storage or disposal of wastes occur; liquid storage tanks; processing areas; and storage areas.

8.P.4.2 *Potential Pollutant Sources.* (See also Part 5.1.3) Assess the potential for the following activities and facility areas to contribute pollutants to stormwater discharges: Onsite waste storage or disposal; dirt/gravel parking areas for vehicles awaiting maintenance; illicit plumbing connections between shop floor drains and the stormwater conveyance system(s); and fueling areas. Describe these activities in the SWPPP.

8.P.4.3 *Description of Good Housekeeping Measures.* You must document in your SWPPP the good housekeeping measures you implement consistent with Part 8.P.3.

8.P.4.4 *Vehicle and Equipment Washwater Requirements.* If applicable, attach to or reference in your SWPPP, a copy of the NPDES permit issued for vehicle/equipment washwater or, if an NPDES permit has not been issued, a copy of the pending application. If an

industrial user permit is issued under a local pretreatment program, attach a copy to your SWPPP. In any case, implement all non-stormwater discharge permit conditions or pretreatment conditions in your SWPPP. If washwater is handled in another manner (e.g., hauled offsite), describe the disposal method and attach all pertinent documentation/information (e.g., frequency, volume, destination, etc.) in the plan.

8.P.5 Additional Inspection Requirements. (See also Part 4.1) Inspect all the following areas/activities: storage areas for vehicles/equipment awaiting maintenance, fueling areas, indoor and outdoor vehicle/equipment maintenance areas, material storage areas, vehicle/equipment cleaning areas and loading/unloading areas.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart Q – Sector Q – Water Transportation.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.Q.1 Covered Stormwater Discharges.

The requirements in Subpart Q apply to stormwater discharges associated with industrial activity from Water Transportation facilities as identified by the SIC Codes specified under Sector Q in Table D-1 of Appendix D of the permit.

8.Q.2 Limitations on Coverage.

8.Q.2.1 *Prohibition of Non-Stormwater Discharges.* (See also Part 1.1.4) Not covered by this permit: bilge and ballast water, sanitary wastes, pressure wash water, and cooling water originating from vessels.

8.Q.3 Additional Technology-Based Effluent Limits.

8.Q.3.1 *Good Housekeeping Measures.* You must implement the following good housekeeping measures in addition to the requirements of part 2.1.2.2:

8.Q.3.1.1 *Pressure Washing Area.* If pressure washing is used to remove marine growth from vessels, the discharge water must be permitted by a separate NPDES permit. Collect or contain the discharges from the pressures washing area so that they are not co-mingled with stormwater discharges authorized by this permit.

8.Q.3.1.2 *Blasting and Painting Area.* Minimize the potential for spent abrasives, paint chips, and overspray to discharge into receiving waters or the storm sewer systems. Consider containing all blasting and painting activities or use other measures to minimize the discharge of contaminants (e.g., hanging plastic barriers or tarpaulins during blasting or painting operations to contain debris). When necessary, regularly clean stormwater conveyances of deposits of abrasive blasting debris and paint chips.

8.Q.3.1.3 *Material Storage Areas.* Store and plainly label all containerized materials (e.g., fuels, paints, solvents, waste oil, antifreeze, batteries) in a protected, secure location away from drains. Minimize the contamination of precipitation or surface runoff from the storage areas. Specify which materials are stored indoors, and consider containment or enclosure for those stored outdoors. If abrasive blasting is performed, discuss the storage and disposal of spent abrasive materials generated at the facility. Consider implementing an inventory control plan to limit the presence of potentially hazardous materials onsite.

8.Q.3.1.4 *Engine Maintenance and Repair Areas.* Minimize the contamination of precipitation or surface runoff from all areas used for engine maintenance and repair. Consider the following (or their equivalents): performing all maintenance activities indoors, maintaining an organized inventory of materials used in the shop, draining all parts of fluid prior to disposal, prohibiting the practice of hosing down the shop floor, using dry cleanup methods, and treating and/or recycling stormwater runoff collected from the maintenance area.

8.Q.3.1.5 *Material Handling Area.* Minimize the contamination of precipitation or surface runoff from material handling operations and areas (e.g., fueling, paint and solvent mixing, disposal of process wastewater streams from vessels). Consider the following (or their equivalents): covering fueling areas, using spill and overflow protection, mixing paints and solvents in a designated area (preferably indoors or under a shed), and minimizing runoff of stormwater to material handling areas.

8.Q.3.1.6 *Drydock Activities.* Routinely maintain and clean the drydock to minimize pollutants in stormwater runoff. Address the cleaning of accessible areas of the drydock prior to flooding, and final cleanup following removal of the vessel and raising the dock. Include procedures for cleaning up oil, grease, and fuel spills occurring on the drydock. Consider the following (or their equivalents): sweeping rather than hosing off debris and spent blasting material from accessible areas of the drydock prior to flooding and making absorbent materials and oil containment booms readily available to clean up or contain any spills.

8.Q.3.2 *Employee Training.* (See also Part 2.1.2.9) As part of your employee training program, address, at a minimum, the following activities (as applicable): used oil management, spent solvent management, disposal of spent abrasives, disposal of vessel wastewaters, spill prevention and control, fueling procedures, general good housekeeping practices, painting and blasting procedures, and used battery management.

8.Q.3.3 *Preventive Maintenance.* (See also Part 2.1.2.3) As part of your preventive maintenance program, perform timely inspection and maintenance of stormwater management devices (e.g., cleaning oil and water separators and sediment traps to ensure that spent abrasives, paint chips, and solids will be intercepted and retained prior to entering the storm drainage system), as well as inspecting and testing facility equipment and systems to uncover conditions that could cause breakdowns or failures resulting in discharges of pollutants to surface waters.

8.Q.4 Additional SWPPP Requirements.

8.Q.4.1 *Drainage Area Site Map.* (See also Part 5.1.2) Document in your SWPPP where any of the following may be exposed to precipitation or surface runoff: fueling; engine maintenance and repair; vessel maintenance and repair; pressure washing; painting; sanding; blasting; welding; metal fabrication; loading and unloading areas; locations used for the treatment, storage, or disposal of wastes; liquid storage tanks; liquid

storage areas (e.g., paint, solvents, resins); and material storage areas (e.g., blasting media, aluminum, steel, scrap iron).

8.Q.4.2 *Summary of Potential Pollutant Sources.* (See also Part 5.1.3) Document in the SWPPP the following additional sources and activities that have potential pollutants associated with them: outdoor manufacturing or processing activities (e.g., welding, metal fabricating) and significant dust or particulate generating processes (e.g., abrasive blasting, sanding, and painting.)

8.Q.5 Additional Inspection Requirements.

(See also Part 4.1) Include the following in all quarterly routine facility inspections: pressure washing area; blasting, sanding, and painting areas; material storage areas; engine maintenance and repair areas; material handling areas; drydock area; and general yard area.

8.Q.6 Sector-Specific Benchmarks. (See also Part 6 of the permit.)

Table 8.Q-1.

Subsector (You may be subject to requirements for more than one sector/subsector)	Parameter	Benchmark Monitoring Concentration
Subsector Q1. Water Transportation Facilities (SIC 4412-4499)	Total Aluminum	0.75 mg/L
	Total Iron	1.0 mg/L
	Total Lead[1]	Hardness Dependent
	Total Zinc[1]	Hardness Dependent

[1] The benchmark values of some metals are dependent on water hardness. For these parameters, permittees must determine the hardness of the receiving water (see Appendix J, "Calculating Hardness in Receiving Waters for Hardness Dependent Metals," for methodology), in accordance with Part 6.2.1.1, to identify the applicable 'hardness range' for determining their benchmark value applicable to their facility. The ranges occur in 25 mg/L increments. Hardness Dependent Benchmarks follow in the table below:

Water Hardness Range	Lead (mg/L)	Zinc (mg/L)
0-25 mg/L	0.014	0.04
25-50 mg/L	0.023	0.05
50-75 mg/L	0.045	0.08
75-100 mg/L	0.069	0.11
100-125 mg/L	0.095	0.13
125-150 mg/L	0.122	0.16
150-175 mg/L	0.151	0.18
175-200 mg/L	0.182	0.20
200-225 mg/L	0.213	0.23
225-250 mg/L	0.246	0.25
250+ mg/L	0.262	0.26

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart R – Sector R – Ship and Boat Building and Repair Yards.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.R.1 Covered Stormwater Discharges.

The requirements in Subpart R apply to stormwater discharges associated with industrial activity from Ship and Boat Building and Repair Yards as identified by the SIC Codes specified under Sector R in Table D-1 of Appendix D of the permit.

8.R.2 Limitations on Coverage.

8.R.2.1 *Prohibition of Non-Stormwater Discharges.* (See also Part 1.1.4) Discharges containing bilge and ballast water, sanitary wastes, pressure wash water, and cooling water originating from vessels are not covered by this permit.

8.R.3 Additional Technology-Based Effluent Limits.

8.R.3.1 *Good Housekeeping Measures.* (See also Part 2.1.2.2)

8.R.3.1.1 *Pressure Washing Area.* If pressure washing is used to remove marine growth from vessels, the discharged water must be permitted as a process wastewater by a separate NPDES permit.

8.R.3.1.2 *Blasting and Painting Area.* Minimize the potential for spent abrasives, paint chips, and overspray to discharging into the receiving water or the storm sewer systems. Consider containing all blasting and painting activities, or use other measures to prevent the discharge of the contaminants (e.g., hanging plastic barriers or tarpaulins during blasting or painting operations to contain debris). When necessary, regularly clean stormwater conveyances of deposits of abrasive blasting debris and paint chips.

8.R.3.1.3 *Material Storage Areas.* Store and plainly label all containerized materials (e.g., fuels, paints, solvents, waste oil, antifreeze, batteries) in a protected, secure location away from drains. Minimize the contamination of precipitation or surface runoff from the storage areas. If abrasive blasting is performed, discuss the storage and disposal of spent abrasive materials generated at the facility. Consider implementing an inventory control plan to limit the presence of potentially hazardous materials onsite.

8.R.3.1.4 *Engine Maintenance and Repair Areas.* Minimize the contamination of precipitation or surface runoff from all areas used for engine maintenance and repair. Consider the following (or their equivalents): performing all maintenance activities indoors, maintaining an organized inventory of

materials used in the shop, draining all parts of fluid prior to disposal, prohibiting the practice of hosing down the shop floor, using dry cleanup methods, and treating and/or recycling stormwater runoff collected from the maintenance area.

8.R.3.1.5 *Material Handling Area.* Minimize the contamination of precipitation or surface runoff from material handling operations and areas (e.g., fueling, paint and solvent mixing, disposal of process wastewater streams from vessels). Consider the following (or their equivalents): covering fueling areas, using spill and overflow protection, mixing paints and solvents in a designated area (preferably indoors or under a shed), and minimizing stormwater run-on to material handling areas.

8.R.3.1.6 *Drydock Activities.* Routinely maintain and clean the drydock to minimize pollutants in stormwater runoff. Clean accessible areas of the drydock prior to flooding and final cleanup following removal of the vessel and raising the dock. Include procedures for cleaning up oil, grease, or fuel spills occurring on the drydock. Consider the following (or their equivalents): sweeping rather than hosing off debris and spent blasting material from accessible areas of the drydock prior to flooding, and having absorbent materials and oil containment booms readily available to clean up and contain any spills.

8.R.3.2 *Employee Training.* (See also Part 2.1.2.9) As part of your employee training program, address, at a minimum, the following activities (as applicable): used oil management, spent solvent management, disposal of spent abrasives, disposal of vessel wastewaters, spill prevention and control, fueling procedures, general good housekeeping practices, painting and blasting procedures, and used battery management.

8.R.3.4 *Preventive Maintenance.* (See also Part 2.1.2.3) As part of your preventive maintenance program, perform timely inspection and maintenance of stormwater management devices (e.g., cleaning oil and water separators and sediment traps to ensure that spent abrasives, paint chips, and solids will be intercepted and retained prior to entering the storm drainage system), as well as inspecting and testing facility equipment and systems to uncover conditions that could cause breakdowns or failures resulting in discharges of pollutants to surface waters.

8.R.4 Additional SWPPP Requirements.

8.R.4.1 *Drainage Area Site Map.* (See also Part 5.1.2) Document in your SWPPP where any of the following may be exposed to precipitation or surface runoff: fueling; engine maintenance or repair; vessel maintenance or repair; pressure washing; painting; sanding; blasting; welding; metal fabrication; loading and unloading areas; treatment, storage, and waste disposal areas; liquid storage tanks; liquid storage areas (e.g., paint, solvents, resins); and material storage areas (e.g., blasting media, aluminum, steel, scrap iron).

8.R.4.2 *Potential Pollutant Sources.* (See also Part 5.1.3) Document in your SWPPP the following additional sources and activities that have potential pollutants associated with them (if applicable): outdoor manufacturing or processing activities (e.g., welding,

metal fabricating) and significant dust or particulate generating processes (e.g., abrasive blasting, sanding, and painting).

8.R.4.3 *Documentation of Good Housekeeping Measures.* Document in your SWPPP any good housekeeping measures implemented to meet the effluent limits in Part 8.R.3.

8.R.4.3.1 *Blasting and Painting Areas.* Document in the SWPPP any standard operating practices relating to blasting and painting (e.g., prohibiting uncontained blasting and painting over open water or prohibiting blasting and painting during windy conditions, which can render containment ineffective).

8.R.4.3.2 *Storage Areas.* Specify in your SWPPP which materials are stored indoors, and consider containment or enclosure for those stored outdoors.

8.R.5 Additional Inspection Requirements.

(See also Part 4.1) Include the following in all quarterly routine facility inspections: pressure washing area; blasting, sanding, and painting areas; material storage areas; engine maintenance and repair areas; material handling areas; drydock area; and general yard area.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart S – Sector S – Air Transportation.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.S.1 Covered Stormwater Discharges.

The requirements in Subpart S apply to stormwater discharges associated with industrial activity from Air Transportation facilities identified by the SIC Codes specified under Sector S in Table D-1 of Appendix D of the permit.

8.S.2 Limitation on Coverage

8.S.2.1 *Limitations on Coverage.* This permit authorizes stormwater discharges from only those portions of the air transportation facility that are involved in vehicle maintenance (including vehicle rehabilitation, mechanical repairs, painting, fueling and lubrication), equipment cleaning operations or deicing operations.

Note: "deicing" will generally be used to imply both deicing (removing frost, snow or ice) and anti-icing (preventing accumulation of frost, snow or ice) activities, unless specific mention is made regarding anti-icing and/or deicing activities.

8.S.2.2 *Prohibition of Non-Stormwater Discharges.* (See also Part 1.1.4 and Part 8.S.3) This permit does not authorize the discharge of aircraft, ground vehicle, runway and equipment washwaters; nor the dry weather discharge of deicing chemicals. Such discharges must be covered by separate NPDES permit(s). Note that a discharge resulting from snowmelt is not a dry weather discharge.

8.S.3 Additional Technology-Based Effluent Limits.

8.S.3.1 *Good Housekeeping Measures.* (See also Part 2.1.2.2)

8.S.3.1.1 Aircraft, Ground Vehicle and Equipment Maintenance Areas. Minimize the contamination of stormwater runoff from all areas used for aircraft, ground vehicle and equipment maintenance (including the maintenance conducted on the terminal apron and in dedicated hangers). Consider the following practices (or their equivalents): performing maintenance activities indoors; maintaining an organized inventory of material used in the maintenance areas; draining all parts of fluids prior to disposal; prohibiting the practice of hosing down the apron or hanger floor; using dry cleanup methods; and collecting the stormwater runoff from the maintenance area and providing treatment or recycling.

8.S.3.1.2 Aircraft, Ground Vehicle and Equipment Cleaning Areas. (See also Part 8.S.3.6) Clearly demarcate these areas on the ground using signage or other

appropriate means. Minimize the contamination of stormwater runoff from cleaning areas.

8.S.3.1.3 Aircraft, Ground Vehicle and Equipment Storage Areas. Store all aircraft, ground vehicles and equipment awaiting maintenance in designated areas only and minimize the contamination of stormwater runoff from these storage areas. Consider the following control measures, including any BMPs (or their equivalents): storing aircraft and ground vehicles indoors; using drip pans for the collection of fluid leaks; and perimeter drains, dikes or berms surrounding the storage areas.

8.S.3.1.4 Material Storage Areas. Maintain the vessels of stored materials (e.g., used oils, hydraulic fluids, spent solvents, and waste aircraft fuel) in good condition, to prevent or minimize contamination of stormwater. Also plainly label the vessels (e.g., "used oil," "Contaminated Jet A," etc.). Minimize contamination of precipitation/runoff from these areas. Consider the following control measures (or their equivalents): storing materials indoors; storing waste materials in a centralized location; and installing berms/dikes around storage areas.

8.S.3.1.5 Airport Fuel System and Fueling Areas. Minimize the discharge of fuel to the storm sewer/surface waters resulting from fuel servicing activities or other operations conducted in support of the airport fuel system. Consider the following control measures (or their equivalents): implementing spill and overflow practices (e.g., placing absorptive materials beneath aircraft during fueling operations); using only dry cleanup methods; and collecting stormwater runoff.

8.S.3.1.6 Source Reduction. Minimize, and where feasible eliminate, the use of urea and glycol-based deicing chemicals, in order to reduce the aggregate amount of deicing chemicals used and/or lessen the environmental impact. Chemical options to replace ethylene glycol, propylene glycol and urea include: potassium acetate; magnesium acetate; calcium acetate; and anhydrous sodium acetate.

8.S.3.1.6.1 Runway Deicing Operation: Minimize contamination of stormwater runoff from runways as a result of deicing operations. Evaluate whether over-application of deicing chemicals occurs by analyzing application rates, and adjust as necessary, consistent with considerations of flight safety. Also consider these control measure options (or their equivalents): metered application of chemicals; pre-wetting dry chemical constituents prior to application; installing a runway ice detection system; implementing anti-icing operations as a preventive measure against ice buildup.

8.S.3.1.6.2 Aircraft Deicing Operations. Minimize contamination of stormwater runoff from aircraft deicing operations. Determine whether excessive application of deicing chemicals occurs and

adjust as necessary, consistent with considerations of flight safety. This evaluation should be carried out by the personnel most familiar with the particular aircraft and flight operations in question (versus an outside entity such as the airport authority). Consider using alternative deicing/anti-icing agents as well as containment measures for all applied chemicals. Also consider these control measure options (or their equivalents) for reducing deicing fluid use: forced-air deicing systems, computer-controlled fixed-gantry systems, infrared technology, hot water, varying glycol content to air temperature, enclosed-basket deicing trucks, mechanical methods, solar radiation, hangar storage, aircraft covers, and thermal blankets for MD-80s and DC-9s. Also consider using ice-detection systems and airport traffic flow strategies and departure slot allocation systems.

8.S.3.1.7 Management of Runoff. (See also 2.1.2.6) Where deicing operations occur, implement a program to control or manage contaminated runoff to minimize the amount of pollutants being discharged from the site. Consider these control measure options (or their equivalents): a dedicated deicing facility with a runoff collection/ recovery system; using vacuum/collection trucks; storing contaminated stormwater/deicing fluids in tanks and releasing controlled amounts to a publicly owned treatment works; collecting contaminated runoff in a wet pond for biochemical decomposition (be aware of attracting wildlife that may prove hazardous to flight operations); and directing runoff into vegetative swales or other infiltration measures. Also consider recovering deicing materials when these materials are applied during non-precipitation events (e.g., covering storm sewer inlets, using booms, installing absorptive interceptors in the drains, etc.) to prevent these materials from later becoming a source of stormwater contamination. Used deicing fluid should be recycled whenever possible.

8.S.3.2 *Deicing Season.* You must determine the seasonal timeframe (e.g., December-February, October - March, etc.) during which deicing activities typically occur at the facility. Implementation of control measures, including any BMPs, facility inspections and monitoring must be conducted with particular emphasis throughout the defined deicing season. If you meet the deicing chemical usage thresholds of 100,000 gallons glycol and/or 100 tons of urea, the deicing season you identified is the timeframe during which you must obtain the four required benchmark monitoring event results for deicing-related parameters, i.e., BOD, COD, ammonia and pH. See also Part 8.S.6.

8.S.4 Additional SWPPP Requirements.

An airport authority and tenants of the airport are encouraged to work in partnership in the development of a SWPPP. If an airport tenant obtains authorization under this permit and develops a SWPPP for discharges from his own areas of the airport, prior to authorization, that SWPPP must be coordinated and integrated with the SWPPP for the entire airport. Tenants of the airport facility include air passenger or cargo companies, fixed based operators and other parties

who have contracts with the airport authority to conduct business operations on airport property and whose operations result in stormwater discharges associated with industrial activity.

8.S.4.1 *Drainage Area Site Map.* (See also Part 5.1.2) Document in the SWPPP the following areas of the facility and indicate whether activities occurring there may be exposed to precipitation/surface runoff: aircraft and runway deicing operations; fueling stations; aircraft, ground vehicle and equipment maintenance/cleaning areas; storage areas for aircraft, ground vehicles and equipment awaiting maintenance.

8.S.4.2 *Potential Pollutant Sources.* (See also Part 5.1.3) In your inventory of exposed materials, describe in your SWPPP the potential for the following activities and facility areas to contribute pollutants to stormwater discharges: aircraft, runway, ground vehicle and equipment maintenance and cleaning; aircraft and runway deicing operations (including apron and centralized aircraft deicing stations, runways, taxiways and ramps). If you use deicing chemicals, you must maintain a record of the types (including the Material Safety Data Sheets [MSDS]) used and the monthly quantities, either as measured or, in the absence of metering, as estimated to the best of your knowledge. This includes all deicing chemicals, not just glycols and urea (e.g., potassium acetate), because large quantities of these other chemicals can still have an adverse impact on receiving waters. Tenants or other fixed-based operations that conduct deicing operations must provide the above information to the airport authority for inclusion with any comprehensive airport SWPPPs.

8.S.4.3 *Vehicle and Equipment Washwater Requirements.* Attach to or reference in your SWPPP, a copy of the NPDES permit issued for vehicle/equipment washwater or, if an NPDES permit has not been issued, a copy of the pending application. If an industrial user permit is issued under a local pretreatment program, include a copy in your SWPPP. In any case, if you are subject to another permit, describe your control measures for implementing all non-stormwater discharge permit conditions or pretreatment requirements in your SWPPP. If washwater is handled in another manner (e.g., hauled offsite, retained onsite), describe the disposal method and attach all pertinent documentation/information (e.g., frequency, volume, destination, etc.) in your SWPPP.

8.S.4.4 *Documentation of Control Measures Used for Management of Runoff:* Document in your SWPPP the control measures used for collecting or containing contaminated melt water from collection areas used for disposal of contaminated snow.

8.S.5 Additional Inspection Requirements.

8.S.5.1 *Inspections.* (See also Part 4.1) At a minimum conduct routine facility inspections at least monthly during the deicing season (e.g., October through April for most mid-latitude airports). If your facility needs to deice before or after this period, expand the monthly inspections to include all months during which deicing chemicals may be used. The Director may specifically require you to increase inspection frequencies.

8.S.5.2 *Comprehensive Site Inspections.* (See also Part 4.3) Using only qualified personnel, conduct your annual site inspection during periods of actual deicing operations, if possible. If not practicable during active deicing because of weather, conduct the

inspection during the season when deicing operations occur and the materials and equipment for deicing are in place.

8.S.6 Sector-Specific Benchmarks. (See also Part 6 of the permit.)

Monitor per the requirements in Table 8.S-1.

Table 8.S-1.

Subsector (You may be subject to requirements for more than one sector/subsector)	Parameter	Benchmark Monitoring Concentration
For airports where a single permittee, or a combination of permitted facilities use more than 100,000 gallons of glycol-based deicing chemicals and/or 100 tons or more of urea on an average annual basis, monitor the first four parameters in ONLY those outfalls that collect runoff from areas where deicing activities occur (SIC 4512-4581).	Biochemical Oxygen Demand (BOD_5)[1]	30 mg/L
	Chemical Oxygen Demand (COD)[1]	120 mg/L
	Ammonia[1]	2.14 mg/L
	pH[1]	6.0 - 9.0 s.u.

[1] These are deicing-related parameters. Collect the four benchmark samples, and any required follow-up benchmark samples, during the timeframe defined in Part 8.S.3.2 when deicing activities are occurring.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart T – Sector T – Treatment Works.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.T.1 Covered Stormwater Discharges.

The requirements in Subpart T apply to stormwater discharges associated with industrial activity from Treatment Works as identified by the Activity Code specified under Sector T in Table D-1 of Appendix D of the permit.

8.T.2 Industrial Activities Covered by Sector T.

The requirements listed under this part apply to all existing point source stormwater discharges associated with the following activities:

8.T.2.1 Treatment works treating domestic sewage, or any other sewage sludge or wastewater treatment device or system used in the storage, treatment, recycling, and reclamation of municipal or domestic sewage, including land dedicated to the disposal of sewage sludge; that are located within the confines of a facility with a design flow of 1.0 million gallons per day (MGD) or more; or are required to have an approved pretreatment program under 40 CFR Part 403.

8.T.2.2 The following are not required to have permit coverage: farm lands, domestic gardens or lands used for sludge management where sludge is beneficially reused and which are not physically located within the facility, or areas that are in compliance with Section 405 of the CWA.

8.T.3 Limitations on Coverage.

8.T.3.1 *Prohibition of Non-Stormwater Discharges.* (See also Part 1.1.4) Sanitary and industrial wastewater and equipment and vehicle washwater are not authorized by this permit.

8.T.4 Additional Technology-Based Effluent Limits.

8.T.4.1 *Control Measures.* (See also the non-numeric effluent limits in Part 2.1.2) In addition to the other control measures, consider the following: routing stormwater to the treatment works; or covering exposed materials (i.e., from the following areas: grit, screenings, and other solids handling, storage, or disposal areas; sludge drying beds; dried sludge piles; compost piles; and septage or hauled waste receiving station).

8.T.4.2 *Employee Training.* (See also Part 2.1.2.9) At a minimum, training must address the following areas when applicable to a facility: petroleum product management; process chemical management; spill prevention and controls; fueling procedures; general good

housekeeping practices; and proper procedures for using fertilizer, herbicides, and pesticides.

8.T.5 Additional SWPPP Requirements.

8.T.5.1 *Site Map.* (See also Part 5.1.2) Document in your SWPPP where any of the following may be exposed to precipitation or surface runoff: grit, screenings, and other solids handling, storage, or disposal areas; sludge drying beds; dried sludge piles; compost piles; septage or hauled waste receiving station; and storage areas for process chemicals, petroleum products, solvents, fertilizers, herbicides, and pesticides.

8.T.5.2 *Potential Pollutant Sources.* (See also Part 5.1.3) Document in your SWPPP the following additional sources and activities that have potential pollutants associated with them, as applicable: grit, screenings, and other solids handling, storage, or disposal areas; sludge drying beds; dried sludge piles; compost piles; septage or hauled waste receiving station; and access roads and rail lines.

8.T.5.3 *Wastewater and Washwater Requirements.* Keep a copy of all your current NPDES permits issued for wastewater and industrial, vehicle and equipment washwater discharges or, if an NPDES permit has not yet been issued, a copy of the pending application(s) with your SWPPP. If the washwater is handled in another manner, the disposal method must be described and all pertinent documentation must be retained onsite.

8.T.6 Additional Inspection Requirements.

(See also Part 4.1) Include the following areas in all inspections: access roads and rail lines; grit, screenings, and other solids handling, storage, or disposal areas; sludge drying beds; dried sludge piles; compost piles; and septage or hauled waste receiving station.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart U – Sector U – Food and Kindred Products.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.U.1 Covered Stormwater Discharges.

The requirements in Subpart U apply to stormwater discharges associated with industrial activity from Food and Kindred Products facilities as identified by the SIC Codes specified in Table D-1 of Appendix D of the permit.

8.U.2 Limitations on Coverage.

8.U.2.1 *Prohibition of Non-Stormwater Discharges.* (See also Part 1.1.4) The following discharges are not authorized by this permit: discharges containing boiler blowdown, cooling tower overflow and blowdown, ammonia refrigeration purging, and vehicle washing and clean-out operations.

8.U.3 Additional Technology-Based Limitations.

8.U.3.1 *Employee Training.* (See also Part 2.1.2.9) Address pest control in your employee training program.

8.U.4 Additional SWPPP Requirements.

8.U.4.1 *Drainage Area Site Map.* (See also Part 5.1.2) Document in your SWPPP the locations of the following activities if they are exposed to precipitation or runoff: vents and stacks from cooking, drying, and similar operations; dry product vacuum transfer lines; animal holding pens; spoiled product; and broken product container storage areas.

8.U.4.2 *Potential Pollutant Sources.* (See also Part 5.1.3) Document in your SWPPP, in addition to food and kindred products processing-related industrial activities, application and storage of pest control chemicals (e.g., rodenticides, insecticides, fungicides) used on plant grounds.

8.U.5 Additional Inspection Requirements.

(See also Part 4.1) Inspect on a quarterly basis, at a minimum, the following areas where the potential for exposure to stormwater exists: loading and unloading areas for all significant materials; storage areas, including associated containment areas; waste management units; vents and stacks emanating from industrial activities; spoiled product and broken product container holding areas; animal holding pens; staging areas; and air pollution control equipment.

8.U.6 Sector-Specific Benchmarks. (See also Part 6 of the permit.)

Table 8.U-1.		
Subsector (You may be subject to requirements for more than one Sector / Subsector)	**Parameter**	**Benchmark Monitoring Concentration**
Subsector U1. Grain Mill Products (SIC 2041-2048)	Total Suspended Solids (TSS)	100 mg/L
Subsector U2. Fats and Oils Products (SIC 2074-2079)	Biochemical Oxygen Demand (BOD_5)	30 mg/L
	Chemical Oxygen Demand (COD)	120 mg/L
	Nitrate plus Nitrite Nitrogen	0.68 mg/L
	Total Suspended Solids (TSS)	100 mg/L

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart V – Sector V – Textile Mills, Apparel, and Other Fabric Products.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.V.1 Covered Stormwater Discharges.

The requirements in Subpart V apply to stormwater discharges associated with industrial activity from Textile Mills, Apparel, and Other Fabric Product manufacturing as identified by the SIC Codes specified under Sector V in Table D-1 of Appendix D of the permit.

8.V.2 Limitations on Coverage.

8.V.2.1 *Prohibition of Non-Stormwater Discharges.* (See also Part 1.1.4) The following are not authorized by this permit: discharges of wastewater (e.g., wastewater resulting from wet processing or from any processes relating to the production process), reused or recycled water, and waters used in cooling towers. If you have these types of discharges from your facility, you must cover them under a separate NPDES permit.

8.V.3 Additional Technology-Based Limitations.

8.V.3.1 *Good Housekeeping Measures.* (See also Part 2.1.2.2)

8.V.3.1.1 *Material Storage Areas.* Plainly label and store all containerized materials (e.g., fuels, petroleum products, solvents, and dyes) in a protected area, away from drains. Minimize contamination of the stormwater runoff from such storage areas. Also consider an inventory control plan to prevent excessive purchasing of potentially hazardous substances. For storing empty chemical drums or containers, ensure that the drums and containers are clean (consider triple-rinsing) and that there is no contact of residuals with precipitation or runoff. Collect and dispose of washwater from these cleanings properly.

8.V.3.1.2 *Material Handling Areas.* Minimize contamination of stormwater runoff from material handling operations and areas. Consider the following (or their equivalents): use of spill and overflow protection; covering fueling areas; and covering or enclosing areas where the transfer of material may occur. When applicable, address the replacement or repair of leaking connections, valves, transfer lines, and pipes that may carry chemicals, dyes, or wastewater.

8.V.3.1.3 *Fueling Areas.* Minimize contamination of stormwater runoff from fueling areas. Consider the following (or their equivalents): covering the fueling area, using spill and overflow protection, minimizing run-on of stormwater to the fueling areas, using dry cleanup methods, and treating and/or recycling stormwater runoff collected from the fueling area.

8.V.3.1.4 *Above-Ground Storage Tank Area.* Minimize contamination of the stormwater runoff from above-ground storage tank areas, including the associated piping and valves. Consider the following (or their equivalents): regular cleanup of these areas; including measures for tanks, piping and valves explicitly in your SPCC program; minimizing runoff of stormwater from adjacent areas; restricting access to the area; inserting filters in adjacent catch basins; providing absorbent booms in unbermed fueling areas; using dry cleanup methods; and permanently sealing drains within critical areas that may discharge to a storm drain.

8.V.3.2 *Employee Training.* (See also Part 2.1.2.9) As part of your employee training program, address, at a minimum, the following activities (as applicable): use of reused and recycled waters, solvents management, proper disposal of dyes, proper disposal of petroleum products and spent lubricants, spill prevention and control, fueling procedures, and general good housekeeping practices.

8.V.4 Additional SWPPP Requirements.

8.V.4.1 *Potential Pollutant Sources. (See also Part 5.1.3) Document in your SWPPP the following additional sources* and activities that have potential pollutants associated with them: industry-specific significant materials and industrial activities (e.g., backwinding, beaming, bleaching, backing bonding, carbonizing, carding, cut and sew operations, desizing, drawing, dyeing locking, fulling, knitting, mercerizing, opening, packing, plying, scouring, slashing, spinning, synthetic-felt processing, textile waste processing, tufting, turning, weaving, web forming, winging, yarn spinning, and yarn texturing).

8.V.4.2 *Description of Good Housekeeping Measures for Material Storage Areas.* Document in the SWPPP your containment area or enclosure for materials stored outdoors in connection with Part 8.V.3.1.1 above.

8.V.5 Additional Inspection Requirements.

(See also Part 4.1) Inspect, at least monthly, the following activities and areas (at a minimum): transfer and transmission lines, spill prevention, good housekeeping practices, management of process waste products, and all structural and nonstructural management practices.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart W – Sector W – Furniture and Fixtures.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.W.1 Covered Stormwater Discharges.

The requirements in Subpart W apply to stormwater discharges associated with industrial activity from Furniture and Fixtures facilities as identified by the SIC Codes specified under Sector W in Table D-1 of Appendix D of the permit.

8.W.2 Additional SWPPP Requirements.

8.W.2.1 *Drainage Area Site Map.* (See also Part 5.1.2) Document in your SWPPP where any of the following may be exposed to precipitation or surface runoff: material storage (including tanks or other vessels used for liquid or waste storage) areas; outdoor material processing areas; areas where wastes are treated, stored, or disposed of; access roads; and rail spurs.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart X – Sector X – Printing and Publishing.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.X.1 Covered Stormwater Discharges.

The requirements in Subpart X apply to stormwater discharges associated with industrial activity from Printing and Publishing facilities as identified by the SIC Codes specified under Sector X in Table D-1 of Appendix D of the permit.

8.X.2 Additional Technology-Based Effluent Limits.

8.X.2.1 *Good Housekeeping Measures.* (See also Part 2.1.2.2)

8.X.2.1.1 *Material Storage Areas.* Plainly label and store all containerized materials (e.g., skids, pallets, solvents, bulk inks, hazardous waste, empty drums, portable and mobile containers of plant debris, wood crates, steel racks, and fuel oil) in a protected area, away from drains. Minimize contamination of the stormwater runoff from such storage areas. Also consider an inventory control plan to prevent excessive purchasing of potentially hazardous substances.

8.X.2.1.2 *Material Handling Area.* Minimize contamination of stormwater runoff from material handling operations and areas (e.g., blanket wash, mixing solvents, loading and unloading materials). Consider the following (or their equivalents): using spill and overflow protection, covering fueling areas, and covering or enclosing areas where the transfer of materials may occur. When applicable, address the replacement or repair of leaking connections, valves, transfer lines, and pipes that may carry chemicals or wastewater.

8.X.2.1.3 *Fueling Areas.* Minimize contamination of stormwater runoff from fueling areas. Consider the following (or their equivalents): covering the fueling area, using spill and overflow protection, minimizing runoff of stormwater to the fueling areas, using dry cleanup methods, and treating and/or recycling stormwater runoff collected from the fueling area.

8.X.2.1.4 *Above Ground Storage Tank Area.* Minimize contamination of the stormwater runoff from above-ground storage tank areas, including the associated piping and valves. Consider the following (or their equivalents): regularly cleaning these areas, explicitly addressing tanks, piping and valves in the SPCC program, minimizing stormwater runoff from adjacent areas, restricting access to the area, inserting filters in adjacent catch basins, providing absorbent booms in unbermed fueling areas, using dry cleanup methods, and permanently sealing drains within critical areas that may discharge to a storm drain.

8.X.2.2 *Employee Training.* (See also Part 2.1.2.9) As part of your employee training program, address, at a minimum, the following activities (as applicable): spent solvent management, spill prevention and control, used oil management, fueling procedures, and general good housekeeping practices.

8.X.3 Additional SWPPP Requirements.

8.X.3.1 *Description of Good Housekeeping Measures for Material Storage Areas.* In connection with Part 8.X.2.1.1, describe in the SWPPP the containment area or enclosure for materials stored outdoors.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart Y – Sector Y – Rubber, Miscellaneous Plastic Products, and Miscellaneous Manufacturing Industries.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.Y.1 Covered Stormwater Discharges.

The requirements in Subpart Y apply to stormwater discharges associated with industrial activity from Rubber, Miscellaneous Plastic Products, and Miscellaneous Manufacturing Industries facilities as identified by the SIC Codes specified under Sector Y in Table D-1 of Appendix D of the permit.

8.Y.2 Additional Technology-Based Effluent Limits.

8.Y.2.1 *Controls for Rubber Manufacturers.* (See also Part 2.1.2) Minimize the discharge of zinc in your stormwater discharges. Parts 8.Y.2.1.1 to 8.Y.2.1.5 give possible sources of zinc to be reviewed and list some specific control measures to be considered for implementation (or their equivalents). Following are some general control measure options to consider: using chemicals purchased in pre-weighed, sealed polyethylene bags; storing in-use materials in sealable containers, ensuring an airspace between the container and the cover to minimize "puffing" losses when the container is opened, and using automatic dispensing and weighing equipment.

8.Y.2.1.1 *Zinc Bags.* Ensure proper handling and storage of zinc bags at your facility. Following are some control measure options: employee training on the handling and storage of zinc bags, indoor storage of zinc bags, cleanup of zinc spills without washing the zinc into the storm drain, and the use of 2,500-pound sacks of zinc rather than 50- to 100-pound sacks.

8.Y.2.1.2 *Dumpsters.* Minimize discharges of zinc from dumpsters. Following are some control measure options: covering the dumpster, moving the dumpster indoors, or providing a lining for the dumpster.

8.Y.2.1.3 *Dust Collectors and Baghouses.* Minimize contributions of zinc to stormwater from dust collectors and baghouses. Replace or repair, as appropriate, improperly operating dust collectors and baghouses.

8.Y.2.1.4 *Grinding Operations.* Minimize contamination of stormwater as a result of dust generation from rubber grinding operations. One control measure option is to install a dust collection system.

8.Y.2.1.5 *Zinc Stearate Coating Operations.* Minimize the potential for stormwater contamination from drips and spills of zinc stearate slurry that may be released

to the storm drain. One control measure option is to use alternative compounds to zinc stearate.

8.Y.2.2 *Controls for Plastic Products Manufacturers.* Minimize the discharge of plastic resin pellets in your stormwater discharges. Control measures to be considered for implementation (or their equivalents) include minimizing spills, cleaning up of spills promptly and thoroughly, sweeping thoroughly, pellet capturing, employee education, and disposal precautions.

8.Y.3 Additional SWPPP Requirements.

8.Y.3.1 *Potential Pollutant Sources for Rubber Manufacturers.* (See also Part 5.1.3) Document in your SWPPP the use of zinc at your facility and the possible pathways through which zinc may be discharged in stormwater runoff.

8.Y.4 Sector-Specific Benchmarks. (See also Part 6 of the permit.)

Table 8.Y-1.

Subsector (You may be subject to requirements for more than one sector/subsector)	Parameter	Benchmark Monitoring Concentration
Subsector Y1. Rubber Products Manufacturing (SIC 3011, 3021, 3052, 3053, 3061, 3069)	Total Zinc[1]	Hardness Dependent

[1] The benchmark values of some metals are dependent on water hardness. For these parameters, permittees must determine the hardness of the receiving water (see Appendix J, "Calculating Hardness in Receiving Waters for Hardness Dependent Metals," for methodology), in accordance with Part 6.2.1.1, to identify the applicable 'hardness range' for determining their benchmark value applicable to their facility. The ranges occur in 25 mg/L increments. Hardness Dependent Benchmarks follow in the table below:

Water Hardness Range	**Zinc** (mg/L)
0-25 mg/L	0.04
25-50 mg/L	0.05
50-75 mg/L	0.08
75-100 mg/L	0.11
100-125 mg/L	0.13
125-150 mg/L	0.16
150-175 mg/L	0.18
175-200 mg/L	0.20
200-225 mg/L	0.23
225-250 mg/L	0.25
250+ mg/L	0.26

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart Z – Sector Z – Leather Tanning and Finishing.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.Z.1 Covered Stormwater Discharges.

The requirements in Subpart Z apply to stormwater discharges associated with industrial activity from Leather Tanning and Finishing facilities as identified by the SIC Code specified under Sector Z in Table D-1 of Appendix D of the permit.

8.Z.2 Additional Technology-Based Effluent Limits.

8.Z.2.3 *Good Housekeeping Measures.* (See also Part 2.1.2.2)

8.Z.2.3.1 *Storage Areas for Raw, Semiprocessed, or Finished Tannery By-products.* Minimize contamination of stormwater runoff from pallets and bales of raw, semiprocessed, or finished tannery by-products (e.g., splits, trimmings, shavings). Consider indoor storage or protection with polyethylene wrapping, tarpaulins, roofed storage, etc. Consider placing materials on an impermeable surface and enclosing or putting berms (or equivalent measures) around the area to prevent stormwater run-on and runoff.

8.Z.2.3.2 *Material Storage Areas.* Label storage containers of all materials (e.g., specific chemicals, hazardous materials, spent solvents, waste materials) minimize contact of such materials with stormwater.

8.Z.2.3.3 *Buffing and Shaving Areas.* Minimize contamination of stormwater runoff with leather dust from buffing and shaving areas. Consider dust collection enclosures, preventive inspection and maintenance programs, or other appropriate preventive measures.

8.Z.2.3.4 *Receiving, Unloading, and Storage Areas.* Minimize contamination of stormwater runoff from receiving, unloading, and storage areas. If these areas are exposed, consider the following (or their equivalents): covering all hides and chemical supplies, diverting drainage to the process sewer, or grade berming or curbing the area to prevent stormwater runoff.

8.Z.2.3.5 *Outdoor Storage of Contaminated Equipment.* Minimize contact of stormwater with contaminated equipment. Consider the following (or their equivalents): covering equipment, diverting drainage to the process sewer, and cleaning thoroughly prior to storage.

8.Z.2.3.6 *Waste Management.* Minimize contamination of stormwater runoff from waste storage areas. Consider the following (or their equivalents): covering

dumpsters, moving waste management activities indoors, covering waste piles with temporary covering material such as tarpaulins or polyethylene, and minimizing stormwater runoff by enclosing the area or building berms around the area.

8.Z.3 Additional SWPPP Requirements.

8.Z.3.1 *Drainage Area Site Map.* (See also Part 5.1.2) Identify in your SWPPP where any of the following may be exposed to precipitation or surface runoff: processing and storage areas of the beamhouse, tanyard, and re-tan wet finishing and dry finishing operations.

8.Z.3.2 *Potential Pollutant Sources.* (See also Part 5.1.3) Document in your SWPPP the following sources and activities that have potential pollutants associated with them (as appropriate): temporary or permanent storage of fresh and brine-cured hides; extraneous hide substances and hair; leather dust, scraps, trimmings, and shavings.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart AA – Sector AA – Fabricated Metal Products

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.AA.1 Covered Stormwater Discharges.

The requirements in Subpart AA apply to stormwater discharges associated with industrial activity from Fabricated Metal Products facilities as identified by the SIC Codes specified under Sector AA in Table D-1 of Appendix D of the permit.

8.AA.2 Additional Technology-Based Effluent Limits.

8.AA.2.1 *Good Housekeeping Measures.* (See also Part 2.1.2.2)

8.AA.2.1.1 *Raw Steel Handling Storage.* Minimize the generation of and/or recover and properly manage scrap metals, fines, and iron dust. Include measures for containing materials within storage handling areas.

8.AA.2.1.2 *Paints and Painting Equipment.* Minimize exposure of paint and painting equipment to stormwater.

8.AA.2.2 *Spill Prevention and Response Procedures.* (See also Part 2.1.2.4) Ensure that the necessary equipment to implement a cleanup is available to personnel. The following areas should be addressed

8.AA.2.2.1 *Metal Fabricating Areas.* Maintain clean, dry, orderly conditions in these areas. Consider using dry clean-up techniques.

8.AA.2.2.2 *Storage Areas for Raw Metal.* Keep these areas free of conditions that could cause, or impede appropriate and timely response to, spills or leakage of materials. Consider the following (or their equivalents): maintaining storage areas so that there is easy access in the event of a spill, and labeling stored materials to aid in identifying spill contents.

8.AA.2.2.3 *Metal Working Fluid Storage Areas.* Minimize the potential for stormwater contamination from storage areas for metal working fluids.

8.AA.2.2.4 *Cleaners and Rinse Water.* Control and clean up spills of solvents and other liquid cleaners, control sand buildup and disbursement from sand-blasting operations, and prevent exposure of recyclable wastes. Substitute environmentally benign cleaners when possible.

8.AA.2.2.5 *Lubricating Oil and Hydraulic Fluid Operations.* Minimize the potential for stormwater contamination from lubricating oil and hydraulic fluid operations. Consider using monitoring equipment or other devices to detect and control

leaks and overflows. Consider installing perimeter controls such as dikes, curbs, grass filter strips, or equivalent measures.

8.AA.2.2.6 *Chemical Storage Areas.* Minimize stormwater contamination and accidental spillage in chemical storage areas. Include a program to inspect containers and identify proper disposal methods.

8.AA.2.3 *Spills and Leaks.* (See also Part 5.1.3.3) In your spill prevention and response procedures, required by Part 2.1.2.4, pay attention to the following materials (at a minimum): chromium, toluene, pickle liquor, sulfuric acid, zinc and other water priority chemicals, and hazardous chemicals and wastes.

8.AA.3 Additional SWPPP Requirements.

8.AA.3.1 *Drainage Area Site Map.* (See also Part 5.1.2) Document in your SWPPP where any of the following may be exposed to precipitation or surface runoff: raw metal storage areas; finished metal storage areas; scrap disposal collection sites; equipment storage areas; retention and detention basins; temporary and permanent diversion dikes or berms; right-of-way or perimeter diversion devices; sediment traps and barriers; processing areas, including outside painting areas; wood preparation; recycling; and raw material storage.

8.AA.3.2 *Potential Pollutant Sources.* (See also Part 5.1.3) Document in your SWPPP the following additional sources and activities that have potential pollutants associated with them: loading and unloading operations for paints, chemicals, and raw materials; outdoor storage activities for raw materials, paints, empty containers, corn cobs, chemicals, and scrap metals; outdoor manufacturing or processing activities such as grinding, cutting, degreasing, buffing, and brazing; onsite waste disposal practices for spent solvents, sludge, pickling baths, shavings, ingot pieces, and refuse and waste piles.

8.AA.4 Additional Inspection Requirements

8.AA.4.1 *Inspections.* (See also Part 4) At a minimum, include the following areas in all inspections: raw metal storage areas, finished product storage areas, material and chemical storage areas, recycling areas, loading and unloading areas, equipment storage areas, paint areas, and vehicle fueling and maintenance areas.

8.AA.4.2 *Comprehensive Site Inspections.* (See also Part 4.3) As part of your inspection, also inspect areas associated with the storage of raw metals, spent solvents and chemicals storage areas, outdoor paint areas, and drainage from roof. Potential pollutants include chromium, zinc, lubricating oil, solvents, aluminum, oil and grease, methyl ethyl ketone, steel, and related materials.

8.AA.5 Sector-Specific Benchmarks. (See also Part 6 of the permit.)

Table 8.AA-1		
Subsector (You may be subject to requirements for more than one sector/subsector)	**Parameter**	**Benchmark Monitoring Concentration**
Subsector AA1. Fabricated Metal Products, except Coating (SIC 3411-3499; 3911-3915)	Total Aluminum	0.75 mg/L
	Total Iron	1.0 mg/L
	Total Zinc[1]	Hardness Dependent
	Nitrate plus Nitrite Nitrogen	0.68 mg/L
Subsector AA2. Fabricated Metal Coating and Engraving (SIC 3479)	Total Zinc[1]	Hardness Dependent
	Nitrate plus Nitrite Nitrogen	0.68 mg/L

[1] The benchmark values of some metals are dependent on water hardness. For these parameters, permittees must determine the hardness of the receiving water (see Appendix J, "Calculating Hardness in Receiving Waters for Hardness Dependent Metals," for methodology), in accordance with Part 6.2.1.1, to identify the applicable 'hardness range' for determining their benchmark value applicable to their facility. The ranges occur in 25 mg/L increments. Hardness Dependent Benchmarks follow in the table below:

Water Hardness Range	**Zinc** (mg/L)
0-25 mg/L	0.04
25-50 mg/L	0.05
50-75 mg/L	0.08
75-100 mg/L	0.11
100-125 mg/L	0.13
125-150 mg/L	0.16
150-175 mg/L	0.18
175-200 mg/L	0.20
200-225 mg/L	0.23
225-250 mg/L	0.25
250+ mg/L	0.26

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart AB – Sector AB – Transportation Equipment, Industrial or Commercial Machinery Facilities.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.AB.1 Covered Stormwater Discharges.

The requirements in Subpart AB apply to stormwater discharges associated with industrial activity from Transportation Equipment, Industrial or Commercial Machinery facilities as identified by the SIC Codes specified under Sector AB in Table D-1 of Appendix D of the permit.

8.AB.2 Additional SWPPP Requirements.

8.AB.2.1 *Drainage Area Site Map.* (See also Part 5.1.2) Identify in your SWPPP where any of the following may be exposed to precipitation or surface runoff: vents and stacks from metal processing and similar operations.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart AC– Sector AC –Electronic and Electrical Equipment and Components, Photographic and Optical Goods.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.AC.1 Covered Stormwater Discharges.

The requirements in Subpart AC apply to stormwater discharges associated with industrial activity from facilities that manufacture Electronic and Electrical Equipment and Components, Photographic and Optical goods as identified by the SIC Codes specified in Table D-1 of Appendix D of the permit.

8.AC.2 Additional Requirements.

No additional sector-specific requirements apply.

Part 8 – Sector-Specific Requirements for Industrial Activity

Subpart AD – Sector AD – Stormwater Discharges Designated by the Director as Requiring Permits.

You must comply with Part 8 sector-specific requirements associated with your primary industrial activity and any co-located industrial activities, as defined in Appendix A. The sector-specific requirements apply to those areas of your facility where those sector-specific activities occur. These sector-specific requirements are in addition to any requirements specified elsewhere in this permit.

8.AD.1 Covered Stormwater Discharges.

Sector AD is used to provide permit coverage for facilities designated by the Director as needing a stormwater permit, and any discharges of stormwater associated with industrial activity that do not meet the description of an industrial activity covered by Sectors A-AC.

8.AD.1.1 *Eligibility for Permit Coverage.* Because this sector is primarily intended for use by discharges designated by the Director as needing a stormwater permit (which is an atypical circumstance), and your facility may or may not normally be discharging stormwater associated with industrial activity, you must obtain the Director's written permission to use this permit prior to submitting an NOI. If you are authorized to use this permit, you will still be required to ensure that your discharges meet the basic eligibility provisions of this permit at Part 1.2.

8.AD.2 Sector-Specific Benchmarks and Effluent Limits. (See also Part 6 of the permit.)

The Director will establish any additional monitoring and reporting requirements for your facility prior to authorizing you to be covered by this permit. Additional monitoring requirements would be based on the nature of activities at your facility and your stormwater discharges.

9. Permit Conditions Applicable to Specific States, Indian Country Lands, or Territories

9.1 Region 1

9.1.1 CTR05000I: Indian Country lands within the State of Connecticut

No additional requirements.

9.1.2 MAR050000: Commonwealth of Massachusetts, except Indian Country lands.

Permittees in Massachusetts must also meet the following conditions.

9.1.2.1 Additional Section 401(a) conditions required by the Commonwealth of Massachusetts. Discharges covered by the general permit must comply with the provisions of 314 CMR 3.00; 314 CMR 4.00; 314 CMR 9.00; and 314 CMR 10.00 and any other related policies adopted under the authority of the Massachusetts Clean Waters Act, MGL c.21, ss. 26-53 and Wetlands Protection Act, MGL s. 40.

New facilities or redevelopment of existing facilities subject to this permit must comply with applicable stormwater performance standards prescribed by state regulation or policy. A permit under 314 CMR 3.04 is not required for existing facilities which meet state stormwater performance standards. An application for a permit under 314 CMR 3.00 is required only when required under 314 CMR 3.04(2)(b) {designation of a discharge on a case-by-case basis} or is otherwise identified in 314 CMR 3.00 or any Department policy as a discharge requiring a permit application. Department regulations and policies may be obtained through the State House Bookstore or online at www.mass.gov/dep.

9.1.2.2 SWPPP Availability. The Department may request a copy of the Stormwater Pollution Prevention Plan (SWPPP) and the permittee is required to submit the SWPPP to the Department within 14 days of such a request.

9.1.2.3 Authorization to Inspect. The Department may conduct an inspection of any facility covered by this permit to ensure compliance with state law requirements, including state water quality standards. The Department may enforce its certification conditions.

9.1.2.4 Submission of Monitoring Data. The results of any monitoring required by this permit must be sent to the appropriate Regional Office of the Department [attention: Bureau of Waste Prevention] where the monitoring identifies exceedances of any effluent limits or benchmarks for any parameter for which monitoring is required under this permit. In addition, any follow-up monitoring and a description of the corrective actions required and undertaken to meet the effluent limits or benchmarks must be sent to the appropriate Department Regional Office.

9.1.2.5 Sector-Specific Requirements. The Massachusetts Coastal Zone Management Program submitted the following conditions to be added to the permit in order to meet the Programs Consistency Review and which will be included in the requirements of this Water Quality Certification:

- In Sector Q [Water Transportation] add copper to the required monitoring parameters.
- In Sector R [Ship and Boat Building and Repair Yards] add aluminum, iron, lead, and copper to the list of required monitoring parameters.
- Modify the monitoring requirements [Part 6.2.1.2 of the permit] such that all four of the quarterly monitoring samples must meet the benchmarks rather than the average of the four before no further monitoring is required.

9.1.3 MAR05000I: Indian Country lands within the Commonwealth of Massachusetts.

No additional requirements.

9.1.5 NHR050000: State of New Hampshire.

Permittees in New Hampshire must also meet the following conditions:

9.1.5.1 On-site Infiltration of Stormwater. In Part 2.1.1 (Control Measure Selection and Design Considerations), you are required to consider opportunities for infiltrating runoff onsite. This is encouraged, but it should only be done if consistent with the statutes and rules of the Department of Environmental Services written to protect groundwater. Infiltration BMPs are not recommended at industrial sites except in areas where industrial activities do not occur, such as at office buildings and their associated parking facilities, or in drainage areas at the facility where a certification of no exposure will always be possible [see 40 CFR 122.26(g)]. Other justifiable reasons for not using on-site infiltration BMPs include the following:

- The facility is located in a wellhead protection area as defined in RSA 485-C:2; or
- The facility is located in an area where groundwater has been reclassified to GAA, GAI or GA2 pursuant to RSA 485-C and Env-Ws 420; or
- Any areas that would be exempt from the groundwater recharge requirements contained in Env-Ws 415.41, including all land uses or activities considered to be a "High-load site."

9.1.5.2 Maintenance of infiltration best management practices. In addition to the requirements in Part 5, the SWPPP must contain the following:

- A description of and the location of each on-site infiltration BMP installed;
- The maintenance procedures that will be followed to ensure proper operation, including the removal of sediment from pretreatment devices;

- The inspection procedures that will be followed at least annually. These should include the procedures for ensuring that the stormwater being infiltrated is not exposed to industrial pollutants and the procedures for ensuring proper drainage to prevent mosquito breeding;
- The employee name (or title of the position) who is a member of the stormwater pollution prevention team (see Part 5.1.1) who will be responsible for the maintenance required in this section, the inspections required in this section, and any necessary corrective actions required in Part 3; and
- Records for all maintenance performed, inspections conducted, and corrective actions taken.

9.1.5.3 Discontinue, Permit or Register On-site Infiltration BMP if Necessary. If at any time a certification of no exposure can no longer be made for any of the stormwater to be infiltrated, then the infiltration BMP must cease for that portion of the runoff or the discharge must be permitted or registered as appropriate. The following may be required:

- Infiltration BMP that meet the definition of a Class V well or that infiltrates stormwater via a subsurface structure (i.e. concrete chambers, dry well, leach field, etc.) will need an underground injection control (UIC) registration from NHDES; and
- Permitting as a groundwater discharge as required in Env-Ws 1500, if the stormwater will or may contain regulated contaminants.

The SWPPP must be modified immediately if new infiltration BMPs are proposed or if existing infiltration BMPs will cease.

9.1.5.4 Required NHDES notification.

- Notify the NHDES Groundwater Discharge Permit Coordinator immediately if you believe that any infiltration BMP may need to be permitted or registered (See Part 9.1.5.3) during the permit term.
- Notify the NHDES Wastewater Engineering Bureau immediately of any plans to discharge any new non-stormwater discharges during the permit term. This does not include the allowable non-stormwater discharges listed in Part 1.1.3.

9.1.5.5 Information that may be requested by NHDES. To ensure compliance with RSA 485-C, RSA 485-A, RSA 485-A:13, I(a), Env-Wq 400 and Env-Ws 401 the following information may be requested by NHDES. This information must be kept on site unless you receive a written request from NHDES that it be sent to the address shown in Part 9.1.5.6.

- A site map required in Part 5.1.2, showing the type and location of all on-site infiltration BMPs utilized at the facility or the reason(s) why none were installed.
- A list of all non-stormwater discharges that occur at the facility, including their source locations and the control measures being used (See Sections 1.1.3 and 5.1.3.4).
- A copy of the Annual Reports required in Part 7.2.

9.1.5.6 Where to Submit Information. All required or requested documents must be sent to: NH Department of Environmental Services, Wastewater Engineering Bureau, Permits & Compliance Section, P.O. Box 95, Concord, NH 03302-0095.

9.1.5.7 Modification of Clean Water Act Section 401 Water Quality Certification. When NHDES determines that additional water quality certification requirements are necessary to the protect water quality, it may require individual dischargers to meet additional conditions to obtain or continue coverage under the MSGP. Any such conditions must be supplied to the permittee in writing. Any required pollutant loading analyses and any designs for structural best management practices necessary to protect water quality must be prepared by a civil or sanitary engineer registered in New Hampshire.

9.1.6 RIR05000I: Indian Country lands within the State of Rhode Island.

No additional requirements.

9.1.7 VTR05000F: Federal Facilities in the State of Vermont.

No additional requirement.

9.2 Region 2

9.2.1 PPR050000: Commonwealth of Puerto Rico

No additional requirements.

9.3 Region 3

9.3.1 DCR050000: The District of Columbia

Permittees in the District of Columbia must also meet the following conditions:

9.3.1.1 Compliance with District of Columbia Laws and Regulations. Discharges covered by the MSGP must comply with the District of Columbia Water Pollution Control Act, (D.C. Code § 8-103.01 *et seq.*) and its implementing regulations in Title 21, Chapters 11 and 19 of the District of Columbia Municipal Regulations. Nothing in this permit will be construed to preclude the institution of any legal action or relieve the permittee from any responsibilities, liabilities, or penalties established pursuant to District of Columbia laws and regulations.

9.3.1.2 Submission of SWPPP. The Stormwater Pollution Prevention Plan (SWPPP) shall be submitted to the District Department of the Environment (Department) at the same time the NOI is submitted to EPA, to ensure compliance with District of Columbia laws and regulations.

9.3.1.3 Submission of No Exposure Certification and NOT. Copies of the No Exposure Certification and Notice of Termination (NOT) shall be submitted to the Department at the same time it is submitted to EPA.

9.3.1.4 Authorization to Inspect. The permittee shall allow the Department to inspect any facilities, equipment, practices, or operations regulated or required under this permit and to access records maintained under the conditions of this permit.

9.3.1.5 Submission of Reports. Signed copies of all reports required under this permit including the reporting requirements of Appendix B.12 shall be submitted to the Department at the same time it is submitted to EPA.

9.3.1.6 Where to Submit Information. All required or requested documents shall be sent to the: District Department of the Environment, Natural Resources Administration, 51 N Street, NE, 5th Floor, Washington, D.C. 20002, Attention: Associate Director, Water Quality Division.

9.3.2 DER05000F: Federal Facilities within the State of Delaware.

No additional requirements.

9.4 Region 4

Permit coverage not available.

9.5 Region 5

9.5.1 MIR05000I: Indian Country Lands within the State of Michigan

No additional requirements.

9.5.2 MNR05000I: Indian Country Lands within the State of Minnesota

9.5.2.1 Fond du Lac Reservation

The following conditions apply only to discharges on the Fond du Lac Reservation.

9.5.2.1.1 Submission of NOI and NOT. Copies of the Notice of Intent (NOI) and Notice of Termination (NOT) shall be submitted to the Office of Water Protection at the same time it is submitted to EPA.

9.5.2.1.2 Submission of SWPPP. A copy of the Stormwater Pollution Plan (SWPPP) shall be submitted to the Office of Water Protection at least thirty (30) days in advance of submitting the NOI to EPA.

9.5.2.1.3 Benchmark Monitoring for TSS. Benchmark Monitoring Concentration (BMC) for Total Suspended Solids (TSS) shall be 10 mg/L for Sector A (Timber Products), Sector J (Mineral Mining and Dressing), and Sector M (Automobile Salvage Yards) that conduct Industrial Activities on the Fond du Lac Reservation.

9.5.2.1.4 Benchmark Monitoring for Nitrate plus Nitrite Nitrogen. Benchmark Monitoring Concentration (BMC) fro Nitrate plus Nitrite Nitrogen shall be 0.12mg/L for Sector J (Mineral Mining and Dressing) that conduct Industrial Activities on the Fond du Lac Reservation.

9.5.2.1.5 Submission of Monitoring Reports. Copies of all Monitoring Reports required by this permit shall be submitted to the Office of Water Protection.

9.5.2.1.6 Where to Submit Information. All required or requested documents shall be sent to the: Fond du Lac Reservation Office of Water Protection (OWP) at Fond du Lac Reservation, Office of Water Protection, 1720 Big Lake Road, Cloquet, Minnesota 55720.

9.5.2.2 Grand Portage Reservation

The following conditions apply only to discharges on the Grand Portage Reservation.

9.5.2.2.1 Compliance with Grand Portage Reservation Laws and Regulations. All industrial stormwater discharges authorized by this permit must comply with the Grand Portage Water Quality Standards, Applicable Federal Standards, and the Grand Portage Water Resources Ordinance, as amended, ("Water Resources Ordinance").

9.5.2.2.2 Additional Monitoring Required by Grand Portage Reservation. The Board must be contacted, at the address in Part 9.5.2.2.10, at the onset of writing the Stormwater Pollution Prevention Plan (SWPPP). Grand Portage may require monitoring of stormwater discharges as determined on a case-by-case basis. If the Board determines that a monitoring plan is necessary, the monitoring plan must be prepared and incorporated in the SWPPP before the Notice of Intent (NOI) is submitted to EPA.

9.5.2.2.3 Submission of SWPPP and NOI. A copy of the SWPPP and NOI must be submitted to the Board for review and approval at least 30 days before submitting the NOI to EPA.

9.5.2.2.4 Submission of NOT. A copy of the Notice of Termination (NOT) must be submitted to the Board at the address in Part 9.5.3.10 at the same time it is submitted to EPA.

9.5.2.2.5 Additional Information. If requested by the Grand Portage Environmental Department, the permittee is required to provide additional information necessary for a case-by-case eligibility determination to assure compliance with the Grand Portage Water Quality Standards and any Applicable Federal Standards.

9.5.2.2.6 ***Submission of Monitoring Data.*** All analytical data (e.g., Discharge Monitoring Reports, etc.) must be submitted to the Board at the same time it is submitted to EPA.

9.5.2.2.7 ***Water Quality Standards.*** Discharges that the Board has determined to be or may reasonably be expected to be contributing to a violation of Grand Portage Water Quality Standards or Applicable Federal Standards are not authorized by this permit. Upon receipt of this determination EPA will notify the permittee to either improve their SWPPP to comply with Grand Portage Water Standards or apply for and obtain an individual NPDES permit for these discharges.

9.5.2.2.8 ***Appeals.*** Appeals related to Tribal decisions actions, or enforcement taken pursuant to any of the preceding conditions will be heard by the Grand Portage Tribal Court.

9.5.2.2.9 ***Definitions.*** The definitions set forth in the Grand Portage Water Resources Ordinance, as amended, govern these certification conditions.

9.5.2.2.10 ***Where to Submit Information.*** All required or requested documents shall be sent to the: Grand Portage Environmental Resources Board, P.O. Box 428, Grand Portage, MN 55605.

9.5.3 WIR05000I: Indian Country lands within the State of Wisconsin, except those on Sokaogon Chippewa Community lands

No additional requirements.

Note: Facilities in the Sokaogon Chippewa Community are not eligible for stormwater discharge coverage under this permit. Contact the EPA Region 5 office for an individual permit application.

9.6 Region 6

9.6.1 LAR05000I: Indian Country Lands within the State of Louisiana

No additional requirements.

9.6.2 The State of New Mexico, except Indian Country lands.

Permittees in New Mexico must also meet the following conditions:

9.6.2.1 ***Certification Requirements.*** Operators are not eligible to obtain authorization under this permit for all new and existing stormwater discharges to outstanding national resource waters (ONRWs) (also referred to as “Tier 3” waters.) As of 2/16/06, the following ONRWs have been designated by the SWQB in New Mexico (see Subsection D of 20.6.4.9 NMAC). (1) Rio Santa Barbara, including the west, middle and east forks from their headwaters downstream to the boundary of the Pecos Wilderness; and (2) the water

within the US forest service Valle Vidal special management unit including: (a) Rio Costilla, including Comanche, La Cueva, Fernandez, Chuckwagon, Little Costilla, Holman, Gold, Grassy, LaBelle, and Vidal creeks, from their headwaters downstream to the boundary of the US forest service Valle Vidal special management unit. (b) Middle Ponil creek, including the waters of Greenwood Canyon, from their headwaters downstream to the boundary of the Elliott S. Barker wildlife management area; (c) Shuree lakes; (d) North Ponil creek, including McCrystal and Seally Canyon creeks, from their headwaters downstream to the boundary of the US forest service Valle Vidal special management unit; and (e) Leandro creek from its headwaters downstream to the boundary of the US forest service Valle Vidal.

9.6.3 Indian Country lands within the State of New Mexico, except Ute Mountain Reservations Lands (see Region 8) and Navajo Reservation Lands (see Region 9).

9.6.3.1 Pueblo of Acoma.

The following condition applies only to discharges on the Pueblo of Acoma:

9.6.3.1.1 ***Submission of NOI and NOT.*** The Pueblo will require the owner/operator of each facility on or bordering the Pueblo of Acoma to submit copies of its Notice of Intent (NOI) and Notice of Termination (NOT) to the Haaku Water Office (HWO) Director at the same time it is submitted to EPA.

9.6.3.1.2 ***SWPPP Availability.*** The HWO may request a copy of the Stormwater Pollution Prevention Plan (SWPPP) and the permittee is required to submit the SWPPP to the HWO upon such request.

9.6.3.1.3 ***Submission of Monitoring Data.*** All analytical data shall also be provided to the HWO at the same time it is submitted to EPA.

9.6.3.1.4 ***Where to Submit Information.*** All required or requested documents shall be sent to: HWO Director, Haaku Water Office, P.O. Box 309, Pueblo of Acoma, NM 87034.

9.6.3.2 Pueblo of Isleta.

The following conditions apply only to discharges on the Pueblo of Isleta:

9.6.3.2.1 ***Submission of SWPPP.*** The Stormwater Pollution Prevention Plan (SWPPP) must be submitted to the Pueblo of Isleta prior to submitting the Notice of Intent (NOI) to EPA.

9.6.3.2.2 ***SWPPP Modification.*** Any update or amendment of the SWPPP shall be submitted to the Pueblo of Isleta within 5 calendar days of its finalization.

9.6.3.2.3 ***Submission of Monitoring Data.*** All monitoring data and reports shall be submitted to the Pueblo of Isleta at the same time they are submitted to EPA.

9.6.3.2.4 Submission of Inspection Reports. All inspection reports, including the Compliance Evaluation Report, shall be submitted to the Pueblo of Isleta within 5 calendar days of their finalization.

9.6.3.2.6 Additional Reporting. Any spill or leak directly to waters designated by the Pueblo of Isleta as 'Primary Contact Recreation' and/or 'Primary Contact Ceremonial' shall be considered significant if it contains toxic or hazardous pollutants, oil or petroleum products. The Pueblo of Isleta shall be notified of any spill containing toxic or hazardous pollutants and of any spill of oil or petroleum product within 8-hours of spill detection.

9.6.3.2.7 Benchmark Monitoring. Following 4 quarters of benchmark monitoring, if the maximum value of the 4 monitoring values does not exceed the benchmark, you have fulfilled your monitoring requirements for that parameter for the permit term. If any of the 4 monitoring values exceeds the benchmark, quarterly monitoring shall continue until no exceedances of the benchmark are detected in four consecutive quarters. Following this determination, you may reduce monitoring for that pollutant to once per year for the duration of the permit period unless an exceedance is again detected at which time quarterly sampling will again be required.

9.6.3.2.8 Corrective Action. You must take corrective action following any benchmark exceedance if you determine as a result of reviewing your SWPPP that your SWPPP does not meet the requirements of Part 5 of this permit.

9.6.3.2.9 Conditions applicable only to Sector G, Metal Mining. (See Part G.4.2.1. Inspection Frequency). Inspections must be conducted at least once every 7 calendar days or at least once every 14 calendar days and within 24 hours of the end of a storm event of 0.25 inches or greater. Inspection frequency may be reduced to at least once every month if the entire site is temporarily stabilized, if runoff is unlikely due to winter conditions (e.g., site is covered with snow, ice, or the ground is frozen), or construction is occurring during seasonal arid periods in arid areas and semi-arid areas.

9.6.3.2.10 Where to Submit Information. All required or requested documents shall be sent to: Director, Environment Department, Pueblo of Isleta, P.O. Box 1270, Isleta, NM 87022.

9.6.3.3 Pueblo of Nambe.

The following conditions apply only to discharges on the Pueblo of Nambe:

9.6.3.3.1 Submission of NOI and NOT. Copies of the Notice of Intent (NOI) and Notice of Termination (NOT) shall be submitted to the Pueblo of Nambe at the same time it is submitted to EPA.

9.6.3.3.2 SWPPP Availability. A copy of the Stormwater Pollution Prevention Plan (SWPPP) must also be submitted to the Pueblo of Nambe, if requested, at the same time the NOI is submitted to EPA.

9.6.3.3.3 Submission of Reports. All analytical data and a copy of all written reports shall be provided to the Pueblo of Nambe at the same time they are provided to the EPA, if requested by the Pueblo of Nambe.

9.6.3.3.4 Where to Submit Information. All required or requested documents shall be sent to: Alan G Hook, Manager, Pueblo of Nambe, Department of Environment and Natural Resources (DENR), Rt. 1 Box 117-BB, Sante Fe, NM 87506.

9.6.3.4 Pueblo of Pojoaque.

The following conditions apply only to discharges on the Pueblo of Pojoaque:

9.6.3.4.1 Submission of NOI and NOT. Copies of the Notice of Intent (NOI) and Notice of Termination (NOT) shall be provided at the same time it is provided to EPA.

9.6.3.4.2 SWPPP Availability. The Pueblo may request a copy of the Stormwater Pollution Prevention Plan (SWPPP) and the permittee is required to submit the SWPPP to the Pueblo upon such request.

9.6.3.4.3 Submission of Monitoring Data. All analytical data (e.g., Discharge Monitoring Reports, etc) shall be submitted to the Pueblo at the same time it is submitted to EPA.

9.6.3.4.4 Where to Submit Information. All required or requested documents shall be sent to: Luke Mario Duran, Director, Environment Department, 5 West Gutierrez, Suite 2B, Sante Fe, NM 87506.

9.6.3.5 Ohkay Owingeh - (formerly known as San Juan Pueblo).

The following condition applies only to discharges on Ohkay Owingeh (formerly known as San Juan Pueblo):

9.6.3.5.1 Submission of NOI and NOT. Copies of the Notice of Intent (NOI) and Notice of Termination (NOT) shall be submitted to Ohkay Owingeh at the same time it is submitted to EPA.

9.6.3.5.2 Submission of Monitoring Data and Additional Reporting. Copies of monitoring data or other documents required under the permit must also be submitted to Ohkay Owingeh upon request.

9.6.3.5.3 Where to Submit Information. All required or requested documents shall be sent to the: Ohkay Owingeh, Office of Environmental Affairs, P.O. Box 1099, San Juan Pueblo, NM 87566.

9.6.3.6 Pueblo of Sandia.

The following conditions apply only to discharges on the Pueblo of Sandia:

9.6.3.6.1 ***Submission of NOI.*** A copy of the Notice of Intent (NOI) must be submitted to the Environment Director at the same time it is submitted to EPA.

9.6.3.6.2 ***Submission of NOT.*** A copy of the Notice of Termination (NOT) must be submitted to the Environment Director at the same time it is submitted to EPA. The Pueblo of Sandia must verify termination of activities prior to EPA's termination of the permit.

9.6.3.6.3 ***SWPPP Availability.*** The Stormwater Pollution Prevention Plan (SWPPP) must be made available to Pueblo of Sandia Environment Department personnel upon request.

9.6.3.6.4 ***Submission of Monitoring Data.*** All analytical data (e.g., Discharge Monitoring Reports, follow-up monitoring reports, Exceedance reports, etc) shall be submitted to the Environment Director at the same time it is submitted to EPA.

9.6.3.6.5 ***Submission of Quarterly Visual Assessments.*** Copies of all "Quarterly Visual Assessments" (Part 4.2) must be submitted to the Environment Director within 7 days of completion.

9.6.3.6.6 ***Submission of Comprehensive Site Inspection Reports.*** Copies of all "Comprehensive Site Inspection Reports" (Part 4.3) must be submitted to the Environment Director within 10 days of completion.

9.6.3.6.7 ***Additional Reporting.*** Any notice of release of oils or hazardous substances shall be provided to the Environment Director within twenty-four (24) hours of becoming aware of the circumstance, followed by the reporting requirements of 40 CFR 110, 40 CFR 302, and 40 CFR 302 relating to spills or other releases of oil or hazardous substances.

The permittee must also telephone the Pueblo of Sandia Environment Department at (505) 867-4533 of any spills or unauthorized discharges that may affect drinking water supplies, ceremonial and recreational surface waters, elicit fish kills, harm wildlife or endangered species or endanger human health or the environment within ten (10) hours of becoming aware of the circumstance, followed by the written report when it is sent to the EPA.

9.6.3.6.8 ***Water Quality Standards.*** If requested by the Pueblo of Sandia Environment Department, the permittee shall provide additional information necessary for a "case by case" eligibility determination to assure compliance with Pueblo of Sandia Water Quality Standards.

Note: Upon receipt of a determination by the Pueblo of Sandia that discharges from a perimittee have reasonable potential to be causing or contributing to a violation of Pueblo of Sandia Water Quality Standards, EPA Region 6 would be notified. EPA Region 6 would then notify the permittee to either improve their Stormwater Pollution Prevention Plan (SWPPP) to achieve compliance with the Pueblo of Sandia Water Quality Standards or apply for and obtain an individual NPDES permit for these discharges per CFR 122.28(b)(3).

9.6.3.6.9 Authorization to Inspect. If requested by the Pueblo of Sandia Environment Department the permittee must allow the Pueblo to perform its own routine or compliance inspection to ensure the permittee is in compliance and any discharge is not contributing to a violation of the Pueblo of Sandia's Water Quality Standard.

9.6.3.6.10 Alternative Permit. Any industry discharging to waters of the United States that has been designated by the EPA as an impaired water shall not be covered under the Multi-Sector General Permit but will be required to obtain an individual permit.

9.6.3.6.11 Where to Submit Information. All required or requested documents shall be sent to: Environment Director, Pueblo of Sandia Environment Department at 481 Sandia Loop, Bernalillo, New Mexico 87004

9.6.3.7 Pueblo of Santa Clara.

The following condition applies only to discharges on the Santa Clara Indian Pueblo:

9.6.3.7.1 Submission of NOI and NOT. The Notice of Intent (NOI) and Notice of Termination (NOT) must be submitted to the Santa Clara Pueblo Governor's Office at the same time it is submitted to EPA

9.6.3.7.2 SWPPP Availability. A copy of the Stormwater Pollution Prevention Plan must be made available to the Pueblo of Santa Clara staff upon request.

9.6.3.7.3 Where to Submit Information. All required or requested documents shall be sent to the: Santa Clara Pueblo, Governor's Office, P.O. Box 580, Espanola, NM 87532.

9.6.3.8 Pueblo of Taos

The following conditions apply only to discharges on the Pueblo of Taos:

9.6.3.8.1 Submission of NOI and NOT. Copies of the Notice of Intent (NOI) and Notice of Termination (NOT) shall be provided at the same time it is provided to EPA.

9.6.3.8.2 Submission of SWPPP. Upon request by the Pueblo, a copy of the Stormwater Pollution Prevention Plan must be provided to the Taos Pueblo Environmental Officer.

9.6.3.8.3 ***Submission of Data and Reports***. All analytical data and a copy of all written reports shall be provided to the Pueblo at the same time it is provided to the EPA.

9.6.3.8.4 ***Where to Submit Information***. All requested materials shall be sent to Program Manager, Taos Pueblo Environmental Office Program Manager, P.O. Box 1846, Taos, NM, 97571.

9.6.3.9 Pueblo of Tesuque.

The following conditions apply only to discharges on the Pueblo of Tesuque:

9.6.3.9.1 ***Submission of NOI and NOT.*** Copies of the Notice of Intent (NOI) and Notice of Termination (NOT) shall be provided at the same time it is provided to EPA.

9.6.3.9.2 ***Submission of SWPPP.*** A copy of the Stormwater Pollution Prevention Plan must also be made available to the Pueblo of Tesuque at the time the NOI submitted.

9.6.3.9.3 ***Submission of Monitoring Data.*** All analytical data (e.g., Discharge Monitoring Reports, etc) shall be provided to the Pueblo at the same time it is provided to the EPA.

9.6.3.9.4 ***Where to Submit Information.*** All required or requested documents shall be sent to: Jennifer Montoya, Director, Pueblo of Tesuque Environment Department, Rt. 42 Box 360-T, Santa Fe, NM 87506.

9.6.4 OKR05000I: Indian Country lands within the State of Oklahoma

9.6.4.1 ***Certification Requirements.*** In order to protect downstream waters subject to the state of Oklahoma's Water Quality Standards (OAC 785:45-5-25) coverage under this permit is not available for any new or proposed discharges located within the watershed of any part of the Oklahoma Scenic Rivers system, including the Illinois River, Flint Creek, Barren Fork Creek, Upper Mountain Fork Creek, Little Lee Creek, and Big Lee Creek or to any water designated as an Outstanding Resource Water (ORW). Existing discharges of stormwater in these watersheds may be permitted under this permit only from point sources existing as of June 25, 1992, whether or not such stormwater discharges were permitted as point sources prior to June 25, 1992. For any such existing discharge, increased load of any pollutant above levels of June 25, 1992 is prohibited. Any new or proposed discharges not eligible for permit coverage under this paragraph must apply for an individual permit.

9.6.4.2 Pawnee Nation of Oklahoma

The following conditions apply only to discharges on the Pawnee Nation of Oklahoma:

9.6.4.2.1 ***Submission of NOI and NOT.*** Copies of the Notice of Intent (NOI) and Notice of Termination (NOT) shall be provided at the same time it is provided to EPA.

9.6.4.2.2 ***Submission of SWPPP.*** Copies of the Stormwater Pollution Prevention Plan must be provided to the Director of the Pawnee Nation Department of Environmental Conservation and Safety (DECS) no later than the same time as submitted to EPA.

9.6.4.2.3 ***Submission of Data and Reports***. All analytical data and a copy of all written reports shall be provided to DECS no later than the same time it is submitted to the EPA.

9.6.4.2.4 ***Spills or Leaks.*** All spills or leaks of any size or amount occurring upon the Pawnee Nation shall be reported to DECS and the Bureau of Indian Affairs – Pawnee Agency, Bureau of Land Management-Moore Office, Oklahoma City, immediately upon detection as required under Title X, Article 6, section 611 (Pawnee Nation Oil Pollution Control Act – Emergency Response/Notification) of the Pawnee Nation Law and Order Code.

9.6.4.2.5 ***Discharges from Secondary Containment.*** Discharge of stormwater from secondary containment is prohibited and shall not be authorized as cited in Title X, Article 6, Section 604(B) (Pawnee National Oil Pollution Control Act – Secondary Containment).

9.6.4.2.6 ***Where to Submit Information.*** All required or requested documents shall be sent to: Director of the Pawnee Nation Department of Environmental Conservation and Safety (DECS), P.O. Box 470, Pawnee, OK 74058.

9.6.5 OKR05000F: Facilities in the State of Oklahoma not under the jurisdiction of the Oklahoma Department of Environmental Quality, except those on Indian Country lands.

9.6.5.1 ***Certification Requirements.*** In accordance with Oklahoma's Water Quality Standards (OAC 785:45-5-25) coverage under this permit is not available for any new or proposed discharges located within the watershed or any part of the Oklahoma Scenic Rivers system, including Illinois River, Flint Creek, Barren Fork Creek, Upper Mountain Fork River, Little Lee Creek, and Big Lee Creek or to any water designated as an Outstanding Resource Water (ORW). Existing discharges of stormwater in these watersheds may be permitted under this permit only from point sources existing as of June 25, 1992, whether or not such stormwater discharges were permitted as point sources prior to June 25, 1992. For any such existing discharge, increased load of any pollutant above levels of June 25, 1992 is prohibited. Any new or proposed discharges not eligible for permit coverage under this paragraph must apply for an individual permit.

9.6.6 TXR05000F: Facilities in the State of Texas not under the jurisdiction of the Texas Commission on Environmental Quality, except those on Indian Country lands.

No additional requirements.

9.6.7 TXR05000I: Indian Country lands within the State of Texas.

No additional requirements.

9.7 Region 7

Permit coverage not available

9.8 Region 8

Permit coverage not available

9.9 Region 9

9.9.1 ASR050000: The islands of American Samoa

The following condition applies only to discharges on the American Samoa:

9.9.1.1 Submission of NOI. All Notices of Intent (NOIs) for stormwater discharges covered under the general permits in American Samoa shall be submitted to the American Samoa Environmental Protection Agency at the same time it is submitted to EPA.

9.9.1.2 Submission of SWPPPs. All SWPPPs for stormwater discharges in American Samoa shall be submitted to the American Samoa Environmental Protection Agency for review and approval.

9.9.2 AZR05000I: Indian Country lands within the State of Arizona, including Navajo Reservation lands in New Mexico and Utah.

9.9.2.1 Hualapai Tribe (Arizona)

The following condition applies only to discharges on the Hualapai Tribe:

9.9.2.1.1 Submission of NOI and SWPPP. All Notices of Intent (NOIs) and Stormwater Pollution Plans (SWPPPs) for stormwater discharges on Hualapai Tribal lands shall be submitted to the Water Resource Program through the Tribal Chairman for review and approval

9.9.2.1.2 Where to Submit Information. All required or requested documents shall be sent to: Water Resource Program through the Tribal Chairman, P.O. Box 179, Peach Springs, AZ 86434.

9.9.2.2 Navajo Nation (Arizona).

The following conditions apply only to discharges on the Navajo Nation:

9.9.2.2.1 ***Submission of NOI.*** Notices of Intent (NOI) must be submitted to Navajo EPA for review, comment and tracking.

9.9.2.2.2 ***Submission of SWPPP.*** Copies of Stormwater Water Pollution Plans (SWPPPs) and supporting Best Management Practices (BMPs) must be submitted to Navajo EPA for review and concurrence.

9.9.2.2.3 ***Submission of Monitoring Data.*** Copies of all monitoring reports must be provided to Navajo EPA.

9.9.2.3 White Mountain Apache Tribe (Arizona).

The following condition applies only to discharges on the White Mountain Apache Tribe:

9.9.2.3.1 ***Submission of NOI.*** All Notices of Intent for proposed stormwater discharges under the MSGP must be submitted to the Tribal Environmental Office.

9.9.2.3.2 ***Where to Submit Information.*** All required or requested documents shall be sent to the: Tribal Environmental Office, Attention: Doreen E. Gatewood, P.O. Box 1000, Whiteriver, AZ 85941.

9.9.3 CAR05000I: Indian Country lands within the State of California.

9.9.3.1 Big Pine Paiute Tribe of the Owens Valley (California).

The following condition applies only to discharges on the Big Pine Paiute Tribe of the Owens Valley:

9.9.3.1.1 ***Submission of NOI.*** Copies of Notices of Intent (NOIs) shall be submitted to the Tribe at the same time (or prior to) it is submitted to EPA.

9.9.3.2 Bishop Paiute Tribe (California).

The following condition applies only to discharges on the Bishop Paiute Tribe:

9.9.3.2.1 ***Submission of NOI.*** Copies of Notices of Intent (NOIs) for proposed stormwater discharges must be submitted to the Tribe's Environmental Management Office for review and comment by the Tribal Environmental Protection Agency (TEPA) Board.

9.9.3.3 Hoopa Valley Tribe (California).

The following conditions apply only to discharges on the Hoopa Valley Tribe:

9.9.3.3.1 Submission of NOI. All Notices of Intent (NOI) submitted for stormwater discharges under the general permits in Hoopa Valley Indian Reservation (HVIR) shall be submitted to the Tribal Environmental Protection Agency (TEPA).

9.9.3.3.2 Submission of SWPPP. All Stormwater Pollution Plans (SWPPPs) for stormwater discharges in HVIR shall be submitted to TEPA for review and approval.

9.9.3.4 Twenty-Nine Palms Band of Mission Indians (California)

The following conditions apply only to discharges on the Twenty-Nine Palms Band of Mission Indians:

9.9.3.4.1 Submission of NOI. Notices of Intent (NOI) must be submitted to the 29 Palms Tribal EPA for review, comment, and tracking.

9.9.3.4.2 Submission of SWPPP. Copies of Stormwater Pollution Prevention Plans (SWPPPs) and supporting best management practices (BMPs) must be submitted to the 29 Palms Tribal EPA for review and compliance.

9.9.3.4.3 Submission of Monitoring Data. Copies of all monitoring reports must be provided to the 29 Palms Tribal EPA.

9.9.4 GUR050000: The Island of Guam.

No additional requirements.

9.9.5 JAR050000: Johnston Atoll.

No additional requirements.

9.9.6 MWR050000: Midway Island and Wake Island.

No additional requirements.

9.9.7 Commonwealth of the Northern Mariana Islands

The following conditions apply only to discharges on the Commonwealth of the Northern Mariana Islands (CNMI):

9.9.7.1 Submission of NOI. Pursuant to Part 10.3(h)(5) of the Standards, every Notice of Intent (NOI) submitted to EPA for activities in the CNMI that are to be covered under this permit must be postmarked no less than seven (7) calendar days prior to any stormwater discharges and a copy must be submitted to the Director of Division of Environmental Quality (DEQ) no later than seven (7) calendar days prior to any stormwater discharges.

9.9.7.2 Submission of SWPPP. Pursuant to Part 10.3(h)(3) of the Standards, for any activity subject to the permit in the CNMI, a Stormwater Pollution Prevention Plan (SWPPP) for stormwater discharges associated with industrial activities must be submitted to DEQ and approved by the Director of DEQ prior to submission of the NOI to EPA.

9.9.7.3 Submission of SWPPP Approval Letter. Pursuant to Part 10.3(h)(4) of the Standards, every NOI submitted to EPA for activities in the CNMI that are to be covered under this permit must be accompanied by a SWPPP approval letter from DEQ.

9.9.7.4 Submission of Monitoring Data. Pursuant to Part 10.3(h)(6) of the Standards, permittees covered under this permit must submit copies of all monitoring reports to DEQ.

9.9.7.5 Certification. Pursuant to Section 10.6 of the Standards, this certification shall be subject to amendment or modification if and to the extent that existing water quality standards are made more stringent, or new water quality standards are adopted, by DEQ.

This certification does not relieve the applicant from obtaining other applicable local or federal permits.

9.9.8 NVR05000I: Indian Country lands within the State of Nevada, including the Duck Valley Reservation in Idaho, the Fort McDermitt Reservation in Oregon and the Confederated Tribes of the Goshute Reservation in Utah

9.9.8.1 Pyramid Lake Paiute Tribe (Nevada)

The following conditions apply only to discharges on the Pyramid Lake Paiute Tribe:

9.9.8.1.1 Submission of NOI. Notice of Intents (NOI) must be submitted to the Tribe for review, comments, and tracking.

9.9.8.1.2 Submission of SWPPP. **C**opies of Stormwater Pollution Prevention Plans (SWPPPs) and supporting best management practices (BMPs) must be submitted to the Pyramid Lake Paiute Tribe for review and concurrence.

9.9.8.1.3 Submission of Monitoring Data. Copies of all monitoring reports must be submitted to the Pyramid Lake Paiute Tribe.

9.10 Region 10

Table 9.10-1
NOI Submittal Deadlines/Discharge Authorization Dates for Facilities within Region 10 Areas

Category	NOI Submission Deadline	Discharge Authorization Date[1]
Existing Dischargers - in operation as of October 30, 2005 and authorized for coverage under MSGP 2000.	No later than May 27, 2009.	30 days after EPA posts your NOI. Your authorization under the MSGP 2000 is automatically continued until you have been granted coverage under this permit or an alternative permit, or coverage is otherwise terminated.
New Dischargers or New Sources - have commenced discharging between October 30, 2005 and May 27, 2009.	As soon as possible but no later than May 27, 2009.	30 days after EPA posts your NOI.
New Dischargers or New Sources - commence discharging after May 27, 2009.	A minimum of 60 days prior to commencing discharge, or a minimum of 30 days if your SWPPP is posted on the Internet during this period and the Internet address (i.e., URL) to your SWPPP is provided on the NOI form.	If you post your SWPPP on the Internet, 30 days after EPA posts your NOI. Otherwise, 60 days after EPA posts your NOI.
New Owner/Operator of Existing Discharger - transfer of ownership and/or operation of a facility whose discharge is authorized under this permit	A minimum of 30 days prior to date that the transfer will take place to the new owner/operator.	30 days after EPA posts your NOI.
Other Eligible Dischargers - in operation prior to October 30, 2005, but not covered under the MSGP 2000 or another NPDES permit.	Immediately, to minimize the time discharges from the facility will continue to be unauthorized.	If you post your SWPPP on the Internet, 30 days after EPA posts your NOI. Otherwise, 60 days after EPA posts your NOI.

[1] Based on a review of your NOI or other information, EPA may delay your authorization for further review, notify you that additional effluent limitations are necessary, or may deny coverage under this permit and require submission of an application for an individual NPDES permit, as detailed in Part 1.6. In these instances, EPA will notify you in writing of the delay, of the need for additional effluent limits, or of the request for submission of an individual NPDES permit application.

9.10.1 AKR050000: The State of Alaska, except Indian Country lands.

Deadlines for the submittal of Notices of Intent are listed in Table 9.10-1. Permittees in Alaska must also meet the following conditions:

9.10.1.1 Submission of NOI, NOT and all other information. A copy of the Notice of Intent, the No Exposure Certification, the Notice of Termination, all information collected and submitted to EPA pursuant to Parts 3.4 and 7, and reports required under Appendix B.12, shall be sent to the Alaska Department of Environmental Conservation (ADEC) at the same time it is submitted to the EPA. Submittals to ADEC shall be made to the following address:

Alaska Department of Environmental Conservation
Wastewater Discharge/Storm Water
555 Cordova St.
Anchorage, AK 99501

9.10.1.2 Plan approval for nondomestic wastewater treatment works. For all new facilities operators who construct, install or operate any part of a nondomestic wastewater treatment works shall submit a copy of the engineering plans to ADEC for review at the address given above (see 18 AAC 72.600), and pay an engineering plan review fee (see 18 AAC 72.955). Engineering plan approval must be obtained from ADEC prior to construction. Nondomestic wastewater includes stormwater runoff.

9.10.1.3 Submission of SWPPP for new dischargers. Operators who have not previously obtained coverage under the MSGP must submit a copy of the Storm Water Pollution Prevention Plan (SWPPP) for the facility, developed by qualified person, to ADEC for review at the time of submittal of the NOI. The SWPPP shall be accompanied by the state-required plan review fee (see 18 AAC 72.955).

9.10.1.4 Submission of SWPPP for existing dischargers. Operators who submitted a SWPPP to ADEC under the previous MSGP must submit copies of any modifications of their SWPPP to meet the requirements of the MSGP 2008 with their NOI.

9.10.1.5 Submission of Additional Information. ADEC reserves the right to request copies of the SWPPP modifications made to comply with Part 5.2. The operator shall submit the SWPPP modification to ADEC within 14 days of such a request.

9.10.1.6 Conditions Applicable to New Dischargers and New Sources under Sector G (Metal Mining) and Sector H (Coal Mining). For new dischargers and new sources operating under Sector G, Metal Mining (specifically, those facilities that are designed to process 500 or more tons per day) and Sector H, Coal Mining, the following conditions apply:

- The operator shall develop a new SWPPP for each phase of the project: i.e., the exploration, construction, active mining, inactive mining, and reclamation phases;

- The operator shall submit the construction phase SWPPP to ADEC for review at least 90 days prior to the start of construction;
- The operator shall submit the active mining phase SWPPP to ADEC for review at least 90 days prior to the start of the active mining;
- The operator shall submit a copy of engineering plans for nondomestic wastewater treatment facilities used during both the construction phase and the active mining phase to ADEC for review at least 90 days prior to the start of construction;
- Representatives of the operator and the prime site construction contractor shall meet with ADEC representatives in a pre-construction conference at least 20 days before the start of the construction phase to discuss the details of the construction phase SWPPP and stormwater management during construction;
- The operator shall have at least one person on-site during construction that is qualified and trained in the principles and practices of erosion and sediment control and that has the authority to direct the maintenance of stormwater control measures.

9.10.1.7 Benchmark Monitoring for pH and turbidity. The benchmark monitoring concentrations, as described in Part 8, may exceed the Alaska water quality standards. In those instances where the benchmark monitoring concentration exceeds the Alaska water quality standard, the Alaska water quality standard shall be used as the benchmark monitoring concentration. The following provides the instances where the Alaska Water Quality Standards shall be used as the benchmark values:

- For Sectors A, D, E, G, J, K, L, O, and S, the acceptable range for pH is 6.5 to 8.5 and may not vary more than 0.5 pH units from natural conditions. See 18 AAC 70.020(b)(6).
- For Sector G, turbidity in fresh water may not exceed 5 nephelometric turbidity units (NTU) above natural conditions when the natural turbidity is 50 NTU or less, and may not have more than 10% increase in turbidity when the natural turbidity is more than 50 NTU, not to exceed a maximum increase of 25 NTU. See 18 AAC 70.020(b)(12)(A)(i).

9.10.2 AKR05000I: Indian Country lands within Alaska

No additional requirements.

9.10.3 IDR050000: The State of Idaho, except Indian Country lands

Deadlines for the submittal of Notices of Intent are listed in Table 9.10-1. Permittees in Idaho must also meet the following conditions:

9.10.3.1 Monitoring Frequency for Numeric Effluent Limitations. Given the inherent variability in stormwater discharges, the monitoring for parameters with numeric effluent limitations as described in Part 6.2.2 must occur twice per year.

9.10.3.2 Follow-up Monitoring for Benchmark Concentrations. If all four quarterly samples do not exceed the benchmark, the permittee is not required to conduct any additional quarterly monitoring for that parameter. If any of the four quarterly samples exceed the benchmark, then the permittee must follow the additional requirements in Part 6.2.1.2 of the MSGP, with the following modifications:

- If the permittee elects to make any necessary modifications and continue quarterly monitoring, such monitoring must occur until the results from four consecutive quarters of monitoring are less than the benchmark concentration.

9.10.3.3 Monitoring of Discharges to Impaired Waters with an applicable WLA in an EPA-approved TMDL. In order to waive any additional monitoring as allowed by Part 6.2.4.2 of the permit, the permittee must also include documentation in their SWPPP that the pollutant(s) of concern is not expected to be present in the discharge. If such documentation can not be made, then the permittee must conduct annual monitoring for the duration of the permit.

9.10.3.4 Stormwater Pollution Prevention Plan (SWPPP) Availability. If requested by Idaho Department of Environmental Quality (DEQ), the permittee must submit a copy of the SWPPP to DEQ within fourteen (14) days of the request.

9.10.3.5 Submission of NOIs, Monitoring Data, and Additional Reporting. Copies of the following information must be sent to the appropriate DEQ regional office at the same time it is submitted to EPA:

- NOIs for facilities with stormwater discharges to impaired waters;
- Monitoring data collected pursuant to Parts 6.2 and 6.3 of this permit, well as any additional monitoring data required by this Part;
- Exceedance Reports as required by Part 6.3.

Both monitoring data and exceedance reports must be sent to the appropriate DEQ regional office with thirty (30) days of receipt of analytical results.

9.10.3.6 Where to Submit Information or to Obtain Additional Information Regarding Impaired Waters and Approved TMDLs. Information regarding impaired waters and approved TMDLs may be obtained from the appropriate regional DEQ office. Contact information for DEQ offices can be obtained from the DEQ website at http://www.deq.idaho.gov/about/contact_us.cfm.

9.10.3.7 Additional Reporting of Discharges Containing Hazardous Materials or Oil. Any unauthorized discharges containing hazardous materials or oil must be reported to the Idaho State Communications Center (1-800-632-8000) or to the appropriate DEQ Regional Office (see IDAPA 58.01.02.850) as follows:.

Regional Office	Phone #	Regional Office	Phone #
Boise	(208) 373-0550	Lewiston	(208) 373-4370
Coeur d'Alene	(208) 769-1422	Pocatello	(208) 236-6168
Idaho Falls	(208) 528-2650	Twin Falls	(208) 736-2190

9.10.3.8 Additional Conditions Applicable to Sector L (Landfills, Land Application Sites and Open Dumps). Stormwater entering a landfill must be managed as leachate, including run off from areas that have received daily cover which may have contacted waste material, and thus is not eligible for coverage under the MSGP (See 40 CFR 258.26 (a)(2); Municipal Solid Waste Landfill Criteria Technical Manual, EPA 530-R-93-017, 1998). Stormwater from a closed landfill or from areas of the landfill that have received final cover is not leachate, and may be covered under the MSGP.

9.10.3.9 .Benchmark Values for Selenium. The benchmark value for selenium (as found in Sectors G and K) is equal to 0.005 mg/L, which is equivalent to the chronic water quality criterion. Given storms are discrete events of relatively short duration, DEQ believes it is more appropriate to use the acute water quality criteria as a benchmark value. Therefore, benchmark values for selenium can be set equal to the acute criteria of 0.02. mg/L and still comply with Idaho WQS.

9.10.4 IDR05000I: Indian Country lands within the State of Idaho, except Duck Valley Reservation lands, which are covered under Nevada permit NVR05000I listed in Part C.9

No additional requirements.

9.10.5 ORR05000I: Indian Country lands within the State of Oregon, except Fort McDermitt Reservation lands, which are covered under Nevada permit NVR05000I listed in Part C.9

9.10.5.1 Confederated Tribes of the Umatilla Indian Reservation

Deadlines for the submittal of Notices of Intent are listed in Table 9.10-1. Permittees located within the Confederated Tribes of the Umatilla Indian Reservation must also meet the following conditions:

9.10.5.1.1 Water Quality Standards. The operator shall be responsible for achieving compliance with Confederated Tribes of the Umatilla Indian Reservation's (CTUIR) Water Quality Standards.

9.10.5.1.2 Submission of NOI. The operator shall submit a copy of the Notice of Intent (NOI to be covered by the general permit to the CTUIR Water Resources Program at the address below, at the same time it is submitted to EPA.

9.10.5.1.3 Submission of SWPPP. The operator shall be responsible for submitting all Stormwater Pollution Prevention Plans (SWPPPs) required under this general permit to

the CTUIR Water Resources Program for review and determination that the SWPPP is sufficient to meet Tribal Water Quality Standards, prior to the beginning of any discharge activities taking place.

9.10.5.1.4 Additional Reporting. The operator shall be responsible for reporting an exceedance to Tribal Water Quality Standards to the CTUIR Water Resources Program at the same time it is reported to EPA.

9.10.5.1.5 Additional Requirements for Historic Properties Preservation. If the project is an undertaking as defined in section 106 of the National Historic Preservation Act (NHPA), a cultural resource investigation must occur. The operator shall provide the CTUIR Tribal Historic Preservation Office (THPO) 30 days to comment on the area of potential effect (APE) as defined in the permit application.

- All fieldwork must be conducted by qualified personnel (as outlined by the Secretary of Interior's Standards and Guidelines) and documented using Oregon Reporting Standards. The resulting report must be submitted to the THPO and the THPO must concur with the findings and recommendations before any ground disturbing work can occur. The THPO requires 30 days to review all reports.
- The operator must obtain THPO concurrence in writing. If historic properties are present, this written concurrence will outline measures to be taken to prevent or mitigate effects to historic properties.

9.10.5.1.6 Where to Submit Information. The NOI, SWPPP, and reports must be sent to:

CTUIR Water Resources Program
P.O. Box 638
Pendleton, OR 97801
(541) 966-2420

All required Historic Properties Preservation information must be sent to:

CTUIR Cultural Resources Protection Program
Tribal Historic Preservation Office
P.O. Box 638
Pendleton, OR 97801
(541) 276-3629

9.10.6 WAR05000I: Indian Country lands within the State of Washington

9.10.6.1 Lummi Nation.

Deadlines for the submittal of Notices of Intent are listed in Table 9.10-1. Permittees located within the Lummi Nation must also meet the following conditions:

9.10.6.1.1 Additional Requirements. Pursuant to Lummi Code of Laws (LCL) 17.05.020(a), the operator must also obtain a land use permit from the Lummi Planning Department as provided in Title 15 of the Lummi Code of Laws and regulations adopted thereunder.

9.10.6.1.2 Submission of SWPPP for Review and Approval. Pursuant to LCL 17.05.020, each operator shall develop and submit a Storm Water Pollution Prevention Plan to the Lummi Water Resources Division for review and approval by the Water Resources Manager prior to beginning any discharge activities.

9.10.6.1.3 Water Quality Standards. Pursuant to LCL Title 17, each operator shall be responsible for achieving compliance with the Water Quality Standards for Surface Waters of the Lummi Indian Reservation (Lummi Administrative Regulations [LAR]17LAR07.010 through 17LAR 07.210).

9.10.6.1.4 Submission of NOI, Monitoring Data, Reports and NOT. Each operator shall submit a copy of the Notice of Intent, analytical monitoring results, and Exceedance Reports, Annual Reports, and Notice of Termination to the Lumni Water Resources Division at the same time it is submitted to the EPA.

9.10.6.1.5 Where to Submit Information or to Obtain Additional Information. All required information shall be submitted to:

Lummi Natural Resources Department
ATTN: Water Resources Manager
2616 Kwina Road
Bellingham, WA 98226

Please see the Lummi Nation website (www.1ummi-nsn.gov)to review a copy of Title 17 of the Lummi Code of Laws and the references upon which the conditions identified above are based.

This certification does not exempt and is provisional upon compliance with other applicable statutes and codes administered by federal and Lummi tribal agencies.

9.10.6.2 Puyallup Tribe of Indians.

Deadlines for the submittal of Notices of Intent are listed in Table 9.10-1. Permittees discharging from tribal trust lands, or to tribal waters of the Puyallup Tribe of Indians (including to the Lower Puyallup River and portions of the Blair and Hylebos waterways) must meet the following conditions:

9.10.6.2.1 Submission of NOI, NOT and No Exposure Certification. Copies of the Notice of Intent (NOI) and Notice of Termination (NOT), and No Exposure Certification shall be submitted to the Puyallup Tribe's Natural Resources Department.

9.10.6.2.2 Submission of the SWPPP. A copy of the Stormwater Pollution Plan (SWPPP) shall be submitted to the Natural Resources Department at least thirty (30) days in advance of submitting the NOI to EPA.

9.10.6.2.3 Compliance with Tribe's Water Quality Standards. Each permittee shall be responsible for achieving compliance with the Tribe's Water Quality Standards, including anti-degradation provisions.

9.10.6.2.4 Submission and Approval of Sampling Plan. A sampling plan shall be submitted to the Natural Resources Department and approved by the Tribe prior to initiation of monitoring required under Part 6 of this permit.

9.10.6.2.5 Submission of Monitoring Data and Reports. The results of any monitoring required by this permit and all reports must be sent to the Natural Resources Department, including a description of the corrective actions required and undertaken to meet effluent limits or benchmarks (as applicable).

9.10.6.2.6 Authorization to Inspect. The Natural Resources Department may conduct an inspection of any facility covered by this permit to ensure compliance with tribal water quality standards. The Department may enforce its certification conditions.

9.10.6.2.7 Tribal Endangered Species Act Consultation. Consultation with the Tribe that addresses the effects of your facility's stormwater discharges, allowable non-stormwater discharges, and stormwater discharge-related activities on federally-listed threatened or endangered species and designated critical habitat. Information required as part of the consultation shall include:

- Basis of the determination that your stormwater discharges, allowable non-stormwater discharges, and stormwater discharge-related activities will not adversely affect federally-listed as endangered or threatened ("listed") under the Endangered Species Act (ESA) and will not result in the adverse modification or destruction of designated critical habitat including appropriate measures to be undertaken to avoid or eliminate the likelihood of adverse effects (under Criterion E in Section 1.1.4.5); and
- Notice of Intent form complete with extent of action area, list of federally-listed threatened or endangered species or designated critical habitat likely to occur in action area, list of potential pollutants (if you are a new discharger) or list of pollutants for which you have ever exceeded an applicable benchmark or effluent limitations guideline, or for which your discharge has ever been found to cause or contribute to an exceedance of an applicable water quality standard (if you are an existing discharger).

9.10.6.2.8 Where to Submit Information. All required or requested documents shall be sent to:

Puyallup Tribe of Indians
Department of Natural Resources
c/o Bill Sullivan and Char Naylor
3009 E. Portland Avenue
Tacoma, Washington 98404

9.10.7 WAR05000F: Federal Facilities in the State of Washington, except those located on Indian Country lands.

No additional requirements. Deadlines for the submittal of Notices of Intent are listed in Table 9.10-1.

Appendix A

Definitions, Abbreviations and Acronyms

Appendix A. Definitions, Abbreviations, and Acronyms (for the purposes of this permit)**.**

Action Area – all areas to be affected directly or indirectly by the stormwater discharges, allowable non-stormwater discharges, and stormwater discharge-related activities, and not merely the immediate area involved in these discharges and activities.

Arid Climate – areas where annual rainfall averages from 0 to 10 inches.

Best Management Practices (BMPs) – schedules of activities, practices (and prohibitions of practices), structures, vegetation, maintenance procedures, and other management practices to prevent or reduce the discharge of pollutants to waters of the United States. BMPs also include treatment requirements, operating procedures, and practices to control plant site runoff, spillage or leaks, sludge or waste disposal, or drainage from raw material storage. See 40 CFR 122.2.

Co-located Industrial Activities – Any industrial activities, excluding your primary industrial activity(ies), located on-site that are defined by the stormwater regulations at 122.26(b)(14)(i)-(ix) and (xi). An activity at a facility is not considered co-located if the activity, when considered separately, does not meet the description of a category of industrial activity covered by the stormwater regulations or identified by the SIC code list in Appendix D.

Control Measure – refers to any BMP or other method (including effluent limitations) used to prevent or reduce the discharge of pollutants to waters of the United States.

Director – a Regional Administrator of the Environmental Protection Agency or an authorized representative. See 40 CFR 122.2.

Discharge – when used without qualification, means the "discharge of a pollutant." See 40 CFR 122.2.

Discharge of a pollutant – any addition of any “pollutant” or combination of pollutants to “waters of the United States” from any “point source,” or any addition of any pollutant or combination of pollutants to the waters of the “contiguous zone” or the ocean from any point source other than a vessel or other floating craft which is being used as a means of transportation. This includes additions of pollutants into waters of the United States from: surface runoff which is collected or channeled by man; discharges through pipes, sewers, or other conveyances, leading into privately owned treatment works. See 40 CFR 122.2.

Discharge-related activities – activities that cause, contribute to, or result in stormwater and allowable non-stormwater point source discharges, and measures such as the siting, construction and operation of BMPs to control, reduce, or prevent pollution in the discharges.

Drought-stricken area – a period of below average water content in streams, reservoirs, ground-water aquifers, lakes and soils.

EPA Approved or Established Total Maximum Daily Loads (TMDLs) – "EPA Approved TMDLs" are those that are developed by a State and approved by EPA. "EPA Established TMDLs" are those that are developed by EPA.

Existing Discharger – an operator applying for coverage under this permit for discharges authorized previously under an NPDES general or individual permit.

Facility or Activity – any NPDES "point source" (including land or appurtenances thereto) that is subject to regulation under the NPDES program. See 40 CFR 122.2.

Federal Facility – any buildings, installations, structures, land, public works, equipment, aircraft, vessels, and other vehicles and property, owned by, or constructed or manufactured for the purpose of leasing to, the federal government.

Impaired Water (or "Water Quality Impaired Water" or "Water Quality Limited Segment") – A water is impaired for purposes of this permit if it has been identified by a State or EPA pursuant to Section 303(d) of the Clean Water Act as not meeting applicable State water quality standards (these waters are called "water quality limited segments" under 40 CFR 30.2(j)). Impaired waters include both waters with approved or established TMDLs, and those for which a TMDL has not yet been approved or established.

Indian Country – (a) all land within the limits of any Indian reservation under the jurisdiction of the United States Government, notwithstanding the issuance of any patent, and including rights-of-way running through the reservation; (b) all dependent Indian communities within the borders of the United States, whether within the original or subsequently acquired territory thereof, and whether within or without the limits of a State, and (c) all Indian allotments, the Indian titles to which have not been extinguished, including rights-of-way running through the same. This definition includes all land held in trust for an Indian tribe. (18 U.S.C. 1151)

Industrial Activity – the 10 categories of industrial activities included in the definition of "stormwater discharges associated with industrial activity" as defined in 40 CFR 122.26(b)(14)(i)-(ix) and (xi).

Industrial Stormwater – stormwater runoff from industrial activity.

Municipal Separate Storm Sewer – a conveyance or system of conveyances (including roads with drainage systems, municipal streets, catch basins, curbs, gutters, ditches, man-made channels, or storm drains):

(i) Owned or operated by a State, city, town, borough, county, parish, district, association, or other public body (created by or pursuant to State law) having jurisdiction over disposal of sewage, industrial wastes, stormwater, or other wastes, including special districts under State law such as a sewer district, flood control district or drainage district, or similar entity, or an Indian tribe or an authorized Indian tribal organization, or a designated and approved management agency under section 208 of the CWA that discharges to waters of the United States;

(ii) Designed or used for collecting or conveying stormwater;

(iii) Which is not a combined sewer; and

(iv) Which is not part of a Publicly Owned Treatment Works (POTW) as defined at 40 CFR 122.2. See 40 CFR 122.26(b)(4) and (b)(7).

New Discharger – a facility from which there is a discharge, that did not commence the discharge at a particular site prior to August 13, 1979, which is not a new source, and which has never received a finally effective NPDES permit for discharges at that site. See 40 CFR 122.2.

New Source – any building, structure, facility, or installation from which there is or may be a "discharge of pollutants," the construction of which commenced:

- after promulgation of standards of performance under section 306 of the CWA which are applicable to such source, or
- after proposal of standards of performance in accordance with section 306 of the CWA which are applicable to such source, but only if the standards are promulgated in accordance with section 306 within 120 days of their proposal. See 40 CFR 122.2.

New Source Performance Standards (NSPS) – technology-based standards for facilities that qualify as new sources under 40 CFR 122.2 and 40 CFR 122.29.

No exposure – all industrial materials or activities are protected by a storm-resistant shelter to prevent exposure to rain, snow, snowmelt, and/or runoff. See 40 CFR 122.26(g).

Operator – any entity with a stormwater discharge associated with industrial activity that meets either of the following two criteria:

(i) The entity has operational control over industrial activities, including the ability to modify those activities; or

(ii) The entity has day-to-day operational control of activities at a facility necessary to ensure compliance with the permit (e.g., the entity is authorized to direct workers at a facility to carry out activities required by the permit).

Person – an individual, association, partnership, corporation, municipality, State or Federal agency, or an agent or employee thereof. See 40 CFR 122.2.

Point source – any discernible, confined, and discrete conveyance, including but not limited to any pipe, ditch, channel, tunnel, conduit, well, discrete fissure, container, rolling stock, concentrated animal feeding operation, landfill leachate collection system, vessel, or other floating craft from which pollutants are or may be discharged. This term does not include return flows from irrigated agriculture or agricultural stormwater runoff. See 40 CFR 122.2.

Pollutant – dredged spoil, solid waste, incinerator residue, filter backwash, sewage, garbage, sewage sludge, munitions, chemical wastes, biological materials, heat, wrecked or discarded equipment, rock, sand, cellar dirt, and industrial, municipal and agricultural waste discharged into water. See 40 CFR 122.2.

Pollutant of concern – A pollutant which causes or contributes to a violation of a water quality standard, including a pollutant which is identified as causing an impairment in a state's 303(d) list.

Primary industrial activity – includes any activities performed on-site which are (1) identified by the facility's primary SIC code; or (2) included in the narrative descriptions of 122.26(b)(14)(i), (iv), (v), or (vii), and (ix). [For co-located activities covered by multiple SIC codes, it is recommended that the primary industrial determination be based on the value of receipts or revenues or, if such information is not available for a particular facility, the number of employees or production rate for each process may be compared. The operation that generates the most revenue or employs the most personnel is the operation in which the facility is primarily engaged. In situations where the vast majority of on-site activity falls within one SIC code, that activity may be the primary industrial activity.] Narrative descriptions in 40 CFR 122.26(b)(14) identified above include: (i) activities subject to stormwater effluent limitations guidelines, new source performance standards, or toxic pollutant effluent standards; (iv) hazardous waste treatment storage, or disposal facilities including those that are operating under interim status or a permit under subtitle C of the Resource Conservation and Recovery Act (RCRA); (v) landfills, land application sites and open dumps that receive or have received industrial wastes; (vii) steam electric power generating facilities; and (ix) sewage treatment works with a design flow of 1.0 mgd or more.

Qualified Personnel – Qualified personnel are those who possess the knowledge and skills to assess conditions and activities that could impact stormwater quality at your facility, and who can also evaluate the effectiveness of control measures.

Reportable Quantity Release – a release of a hazardous substance at or above the established legal threshold that requires emergency notification. Refer to 40 CFR Parts 110, 117, and 302 for complete definitions and reportable quantities for which notification is required.

Runoff coefficient – the fraction of total rainfall that will appear at the conveyance as runoff. See 40 CFR 122.26(b)(11).

Semi-Arid Climate – areas where annual rainfall averages from 10 to 20 inches.

Significant materials – includes, but is not limited to: raw materials; fuels; materials such as solvents, detergents, and plastic pellets; finished materials such as metallic products; raw materials used in food processing or production; hazardous substances designated under section 101(14) of CERCLA; any chemical the facility is required to report pursuant to section 313 of Title III of SARA; fertilizers; pesticides; and waste products such as ashes, slag and sludge that have the potential to be released with stormwater discharges. See 40 CFR 122.26(b)(12).

Special Aquatic Sites – sites identified in 40 CFR 230 Subpart E. These are geographic areas, large or small, possessing special ecological characteristics of productivity, habitat, wildlife protection, or other important and easily disrupted ecological values. These areas are generally recognized as significantly influencing or positively contributing to the general overall environmental health or vitality of the entire ecosystem of a region.

Stormwater – stormwater runoff, snow melt runoff, and surface runoff and drainage. See 40 CFR 122.26(b)(13).

Stormwater Discharges Associated with Construction Activity – a discharge of pollutants in stormwater runoff from areas where soil disturbing activities (e.g., clearing, grading, or excavating), construction materials, or equipment storage or maintenance (e.g., fill piles, borrow areas, concrete truck washout, fueling), or other industrial stormwater directly related to the construction process (e.g., concrete or asphalt batch plants) are located. See 40 CFR 122.26(b)(14)(x) and 40 CFR 122.26(b)(15).

Stormwater Discharges Associated with Industrial Activity – the discharge from any conveyance that is used for collecting and conveying stormwater and that is directly related to manufacturing, processing or raw materials storage areas at an industrial plant. The term does not include discharges from facilities or activities excluded from the NPDES program under Part 122. For the categories of industries identified in this section, the term includes, but is not limited to, stormwater discharges from industrial plant yards; immediate access roads and rail lines used or traveled by carriers of raw materials, manufactured products, waste material, or by-products used or created by the facility; material handling sites; refuse sites; sites used for the application or disposal of process waste waters (as defined at part 401 of this chapter); sites used for the storage and maintenance of material handling equipment; sites used for residual treatment, storage, or disposal; shipping and receiving areas; manufacturing buildings; storage areas (including tank farms) for raw materials, and intermediate and final products; and areas where industrial activity has taken place in the past and significant materials remain and are exposed to stormwater. For the purposes of this paragraph, material handling activities include storage, loading and unloading, transportation, or conveyance of any raw material, intermediate product, final product, by-product or waste product. The term excludes areas located on plant lands separate from the plant's industrial activities, such as office buildings and accompanying parking lots as long as the drainage from the excluded areas is not mixed with stormwater drained from the above described areas. Industrial facilities include those that are federally, State, or municipally owned or operated that meet the description of the facilities listed in 40 CFR 122.26(b)(14). The term also includes those facilities designated under the provisions of 40 CFR 122.26(a)(1)(v). See 40 CFR 122.26(b)(14).

Tier 2 Waters – For antidegradation purposes, pursuant to 40 CFR 131.12(a)(2), Tier 2 waters are characterized as having water quality that exceeds the levels necessary to support propagation of fish, shellfish, and wildlife and recreation in and on the water.

Tier 2.5 Waters – For antidegradation purposes, Tier 2.5 waters are those waters designated by States or Tribes as neither Tier 2 nor Tier 3. States have special requirements for these waters.

These waters are given a level of protection equal to and above that given to Tier 2 waters, but less than that given Tier 3 waters.

Tier 3 Waters – For antidegradation purposes, pursuant to 40 CFR 131.12(a)(3), Tier 3 waters are identified by states as having high quality waters constituting an Outstanding Natural Resource Water (ONRW), such as waters of National Parks and State Parks, wildlife refuges, and waters of exceptional recreational or ecological significance.

Total Maximum Daily Loads (TMDLs) – A TMDL is a calculation of the maximum amount of a pollutant that a waterbody can receive and still meet water quality standards, and an allocation of that amount to the pollutant's sources. A TMDL includes wasteload allocations (WLAs) for point source discharges; load allocations (LAs) for nonpoint sources and/or natural background, and must include a margin of safety (MOS) and account for seasonal variations. (See section 303(d) of the Clean Water Act and 40 CFR 130.2 and 130.7).

Water Quality Impaired – See 'Impaired Water'.

Water Quality Standards – A water quality standard defines the water quality goals of a water body, or portion thereof, by designating the use or uses to be made of the water and by setting criteria necessary to protect the uses. States and EPA adopt water quality standards to protect public health or welfare, enhance the quality of water and serve the purposes of the Clean Water Act (See CWA sections 101(a)2 and 303(c)). Water quality standards also include an antidegradation policy. See P.U.D. o. 1 of Jefferson County et al v. Wash Dept of Ecology et al, 511 US 701, 705 (1994).

"You" and "Your" – as used in this permit are intended to refer to the permittee, the operator, or the discharger as the context indicates and that party's facility or responsibilities. The use of "you" and "your" refers to a particular facility and not to all facilities operated by a particular entity. For example, "you must submit" means the permittee must submit something for that particular facility. Likewise, "all your discharges" would refer only to discharges at that one facility.

A.2. ABBREVIATIONS AND ACRONYMS

BAT – Best Available Technology Economically Achievable

BOD5 – Biochemical Oxygen Demand (5-day test)

BMP – Best Management Practice

BPJ – Best Professional Judgment

BPT – Best Practicable Control Technology Currently Available

CERCLA – Comprehensive Environmental Response, Compensation and Liability Act

CGP – Construction General Permit

COD – Chemical Oxygen Demand

CWA – Clean Water Act (or the Federal Water Pollution Control Act, 33 U.S.C. §1251 *et seq*)

CWT – Centralized Waste Treatment

DMR – Discharge Monitoring Report

EPA – U. S. Environmental Protection Agency

ESA – Endangered Species Act

FWS – U. S. Fish and Wildlife Service

LA – Load Allocations

MDMR – MSGP Discharge Monitoring Report

MGD – Million Gallons per Day

MOS – Margin of Safety

MS4 – Municipal Separate Storm Sewer System

MSDS – Material Safety Data Sheet

MSGP – Multi-Sector General Permit

NAICS – North American Industry Classification System

NEPA – National Environmental Policy Act

NHPA – National Historic Preservation Act

NMFS – U. S. National Marine Fisheries Service

NOI – Notice of Intent

NOT – Notice of Termination

NPDES – National Pollutant Discharge Elimination System

NRC – National Response Center

NRHP – National Register of Historic Places

NSPS – New Source Performance Standard

NTU – Nephelometric Turbidity Unit

OMB – U. S. Office of Management and Budget

ORW – Outstanding Resource Water

OSM – U. S. Office of Surface Mining

POTW – Publicly Owned Treatment Works

RCRA – Resource Conservation and Recovery Act

RQ – Reportable Quantity

SARA – Superfund Amendments and Reauthorization Act

SHPO – State Historic Preservation Officer

SIC – Standard Industrial Classification

SMCRA – Surface Mining Control and Reclamation Act

SPCC – Spill Prevention, Control, and Countermeasures

SWPPP – Stormwater Pollution Prevention Plan

THPO – Tribal Historic Preservation Officer

TMDL – Total Maximum Daily Load

TSDF – Treatment, Storage, or Disposal Facility

TSS – Total Suspended Solids

USGS – United States Geological Survey

WLA – Wasteload Allocation

WQS – Water Quality Standard

Appendix B
Standard Permit Conditions

Appendix B. Standard Permit Conditions.

Standard permit conditions in Appendix B are consistent with the general permit provisions required under 40 CFR 122.41.

B.1 Duty To Comply.

You must comply with all conditions of this permit. Any permit noncompliance constitutes a violation of the Clean Water Act and is grounds for enforcement action; for permit termination, revocation and reissuance, or modification; or for denial of a permit renewal application.

A. You must comply with effluent standards or prohibitions established under section 307(a) of the Clean Water Act for toxic pollutants within the time provided in the regulations that establish these standards, even if the permit has not yet been modified to incorporate the requirement.

B. Penalties for Violations of Permit Conditions: The Director will adjust the civil and administrative penalties listed below in accordance with the Civil Monetary Penalty Inflation Adjustment Rule (61 FR 252, December 31, 1996, pp. 69359-69366, as corrected in 62 FR 54, March 20, 1997, pp.13514-13517) as mandated by the Debt Collection Improvement Act of 1996 for inflation on a periodic basis. This rule allows EPA's penalties to keep pace with inflation. The Agency is required to review its penalties at least once every 4 years thereafter and to adjust them as necessary for inflation according to a specified formula. The civil and administrative penalties following were adjusted for inflation starting in 1996.

1. Criminal Penalties.

1.1 Negligent Violations. The CWA provides that any person who negligently violates permit conditions implementing Sections 301, 302, 306, 307, 308, 318, or 405 of the Act is subject to criminal penalties of not less than $2,500 nor more than $25,000 per day of violation, or imprisonment of not more than one year, or both. In the case of a second or subsequent conviction for a negligent violation, a person shall be subject to criminal penalties of not more than $50,000 per day of violation or by imprisonment of not more than two years, or both.

1.2. *Knowing Violations.* The CWA provides that any person who knowingly violates permit conditions implementing Sections 301, 302, 306, 307, 308, 318, or 405 of the Act is subject to a fine of not less than $5,000 nor more than $50,000 per day of violation, or by imprisonment for not more than 3 years, or both. In the case of a second or subsequent conviction for a knowing violation, a person shall be subject to criminal penalties of not more than $100,000 per day of violation, or imprisonment of not more than 6 years, or both.

1.3. *Knowing Endangerment.* The CWA provides that any person who knowingly violates permit conditions implementing Sections 301, 302, 306, 307, 308, 318, or 405 of the Act and who knows at that time that he or she is placing another person in imminent danger of death or serious bodily injury shall upon conviction be subject to a fine of not more than $250,000 or by imprisonment of not more than 15 years, or both. In the case of a second or subsequent conviction for a knowing endangerment violation, a person shall be subject to a fine of not more than $500,000 or by imprisonment of not more than 30 years, or both. An organization, as defined in section 309(c)(3)(B)(iii) of the Act, shall, upon conviction of violating the imminent danger provision be subject to a fine of not more than $1,000,000 and can fined up to $2,000,000 for second or subsequent convictions.

1.4. *False Statement.* The CWA provides that any person who falsifies, tampers with, or knowingly renders inaccurate any monitoring device or method required to be maintained under this permit shall, upon conviction, be punished by a fine of not more than $10,000, or by imprisonment for not more than 2 years, or both. If a conviction of a person is for a violation committed after a first conviction of such person under this paragraph, punishment is a fine of not more than $20,000 per day of violation, or by imprisonment of not more than 4 years, or both. The Act further provides that any person who knowingly makes any false statement, representation, or certification in any record or other document submitted or required to be maintained under this permit, including monitoring reports or reports of compliance or non-compliance shall, upon conviction, be punished by a fine of not more than $10,000 per violation, or by imprisonment for not more than 6 months per violation, or by both.

2. *Civil Penalties.* The CWA provides that any person who violates a permit condition implementing Sections 301, 302, 306, 307, 308, 318, or 405 of the Act is subject to a civil penalty not to exceed the maximum amounts authorized by Section 309(d) of the Act and the Federal Civil Penalties Inflation Adjustment Act (28 U.S.C. § 2461 note) as amended by the Debt Collection Improvement Act (31 U.S.C. § 3701 note) (currently $32,500 per day for each violation).

3. *Administrative Penalties.* The CWA provides that any person who violates a permit condition implementing Sections 301, 302, 306, 307, 308, 318, or 405 of the Act is subject to an administrative penalty, as follows

3.1. *Class I Penalty*. Not to exceed the maximum amounts authorized by Section 309(g)(2)(A) of the Act and the Federal Civil Penalties Inflation Adjustment Act (28 U.S.C. § 2461 note) as amended by the Debt Collection Improvement Act (31 U.S.C. § 3701 note) (currently $11,000 per violation, with the maximum amount of any Class I penalty assessed not to exceed $32,500).

3.2. *Class II Penalty.* Not to exceed the maximum amounts authorized by Section 309(g)(2)(B) of the Act and the Federal Civil Penalties Inflation Adjustment Act (28 U.S.C. § 2461 note) as amended by the Debt Collection Improvement Act (31 U.S.C. § 3701 note) (currently $11,000 per day for each day during which the violation continues, with the maximum amount of any Class II penalty not to exceed $157,500).

B.2 Duty to Reapply.

If you wish to continue an activity regulated by this permit after the expiration date of this permit, you must apply for and obtain authorization as required by the new permit once EPA issues it.

B.3 Need to Halt or Reduce Activity Not a Defense.

It shall not be a defense for you in an enforcement action that it would have been necessary to halt or reduce the permitted activity in order to maintain compliance with the conditions of this permit.

B.4 Duty to Mitigate.

You must take all reasonable steps to minimize or prevent any discharge in violation of this permit which has a reasonable likelihood of adversely affecting human health or the environment.

B.5 Proper Operation and Maintenance.

You must at all times properly operate and maintain all facilities and systems of treatment and control (and related appurtenances) which are installed or used by you to achieve compliance with the conditions of this permit. Proper operation and maintenance also includes adequate laboratory controls and appropriate quality assurance procedures. This provision requires the operation of backup or auxiliary facilities or similar systems which are installed by you only when the operation is necessary to achieve compliance with the conditions of this permit.

B.6 Permit Actions.

This permit may be modified, revoked and reissued, or terminated for cause. Your filing of a request for a permit modification, revocation and reissuance, or termination, or a notification of planned changes or anticipated noncompliance does not stay any permit condition.

B.7 Property Rights.

This permit does not convey any property rights of any sort, or any exclusive privileges.

B.8 Duty to Provide Information.

You must furnish to EPA or an authorized representative (including an authorized contractor acting as a representative of EPA), within a reasonable time, any information which EPA may request to determine whether cause exists for modifying, revoking and reissuing, or terminating this permit or to determine compliance with this permit. You must also furnish to EPA or an authorized representative upon request, copies of records required to be kept by this permit.

B.9 Inspection and Entry.

You must allow EPA or an authorized representative (including an authorized contractor acting as a representative of EPA), upon presentation of credentials and other documents as may be required by law, to:

A. Enter upon your premises where a regulated facility or activity is located or conducted, or where records must be kept under the conditions of this permit;

B. Have access to and copy, at reasonable times, any records that must be kept under the conditions of this permit;

C. Inspect at reasonable times any facilities, equipment (including monitoring and control equipment), practices, or operations regulated or required under this permit; and

D. Sample or monitor at reasonable times, for the purposes of assuring permit compliance or as otherwise authorized by the Clean Water Act, any substances or parameters at any location.

B.10 Monitoring and Records.

A. Samples and measurements taken for the purpose of monitoring must be representative of the volume and nature of the monitored activity.

B. You must retain records of all monitoring information, including all calibration and maintenance records and all original strip chart recordings for continuous monitoring instrumentation, copies of all reports required by this permit, and records of all data used to complete the application for this permit, for a period of at least three years from the date the permit expires or the date the permittee's authorization is terminated. This period may be extended by request of EPA at any time.

C. Records of monitoring information must include:

1. The date, exact place, and time of sampling or measurements;

2. The individual(s) who performed the sampling or measurements;

3. The date(s) analyses were performed

4. The individual(s) who performed the analyses;

5. The analytical techniques or methods used; and

6. The results of such analyses.

D. Monitoring must be conducted according to test procedures approved under 40 CFR Part 136, unless other test procedures have been specified in the permit.

E. The Clean Water Act provides that any person who falsifies, tampers with, or knowingly renders inaccurate any monitoring device or method required to be maintained under this permit shall, upon conviction, be punished by a fine of not more than $10,000, or by imprisonment for not more than 2 years, or both. If a conviction of a person is for a violation committed after a first conviction of such person under this paragraph, punishment is a fine of not more than $20,000 per day of violation, or by imprisonment of not more than 4 years, or both.

B.11 Signatory Requirements.

A. All applications, including NOIs, must be signed as follows:

1. For a corporation: By a responsible corporate officer. For the purpose of this subsection, a responsible corporate officer means: (i) a president, secretary, treasurer, or vice-president of the corporation in charge of a principal business function, or any other person who performs similar policy- or decision-making functions for the corporation, or (ii) the manager of one or more manufacturing, production, or operating facilities, provided, the manager is authorized to make management decisions which govern the operation of the regulated facility including having the explicit or implicit duty of making major capital investment recommendations, and initiating and directing other comprehensive measures to assure long term environmental compliance with environmental laws and regulations; the manager can ensure that the necessary systems are established or actions taken to gather complete and accurate information for permit application requirements; and where authority to sign documents has been assigned or delegated to the manager in accordance with corporate procedures.

2. For a partnership or sole proprietorship: By a general partner or the proprietor, respectively; or

3. For a municipality, state, federal, or other public agency: By either a principal executive officer or ranking elected official. For purposes of this subsection, a principal executive officer of a federal agency includes (i) the chief executive officer of the agency, or (ii) a senior executive officer having responsibility for

the overall operations of a principal geographic unit of the agency (e.g., Regional Administrator of EPA).

B. Your SWPPP, including changes to your SWPPP to document any corrective actions taken as required by Part 3.1, and all reports submitted to EPA, must be signed by a person described in Appendix B, Subsection 11.A above or by a duly authorized representative of that person. A person is a duly authorized representative only if:

1. The authorization is made in writing by a person described in Appendix B, Subsection 11.A;

2. The authorization specifies either an individual or a position having responsibility for the overall operation of the regulated facility or activity such as the position of plant manager, operator of a well or a well field, superintendent, position of equivalent responsibility, or an individual or position having overall responsibility for environmental matters for the company. (A duly authorized representative may thus be either a named individual or any individual occupying a named position); and

3. The signed and dated written authorization is included in the SWPPP. A copy must be submitted to EPA, if requested.

C. All other changes to your SWPPP, and other compliance documentation required under Part 5.4, must be signed and dated by the person preparing the change or documentation.

D. Changes to Authorization. If an authorization under Appendix B, Subsection 11.B is no longer accurate because the industrial facility has been purchased by a different entity, a new NOI satisfying the requirements of Subsection 11.B must be submitted to EPA. See Table 1-2 in Part 1.3.1 of the permit. However, if the only change that is occurring is a change in contact information or a change in the facility's address, the operator need only make a modification to the existing NOI submitted for authorization.

E. Any person signing documents in accordance with Appendix B, Subsections 11.A or 11.B above must include the following certification:

> "I certify under penalty of law that this document and all attachments were prepared under my direction or supervision in accordance with a system designed to assure that qualified personnel properly gathered and evaluated the information contained therein. Based on my inquiry of the person or persons who manage the system, or those persons directly responsible for gathering the information, the information contained is, to the best of my knowledge and belief, true, accurate, and complete. I am aware that there are significant penalties for submitting false information, including the possibility of fine and imprisonment for knowing violations."

F. The CWA provides that any person who knowingly makes any false statement, representation, or certification in any record or other document submitted or required to be maintained under this permit, including monitoring reports or reports of compliance or non-compliance shall, upon conviction, be punished by a fine of not more than $10,000 per violation, or by imprisonment for not more than 6 months per violation, or by both.

B.12 Reporting Requirements.

A. Planned changes. You must give notice to EPA as soon as possible of any planned physical alterations or additions to the permitted facility. Notice is required only when:

1. The alteration or addition to a permitted facility may meet one of the criteria for determining whether a facility is a new source in 40 CFR 122.29(b); or

2. The alteration or addition could significantly change the nature or increase the quantity of pollutants discharged. This notification applies to pollutants which are subject neither to effluent limitations in the permit, nor to notification requirements under 40 CFR 122.42(a)(1).

B. Anticipated noncompliance. You must give advance notice to EPA of any planned changes in the permitted facility or activity which may result in noncompliance with permit requirements.

C. Transfers. This permit is not transferable to any person except after notice to EPA. Where a facility wants to change the name of the permittee, the original permittee (the first owner or operators) must submit a Notice of Termination pursuant to Part 1.4. The new owner or operator must submit a Notice of Intent in accordance with Part 1.3.1 and Table 1-2. See also requirements in Appendix B, Subsections 11.B and 11.D.

D. Monitoring reports. Monitoring results must be reported at the intervals specified elsewhere in this permit.

1. Pursuant to Part 7.1, all monitoring data collected pursuant to Part 6.2 and 6.3 must be submitted to EPA using EPA's online eNOI system (www.epa.gov/npdes/eNOI). Alternatively, if you cannot access eNOI, monitoring results should be reported on the MSGP Discharge Monitoring Report (MDMR) form, available at www.epa.gov/npdes/stormwater/msgp, and submitted to EPA.

2. If you monitor any pollutant more frequently than required by the permit using test procedures approved under 40 CFR Part 136 or as specified in the permit, the results of this monitoring must be included in the calculation and reporting of the data submitted in the MDMR.

3. Calculations for all limitations which require averaging of measurements must use an arithmetic mean. For averaging purposes, use a value of zero for any

individual sample parameter, which is determined to be less than the method detection limit. For sample values that fall between the method detection level and the quantitation limit (i.e., a confirmed detection but below the level that can be reliably quantified), use a value halfway between zero and the quantitation limit.

E. Compliance schedules. Reports of compliance or noncompliance with, or any progress reports on, interim and final requirements contained in any compliance schedule of this permit must be submitted no later than 14 days following each schedule date.

F. Twenty-four hour reporting.

1. You must report any noncompliance which may endanger health or the environment. Any information must be provided orally within 24 hours from the time you become aware of the circumstances. A written submission must also be provided within five days of the time you become aware of the circumstances. The written submission must contain a description of the noncompliance and its cause; the period of noncompliance, including exact dates and times, and if the noncompliance has not been corrected, the anticipated time it is expected to continue; and steps taken or planned to reduce, eliminate, and prevent reoccurrence of the noncompliance.

2. The following shall be included as information which must be reported within 24 hours under this paragraph.

a. Any unanticipated bypass which exceeds any effluent limitation in the permit. (See 40 CFR 122.41(m)(3)(ii))

b. Any upset which exceeds any effluent limitation in the permit

c. Violation of a maximum daily discharge limit for any numeric effluent limitation. (See 40 CFR 122.44(g).)

3. EPA may waive the written report on a case-by-case basis for reports under Appendix B, Subsection 12.F.2 if the oral report has been received within 24 hours.

G. Other noncompliance. You must report all instances of noncompliance not reported under Appendix B, Subsections 12.D, 12.E, and 12.F, at the time monitoring reports are submitted. The reports must contain the information listed in Appendix B, Subsection 12.F.

H. Other information. Where you become aware that you failed to submit any relevant facts in a permit application, or submitted incorrect information in a permit application or in any report to the Permitting Authority, you must promptly submit such facts or information.

B.13 Bypass.

A. Definitions.

1. Bypass means the intentional diversion of waste streams from any portion of a treatment facility See 40 CFR 122.41(m)(1)(i).

2. Severe property damage means substantial physical damage to property, damage to the treatment facilities which causes them to become inoperable, or substantial and permanent loss of natural resources which can reasonably be expected to occur in the absence of a bypass. Severe property damage does not mean economic loss caused by delays in production. See 40 CFR 122.41(m)(1)(ii).

B. Bypass not exceeding limitations. You may allow any bypass to occur which does not cause effluent limitations to be exceeded, but only if it also is for essential maintenance to assure efficient operation. These bypasses are not subject to the provisions of Appendix B, Subsections 13.C and 13.D. See 40 CFR 122.41(m)(2).

C. Notice.

1. Anticipated bypass. If you know in advance of the need for a bypass, you must submit prior notice, if possible at least ten days before the date of the bypass. See 40 CFR 122.41(m)(3)(i).

2. Unanticipated bypass. You must submit notice of an unanticipated bypass as required in Appendix B, Subsection 12.F (24-hour notice). See 40 CFR 122.41(m)(3)(ii).

D. Prohibition of bypass. See 40 CFR 122.41(m)(4).

1. Bypass is prohibited, and EPA may take enforcement action against you for bypass, unless:

a. Bypass was unavoidable to prevent loss of life, personal injury, or severe property damage;

b. There were no feasible alternatives to the bypass, such as the use of auxiliary treatment facilities, retention of untreated wastes, or maintenance during normal periods of equipment downtime. This condition is not satisfied if adequate back-up equipment should have been installed in the exercise of reasonable engineering judgment to prevent a bypass which occurred during normal periods of equipment downtime or preventive maintenance; and

c. You submitted notices as required under Appendix B, Subsection 13.C.

2. EPA may approve an anticipated bypass, after considering its adverse effects, if EPA determines that it will meet the three conditions listed above in Appendix B, Subsection 13.D.1.

B.14 Upset.

A. Definition. Upset means an exceptional incident in which there is unintentional and temporary noncompliance with technology based permit effluent limitations because of factors beyond your reasonable control. An upset does not include noncompliance to the extent caused by operational error, improperly designed treatment facilities, inadequate treatment facilities, lack of preventive maintenance, or careless or improper operation. See 40 CFR 122.41(n)(1).

B. Effect of an upset. An upset constitutes an affirmative defense to an action brought for noncompliance with such technology based permit effluent limitations if the requirements of Appendix B, Subsection 14.C are met. No determination made during administrative review of claims that noncompliance was caused by upset, and before an action for noncompliance, is final administrative action subject to judicial review. See 40 CFR 122.41(n)(2).

C. Conditions necessary for a demonstration of upset. See 40 CFR 122.41(n)(3). A permittee who wishes to establish the affirmative defense of upset must demonstrate, through properly signed, contemporaneous operating logs, or other relevant evidence that:

1. An upset occurred and that you can identify the cause(s) of the upset;

2. The permitted facility was at the time being properly operated; and

3. You submitted notice of the upset as required in Appendix B, Subsection 12.F.2.b (24 hour notice).

4. You complied with any remedial measures required under Appendix B, Subsection 4.

D. Burden of proof. In any enforcement proceeding, you, as the one seeking to establish the occurrence of an upset, have the burden of proof. See 40 CFR 122.41(n)(4).

Appendix C
Areas Covered

Appendix C. Permit Area.

EPA can only provide permit coverage in these areas and for classes of discharges that are outside the scope of a State's NPDES program authorization.

C.1 EPA Region 1: Connecticut, Massachusetts, Maine, New Hampshire, Rhode Island, Vermont.

This permit offers NPDES permit coverage for stormwater discharges associated with industrial activity from the following areas in EPA Region 1:

Permit Number	Areas of Coverage/Where EPA Is Permitting Authority
CTR05000I	Indian Country within the State of Connecticut
MAR050000	Commonwealth of Massachusetts, except Indian Country
MAR05000I	Indian Country within the Commonwealth of Massachusetts
NHR050000	State of New Hampshire
RIR05000I	Indian Country within the State of Rhode Island
VTR05000F	Federal facilities in the State of Vermont

For stormwater discharges in EPA Region 1 outside the areas of coverage identified above, please contact your State NPDES permitting authority to obtain coverage under a State-issued NPDES permit.

C.2 EPA Region 2: New Jersey, New York, Puerto Rico, Virgin Islands.

This permit offers NPDES permit coverage for stormwater discharges associated with industrial activity from the following areas in EPA Region 2:

Permit Number	Areas of Coverage/Where EPA Is Permitting Authority
PRR050000	Commonwealth of Puerto Rico

For stormwater discharges in EPA Region 2 outside the areas of coverage identified above, please contact your State NPDES permitting authority to obtain coverage under a State-issued NPDES permit.

C.3 EPA Region 3: Delaware, District of Columbia, Maryland, Pennsylvania, Virginia, West Virginia.

This permit offers NPDES permit coverage for stormwater discharges associated with industrial activity from the following areas in EPA Region 3:

Permit Number	Areas of Coverage/Where EPA Is Permitting Authority
DCR050000	District of Columbia
DER05000F	Federal facilities in the State of Delaware

For stormwater discharges in EPA Region 3 outside the areas of coverage identified above, please contact your State NPDES permitting authority to obtain coverage under a State-issued NPDES permit.

C.4 EPA Region 4: Alabama, Florida, Georgia, Kentucky, Mississippi, North Carolina, South Carolina, Tennessee (Coverage not available under this permit).

For stormwater discharges in EPA Region 4, please contact your State NPDES permitting authority to obtain coverage under a State-issued NPDES permit.

C.5 EPA Region 5: Illinois, Indiana, Michigan, Minnesota, Ohio, Wisconsin.

This permit offers NPDES permit coverage for stormwater discharges associated with industrial activity from the following areas in EPA Region 5:

Permit Number	Areas of Coverage/Where EPA Is Permitting Authority
MIR05000I	Indian Country within the State of Michigan
MNR05000I	Indian Country within the State of Minnesota
WIR05000I	Indian Country within the State of Wisconsin, except those on Sokaogon Chippewa Community lands

For stormwater discharges in EPA Region 5 outside the areas of coverage identified above, please contact your State NPDES permitting authority to obtain coverage under a State-issued NPDES permit.

C.6 EPA Region 6: Arkansas, Louisiana, Oklahoma, Texas, and New Mexico (except see Region 9 for Navajo lands, and see Region 8 for Ute Mountain Reservation lands).

This permit offers NPDES permit coverage for stormwater discharges associated with industrial activity from the following areas in EPA Region 6:

Permit Number	Areas of Coverage/Where EPA Is Permitting Authority
LAR05000I	Indian Country within the State of Louisiana
NMR050000	The State of New Mexico, except Indian Country
NMR05000I	Indian Country within the State of New Mexico, except Ute Mountain Reservation lands that are covered under Colorado permit COR05000I listed in Part C.8 and Navajo Reservation lands that are covered under Arizona permit AZR05000I listed in Part C.9.
OKR05000I	Indian Country within the State of Oklahoma
OKR05000F	Facilities in the State of Oklahoma not under the jurisdiction of the Oklahoma Department of Environmental Quality, except those on Indian Country. EPA jurisdiction facilities include SIC Codes 1311, 1381, 1382, 1389, and 5171 and point source (but not nonpoint source) discharges associated with agricultural production, services, and silviculture.

Permit Number	Areas of Coverage/Where EPA Is Permitting Authority
TXR05000F	Facilities in the State of Texas not under the jurisdiction of the Texas Commission on Environmental Quality, except those on Indian Country. EPA-jurisdiction facilities include SIC Codes 1311, 1321, 1381, 1382, and 1389 (other than oil field service company "home base" facilities).
TXR05000I	Indian Country within the State of Texas

For stormwater discharges in EPA Region 6 outside the areas of coverage identified above, please contact your State NPDES permitting authority to obtain coverage under a State-issued NPDES permit.

C.7 EPA Region 7: Iowa, Kansas, Missouri, Nebraska (Coverage not available under this permit).

For stormwater discharges in EPA Region 7, please contact EPA Region 7 or your State NPDES permitting authority to obtain coverage under a State-issued NPDES permit.

C.8 EPA Region 8: Colorado, Montana, North Dakota, South Dakota, Wyoming, Utah (Coverage not available under this permit).

For stormwater discharges in EPA Region 8 please contact EPA Region 8 or your State NPDES permitting authority to obtain coverage under an NPDES permit.

C.9 EPA Region 9: California, Hawaii, Nevada, Guam, American Samoa, the Commonwealth of the Northern Mariana Islands, the Confederated Tribes of the Goshute Reservation in Utah and Nevada, Indian Country within the State of Arizona including the Navajo Reservation in Utah and New Mexico and Arizona, the Duck Valley Reservation in Idaho, and the Fort McDermitt Reservation in Oregon.

This permit offers NPDES permit coverage for stormwater discharges associated with industrial activity from the following areas in EPA Region 9:

Permit Number	Areas of Coverage/Where EPA Is Permitting Authority
ASR050000	The islands of American Samoa
AZR05000I	Indian Country within the State of Arizona, including Navajo Reservation lands in New Mexico and Utah
CAR05000I	Indian Country within the State of California
GUR050000	The island of Guam
JAR050000	Johnston Atoll
MWR050000	Midway Island and Wake Island
NIR050000	Commonwealth of the Northern Mariana Islands
NVR05000I	Indian Country within the State of Nevada, including the Duck Valley Reservation in Idaho, the Fort McDermitt Reservation in Oregon and the Confederated Tribes of the Goshute Reservation in Utah

For stormwater discharges in EPA Region 9 outside the areas of coverage identified above, please contact your State NPDES permitting authority to obtain coverage under a State-issued NPDES permit.

C.10 Region 10: Alaska, Idaho (except see Region 9 for Duck Valley Reservation lands), Oregon (except see Region 9 for Fort McDermitt Reservation), Washington.

This permit offers NPDES permit coverage for stormwater discharges associated with industrial activity from the following areas in EPA Region 10:

Permit Number	Areas of Coverage/Where EPA Is Permitting Authority
AKR050000	The State of Alaska, except Indian Country lands
AKR05000I	Indian Country lands within Alaska
IDR050000	The State of Idaho, except Indian Country lands
IDR05000I	Indian Country lands within the State of Idaho, except Duck Valley Reservation lands, which are covered under Nevada permit NVR05000I listed in Part C.9
ORR05000I	Indian Country lands within the State of Oregon, except Fort McDermitt Reservation lands, which are covered under Nevada permit NVR05000I listed in Part C.9
WAR05000I	Indian Country lands within the State of Washington
WAR05000F	Federal facilities in the State of Washington, except those located on Indian Country lands

For stormwater discharges in EPA Region 10 outside the areas of coverage identified above, please contact your State NPDES permitting authority to obtain coverage under a State-issued NPDES permit.

Appendix D
Activities Covered

Appendix D. Facilities and Activities Covered

Your permit eligibility is limited to discharges from facilities in the "sectors" of industrial activity summarized in Table D-1. These sector descriptions are based on Standard Industrial Classification (SIC) Codes and Industrial Activity Codes. References to "sectors" in this permit (e.g., sector-specific monitoring requirements) refer to these groupings.

Table D-1. Sectors of Industrial Activity Covered by This Permit

Subsector (May be subject to more than one sector/subsector)	SIC Code or Activity Code[1]	Activity Represented
SECTOR A: TIMBER PRODUCTS		
A1	2421	General Sawmills and Planing Mills
A2	2491	Wood Preserving
A3	2411	Log Storage and Handling
A4	2426	Hardwood Dimension and Flooring Mills
	2429	Special Product Sawmills, Not Elsewhere Classified
	2431-2439 (except 2434)	Millwork, Veneer, Plywood, and Structural Wood (see Sector W)
	2448	Wood Pallets and Skids
	2449	Wood Containers, Not Elsewhere Classified
	2451, 2452	Wood Buildings and Mobile Homes
	2493	Reconstituted Wood Products
	2499	Wood Products, Not Elsewhere Classified
A5	2441	Nailed and Lock Corner Wood Boxes and Shook
SECTOR B: PAPER AND ALLIED PRODUCTS		
B1	2631	Paperboard Mills
B2	2611	Pulp Mills
	2621	Paper Mills
	2652-2657	Paperboard Containers and Boxes
	2671-2679	Converted Paper and Paperboard Products, Except Containers and Boxes
SECTOR C: CHEMICALS AND ALLIED PRODUCTS		
C1	2873-2879	Agricultural Chemicals
C2	2812-2819	Industrial Inorganic Chemicals
C3	2841-2844	Soaps, Detergents, and Cleaning Preparations; Perfumes, Cosmetics, and Other Toilet Preparations
C4	2821-2824	Plastics Materials and Synthetic Resins, Synthetic Rubber, Cellulosic and Other Manmade Fibers Except Glass
C5	2833-2836	Medicinal Chemicals and Botanical Products; Pharmaceutical Preparations; in vitro and in vivo Diagnostic Substances; and Biological Products, Except Diagnostic Substances
	2851	Paints, Varnishes, Lacquers, Enamels, and Allied Products

Table D-1. Sectors of Industrial Activity Covered by This Permit		
Subsector (May be subject to more than one sector/subsector)	**SIC Code or Activity Code[1]**	**Activity Represented**
	2861-2869	Industrial Organic Chemicals
	2891-2899	Miscellaneous Chemical Products
	3952 (limited to list of inks and paints)	Inks and Paints, Including China Painting Enamels, India Ink, Drawing Ink, Platinum Paints for Burnt Wood or Leather Work, Paints for China Painting, Artist's Paints and Artist's Watercolors
	2911	Petroleum Refining
SECTOR D: ASPHALT PAVING AND ROOFING MATERIALS AND LUBRICANTS		
D1	2951, 2952	Asphalt Paving and Roofing Materials
D2	2992, 2999	Miscellaneous Products of Petroleum and Coal
SECTOR E: GLASS, CLAY, CEMENT, CONCRETE, AND GYPSUM PRODUCTS		
E1	3251-3259	Structural Clay Products
	3261-3269	Pottery and Related Products
E2	3271-3275	Concrete, Gypsum, and Plaster Products
E3	3211	Flat Glass
	3221, 3229	Glass and Glassware, Pressed or Blown
	3231	Glass Products Made of Purchased Glass
	3241	Hydraulic Cement
	3281	Cut Stone and Stone Products
	3291-3299	Abrasive, Asbestos, and Miscellaneous Nonmetallic Mineral Products
SECTOR F: PRIMARY METALS		
F1	3312-3317	Steel Works, Blast Furnaces, and Rolling and Finishing Mills
F2	3321-3325	Iron and Steel Foundries
F3	3351-3357	Rolling, Drawing, and Extruding of Nonferrous Metals
F4	3363-3369	Nonferrous Foundries (Castings)
F5	3331-3339	Primary Smelting and Refining of Nonferrous Metals
	3341	Secondary Smelting and Refining of Nonferrous Metals
	3398, 3399	Miscellaneous Primary Metal Products
SECTOR G: METAL MINING (ORE MINING AND DRESSING)		
G1	1021	Copper Ore and Mining Dressing Facilities
G2	1011	Iron Ores
	1021	Copper Ores
	1031	Lead and Zinc Ores
	1041, 1044	Gold and Silver Ores
	1061	Ferroalloy Ores, Except Vanadium
	1081	Metal Mining Services
	1094, 1099	Miscellaneous Metal Ores

Table D-1. Sectors of Industrial Activity Covered by This Permit		
Subsector (May be subject to more than one sector/subsector)	**SIC Code or Activity Code[1]**	**Activity Represented**
SECTOR H: COAL MINES AND COAL MINING-RELATED FACILITIES		
H1	1221-1241	Coal Mines and Coal Mining-Related Facilities
SECTOR I: OIL AND GAS EXTRACTION AND REFINING		
I1	1311	Crude Petroleum and Natural Gas
	1321	Natural Gas Liquids
	1381-1389	Oil and Gas Field Services
SECTOR J: MINERAL MINING AND DRESSING		
J1	1442	Construction Sand and Gravel
	1446	Industrial Sand
J2	1411	Dimension Stone
	1422-1429	Crushed and Broken Stone, Including Rip Rap
	1481	Nonmetallic Minerals Services, Except Fuels
	1499	Miscellaneous Nonmetallic Minerals, Except Fuels
J3	1455, 1459	Clay, Ceramic, and Refractory Materials
	1474-1479	Chemical and Fertilizer Mineral Mining
SECTOR K: HAZARDOUS WASTE TREATMENT, STORAGE, OR DISPOSAL FACILITIES		
K1	HZ	Hazardous Waste Treatment, Storage, or Disposal Facilities, including those that are operating under interim status or a permit under subtitle C of RCRA
SECTOR L: LANDFILLS, LAND APPLICATION SITES, AND OPEN DUMPS		
L1	LF	All Landfill, Land Application Sites and Open Dumps
L2	LF	All Landfill, Land Application Sites and Open Dumps, except Municipal Solid Waste Landfill (MSWLF) Areas Closed in Accordance with 40 CFR 258.60
SECTOR M: AUTOMOBILE SALVAGE YARDS		
M1	5015	Automobile Salvage Yards
SECTOR N: SCRAP RECYCLING FACILITIES		
N1	5093	Scrap Recycling and Waste Recycling Facilities except Source-Separated Recycling
N2	5093	Source-separated Recycling Facility
SECTOR O: STEAM ELECTRIC GENERATING FACILITIES		
O1	SE	Steam Electric Generating Facilities, including coal handling sites
SECTOR P: LAND TRANSPORTATION AND WAREHOUSING		
P1	4011, 4013	Railroad Transportation
	4111-4173	Local and Highway Passenger Transportation
	4212-4231	Motor Freight Transportation and Warehousing
	4311	United States Postal Service

Table D-1. Sectors of Industrial Activity Covered by This Permit		
Subsector (May be subject to more than one sector/subsector)	**SIC Code or Activity Code[1]**	**Activity Represented**
	5171	Petroleum Bulk Stations and Terminals
SECTOR Q: WATER TRANSPORTATION		
Q1	4412-4499	Water Transportation Facilities
SECTOR R: SHIP AND BOAT BUILDING AND REPAIRING YARDS		
R1	3731, 3732	Ship and Boat Building or Repairing Yards
SECTOR S: AIR TRANSPORTATION FACILITIES		
S1	4512-4581	Air Transportation Facilities
SECTOR T: TREATMENT WORKS		
T1	TW	Treatment Works treating domestic sewage or any other sewage sludge or wastewater treatment device or system, used in the storage, treatment, recycling, and reclamation of municipal or domestic sewage, including land dedicated to the disposal of sewage sludge that are located within the confines of the facility, with a design flow of 1.0 mgd or more, or required to have an approved pretreatment program under 40 CFR Part 403. Not included are farm lands, domestic gardens or lands used for sludge management where sludge is beneficially reused and which are not physically located in the confines of the facility, or areas that are in compliance with section 405 of the CWA
SECTOR U: FOOD AND KINDRED PRODUCTS		
U1	2041-2048	Grain Mill Products
U2	2074-2079	Fats and Oils Products
U3	2011-2015	Meat Products
	2021-2026	Dairy Products
	2032-2038	Canned, Frozen, and Preserved Fruits, Vegetables, and Food Specialties
	2051-2053	Bakery Products
	2061-2068	Sugar and Confectionery Products
	2082-2087	Beverages
	2091-2099	Miscellaneous Food Preparations and Kindred Products
	2111-2141	Tobacco Products
SECTOR V: TEXTILE MILLS, APPAREL, AND OTHER FABRIC PRODUCT MANUFACTURING; LEATHER AND LEATHER PRODUCTS		
V1	2211-2299	Textile Mill Products
	2311-2399	Apparel and Other Finished Products Made from Fabrics and Similar Materials
	3131-3199	Leather and Leather Products (note: see Sector Z1 for Leather Tanning and Finishing)
SECTOR W: FURNITURE AND FIXTURES		
W1	2434	Wood Kitchen Cabinets
	2511-2599	Furniture and Fixtures

Table D-1. Sectors of Industrial Activity Covered by This Permit		
Subsector (May be subject to more than one sector/subsector)	**SIC Code or Activity Code[1]**	**Activity Represented**
SECTOR X: PRINTING AND PUBLISHING		
X1	2711-2796	Printing, Publishing, and Allied Industries
SECTOR Y: RUBBER, MISCELLANEOUS PLASTIC PRODUCTS, AND MISCELLANEOUS MANUFACTURING INDUSTRIES		
Y1	3011	Tires and Inner Tubes
	3021	Rubber and Plastics Footwear
	3052, 3053	Gaskets, Packing and Sealing Devices, and Rubber and Plastic Hoses and Belting
	3061, 3069	Fabricated Rubber Products, Not Elsewhere Classified
Y2	3081-3089	Miscellaneous Plastics Products
	3931	Musical Instruments
	3942-3949	Dolls, Toys, Games, and Sporting and Athletic Goods
	3951-3955 (except 3952 – see Sector C)	Pens, Pencils, and Other Artists' Materials
	3961, 3965	Costume Jewelry, Costume Novelties, Buttons, and Miscellaneous Notions, Except Precious Metal
	3991-3999	Miscellaneous Manufacturing Industries
SECTOR Z: LEATHER TANNING AND FINISHING		
Z1	3111	Leather Tanning and Finishing
SECTOR AA: FABRICATED METAL PRODUCTS		
AA1	3411-3499 (except 3479)	Fabricated Metal Products, Except Machinery and Transportation Equipment, and Coating, Engraving, and Allied Services.
	3911-3915	Jewelry, Silverware, and Plated Ware
AA2	3479	Fabricated Metal Coating and Engraving
SECTOR AB: TRANSPORTATION EQUIPMENT, INDUSTRIAL OR COMMERCIAL MACHINERY		
AB1	3511-3599 (except 3571-3579)	Industrial and Commercial Machinery, Except Computer and Office Equipment (see Sector AC)
	3711-3799 (except 3731, 3732)	Transportation Equipment Except Ship and Boat Building and Repairing (see Sector R)
SECTOR AC: ELECTRONIC, ELECTRICAL, PHOTOGRAPHIC, AND OPTICAL GOODS		
AC1	3571-3579	Computer and Office Equipment
	3812-3873	Measuring, Analyzing, and Controlling Instruments; Photographic and Optical Goods, Watches, and Clocks
	3612-3699	Electronic and Electrical Equipment and Components, Except Computer Equipment

Table D-1. Sectors of Industrial Activity Covered by This Permit		
Subsector (May be subject to more than one sector/subsector)	**SIC Code or Activity Code[1]**	**Activity Represented**
SECTOR AD: NON-CLASSIFIED FACILITIES		
AD1		Other stormwater discharges designated by the Director as needing a permit (see 40 CFR 122.26(a)(9)(i)(C) & (D)) or any facility discharging stormwater associated with industrial activity not described by any of Sectors A-AC. NOTE: Facilities may not elect to be covered under Sector AD. Only the Director may assign a facility to Sector AD.

[1] A complete list of SIC Codes (and conversions from the newer North American Industry Classification System" (NAICS)) can be obtained from the Internet at www.census.gov/epcd/www/naics.html or in paper form from various locations in the document titled *Handbook of Standard Industrial Classifications*, Office of Management and Budget, 1987.

Appendix E
Procedures Relating to Endangered Species Protection

Appendix E. Procedures Relating to Endangered Species Protection

E.1 Assessing the Effects of Your Discharge and Discharge-Related Activities

You must follow the procedures in this appendix to assess the potential effects of applicable stormwater discharges, discharge-related activities, and allowable non-stormwater discharges on listed species and their critical habitat and determine which of the eligibility criterion (see Part E.2), if any, you qualify under. In accordance with Part 5.1.6.1 of this permit, you must keep documentation with your SWPPP to support your determination of eligibility under Part 1.1.4.5, including the process employed and results of the endangered species investigation.

If you are seeking renewal of coverage under the MSGP, you must complete this analysis using any data collected when your site was fully active and operational, even if you are now claiming that your site is inactive and no industrial materials or activities are exposed to stormwater. If no such data exist for your facility, you should utilize the best available information from any industrial facility(ies) expected to discharge substantially similar effluents, based on the similarities of the general industrial activity, control measures, and runoff coefficients of their drainage areas. You should contact EPA if you need assistance in obtaining data from a facility with a substantially similar effluent.

When evaluating the potential effects of your activities, you must consider effects to listed species or critical habitats within the "action area." Action area is defined in Appendix B as all areas affected directly or indirectly by the stormwater discharges, allowable non-stormwater discharges, and stormwater discharge-related activities, and not merely the immediate area involved in these discharges and activities. This includes areas beyond the footprint of the facility that are likely to be affected by stormwater discharges, discharge-related activities, and allowable non-stormwater discharges. For example, normal construction, operations and maintenance activities can result in noise impacts and discharges of pollutants into downstream areas which can increase the "action area" beyond the footprint of the facility. "Facility" is defined in Appendix A.

Step One: *Determine if the Eligibility Requirements of Criterion B, C, or F Can Be Met.*

You should first determine whether you are eligible under Criteria B, C, or F because of a previously completed ESA section 7 consultation, a previously issued ESA Section 10 permit, or because your activities were already addressed in another discharger's certification of eligibility as follows:

i. The effects of your activities have been addressed in a consultation under ESA Section 7 on a separate Federal action (check box B corresponding to Criterion B).

ii. The effects of your activities have been addressed through approval of a Habitat Conservation Plan under Section 10 of the ESA (check box C corresponding to Criterion C). Stormwater discharges from your industrial facility may be

authorized by this MSGP if some activity is authorized through the issuance of a permit under section 10 of the ESA and that authorization addressed the effects of your stormwater discharges on federally-listed species and designated critical habitat. You must follow U.S. Fish and Wildlife Service (FWS) and/or National Marine Fisheries Service, also known as NOAA Fisheries (NMFS) procedures when applying for an ESA Section 10 permit (see 50 CFR 17.22(b)(1) for FWS and 222.22 for NMFS). Application instructions for section 10 permits for FWS and NMFS can be obtained by accessing the FWS and NMFS websites (www.fws.gov and www.nmfs.noaa.gov) or by contacting the appropriate FWS and NMFS regional office.

iii. You are covered under the eligibility certification of another operator for the project area (check box F corresponding to Criterion F). Your stormwater discharges, discharge-related activities, and allowable non-stormwater discharges were already addressed in another discharger's certification of eligibility under Criteria A, B, C, D, or E, which also included your facility and determined that federally listed endangered or threatened species or designated critical habitat would not be jeopardized. To certify eligibility under this criterion there must be no lapse of coverage in the other operator's certification. By certifying eligibility under Criterion F, you agree to comply with any measures or controls upon which the other discharge certification under Criterion B, C, or D was based. If your certification is based on another operator's certification under Criterion E, that certification is valid only if you have documentation showing that the other operator had certified under Criterion E, and you provide EPA with the relevant supporting information in your NOI form. Certification under Criterion F is discussed in more detail in the Fact Sheet that accompanies this permit.

Step Two: *Determine if Listed Threatened or Endangered Species and Critical Habitat are Present in the Action Area.*

Next, you should first determine whether federally-listed species are likely to occur in your action area. If you determine that there is a federally-listed species likely to occur in your action area, follow Step 3. If you determine that there are no federally-listed species likely to occur in your action area, you can certify that the facility meets Criteria A (check box A corresponding to Criteria A).

You can do this by obtaining a list of threatened and endangered species that are likely to occur in your general area, including the appropriate receiving water for your discharges. County-specific or sometimes township-specific lists of Federally threatened and endangered species are available from the local offices of FWS, and NMFS, or on their internet sites. The types of species that are likely to be present determine which Service office you should contact (in general, NMFS has jurisdiction over marine, estuarine, and anadromous species). Visit www.epa.gov/npdes/stormwater/cgp to find the appropriate site for your state or check with your local Service office. If there are listed species in your county or township, you must then determine, as best you are able, whether any of the species are likely to occur in your action area

(use the Services or State and Tribal Heritage Centers, as necessary). General species information can be found at www.fws.gov/endangered.wildlife.html.

You must also check to see if critical habitat has been designated and whether such areas overlap your action area. Critical habitat should be listed on the species list for your county or township available from the appropriate Service office. You can also find critical habitat designations at 50 CFR Parts 17 and 226 www.access.gpo.gov and at www.fws.gov/endangered/wildlife.html.

If there are no listed species and no critical habitat areas that overlap your action area, or if your local FWS or NMFS indicates that listed species are not likely to occur in your action area, you have satisfied your eligibility obligations under Criterion A (check box A on the Notice of Intent Form). If there are listed species and if you determine or your local FWS, NMFS, or State or Tribal Heritage Center indicates that these species could occur in the action area, you will need to evaluate whether your action area supports habitat(s) that are suitable for listed species or the constituent elements of critical habitat. Your evaluation may utilize one or more of the following approaches:

Gather information about the species and critical habitat that are likely to occur in your action area (www.fws.gov/endangered/wildlife.html). Conduct a visual inspection of the action area to assess the potential presence of listed species and their habitats. Compare the size and types of habitats available in your action area and adjacent areas with the size and types of habitats used by listed species and constituent elements of critical habitat. This method may be particularly suitable for facilities where the action area is smaller in size or located in non-natural settings such as highly urbanized areas or industrial parks where there is little or no natural habitat, or for facilities that discharge directly into municipal separate storm sewer systems.

Conduct a formal biological survey (typically performed by environmental consulting firms). In some cases, biological surveys may be an appropriate way to assess whether species are likely to be located in the action area and whether there could be adverse effects to such species. A biological survey may in some cases be useful in conjunction with Steps Two, Three or Four of these instructions. However, biological surveys can often be inconclusive and some survey methods may require a special State or Federal permit. You should coordinate with the appropriate Service office before conducting biological surveys for threatened and endangered species.

Reference an environmental assessment completed for the site under the National Environmental Policy Act (NEPA). Such assessments may indicate whether listed species and critical habitats are likely to occur in the action area. Coverage under this MSGP may trigger a requirement for such an assessment for new sources (that is, dischargers subject to New Source Performance Standards under section 306 of the Clean Water Act). Other facilities might require an assessment under NEPA for other reasons, such as federal funding or other federal involvement in the facility. If the action area likely supports listed threatened or endangered species or critical habitat, you must evaluate the potential for impacts to species and/or habitat when following Steps Three through Five. Note that many but not all measures implemented to protect listed species under these steps will also protect critical habitat. Thus, meeting the

eligibility requirements of this MSGP may require measures to protect critical habitat that are separate from those to protect listed species.

Step Three: *Determine if your Activities Are Not Likely to Adversely Affect Listed Threatened or Endangered Species or Designated Critical Habitat*

To receive MSGP coverage, you must analyze the effects of your activities, which may include not only your discharge, but also any construction, operation, and maintenance activities related to stormwater management. You must be able to conclude that your discharge and stormwater management related activities are not likely to adversely affect threatened or endangered species or designated critical habitat that are likely to occur in your action area. To arrive at this conclusion, you should be able to conclude that listed species and critical habitat are not likely to be exposed to the effects of your activities, or if they are exposed, they are not likely to respond to the effects, or if they do respond, the responses are not sufficient to reduce an individual's chances of surviving and reproducing or diminish the amount or suitability of constituent elements of critical habitat. Construction, operation, and maintenance of facilities related to your stormwater discharge can potentially result in the following adverse effects:

- **Hydrological.** Stormwater discharges may adversely affect receiving waters from pollutant parameters such as temperature, salinity or pH. These effects will vary with the amount of stormwater discharged and the volume and condition of the receiving water. Where a stormwater discharge constitutes a minute portion of the total volume of the receiving water, adverse hydrological effects are less likely. Industrial activity itself may also alter drainage patterns on a site where construction occurs, which can impact listed species, their habitat, and critical habitat.

- **Habitat.** Outdoor activities, such as storage of materials and land disturbances associated with stormwater management-related activities, such as the installation or placement of stormwater control measures, may adversely affect listed species, their habitat, and critical habitat. Stormwater may drain or inundate listed species habitat.

- **Toxicity.** Pollutants in stormwater may have toxic effects on listed species and adversely affect critical habitat. Exceedances of benchmarks, effluent limitation guidelines, or State or Tribal water quality requirements may be indicative of potential adverse affects on listed species or critical habitat.

The scope of effects to consider will vary with each site. If you are having difficulty determining whether your facility is likely to adversely affect listed species or critical habitat, or one of the Services has already raised concerns to you, you must contact the appropriate office of the FWS or NMFS for assistance. If adverse effects are not likely, you have satisfied your eligibility obligations under Criterion E and you may proceed to submitting your NOI for coverage under the MSGP (check box E corresponding to Criterion E). As part of certifying your compliance with Criterion E, you must submit information to support your findings. If you are an existing discharger, you are required to (1) identify any pollutant parameters for which you have ever exceeded the benchmark or effluent limitations guideline, or have ever been found to have caused or contributed to an exceedance of an applicable water quality standard, or

violated a State or Tribal water quality requirement; (2) provide a list of the federally-listed threatened or endangered species or their designated critical habitat that are likely to occur in the action area; and (3) provide your rationale supporting your determination that you qualify under Criterion E. If you are a new discharger, you must provide the list of species or critical habitat and the technical evaluation (described in (2) and (3) above, respectively), and you must also include a list of the potential pollutants in your discharge.

If you can not yet conclude your stormwater discharge is not likely to adversely affect listed species or critical habitat, or if you conclude that your stormwater discharge could potentially adversely affect listed species or critical habitat, you must follow Step Four.

Step Four: *Determine if Measures Can Be Implemented to Avoid Adverse Effects or If Further Analysis Supports the Conclusion that Adverse Effects Are Not Likely.*

If you could not make a preliminary determination in Step 3 that adverse effects to listed species and/or critical habitat are not likely to occur, you can still receive coverage under Criterion E if appropriate measures are undertaken to avoid or eliminate the likelihood of adverse effects prior to applying for MSGP coverage. These measures may be relatively simple, e.g., re-routing a stormwater discharge to bypass an area where species are located, relocating control measures, or changing the "footprint" of the industrial activity. Provided you are able to install and implement appropriate measures, you may proceed to submitting your NOI for coverage under the MSGP (check box E corresponding to Criterion E). As part of certifying your compliance with Criterion E, you must submit information to support your findings. If you are an existing discharger, you are first required to (1) identify any pollutant parameters for which you have ever exceeded a benchmark or an effluent limitations guideline, or have ever been found to have caused or contributed to an exceedance of an applicable water quality standard, or violated a State or Tribal water quality requirement; (2) provide a list of the federally-listed threatened or endangered species or their designated critical habitat that are likely to occur in the action area; and (3) provide your rationale supporting your determination that you qualify under Criterion E, including a description of measures you will implement to avoid or eliminate the likelihood of adverse effects. If you are a new discharger, you must provide the list of species or critical habitat and the technical evaluation (described in (2) and (3) above, respectively), and you must also include a list of the potential pollutants in your discharge.

If you cannot ascertain which measures to implement to avoid the likelihood of adverse effects, you must follow Step Five.

Step Five: *Determine if the Eligibility Requirements of Criteria D Can Be Met.*

Where adverse effects are likely and you are unable to avoid or eliminate the likelihood of adverse effects, you must contact the FWS and/or NMFS. However, you may still be eligible for MSGP coverage if any likely adverse effects can be addressed through meeting Criteria D as follows:

You have coordinated your activities with the appropriate Service office (see Criterion D). In the absence of any other conditions set forth in Step Four, you may still be able to

qualify for coverage under this MSGP if you coordinate with the FWS or NMFS and the Service provides a letter or memorandum concluding that permitting your stormwater discharges under the MSGP is consistent with the "not likely to adversely affect" determination for the MSGP. If you adopt measures to avoid or eliminate adverse effects, per the Service's requirements or recommendations, you must abide by those measures for the duration of your coverage under the MSGP. Any such measures must be described in the Stormwater Pollution Prevention Plan and are enforceable MSGP conditions and/or conditions for meeting the eligibility criteria in Part 1.1.4.5.

You must comply with any terms and conditions imposed under the eligibility requirements to ensure that your stormwater discharges, discharge-related activities, and allowable non-stormwater discharges are protective of listed species and/or critical habitat. See Part 2.3 of the permit. If the eligibility requirements cannot be met, and maintained, then you are not eligible for coverage under this MSGP. In these instances, you may consider applying to EPA for an individual permit.

E.2 Eligibility Criterion

As required by Part 1.1.4.5, you must meet one or more of the following six criteria (A-F) to be eligible for coverage under the permit for your stormwater discharge, discharge-related activities, and allowable non-stormwater discharges:

Criterion A. No federally-listed threatened or endangered species or their designated critical habitat are likely to occur in the "action area"; or

Criterion B. Consultation between a Federal agency and the U.S. Fish and Wildlife Service and/or the National Marine Fisheries Service (together, the "Services") under section 7 of the ESA has been concluded. Consultations can be either formal or informal, and would have occurred only as a result of a separate federal action (e.g., during application for an individual wastewater discharge permit or the issuance of a wetlands dredge and fill permit).

The consultation must have addressed the effects of your facility's stormwater discharges, allowable non-stormwater discharges, and stormwater discharge-related activities on federally-listed threatened or endangered species and federally-designated critical habitat, and must have resulted in either:

i. a biological opinion finding no jeopardy to federally-listed species or destruction/adverse modification of federally-designated critical habitat; or

ii. written concurrence from the Service(s) with a finding that the facility's stormwater discharges associated with industrial activity, discharge-related activities and allowable non-stormwater discharges are not likely to adversely affect federally-listed species or federally-designated critical habitat; or

Criterion C. Your industrial activities are authorized through the issuance of a permit under section 10 of the ESA, and authorization addresses the effects of the stormwater discharges associated with industrial activity, discharge-related activities, and allowable non-stormwater discharges on federally-listed species and federally-designated critical habitat; or

Criterion D. Coordination between you and the U.S. Fish and Wildlife Service and/or the National Marine Fisheries Service has been concluded. The coordination must have addressed the effects of the facility's stormwater discharges associated with industrial activity, discharge-related activities, and allowable non-stormwater discharges on federally-listed threatened or endangered species and federally-designated critical habitat. The result of the coordination must be a written statement from the Service concluding that authorizing your stormwater discharges, discharge-related activities, and allowable non-stormwater discharges is consistent with the determination that the issuance of the MSGP is not likely to adversely affect federally-listed threatened or endangered species and federally-designated critical habitat. Any conditions or prerequisites deemed necessary to achieve consistency with the "not likely to adversely effect" determination become eligibility conditions for MSGP coverage, and permit requirements under Part 2.3; or

Criterion E. Authorizing your stormwater discharges associated with industrial activity, discharge-related activities, and allowable non-stormwater discharges is consistent with the determination that the issuance of the MSGP is not likely to adversely affect any federally-listed endangered and threatened ("listed") species or designated critical habitat ("critical habitat"). To support your determination that you meet Criterion E, you must provide supporting documentation for your determination.

i. If you are an existing discharger, you must provide the following information with your completed Notice of Intent (NOI) form: (1) a list of the federally-listed threatened or endangered species or their designated critical habitat that are likely to occur in the "action area"; (2) a list of the pollutant parameters for which you have ever exceeded the benchmark or applicable effluent limitations guideline, or for which you have ever been found to have caused or contributed to an exceedance of an applicable water quality standard or to have violated a State or Tribal water quality requirement (Part 9); and (3) your rationale supporting your determination that you meet Criterion E, including appropriate measures to be undertaken to avoid or eliminate the likelihood of adverse effects.

ii. If you are a new discharger, you must provide the following information with your completed NOI form: (1) a list of the federally-listed threatened or endangered species or their designated critical habitat that are likely to occur in the "action area"; (2) a list of the potential pollutants in your discharge; and (3) your rationale supporting your determination that you meet Criterion E, including

appropriate measures to be undertaken to avoid or eliminate the likelihood of adverse effects; or

Criterion F. The facility's stormwater discharges associated with industrial activity, discharge-related activities, and allowable non-stormwater discharges were already addressed in another operator's valid certification of eligibility that included the industrial activities and there is no reason to believe that federally-listed species or federally-designated critical habitat not considered in the prior certification may be present or located in the "action area". To certify eligibility under this criterion there must be no lapse of coverage in the other operator's certification. By certifying eligibility under this criterion, you agree to comply with any measures or controls upon which the other operator's certification was based. You must comply with any applicable terms, conditions, or other requirements developed in the process of meeting the eligibility requirements of the criteria in this section to remain eligible for coverage under this permit. Documentation must be kept with your SWPPP. If your certification is based on another operator's certification under Criterion E, that certification is valid only if you have documentation showing that the other operator had certified under Criterion E, and you provide EPA with the relevant supporting information required of existing dischargers in Criterion E (above, under subparagraph (i)) in your NOI form.

Appendix F
Procedures Relating to Historic Properties Preservation

Appendix F – Procedures Relating to Historic Properties Preservation

Section 106 of the National Historic Preservation Act (NHPA) requires Federal agencies to take into account the effects of Federal "undertakings" on historic properties that are either listed on, or eligible for listing on, the National Register of Historic Places. The term Federal "undertaking" is defined in the NHPA regulations to include a project, activity, or program of a Federal agency including those carried out by or on behalf of a Federal agency, those carried out with Federal financial assistance, and those requiring a Federal permit, license or approval. See 36 CFR 800.16(y). Historic properties are defined in the NHPA regulations to include prehistoric or historic districts, sites, buildings, structures, or objects that are included in, or are eligible for inclusion in, the National Register of Historic Places. This term includes artifacts, records, and remains that are related to and located within such properties. See 36 CFR 800.16(1).

EPA's issuance of the Multi-Sector General Permit is a Federal undertaking within the meaning of the NHPA regulations. To address any issues relating to historic properties in connection with issuance of the permit, EPA has included criteria for applicants to certify that potential impacts of their covered activities on historic properties have been appropriately considered and addressed. Although individual applications for coverage under the general permit do not constitute separate Federal undertakings, the screening criteria and certifications provide an appropriate site-specific means of addressing historic property issues in connection with EPA's issuance of the permit. Applicants seeking coverage under the MSGP are thus required to make certain certifications regarding the potential effects of their stormwater discharge, allowable non-stormwater discharge, and discharge-related activities on properties listed or eligible for listing on the National Register of Historic Places.

You must meet one or more of the four criteria (A-D), which are also included in Part 1.1.4.6, to be eligible for coverage under this permit.

Criterion A. Your stormwater discharges and allowable non-stormwater discharges do not have the potential to have an effect on historic properties and you are not constructing or installing new stormwater control measures on your site that cause subsurface disturbance; or

Criterion B. Your discharge-related activities (i.e., construction and/or installation of stormwater control measures that involve subsurface disturbance) will not affect historic properties; or

Criterion C. Your stormwater discharges, allowable non-stormwater discharges, and discharge-related activities have the potential to have an effect on historic properties, and you have obtained and are in compliance with a written agreement with the State Historic Preservation Officer (SHPO), Tribal Historic Preservation Officer (THPO), or other tribal representative regarding measures to mitigate or prevent any adverse effects on historic properties, and you have either (1) obtained and are in compliance with a written agreement that outlines all such measures, or (2) been unable to reach agreement on such measures; or

Criterion D. You have contacted the State Historic Preservation Officer, Tribal Historic Preservation Officer, or other tribal representative and EPA in writing informing them that you have the potential to have an effect on historic properties and you did not receive a response from the SHPO, THPO, or tribal representative within 30 days of receiving your letter.

If you have been unable to reach agreement with a SHPO, THPO, or other tribal representative regarding appropriate measures to mitigate or prevent adverse effects, EPA may notify you of additional measures you must implement in order to be eligible for coverage under this permit.

Activities with No Potential to Have an Effect on Historic Properties

A determination that a Federal undertaking has no potential to have an effect on historic properties fulfills an agency's obligations under the NHPA. EPA has reason to believe that the vast majority of activities authorized under the MSGP have no potential to have effects on historic properties. The purpose of this permit is to control pollutants that may be transported in stormwater runoff from industrial facilities. EPA does not anticipate effects on historic properties from the pollutants in the stormwater and allowable non-stormwater discharges from these industrial facilities. Thus, to the extent EPA's issuance of this general permit authorizes discharges of such constituents, confined to existing stormwater channels or natural drainage areas; the permitting action does not have the potential to cause effects on historic properties.

In addition, the overwhelming majority of sources covered under this permit will be facilities that are seeking renewal of previous permit coverage. These existing dischargers should have already addressed NHPA issues in the 2000 MSGP as they were required to certify that they were either not affecting historic properties or they had obtained written agreement from the applicable State Historic Preservation Officer (SHPO) or Tribal Historic Preservation Officer (THPO) regarding methods of mitigating potential impacts. Both existing and new dischargers must follow the historic property screening procedures to determine their eligibility. EPA is not aware of any impacts on historic properties from activities covered under the 2000 MSGP, or, for that matter, any need for a written agreement. Therefore, to the extent this permit authorizes renewal of prior coverage without relevant changes in operations, it has no potential to have an effect on historic properties.

Activities with Potential to Have an Effect on Historic Properties

EPA believes this permit may have some potential to have an effect on historic properties where permittees construct and/or install stormwater control measures that involve subsurface disturbance and impact less than one (1) acre of land to comply with this permit. (Ground disturbances of one (1) acre or more require coverage under a different permit, the Construction General Permit.) Where you have to disturb the land through the construction and/or installation of control measures, there is a possibility that artifacts, records, or remains associated with historic properties could be impacted. Therefore, if you are establishing new or altering existing control measures to manage your stormwater that will involve subsurface ground disturbance of less than one (1) acre, you will need to ensure (1) that historic properties will not be impacted by

your activities or (2) that you have consulted with the appropriate SHPO, THPO, or other tribal representative regarding measures that would mitigate or prevent any adverse effects on historic properties.

Examples of Control Measures Which Involve Subsurface Disturbance

EPA reviewed typical control measures currently employed to determine which practices involve some level of earth disturbance. The types of control measures that are presumptively expected to cause subsurface ground disturbance include:

- Dikes
- Berms
- Catch Basins
- Ponds
- Ditches
- Trenches
- Culverts
- Land manipulation: contouring, sloping, and grading
- Channels
- Perimeter Drains
- Swales

EPA cautions dischargers that this list is non-inclusive. Other control measures that involve earth disturbing activities that are not on this list must also be examined for the potential to affect historic properties.

Historic Property Screening Process

You should follow the following screening process in order to certify your compliance with historic property eligibility requirements under this permit (see Part 1.1.4.6). The following four steps describe how applicants can meet the permit eligibility criteria for protection of historic properties under this permit:

Step One: *Are you an existing facility that is reapplying for certification under the 2008 MSGP?*

If you are an existing facility you should have already addressed NHPA issues. To gain coverage under the 2000 MSGP you were required to certify that you were either not affecting historic properties or had obtained written agreement from the relevant SHPO or THPO regarding methods of mitigating potential impacts. As long as you are not constructing or installing any new stormwater control measures then you have met eligibility Criterion A of the MSGP. After you submit your NOI, there is a 30-day waiting period during which the SHPO, THPO, or other tribal representative may review your NOI. The SHPO, THPO, or other tribal representative may request that EPA hold up authorization based on concerns about potential adverse impacts to historic properties. EPA will evaluate any such request and notify you if any additional measures to address adverse impacts to historic properties are necessary.

If you are an existing facility and will construct or install stormwater control measures that require subsurface disturbance of less than one (1) acre then you should proceed to Step Three. (Note: Construction activities disturbing one (1) acre or more are not eligible for coverage under this permit.)

If you are a new facility then you should proceed to Step Two.

Step Two: *Are you constructing or installing any stormwater control measures that require subsurface disturbance of less than one (1) acre?*

If, as part of your coverage under this permit, you are not building or installing control measures on your site that cause less than one (1) acre of subsurface disturbance, then your discharge-related activities do not have the potential to have an effect on historic properties. You have no further obligations relating to historic properties. You have met eligibility Criterion A of the MSGP. After you submit your NOI, there is a 30-day waiting period during which the SHPO, THPO, or other tribal representative may review your NOI. The SHPO, THPO, or other tribal representative may request that EPA hold up authorization based on concerns about potential adverse impacts to historic properties. EPA will evaluate any such request and notify you if any additional measures to address adverse impacts to historic properties are necessary.

If the answer to the Step Two question is yes, then you should proceed to Step Three.

Step Three: *Have prior earth disturbances determined that historic properties do not exist, or have prior disturbances precluded the existence of historic properties?*

If previous construction either revealed the absence of historic properties or prior disturbances preclude the existence of historic properties, then you have no further obligations relating to historic properties. You have met eligibility Criterion B of the MSGP. After you submit your NOI, there is a 30-day waiting period during which the SHPO, THPO, or other tribal representative may review your NOI. The SHPO, THPO, or other tribal representative may request that EPA hold up authorization based on concerns about potential adverse impacts to historic properties. EPA will evaluate any such request and notify you if any additional measures to address adverse impacts to historic properties are necessary.

If the answer to the Step Three question is no, then you should proceed to Step Four.

Step Four: *Contact the appropriate historic preservation authorities*

Where you are building and/or installing control measures affecting less than one (1) acre of land to control stormwater or allowable non-stormwater discharges associated with this permit, and the answer to Step Three is no, then you should contact the relevant SHPO, THPO, or other tribal representative to determine the likelihood that artifacts, records, or remains are potentially present on your site. This may involve examining local records to determine if historic artifacts have been found in nearby areas, as well as limited surface and subsurface examination carried out by qualified professionals.

If through this process it is determined that such historic properties potentially exist and may be impacted by your construction or installation of control measures, you should contact the relevant SHPO, THPO, or tribal representative in writing and request to discuss mitigation or prevention of any adverse effects. The letter should describe your facility, the nature and location of subsurface disturbance activities that are contemplated, any known or suspected historic properties in the area, and any anticipated effects on such properties. The letter should state that if the SHPO, THPO, or tribal representative does not respond within 30 days of receiving your letter, you may submit your NOI without further consultation. EPA encourages applicants to contact the appropriate authorities as soon as possible in the event of a potential adverse effect to an historic property.

If the SHPO, THPO, or tribal representative sent you a response within 30 days of receiving your letter and you enter into, and comply with, a written agreement with the SHPO, THPO, or other tribal representative regarding how to address any adverse impacts on historic properties, you have met eligibility Criterion C. In this case, you should retain a copy of the written agreement consistent with Part 5.1.6.2 of the MSGP. After you submit your NOI, there is a 30-day waiting period during which the SHPO, THPO, or other tribal representative may review your NOI. The SHPO, THPO, or other tribal representative may request that EPA delay authorization based on concerns about potential adverse impacts to historic properties. However, EPA would generally accept any written agreement as fully addressing such concerns unless new information was brought to the Agency's attention that was not considered in your previous discussions with the SHPO, THPO or other tribal representative.

If you receive a response within 30 days after the SHPO, THPO, or tribal representative received your letter and you consult with the SHPO, THPO or tribal representative regarding adverse impacts to historic properties and measures to mitigate them but an agreement cannot be reached between you and the SHPO, THPO, or other tribal representative, you have still met the eligibility for Criterion C. In this case you should include in your SWPPP a brief description of potential effects to historic properties, the consultation process, any measures you will adopt to address the potential adverse impacts, and any significant remaining disagreements between you and the SHPO, THPO or other tribal representative. After you submit your NOI, there is a 30-day waiting period during which the SHPO, THPO, or other tribal representative may review your NOI. The SHPO, THPO, or other tribal representative may request that EPA delay authorization based on concerns about potential adverse impacts to historic properties. EPA will evaluate any such request and notify you if any additional measures to address adverse impacts to historic properties are necessary.

If you have contacted the SHPO, THPO, or tribal representative in writing regarding your potential to have an effect on historic properties and the SHPO, THPO, or tribal representative did not respond within 30 days of receiving your letter, you have met eligibility Criterion D. You are advised to get a receipt from the post office or other carrier confirming the date on which your letter was received. In this case, you should submit a copy of your letter notifying the SHPO, THPO or tribal representative of potential impacts with your NOI. After you submit your NOI, there is a 30-day waiting period during which the SHPO, THPO, or other tribal representative may review your NOI. The SHPO, THPO, or other tribal representative may

request that EPA hold up authorization based on concerns about potential adverse impacts to historic properties. EPA will evaluate any such request and notify you if any additional measures to address adverse impacts to historic properties are necessary.

Addresses for State Historic Preservation Officers and Tribal Historic Preservation Officers may be found on the Advisory Council on Historic Preservation's website (www.achp.gov/programs.html). In instances where a Tribe does not have a Tribal Historic Preservation Officer, you should contact the appropriate Tribal government office when responding to this permit eligibility condition.

Appendix G
Notice of Intent (NOI) Form

Appendix G –Notice of Intent (NOI) Form

To obtain coverage under this permit, you must submit a Notice of Intent (NOI). You must submit an NOI using either (1) EPA's Electronic Notice of Intent (eNOI) system, available at www.epa.gov/npdes/eNOI, or (2) file a paper copy of the NOI, a copy of which follows.

NPDES FORM 3510 -6

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460
NOTICE OF INTENT (NOI) FOR STORMWATER DISCHARGES ASSOCIATED WITH INDUSTRIAL ACTIVITY UNDER THE NPDES MULTI-SECTOR GENERAL PERMIT

Form Approved.
OMB No. 2040-0086

Submission of this completed Notice of Intent (NOI) constitutes notice that the operator identified in Section B of this form requests authorization to discharge pollutants to waters of the United States from the facility or site identified in Section C under EPA's NPDES Stormwater Multi-Sector General Permit (MSGP) for industrial stormwater. Submission of this NOI constitutes your notice to EPA that the facility identified in Section C of this form meets the eligibility conditions of Part 1.1 of the MSGP. Please read and make sure you comply with all eligibility requirements, including the requirement to prepare a stormwater pollution prevention plan. Refer to the instructions at the end of this form to complete your NOI.

A. Permit Number: | | R | | | | | | (see Appendix C of the MSGP for the list of eligible permit numbers)

Tracking Number (EPA Use Only): | | | | | | | |

B. Facility Operator Information

1. Name:

2. IRS Employer Identification Number (EIN): | | – | | | | | | |

3. Mailing Address:

a. Street:

b. City: c. State: d. Zip Code: -

e. Phone: - - f. Fax (optional): - - g. E-mail:

C. Facility Information

1. Facility Name:

2. Have stormwater discharges from your site been covered previously under an NPDES permit? ☐ YES ☐ NO

a. If yes, provide the Tracking Number if you had coverage under EPA's MSGP 2000 or the NPDES permit number if you had coverage under an EPA individual permit.

b.1 If no, was your facility in operation and discharging stormwater prior to October 30, 2005? ☐ YES ☐ NO

b.2 If no to C.2.b.1, did your facility commence discharging after October 30, 2005 and before January 5, 2009? ☐ YES ☐ NO

3. Location Address:

a. Street

b. City:

c. County or similar government subdivision: d. State: e. Zip Code: -

f. Latitude: (use any one of the three formats provided.)
1. ___ ___° ___ ___′ ___ ___″ N (degrees, minutes, seconds)
2. ___ ___° ___ ___. ___ ___′ N (degrees, minutes, decimal)
3. ___ ___. ___ ___ ___ ___° N (degrees decimal)

g. Longitude: (use any of these 3 formats)
1. ___ ___ ___° ___ ___′ ___ ___″ W (degrees, minutes, seconds)
2. ___ ___ ___° ___ ___. ___ ___′ W (degrees, minutes, decimal)
3. ___ ___ ___. ___ ___ ___ ___° W (degrees decimal)

h. Lat/Long Data Source: ☐ USGS topographic map ☐ EPA web site ☐ GPS ☐ Other:

If you used a USGS topographic map, what was the scale?

4. Estimated area of industrial activity at your site exposed to stormwater: _____ (acres)

5. Is this a federal facility? ☐ YES ☐ NO

6. Is your facility located on Indian Country lands? ☐ YES ☐ NO

If yes, name of reservation, or if not part of a reservation, put "Not Applicable:"

D. Discharge information

1. Does your facility discharge stormwater into a Municipal Separate Storm Sewer System (MS4)? ☐ YES ☐ NO

If yes, name of MS4 operator: ____________________

2. Receiving Waters and Wetlands (**Note**: If additional space is needed for this question, fill out Attachment 1.)

a. What is the name(s) of your receiving water(s) that receive stormwater directly and/or through an MS4)? If your receiving water is impaired then identify the name of the impaired segment, if applicable, in parentheses following the receiving water name.	b. Are any of your discharges directly into any segment of an "impaired" water?	If you answered yes to question D.2.b, then answer the following three questions:		
		b.1. What pollutant(s) are causing the impairment?	b.2. Are the pollutant(s) causing the impairment present in your discharge?	b.3. Has a TMDL been completed for the pollutant(s) causing the impairment?
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO

3. Water Quality Standards (for new dischargers only)

a. Are any of your discharges into any portion of a receiving water designated by the state or tribal authority under its antidegradation policy as a Tier 2 (or Tier 2.5) water (water quality exceeds levels necessary to support propagation of fish, shellfish, and wildlife and recreation in and on the water)? ☐ YES ☐ NO

b. Has the receiving water(s) been designated by the state or tribal authority under its antidegradation policy as a Tier 3 water (Outstanding Natural Resource Water)? ☐ YES ☐ NO

4. Federal Effluent Limitation Guidelines and Sector-Specific Requirements

a. Are you requesting permit coverage for any stormwater discharges subject to effluent limitation guidelines? ☐ YES ☐ NO

b. If yes, which effluent limitation guidelines apply to your stormwater discharges?

40 CFR Part/Subpart	Eligible Discharges	Affected MSGP Sector	Check if Applicable
Part 411, Subpart C	Runoff from material storage piles at cement manufacturing facilities	E	☐
Part 418 Subpart A	Runoff from phosphate fertilizer manufacturing facilities that comes into contact with any raw materials, finished product, by-products or waste products (SIC 2874)	C	☐
Part 423	Coal pile runoff at steam electric generating facilities	O	☐
Part 429, Subpart I	Discharges resulting from spray down or intentional wetting of logs at wet deck storage areas	A	☐
Part 436, Subpart B, C, or D	Mine dewatering discharges at crushed stone mines, construction sand and gravel mines, or industrial sand mines	J	☐
Part 443, Subpart A	Runoff from asphalt emulsion facilities	D	☐
Part 445, Subparts A & B	Runoff from hazardous waste and non-hazardous waste landfills	K, L	☐

c. If you are a Sector S (Air Transportation) facility, do you anticipate using more than 100,000 gallons of glycol-based deicing/anti-icing chemicals and/or 100 tons or more of urea on an average annual basis? ☐ YES ☐ NO

5. Identify the 4-digit Standard Industrial Classification (SIC) code or 2-letter Activity Code that best represents the products produced or services rendered for which your facility is primarily engaged, as defined in MSGP:

Primary SIC Code: ☐☐☐☐ OR Primary Activity Code ☐☐

6. Identify the applicable sector(s) and subsector(s) of industrial activity, including co-located industrial activity, for which you are requesting permit coverage:

a. Sector ☐☐ Subsector ☐ b. Sector ☐☐ Subsector ☐ c. Sector ☐☐ Subsector ☐

d. Sector ☐☐ Subsector ☐ e. Sector ☐☐ Subsector ☐ f. Sector ☐☐ Subsector ☐

7.a. Is your site presently inactive and unstaffed? ☐ YES ☐ NO

b1. If yes, is your site expected to be inactive and unstaffed for the entire permit term? ☐ YES ☐ NO

b2. If you select "no" in 7.b1 above, then indicate the length of time that you expect your facility to be inactive and unstaffed ____________________

E. Stormwater Pollution Prevention Plan (SWPPP) Contact Information

1a. SWPPP Contact Name:

b. Phone: - - Ext. c. E-mail: _____

2. URL of SWPPP (if applicable): _____

F. Endangered Species Protection

1. Using the instructions in Appendix E of the MSGP, under which criterion listed in Part 1.1.4.5 are you eligible for coverage under this permit?

☐ A ☐ B ☐ C ☐ D ☐ E ☐ F

2. If you select criterion E from Part 1.1.4.5:

a. What federally-listed species or federally-designated critical habitat are in your "action area?"_____

_____

b. List the pollutants expected to be present in your discharge _____

_____

c. If you are an existing discharger, do you have effluent monitoring data from EPA's MSGP 2000, or another previous NPDES permit? ☐ YES ☐ NO

c.1 If no, why not? ☐ No monitoring required for my sector ☐ Inactive/unstaffed site ☐ Other _____

c.2 Do you have any other data characterizing pollutants in your stormwater (describe)? _____

c.3 If you have benchmark monitoring data, did you exceed any of the applicable benchmarks? ☐ YES ☐ NO

c.4 Did you exceed any applicable effluent limitation guideline or cause or contribute to an exceedance of a State or Tribal water quality standard? ☐ YES ☐ NO

c.5 If you answered "yes" to either question F.2.c.3 or F.2.c.4 above, for what pollutant(s)? _____

d. Attach documentation supporting criterion E eligibility. Documentation should address species and habitat listed in F.2.a and the potential effects of pollutants listed in F.2.b (including any monitoring data for these pollutants) on the listed species and habitat.

3. If you select criterion F from Part 1.1.4.5, provide the operator's NPDES Tracking Number under which you are certifying eligibility:

G. Historic Preservation

Using the instructions in Appendix F of the MSGP, under which criterion listed in Part 1.1.4.6 are you eligible for coverage under this permit?

☐ A ☐ B ☐ C ☐ D

H. Certifier Name and Title

I certify under penalty of law that I meet the eligibility conditions of this permit and that this document and all attachments were prepared under my direction or supervision in accordance with a system designed to assure that qualified personnel properly gather and evaluate the information submitted. Based on my inquiry of the person or persons who manage the system, or those persons directly responsible for gathering the information, I certify that the information submitted is, to the best of my knowledge and belief, true, accurate, and complete. I certify that I am aware that there are significant penalties for submitting false information, including the possibility of fines and imprisonment for knowing violations.

Print Name:

Title:

Signature: _____ Date:

E-mail: _____

NOI Preparer (Complete if NOI was prepared by someone other than the certifier)

Prepared by:

Organization:

Phone: - - Ext. E-mail: _____

Attachment 1. (Fill in as necessary if more space is required for D.2 a-e)

a. What is the name(s) of your receiving water(s) that receive stormwater from your facility (directly and/or through an MS4)? If your receiving water is impaired then identify the name of the impaired segment, if applicable, in parentheses following the receiving water name.	b. Are any of your discharges directly into any segment of an "impaired" water?	If you answered yes to question D.2.b, then answer the following three questions:		
		b.1. What pollutant(s) are causing the impairment?	b.2. Are the pollutant(s) causing the impairment present in your discharge?	b.3. Has a TMDL been completed for the pollutant(s) causing the impairment?
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
	☐ YES ☐ NO		☐ YES ☐ NO	☐ YES ☐ NO
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NOI Submittal Deadlines/Discharge Authorization Dates		
Category	**NOI Deadline**	**Discharge Authorization Date**[1]
Existing Dischargers - in operation as of October 30, 2005 and authorized for coverage under MSGP 2000.	No later than January 5, 2009.	30 days after EPA posts your NOI. Your authorization under the MSGP 2000 is automatically continued until you have been granted coverage under this permit or an alternative permit, or coverage is otherwise terminated.
New Dischargers or New Sources - have commenced discharging between October 30, 2005 and January 5, 2009.	As soon as possible but no later than January 5, 2009.	30 days after EPA posts your NOI.
New Dischargers or New Sources - commence discharging after January 5, 2009.	A minimum of 60 days prior to commencing operation of the facility, or a minimum of 30 days if your SWPPP is posted on the Internet during this period and the Internet address (i.e., URL) to your SWPPP is provided on the NOI form.	If you post your SWPPP on the Internet, 30 days after EPA posts your NOI. Otherwise, 60 days after EPA posts your NOI.
New Owner/Operator of Existing Discharger - transfer of ownership and/or operation of a facility whose discharge is authorized under this permit	A minimum of 30 days prior to date that the transfer will take place to the new owner/operator.	30 days after EPA posts your NOI.
Other Eligible Dischargers - in operation prior to October 30, 2005 but not covered under the MSGP 2000 or another NPDES permit.	Immediately, to minimize the time discharges from the facility will continue to be unauthorized.	If you post your SWPPP on the Internet, 30 days after EPA posts your NOI. Otherwise, 60 days after EPA posts your NOI.

[1] Based on a review of your NOI or other information, EPA may delay your authorization for further review, notify you that additional effluent limitations are necessary, or may deny coverage under this permit and require submission of an application for an individual NPDES permit, as detailed in MSGP Part 1.6. In these instances, EPA will notify you in writing of the delay or the request for submission of an individual NPDES permit application. EPA will post these NOIs on its website at www.epa.gov/npdes/enoi.

Who Must File a Notice of Intent with EPA?

Under section 402(p) of the Clean Water Act (CWA) and regulations at 40 CFR Part 122, stormwater discharges associated with industrial activity are prohibited to waters of the United States unless authorized under a National Pollutant Discharge Elimination System (NPDES) permit. You can obtain coverage under the MSGP by submitting a completed NOI if you operate a facility:

- that is located in a jurisdiction where EPA is the permitting authority, listed in Appendix C of the MSGP,
- that discharges stormwater associated with industrial activities, identified in Appendix D of the MSGP,
- that meets the eligibility requirements in Part 1.1 of the permit,
- that develops a stormwater pollution prevention plan (SWPPP) in accordance with Part 5 of the MSGP; and
- that installs and implements control measures in accordance with Part 2 to meet numeric and non-numeric effluent limits.

If you are unsure if you need an NPDES stormwater permit, contact your EPA or State NPDES stormwater permit program. Contacts are listed at www.epa.gov/npdes/stormwatercontacts.

One NOI must be submitted for each facility or site for which you are seeking permit coverage. You do not need to submit separate NOIs for each type of industrial activity present at your facility, provided your SWPPP covers all activities.

When to File the NOI Form

Do not file your NOI until you have obtained and thoroughly read a copy of the MSGP. A copy of the MSGP is located on the EPA website (www.epa.gov/npdes/stormwater/msgp). The MSGP describes procedures to ensure your eligibility, prepare your SWPPP, install and implement appropriate stormwater control measures, and complete the NOI form questions – all of which must be done before you sign the NOI certification statement attesting to the accuracy and completeness of your NOI. You will also need a copy of the MSGP once you have obtained coverage so that you can comply with the implementation requirements of the permit.

Where to File the NOI Form

EPA encourages you to complete the NOI form electronically via the Internet. EPA's Electronic Notice of Intent System (eNOI) can be found at www.epa.gov/npdes/enoi. Filing electronically is the fastest way to obtain permit coverage and help ensure that your NOI is complete. If you choose not to file electronically, you must send the NOI to one of the addresses listed below.

NOIs sent regular mail:
Stormwater Notice Processing Center (4203M)
USEPA
1200 Pennsylvania Avenue, NW
Washington, DC 20460

NOIs sent overnight/express mail:
Stormwater Notice Processing Center
EPA East Building, Rm. 7420
1201 Constitution Avenue, NW
Washington, DC 20004
202-564-9545

If you have questions, please contact EPA's Stormwater Notice Processing Center toll free at (866) 352-7755.

- If you file a paper NOI, please submit the original with a signature in ink – Do Not Send Copies. Also, faxed copies will not be accepted.
- Your SWPPP does not need to be submitted for review unless specifically requested by EPA or as otherwise required in Part 9 of the MSGP (State, Territory, and Tribal requirements). You must keep a copy of your SWPPP on-site or otherwise make it available to facility personnel responsible for implementing provisions of the permit.

Completing the NOI Form

To complete this form, type or print in uppercase letters in the appropriate areas only. Please make sure you complete all questions. Make sure you make a photocopy for your records before you send the completed original form to the address above. You may also use this paper form as a checklist for the information you will need when filing an NOI electronically via EPA's eNOI system.

Section A. Permit Number

Appendix C of the MSGP 2008 contains a list of geographic areas covered by the permit. If your facility is located in one of the listed areas, include the appropriate permit number in this section. (For example, if you facility is located in Massachusetts, and not on Indian Lands, you would write MAR050000 in this space.) If your facility is located in an area not covered by the MSGP, please contact your EPA Region, state or territorial NPDES stormwater coordinator (see www.epa.gov/npdes/stormwatercontacts for a list of contacts).

Section B. Facility Operator Information

1. Provide the legal name of the person, firm, public organization or any other public entity that operates the facility described in this application. An operator of a facility is a legal entity that controls the operation of the facility.
2. Provide the Employer Identification Number (EIN from the Internal Revenue Service (IRS)), commonly referred to as your taxpayer ID number. If the operator does not have an EIN, enter "NA" in the space provided.
3. Provide the operator's mailing address, telephone number, fax number (optional), and email address. Correspondence will be sent to this address.

Section C. Facility Information

1. Enter the facility's official or legal name. Unless the name of your facility has changed, please use the same name provided on prior NOIs or permit applications. You can use EPA's NOI Search website (www.epa.gov/npdes/noisearch) to view your previous NOI.

2. Indicate if industrial stormwater discharges from your facility were previously covered by an NPDES permit.

2a. If your facility was covered by EPA's MSGP-2000, please include the tracking number that you received in your confirmation letter or email from EPA's Stormwater Notice Processing Center. You can find the tracking number assigned to your previous NOI on EPA's NOI Search website (www.epa.gov/npdes/noisearch).

2b1. If your facility was not previously covered by an NPDES permit and discharged industrial stormwater, then indicate if it was in operation before October 30, 2005 and not covered under the MSGP 2000. If you select "yes" to this question then you have a 30 day waiting period before you are authorized to discharge.

2b2. If you select "no" in C.2.b.1, then indicate if your facility discharged stormwater between October 30, 2005 and January 5, 2009. If you select "yes" to this

question then you have a 30 day waiting period before you are authorized to discharge. If you select "no" to this question and you post your SWPPP on the Internet and provide EPA the URL in E.2, then you have a 30 day waiting period before you are authorized to discharge. If you select "no" to this question, but do not post your SWPPP on the Internet and therefore do not answer E.2, then you have a 60 day waiting period before you are authorized to discharge.

3.a-e. Enter the street address, including city, state, zip code, county or similar government subdivision of the actual physical location of the facility. Do not use a P.O. Box.

3.f-g. Provide the facility latitude and longitude in one of three formats: (1) degrees, minutes, seconds; (2) degrees, minutes, decimal; or(3) degrees decimal. You can obtain your facility's latitude and longitude though Global Positioning System (GPS) receivers, U.S. Geological Survey (USGS) quadrangle or topographic maps, and EPA's web-based siting-tools, among other methods. Refer to www.epa.gov/npdes/stormwater/msgp for guidance on the use of these methods. For consistency, EPA requests you take measurements from the location of your facility's stormwater outfall. Outfalls are locations where the stormwater exits the facility, including pipes, ditches, swales, and other structures that transport stormwater. If there is more than one outfall present, measure at the primary outfall (i.e., the outfall with the largest volume of stormwater discharge associated with industrial activity).

3.h. Identify the data source that you used to determine the facility latitude and longitude. If you did not use a USGS quadrangle or topographic map, the EPA website, or GPS receivers, then select "Other" and write the method used on the line provided. If you used a USGS quadrangle or topographic map, write the map scale on the line provided. Scale should be identified on the map.

4. Enter the estimated area of industrial activity at your site exposed to stormwater, in acres.

5. Indicate if the facility is considered a "federal facility" - Federal facilities include any buildings, installations, structures, land, public works, equipment, aircraft, vessels, and other vehicles and property, owned or leased by the federal government.

6. Indicate whether the facility is located in Indian Country, and, if so, provide the name of the reservation, if applicable.

Section D. Discharge Information

1. Indicate whether stormwater from your site will be discharged into a municipal separate storm sewer system (MS4). An MS4 is a conveyance or system of conveyances, including roads with drainage systems, municipal streets, catch basins, storm drains, curbs and gutters, ditches and man-made channels, owned or operated by a state, city, town, borough, county, parish, district, association or other public body, used to collect or convey stormwater. If you check "Yes" then identify the name of the MS4 operator on the line provided. If you are uncertain of the MS4 operator, contact your local government for that information. MS4s are different than combined sewers, which are designed to convey both stormwater and sanitary wastewater. Discharges to combined sewers do not require an NPDES permit but may be subject to other CWA requirements (contact the combined sewer operator for more information).

2. Enter information regarding your discharge. If additional space is needed fill out Attachment 1.

2a. Indicate in column "a" of the table the name(s) of the receiving water(s) into which stormwater from your facility will discharge. Also provide in parentheses the name of the impaired water (and segment, if applicable) into which your stormwater is discharged. If you identified more than on receiving water for your facility, indicate the first receiving water and complete question 2b and 2.b.1-3 (if applicable), before entering the next receiving water. The EPA's Water Locator Tool can help you identify the closest receiving water to your facility (www.epa.gov/npdes/msgp). Your receiving water may be a lake, stream, river, ocean, wetland or other waterbody, and may or may not be located adjacent to your facility. Your stormwater may discharge directly to the receiving water or indirectly via a storm sewer system, an open drain or ditch, or other conveyance structure. Do NOT list a man-made conveyance, such as a storm sewer system, as your receiving water. Indicate the first receiving water your stormwater discharge enters. For example, if your discharge enters a storm sewer system, that empties into Trout Creek, which flows into Pine River, your receiving water is Trout Creek, because it is the first waterbody your discharge will reach. Similarly, a discharge into a ditch that feeds Spring Creek should be identified as "Spring Creek" since the ditch is a manmade conveyance. If you discharge into a municipal separate storm sewer system (MS4), you must identify the waterbody into which that portion of the storm sewer discharges. That information should be readily available from the operator of the MS4.

2b. Indicate in column "b" of the table whether you discharge directly to an impaired water (lake, stream segment, estuary, etc), listed as "impaired" under section 303(d) of the Clean Water Act. Each state water quality agency maintains a list of waters that are impaired. Most state agencies publish these lists online. The EPA's Water Locator Tool may also help you identify if the nearest receiving water is impaired (www.epa.gov/npdes/msgp). If you discharge into a stream segment that is upstream of a listed impaired water but which is not itself on the State's impaired waters list, answer "no" to this question. In this case, requirements in the MSGP for discharges into impaired waters do not apply to you, unless notified otherwise by EPA.

Answer the following three questions only if you answered "Yes" to D 2.b:

2b1. Provide the pollutant(s) listed as causing the impairment in the water identified in D.2.b.1 above. Enter each pollutant individually on a separate row in the table.

2b2. Out of the pollutant(s) that you identified in D.2.b.1 above, indicate which pollutants you believe will be present in your discharge. If you do not expect the pollutant(s) to be in your discharge, then select "no."

2b3.Indicate the pollutant(s) that have a Total Maximum Daily Load (TMDL) for the impaired stream segment that you identified in D.2.b.2 above. Check with your state water quality agency for lists of waters with approved or established TMDLs. See www.epa.gov/npdes/msgp for more information.

3. Water Quality Standards

3a.If you selected "no" in C.2 indicating that stormwater discharges from your facility have not been previously covered under an NPDES permit, then you are considered a new discharger and must answer this question; otherwise you are considered an existing discharger and may skip this question. State water quality agencies are responsible for setting water quality standards for waters within the state's boundaries. Check EPA's website (www.epa.gov/npdes/msgp) to determine if the water(s) that you discharge into are designated as a "Tier 2 (or Tier 2.5) water" (See Appendix A of the MSGP 2008 for definitions of "Tier 2 water" and "Tier 2.5 water"). If you discharge into these waters, EPA may impose additional permit conditions to ensure that you do not violate the State's antidegradation policy.

3.b Idenitfy whether your receiving water is designated as a Tier 3 waterbody. Go to www.epa.gov/npdes/msgp for a list of Tier 3 waterbodies. Note that new discharges into designated Tier 3 waters are not eligible for coverage under the MSGP 2008.

4. Federal Effluent Limitation Guidelines and Sector-Specific Requirements

4.a-b. Depending on your industrial activities, your facility may be subject to effluent limitation guidelines which include additional effluent limits and monitoring requirements for your facility. Please review these requirements, described in Part 2.1.3 of the MSGP, and check any appropriate boxes on the NOI form.

4.c. For Sector S facilities (Air Transportation), indicate whether you anticipate that the entire airport facility will use more than 100,000 gallons of glycol-based deicing/anti-icing chemicals and/or 100 tons or more of urea on an average annual basis. If so, additional effluent limits and monitoring conditions apply to your discharge (see Part 8 Sector S of the MSGP 2008).

5. List the four-digit Standard Industrial Classification (SIC) code and/or two character activity code that best describes the primary industrial activities performed by your facility under which you are required to obtain permit coverage. Your primary industrial activity includes any activities performed on-site which are (1) identified by the facility's one SIC code for which the facility is primarily engaged; and (2) included in the narrative descriptions of 40 CFR 122.26(b)(14)(i), (iv), (v), or (vii), and (ix). See Appendix D of the MSGP for a complete list of SIC codes and activities codes.

6. If your site has co-located industrial activities that are not identified as your primary industrial activity, identify the sector and subsector codes that describe these other industrial activities. For a complete list of sector and subsector codes, see Appendix D of the MSGP.

7.a-b Indicate whether your facility is currently inactive and unstaffed. If so then indicate whether your facility will be inactive and unstaffed for the entire permit term, or if not, specify the specific length of time in units of days, weeks, months, or years (e.g. 3 months) that you expect the facility to be inactive and unstaffed.

Section E. Facility Contact Information and SWPPP Location

1.a-c. Identify the name, telephone number, and email address of the person who will serve as a contact for EPA on issues related to stormwater management at your facility. This person should be able to answer questions related to stormwater discharges, the SWPPP, and other issues related to stormwater permit coverage, or have immediate access to individuals with that knowledge. This person does not have to be the facility operator, but should have intimate knowledge of stormwater management activities at the facility.

2. If you are making your Stormwater Pollution Prevention Plan publicly available on a website provide the appropriate Internet URL address. (Please note that by posting your SWPPP on the web, you may qualify for a shortened authorization waiting period. See Table 1-2 of the MSGP for more information.)

Section F. Endangered Species Protection

1. Based on the instruction provided in Appendix E of the MSGP 2008, indicate which permit criterion (A,B,C,D,E, or F) listed in Part 1.1.4.5 you are using to satisfy your eligibility obligations for protection of endangered and threatened species, and designated critical habitat.

2.a. If you select criterion E (not likely to adversely affect), list those federally-listed endangered or threatened species and any federally-listed designated critical habitat expected to exist in proximity to your facility.

2.b List the pollutants that you expect to be present in your stormwater discharge. Include any pollutants that you may have included in D.2.b.3 above.

2.c If you selected "yes" in C.2 then you are considered an existing discharger and must answer all the questions in F.2.c.1--5; otherwise you are considered a new discharger and may skip the questions under F.2.c. If you are an existing discharger who was previously covered under the MSGP 2000, indicate whether you have any previous effluent monitoring data.

2.c1-2.If you select "No," to F.2.c then indicate why you don't have any data. Also indicate if you have any other data characterizing pollutants in your stormwater discharge.

2.c.3. If you select "Yes," to F.2.c then indicate whether you exceeded any benchmark.

2.c.4 Indicate whether you have exceeded any applicable effluent limitation guideline, or caused or contributed to an exceedance of state or tribal water quality requirement(s).

2.c.5. If you select "Yes" to F.2.c.3.and/or F.2.c.4 then indicate the pollutant parameters for which you exceeded the benchmark, applicable effluent limitation guideline, or State or Tribal water quality requirement(s).

2.d. Attach your supporting rationale for your determination of the applicability of Criterion E for your facility (applies to both new and existing dischargers). Your documentation should address species and habitat listed in F.2.a and the potential effects of pollutants listed in F.2.b on the listed species and habitat. This should include consideration of any available data characterizing pollutants in your stormwater discharge, or in the discharge of similar facilities if data for you facility is not available, that may be of concern to listed species.

3. If you select Criterion F (already addressed in another operator's valid certification), provide the tracking number that the operator received in their confirmation letter or email from EPA's NOI Processing Center (see Appendix E). You can find the tracking number assigned to your previous NOI on EPA's NOI Search website (www.epa.gov/npdes/noisearch). An example where criterion F may apply includes airports where several individual airlines have applied for coverage under the MSGP, and the entire airport also has applied for or obtained coverage. If the airport has already certified under Appendix E, and that certification addresses any potential impacts from the individual airlines, then the airlines may reference the airport's permit tracking number.

Section G. Historic Preservation

Based on the instruction provided in Appendix F of the MSGP 2008, indicate which permit criterion (A, B, C, or D) listed in Part 1.1.4.6 of the MSGP you used to satisfy your eligibility obligations for protection of historic properties.

Section H. Certification

Certification statement and signature (see Section B.11 of Appendix B of the MSGP for more information). Enter certifier's printed name, title and email address. Sign and date the form. (CAUTION: An unsigned or undated NOI form will prevent the granting of permit coverage.) Federal statutes provide for severe penalties for submitting false information on this application form. Federal regulations require this application to be signed as follows:

For a corporation: by a responsible corporate officer, which means:

(i) president, secretary, treasurer, or vice-president of the corporation in charge of a principal business function, or any other person who performs similar policy or decision making functions for the corporation, or

(ii) the manager of one or more manufacturing, production, or operating facilities, provided the manager is authorized to make management decisions which govern the operation of the regulated facility including having the explicit or implicit duty of making major capital investment recommendations, and initiating and directing other comprehensive measures to assure long term environmental compliance with environmental laws and regulations; the manager can ensure that the necessary systems are established or actions taken to gather complete and accurate information for permit application requirements; and where authority to sign documents has been assigned or delegated to the manager in accordance with corporate procedures;

For a partnership or sole proprietorship: by a general partner or the proprietor; or

For a municipal, State, Federal, or other public facility: by either a principal executive or ranking elected official.

If the NOI was prepared by someone other than the certifier (for example, if the NOI was prepared by the facility SWPPP contact or a consultant for the certifier's signature), include the name, organization, phone number and email address of the NOI preparer.

Paperwork Reduction Act Notice

Public reporting burden for this certification is estimated to average 3.7 hours per certification, including time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Burden means the total time, effort, or financial resources expended by persons to generate, maintain, retain, or disclose to provide information to or for a Federal agency. This includes the time needed to review instructions; develop, acquire, install, and utilize technology and systems for the purposes of collecting, validating, and verifying information, processing and maintaining information, and disclosing and providing information; adjust the existing ways to comply with any previously applicable instructions and requirements; train personnel to be able to respond to a collection of information; search data sources; complete and review the collection of information; and transmit or otherwise disclose the information. An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number. Send comments regarding the burden estimate, any other aspect of the collection of information, or suggestions for improving this form, including any suggestions which may increase or reduce this burden to: Director, Office of Environmental Information Services, Collection Services Division (2823), USEPA, 1200 Pennsylvania Avenue, NW, Washington, DC 20460. Include the OMB control number of this form on any correspondence. Do not send the completed NOI form to this address.

Appendix H
Notice of Termination (NOT) Form

Appendix H – Notice of Termination (NOT) Form

To terminate coverage under this permit, you must submit a Notice of Termination (NOT). You must either (1) terminate coverage using EPA's online eNOI system, available at www.epa.gov/npdes/eNOI or (2) file a paper copy of the NOT, a copy of which follows.

This Form Replaces Previous Form 2040-0086 (Please See Instructions Before Completing This Form)

NPDES FORM 3510-7

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460
NOTICE OF TERMINATION (NOT) OF COVERAGE UNDER A NPDES GENERAL PERMIT FOR STORMWATER DISCHARGES ASSOCIATED WITH INDUSTRIAL ACTIVITY

Form Approved.
OMB No. 2040-0086

Submission of this Notice of Termination (NOT) constitutes notice that the party identified in Section B of this form is no longer authorized to discharge stormwater associated with industrial activity under the NPDES program for the facility identified in Section C of this form. All necessary information must be included on this form. Refer to the instructions at the end of this form.

A. Permit Number:

1. NPDES Permit Tracking Number:

2. Reason for Termination (check one only):
   - a. ☐ You transferred operational control to another operator.
   - b. ☐ You no longer have a stormwater discharge associated with industrial activity subject to regulation under the NPDES program, and you have already implemented necessary sediment and erosion controls as required by Part 2.1.2.5.
   - c. ☐ You are a Sector G, H, or J facility and you have met the applicable termination requirements.
   - d. ☐ You obtained coverage under an alternative NPDES permit.

B. Facility Operator Information

1. Name:

2. IRS Employer Identification Number (EIN): –

3. Mailing Address:

a. Street:

b. City: c. State: d. Zip Code: -

e. Phone: - - f. Fax (optional): - - g. E-mail: _____

C. Facility Information

1. Facility Name:

2. Location Address:

a. Street

b. City:

c. County or similar government subdivision: d. State: e. Zip Code: -

D. Certifier Name and Title

I certify under penalty of law that I have met at least one of the reasons for terminating permit coverage listed in Section A.2 above. I understand that by submitting this Notice of Termination, I am no longer authorized to discharge stormwater associated with industrial activity under this general permit, and that discharging pollutants in stormwater associated with industrial activity to waters of the United States is unlawful under the Clean Water Act where the discharge is not authorized by a NPDES permit. I also understand that the submittal of this Notice of Termination does not release an operator from liability for any violations of this permit or the Clean Water Act.

Print Name:

Title:

Signature: _____ Date:

E-mail: _____

Instructions for Completing the Notice of Termination for Stormwater Discharges Associated with INDUSTRIAL ACTIVITY under the Multi-Sector General Permit (MSGP)

Who May File Notice of Termination (NOT) Form

Permittees currently covered by EPA's NPDES Stormwater Multi-Sector General Permit may submit a Notice of Termination (NOT) form. You must submit an NOT within 30 days after one or more of the following conditions have been met:

- a new owner or operator has assumed responsibility for the facility; or
- you have ceased operations at the facility and there are not or no longer will be discharges of stormwater associated with industrial activity from the facility, and you have already implemented necessary sediment and erosion controls as required by Part 2.1.2.5;
- you are a Sector G, H, or J facility and you have met the applicable termination requirements; or
- you have obtained coverage under an individual or alternative general permit for all discharges required to be covered by an NPDES permit.

See the MSGP Part 1.4 for more information.

Where to File NOT form

EPA encourages you to complete the NOT form online, via the Internet. The Electronic Notice of Intent System (eNOI) is found at www.epa.gov/npdes/eNOI. If you cannot access the electronic system, you must send the NOT to the address listed below.

NOTs sent regular mail:
Stormwater Notice of Termination (4203M)
USEPA
1200 Pennsylvania Avenue, NW
Washington, D.C. 20460

NOTs sent overnight/express
Stormwater Notice of Termination
US EPA East Building, Rm 7420
1201 Constitution Avenue, NW
Washington, D.C. 20004
(202) 564-9545

Completing the Form

To complete this form, type or print in uppercase letters in the appropriate areas only. Please make sure you complete all questions. Make sure you make a photocopy for your records before you send the completed original form to the address above. Please use ink when you sign the original document – DO NOT send copies. If you have any questions about this form, you may call the EPA's Stormwater Notice Processing Center at (866) 352-7755.

Section A. Permit Information

1. Enter the NPDES tracking number assigned by EPA's Stormwater Notice Processing Center to the facility. If you do not know the tracking number, you can find the tracking number assigned to your previous NOI on EPA's NOI Search website (www.epa.gov/npdes/noisearch).

2. Indicate your reason for submitting this Notice of Termination by checking the appropriate box (see MSGP Part 1.4 for more information).

Section B. Facility Operator Information

1. Give the legal name of the person, firm, public organization, or any other entity that operates the facility described in this application. The operator of the facility is the legal entity which controls the facility's operation, rather than the plant or site manager. Do not use a colloquial name.

2-3. Enter the facility operator's IRS Employer Identification Number (also know as the tax payer ID number). Enter the complete mailing address, email address and telephone number of the operator. This address will be used for any future correspondence between EPA and the facility operator.

Section C. Facility Information

1-2. Enter the facility's official or legal name and complete address, including city, county or similar government subdivision, state, and ZIP code.

Section D. Certification

Certification statement and signature (see Section B.11 of Appendix B of the MSGP for more information). Enter certifier's printed name, title and email address. Sign and date the form. Federal statutes provide for severe penalties for submitting false information on this application form. Federal regulations require this application to be signed as follows:

For a corporation: by a responsible corporate officer, which means: (i) president, secretary, treasurer, or vice-president of the corporation in charge of the principal business function, or any other person who performs similar policy or decision making functions, or (ii) the manager of one or more manufacturing, production, or operating facilities employing more than 250 persons or having gross annual sales or expenditures exceeding $25 million (in second-quarter 1980 dollars), if authority to sign documents has been assigned or delegated to the manager in accordance with corporate procedures;

For a partnership or sole proprietorship: by a general partner or the proprietor; or

For a municipality State, Federal, or other facility: by either a principal executive office or ranking elected official.

Paperwork Reduction Act Notice

Public reporting burden for this application is estimated to average 0.5 hours per application, including time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding the burden estimate, any other aspect of the collection of information, or suggestions for improving this form, including any suggestions which may increase or reduce this burden to: Director, Office of Environmental Information Services, Collection Services Division (2823), USEPA, 1200 Pennsylvania Avenue, NW, Washington, DC 20460. Include the OMB control number of this form on any correspondence. Do not send the completed NOT form to this address.

Appendix I
Annual Reporting Form

NPDES Permit Tracking No.:

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

Annual Reporting Form

A. GENERAL INFORMATION

1. Facility Name:

2. NPDES Permit Tracking No.:

3. Facility Physical Address:

a. Street:

b. City: c. State: d. Zip Code: -

4. Lead Inspectors Name: Title:

Additional Inspectors Name(s):

5. Contact Person: Title:

Phone: - - Ext. E-mail:

6. Inspection Date: / /

B. GENERAL INSPECTION FINDINGS

1. As part of this comprehensive site inspection, did you inspect all potential pollutant sources, including areas where industrial activity may be exposed to stormwater?
☐ YES ☐ NO

If NO, describe why not:

NOTE: *Complete Section C of this form for each industrial activity area inspected and included in your SWPPP or as newly identified in B.2 or B.3 below where pollutants may be exposed to stormwater.*

2. Did this inspection identify any stormwater or non-stormwater outfalls not previously identified in your SWPPP? ☐ YES ☐ NO

If YES, for each location, describe the sources of those stormwater and non-stormwater discharges and any associated control measures in place:

NPDES Permit Tracking No.:

3. Did this inspection identify any sources of stormwater or non-stormwater discharges not previously identified in your SWPPP? ☐ YES ☐ NO

If YES, describe these sources of stormwater or non-stormwater pollutants expected to be present in these discharges, and any control measures in place:

4. Did you review stormwater monitoring data as part of this inspection to identify potential pollutant hot spots? ☐ YES ☐ NO ☐ NA, no monitoring performed

If YES, summarize the findings of that review and describe any additional inspection activities resulting from this review:

5. Describe any evidence of pollutants entering the drainage system or discharging to surface waters, and the condition of and around outfalls, including flow dissipation measures to prevent scouring:

6. Have you taken or do you plan to take any corrective actions, as specified in Part 3 of the permit, since your last annual report submission (or since you received authorization to discharge under this permit if this is your first annual report), including any corrective actions identified as a result of this annual comprehensive site inspection?

☐ YES ☐ NO

If YES, how many conditions requiring review for correction action as specified in Parts 3.1 and 3.2 were addressed by these corrective actions? ☐☐

NOTE: *Complete the attached Corrective Action Form (Section D) for each condition identified, including any conditions identified as a result of this comprehensive stormwater inspection.*

NPDES Permit Tracking No.:

C. INDUSTRIAL ACTIVITY AREA SPECIFIC FINDINGS

Complete one block for each industrial activity area where pollutants may be exposed to stormwater. Copy this page for additional industrial activity areas.

In reviewing each area, you should consider:

- Industrial materials, residue, or trash that may have or could come into contact with stormwater;
- Leaks or spills from industrial equipment, drums, tanks, and other containers;
- Offsite tracking of industrial or waste materials from areas of no exposure to exposed areas; and
- Tracking or blowing of raw, final, or waste materials from areas of no exposure to exposed areas.

INDUSTRIAL ACTIVITY AREA _____:

1. Brief Description**:**

2. Are any control measures in need of maintenance or repair? ☐ YES ☐ NO

3. Have any control measures failed and require replacement? ☐ YES ☐ NO

4. Are any additional/revised control measures necessary in this area? ☐ YES ☐ NO

If YES to any of these three questions, provide a description of the problem: (Any necessary corrective actions should be described on the attached Corrective Action Form)

INDUSTRIAL ACTIVITY AREA _____:

1. Brief Description:

2. Are any control measures in need of maintenance or repair? ☐ YES ☐ NO

3. Have any control measures failed and require replacement? ☐ YES ☐ NO

4. Are any additional/revised c necessary in this area? ☐ YES ☐ NO

If YES to any of these three questions, provide a description of the problem: (Any necessary corrective actions should be described on the attached Corrective Action Form)

INDUSTRIAL ACTIVITY AREA _____:

Brief Description:

2. Are any control measures in need of maintenance or repair? ☐ YES ☐ NO

3. Have any control measures failed and require replacement? ☐ YES ☐ NO

4. Are any additional/revised BMPs necessary in this area? ☐ YES ☐ NO

If YES to any of these three questions, provide a description of the problem: (Any necessary corrective actions should be described on the attached Corrective Action Form)

NPDES Permit Tracking No.: ☐☐☐☐☐☐☐☐☐☐

NOTE: *Copy this page and attach additional pages as necessary*

INDUSTRIAL ACTIVITY AREA ______:

1. Brief Description:

2. Are any control measures in need of maintenance or repair? ☐ YES ☐ NO
3. Have any control measures failed and require replacement? ☐ YES ☐ NO
4. Are any additional/revised BMPs necessary in this area? ☐ YES ☐ NO

If YES to any of these three questions, provide a description of the problem: (Any necessary corrective actions should be described on the attached Corrective Action Form)

INDUSTRIAL ACTIVITY AREA ______:

1. Brief Description:

2. Are any control measures in need of maintenance or repair? ☐ YES ☐ NO
3. Have any control measures failed and require replacement? ☐ YES ☐ NO
4. Are any additional/revised BMPs necessary in this area? ☐ YES ☐ NO

If YES to any of these three questions, provide a description of the problem: (Any necessary corrective actions should be described on the attached Corrective Action Form)

INDUSTRIAL ACTIVITY AREA ______:

1. Brief Description:

2. Are any control measures in need of maintenance or repair? ☐ YES ☐ NO
3. Have any control measures failed and require replacement? ☐ YES ☐ NO
4. Are any additional/revised BMPs necessary in this area? ☐ YES ☐ NO

If YES to any of these three questions, provide a description of the problem: (Any necessary corrective actions should be described on the attached Corrective Action Form)

NPDES Permit Tracking No.:

D. CORRECTIVE ACTIONS

Complete this page for each specific condition requiring a corrective action or a review determining that no corrective action is needed. Copy this page for additional corrective actions or reviews.

Include both corrective actions that have been initiated or completed since the last annual report, and future corrective actions needed to address problems identified in this comprehensive stormwater inspection. Include an update on any outstanding corrective actions that had not been completed at the time of your previous annual report.

1. Corrective Action # ☐☐ of ☐☐ for this reporting period.

2. Is this corrective action:

- ☐ An update on a corrective action from a previous annual report; or
- ☐ A new corrective action?

3. Identify the condition(s) triggering the need for this review:

- ☐ Unauthorized release or discharge
- ☐ Numeric effluent limitation exceedance
- ☐ Control measures inadequate to meet applicable water quality standards
- ☐ Control measures inadequate to meet non-numeric effluent limitations
- ☐ Control measures not properly operated or maintained
- ☐ Change in facility operations necessitated change in control measures
- ☐ Average benchmark value exceedance
- ☐ Other (describe): _____

4. Briefly describe the nature of the problem identified:

5. Date problem identified: ☐☐/☐☐/☐☐☐☐

6. How problem was identified:

- ☐ Comprehensive site inspection
- ☐ Quarterly visual assessment
- ☐ Routine facility inspection
- ☐ Benchmark monitoring
- ☐ Notification by EPA or State or local authorities
- ☐ Other (describe): _____

7. Description of corrective action(s) taken or to be taken to eliminate or further investigate the problem (e.g., describe modifications or repairs to control measures, analyses to be conducted, etc.) or if no modifications are needed, basis for that determination:

8. Did/will this corrective action require modification of your SWPPP? ☐ YES ☐ NO

9. Date corrective action initiated: ☐☐/☐☐/☐☐☐☐

10. Date correction action completed: ☐☐/☐☐/☐☐☐☐ or expected to be completed: ☐☐/☐☐/☐☐☐☐

11. If corrective action not yet completed, provide the status of corrective action at the time of the comprehensive site inspection and describe any remaining steps (including timeframes associated with each step) necessary to complete corrective action:

NPDES Permit Tracking No.:

E. ANNUAL REPORT CERTIFICATION

1. Compliance Certification

Do you certify that your annual inspection has met the requirements of Part 4.2 of the permit, and that, based upon the results of this inspection, to the best of your knowledge, you are in compliance with the permit? ☐ YES ☐ NO

If NO, summarize why you are not in compliance with the permit:

2. Annual Report Certification

I certify under penalty of law that this document and all attachments were prepared under my direction or supervision in accordance with a system designed to assure that qualified personnel properly gathered and evaluated the information submitted. Based on my inquiry of the person or persons who manage the system, or those persons directly responsible for gathering the information, the information submitted is, to the best of my knowledge and belief, true, accurate, and complete. I am aware that there are significant penalties for submitting false information, including the possibility of fine and imprisonment for knowing violations.

Authorized Representative Printed Name: Title:

Signature: Date Signed:

Appendix J
Calculating Hardness in Receiving Waters for Hardness Dependent Metals

Appendix J. Calculating Hardness in Receiving Waters for Hardness Dependent Metals

Overview

EPA adjusted the benchmarks for six hardness-dependent metals (i.e., cadmium, copper, lead, nickel, silver, and zinc) to further ensure compliance with water quality standards and provide additional protection for endangered species and their critical habitat. For any sectors required to conduct benchmark samples for a hardness-dependent metal, EPA includes 'hardness ranges' from which benchmark values are determined. To determine which hardness range to use, you must collect data on the hardness of your receiving water(s). Once the site-specific hardness data have been collected, the corresponding benchmark value for each metal is determined by comparing where the hardness data fall within 25 mg/L ranges, as shown in Table 1.

Table 1. ***Hardness Ranges to Be Used to Determine Benchmark Values for Cadmium, Copper, Lead, Nickel, Silver, and Zinc.***

All Units mg/L	Benchmark Values (mg/L, total)					
	Cadmium	Copper	Lead	Nickel	Silver	Zinc
0-25 mg/L	0.0005	0.0038	0.014	0.15	0.0007	0.04
25-50 mg/L	0.0008	0.0056	0.023	0.20	0.0007	0.05
50-75 mg/L	0.0013	0.0090	0.045	0.32	0.0017	0.08
75-100 mg/L	0.0018	0.0123	0.069	0.42	0.0030	0.11
100-125 mg/L	0.0023	0.0156	0.095	0.52	0.0046	0.13
125-150 mg/L	0.0029	0.0189	0.122	0.61	0.0065	0.16
150-175 mg/L	0.0034	0.0221	0.151	0.71	0.0087	0.18
175-200 mg/L	0.0039	0.0253	0.182	0.80	0.0112	0.20
200-225 mg/L	0.0045	0.0285	0.213	0.89	0.0138	0.23
225-250 mg/L	0.0050	0.0316	0.246	0.98	0.0168	0.25
250+ mg/L	0.0053	0.0332	0.262	1.02	0.0183	0.26

How to Determine Hardness for Hardness-Dependent Parameters.

You may select one of three methods to determine hardness, including; individual grab sampling, grab sampling by a group of operators which discharge to the same receiving water, or using third-party data. Regardless of the method used, you are responsible for documenting the procedures used for determining hardness values. Once the hardness value is established, you are required to include this information in your first benchmark report submitted to EPA so that the Agency can make appropriate comparisons between your benchmark monitoring results and the corresponding benchmark. You must retain all report and monitoring data in accordance with Part 7.5 of the permit. The three method options for determining hardness are detailed in the following sections.

(1) Permittee Samples for Receiving Stream Hardness

This method involves collecting samples in the receiving water and submitting these to a laboratory for analysis. If you elect to sample your receiving water(s) and submit samples for

analysis, hardness must be determined from the closest intermittent or perennial stream downstream of your point of discharge. The sample can be collected during either dry or wet weather. Collection of the sample during wet weather is more representative of conditions during stormwater discharges; however, collection of in-stream samples during wet weather events may be impracticable or present safety issues.

Hardness must be sampled and analyzed using approved methods as described in 40 CFR Part 136 (Guidelines Establishing Test Procedures for the Analysis of Pollutants).

(2) Group Monitoring for Receiving Stream Hardness

You can be part of a group of permittees discharging to the same receiving waters and collect samples that are representative of the hardness values for all members of the group. In this scenario, hardness of the receiving water must be determined using 40 CFR Part 136 procedures and the results shared by group members. To use the same results, hardness measurements must be taken on a stream reach within a reasonable distance of the discharge points of each of the group members.

(3) Collection of Third-Party Hardness Data

You can submit receiving stream hardness data collected by a third party provided the results are collected consistent with the approved 40 CFR Part 136 methods. These data may come from a local water utility, previously conducted stream reports, TMDLs, peer reviewed literature, other government publications, or data previously collected by the permittee. Data should be less than 10 years old.

Water quality data for many of the nation's surface waters are available on-line or by contacting EPA or a state environmental agency. EPA's data system STORET, short for STOrage and RETrieval, is a repository for receiving water quality, biological, and physical data and is used by state environmental agencies, EPA and other federal agencies, universities, private citizens, and many others. Similarly, state environmental agencies and the U.S. Geological Service (USGS) also have water quality data available that, in some instances, can be accessed online. "Legacy STORET" codes for hardness include: 259 hardness, carbonate; 260 hardness, noncarbonated; and 261 calcium + magnesium, while more recent, "Modern STORET" data codes include: 00900 hardness, 00901 carbonate hardness, and 00902 noncarbonate hardness; or the discrete measurements of calcium (00915) and magnesium (00925) can be used to calculate hardness. Hardness data historically has been reported as "carbonate," "noncarbonate," or "Ca + Mg." If these are unavailable, then individual results for calcium (Ca) and magnesium (Mg) may be used to calculate hardness using the following equation:

$$\text{mg/L } CaCO_3 = 2.497 \text{ (Ca mg/L)} + 4.118 \text{ (Mg mg/L)}$$

When interpreting the data for carbonate and non-carbonate hardness, note that total hardness is equivalent to the sum of carbonate and noncarbonate hardness if both forms are reported. If only carbonate hardness is reported, it is more than likely that noncarbonate hardness is absent and the total hardness is equivalent to the available carbonate hardness.

Appendix K
No Exposure Certification Form

NPDES FORM 3510-11

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

NO EXPOSURE CERTIFICATION FOR EXCLUSION FROM NPDES STORMWATER PERMITTING

Form Approved
OMB No. 2040-0211

Submission of this No Exposure Certification constitutes notice that the entity identified in Section A does not require permit authorization for its stormwater discharges associated with industrial activity in the State identified in Section B under EPA's Stormwater Multi Sector General Permit due to the existence of a condition of no exposure.

A condition of no exposure exists at an industrial facility when all industrial materials and activities are protected by a storm resistant shelter to prevent exposure to rain, snow, snowmelt, and/or runoff. Industrial materials or activities include, but are not limited to, material handling equipment or activities, industrial machinery, raw materials, intermediate products, by-products, final products, or waste products. Material handling activities include the storage, loading and unloading, transportation, or conveyance of any raw material, intermediate product, final product or waste product. A storm resistant shelter is not required for the following industrial materials and activities:

- drums, barrels, tanks, and similar containers that are tightly sealed, provided those containers are not deteriorated and do not leak. "Sealed" means banded or otherwise secured and without operational taps or valves;
- adequately maintained vehicles used in material handling; and
- final products, other than products that would be mobilized in stormwater discharges (e.g., rock salt).

A No Exposure Certification must be provided for each facility qualifying for the no exposure exclusion. In addition, the exclusion from NPDES permitting is available on a facility-wide basis only, not for individual outfalls. If any industrial activities or materials are or will be exposed to precipitation, the facility is not eligible for the no exposure exclusion.

By signing and submitting this No Exposure Certification form, the entity in Section A is certifying that a condition of no exposure exists at its facility or site, and is obligated to comply with the terms and conditions of 40 CFR 122.26(g).

ALL INFORMATION MUST BE PROVIDED ON THIS FORM.

Detailed instructions for completing this form and obtaining the no exposure exclusion are provided on pages 3 and 4.

A. Facility Operator Information

1. Name: 2. Phone: ___ - ___ - ____

3. Email:

4. Mailing Address: a. Street

b. City: c. State d. Zip Code: ____ – ____

B. Facility/Site Location Information

1. Facility Name:

2. a. Street Address:

b. City: c. County:

d. State: e. Zip Code: ____ – ____

3. Is the facility located on Indian Lands? ☐ YES ☐ NO

4. Is this a Federal facility? ☐ YES ☐ NO

5. a. Latitude: ° ' " b. Longitude: ° ' "

6. a. Was the facility or site previously covered under an NPDES stormwater permit? ☐ YES ☐ NO

b. If yes, enter NPDES permit number or tracking number:

7. SIC/Activity Codes: Primary: Secondary (if applicable):

8. Total size of site associated with industrial activity: acres

9. a. Have you paved or roofed over a formerly exposed, pervious area in order to qualify for the no exposure exclusion? ☐ YES ☐ NO

b. If yes, please indicate approximately how much area was paved or roofed over. Completing this question does not disqualify you for the no exposure exclusion. However, your permitting authority may use this information in considering whether stormwater discharges from your site are likely to have an adverse impact on water quality, in which case you could be required to obtain permit coverage.

Less than one acre ☐ One to five acres ☐ More than five acres ☐

C. Exposure Checklist

Are any of the following materials or activities exposed to precipitation, now or in the foreseeable future? (Please check either "Yes" or "No" in the appropriate box.) **If you answer "Yes" to any of these questions (1) through (11), you are <u>not</u> eligible for the no exposure exclusion.**

	Yes	No
1. Using, storing or cleaning industrial machinery or equipment, and areas where residuals from using, storing or cleaning industrial machinery or equipment remain and are exposed to stormwater	☐	☐
2. Materials or residuals on the ground or in stormwater inlets from spills/leaks	☐	☐
3. Materials or products from past industrial activity	☐	☐
4. Material handling equipment (except adequately maintained vehicles)	☐	☐
5. Materials or products during loading/unloading or transporting activities	☐	☐
6. Materials or products stored outdoors (except final products intended for outside use [e.g., new cars] where exposure to stormwater does not result in the discharge of pollutants)	☐	☐
7. Materials contained in open, deteriorated or leaking storage drums, barrels, tanks, and similar containers	☐	☐
8. Materials or products handled/stored on roads or railways owned or maintained by the discharger	☐	☐
9. Waste material (except waste in covered, non leaking containers [e.g., dumpsters])	☐	☐
10. Application or disposal of process wastewater (unless otherwise permitted)	☐	☐
11. Particulate matter or visible deposits of residuals from roof stacks and/or vents not otherwise regulated (i.e., under an air quality control permit) and evident in the stormwater outflow	☐	☐

D. Certification Statement

I certify under penalty of law that I have read and understand the eligibility requirements for claiming a condition of "no exposure" and obtaining an exclusion from NPDES stormwater permitting.

I certify under penalty of law that there are no discharges of stormwater contaminated by exposure to industrial activities or materials from the industrial facility or site identified in this document (except as allowed under 40 CFR 122.26(g)(2)).

I understand that I am obligated to submit a no exposure certification form once every five years to the NPDES permitting authority and, if requested, to the operator of the local municipal separate storm sewer system (MS4) into which the facility discharges (where applicable). I understand that I must allow the NPDES permitting authority, or MS4 operator where the discharge is into the local MS4, to perform inspections to confirm the condition of no exposure and to make such inspection reports publicly available upon request. I understand that I must obtain coverage under an NPDES permit prior to any point source discharge of stormwater from the facility.

Additionally, I certify under penalty of law that this document and all attachments were prepared under my direction or supervision in accordance with a system designed to assure that qualified personnel properly gathered and evaluated the information submitted. Based on my inquiry of the person or persons who manage the system, or those persons directly responsible for gathering the information, the information submitted is to the best of my knowledge and belief true, accurate and complete. I am aware that there are significant penalties for submitting false information, including the possibility of fine and imprisonment for knowing violations.

Print Name: _____

Print Title: _____

Signature: _____

Date: ___ / ___ / ____
Mo Day Year

Email: _____

Instructions for the NO EXPOSURE CERTIFICATION for Exclusion from NPDES Stormwater Permitting

Who May File a No Exposure Certification

Federal law at 40 CFR Part 122.26 prohibits point source discharges of stormwater associated with industrial activity to waters of the U.S. without a National Pollutant Discharge Elimination System (NPDES) permit. However, NPDES permit coverage is not required for discharges of stormwater associated with industrial activities identified at 40CFR 122.26(b)(14)(i)-(ix) and (xi) if the discharger can certify that a condition of "no exposure" exists at the industrial facility or site.

Stormwater discharges from construction activities identified in 40 CFR 122.26(b)(14)(x) and (b)(15) are not eligible for the no exposure exclusion.

Obtaining and Maintaining the No Exposure Exclusion

This form is used to certify that a condition of no exposure exists at the industrial facility or site described herein. This certification is only applicable in jurisdictions where EPA is the NPDES permitting authority and must be re-submitted at least once every five years.

The industrial facility operator must maintain a condition of no exposure at its facility or site in order for the no exposure exclusion to remain applicable. If conditions change resulting in the exposure of materials and activities to stormwater, the facility operator must obtain coverage under an NPDES stormwater permit immediately.

Where to File the No Exposure Certification Form

No Exposure Forms sent regular mail:

SW No Exposure Certification (4203M)
USEPA
1200 Pennsylvania Avenue, NW
Washington, D.C. 20460

Forms sent overnight/express:

SW No Exposure Certification
US EPA East Building, Rm. 7420
1201 Constitution Avenue, NW
Washington, D.C. 20004
(202) 564-9545

Completing the Form

You must type or print, using uppercase letters, in appropriate areas only. Enter only one character per space (i.e., between the marks). Abbreviate if necessary to stay within the number of characters allowed for each item. Use one space for breaks between words. One form must be completed for each facility or site for which you are seeking to certify a condition of no exposure. Additional guidance on completing this form can be accessed at EPA's website: www.epa.gov/npdes/stormwater. Please make sure you have addressed all applicable questions and have made a photocopy for your records before sending the completed form to the above address.

Section A. Facility Operator Information

1. Provide the legal name of the person, firm, public organization, or any other entity that operates the facility or site described in this certification. The name of the operator may or may not be the same as the name of the facility. The operator is the legal entity that controls the facility's operation, rather than the plant or site manager.
2. Provide the telephone number of the facility operator.
3. Provide the email address of the facility operator.
4. Provide the mailing address of the operator (P.O. Box numbers may be used). Include the city, state, and zip code. All correspondence will be sent to this address.

Section B. Facility/Site Location Information

1. Enter the official or legal name of the facility or site.
2. Enter the complete street address (if no street address exists, provide a geographic description [e.g., Intersection of Routes 9 and 55]), city, county, state, and zip code. Do not use a P.O. Box number.
3. Indicate whether the facility is located on Indian Lands.
4. Indicate whether the industrial facility is operated by a department or agency of the Federal Government (see also Section 313 of the Clean Water Act).
5. Enter the latitude and longitude of the approximate center of the facility or site in degrees/minutes/seconds. Latitude and longitude can be obtained from United States Geological Survey (USGS) quadrangle or topographic maps, by calling 1-(888) ASK-USGS, or by accessing the Census Bureau at: www.census.gov/cgi-bin/gazetteer

   Latitude and longitude for a facility in decimal form must be converted to degrees (°), minutes ('), and seconds (") for proper entry on the certification form. To convert decimal latitude or longitude to degrees/minutes/seconds, follow the steps in the following example.

   Example: Convert decimal latitude 45.1234567 to degrees (°), minutes ('), and seconds (").

   a) The numbers to the left of the decimal point are the degrees: 45°.

   b) To obtain minutes, multiply the first four numbers to the right of the decimal point by 0.006: 1234 x 0.006 = 7.404.

   c) The numbers to the left of the decimal point in the result obtained in (b) are the minutes: 7'.

   d) To obtain seconds, multiply the remaining three numbers to the right of the decimal from the result obtained in (b) by 0.06: 404 x 0.06 = 24.24. Since the numbers to the right of the decimal point are not used, the result is 24".

   e) The conversion for 45.1234567 = 45° 7' 24".
6. Indicate whether the facility was previously covered under an NPDES stormwater permit. If so, include the permit number or permit tracking number.
7. Enter the 4-digit SIC code which identifies the facility's primary activity and second 4-digit SIC code identifying the facility's secondary activity, if applicable. SIC codes can be obtained from the Standard Industrial Classification Manual, 1987.
8. Enter the total size of the site associated with industrial activity in acres. Acreage may be determined by dividing square footage by 43,560, as demonstrated in the following example.

   Example: Convert 54,450 ft^2 to acres

   Divide 54,450 ft^2 by 43,450 square feet per acre:
   54, 450 ft^2 ÷ 43,560 ft^2/acre = 1.25 acres.
9. Check "Yes" or "No" as appropriate to indicate whether you have paved or roofed over a formerly exposed, pervious area (i.e., lawn, meadow, dirt or gravel road/parking lot) in order to qualify for no exposure. If yes, also indicate approximately how much area was paved or roofed over and is now impervious area.

Instructions for the NO EXPOSURE CERTIFICATION for Exclusion from NPDES Stormwater Permitting

Section C. Exposure Checklist

Check "Yes" or "No" as appropriate to describe the exposure condition at your facility. If you answer "Yes" to **ANY** of the questions (1) through (11) in this section, a potential for exposure exists at your site and you cannot certify to a condition of no exposure. You must obtain (or already have) coverage under an NPDES stormwater permit. After obtaining permit coverage, you can institute modifications to eliminate the potential for a discharge of stormwater exposed to industrial activity, and then certify to a condition of no exposure.

Section D. Certification Statement

Federal statutes provide for severe penalties for submitting false information on this application form. Federal regulations require this application to be signed as follows:

For a corporation: by a responsible corporate officer, which means:

(i) president, secretary, treasurer, or vice-president of the corporation in charge of a principal business function, or any other person who performs similar policy or decision making functions for the corporation, or

(ii) the manager of one or more manufacturing, production, or operating facilities, provided the manager is authorized to make management decisions which govern the operation of the regulated facility including having the explicit or implicit duty of making major capital investment recommendations, and initiating and directing other comprehensive measures to assure long term environmental compliance with environmental laws and regulations; the manager can ensure that the necessary systems are established or actions taken to gather complete and accurate information for permit application requirements; and where authority to sign documents has been assigned or delegated to the manager in accordance with corporate procedures;

For a partnership or sole proprietorship: by a general partner or the proprietor, or

For a municipal, State, Federal, or other public facility: by either a principal executive or ranking elected official.

Paperwork Reduction Act Notice

Public reporting burden for this certification is estimated to average 1.0 hour per certification, including time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Burden means the total time, effort, or financial resources expended by persons to generate, maintain, retain, or disclose to provide information to or for a Federal agency. This includes the time needed to review instructions; develop, acquire, install, and utilize technology and systems for the purposes of collecting, validating, and verifying information, processing and maintaining information, and disclosing and providing information; adjust the existing ways to comply with any previously applicable instructions and requirements; train personnel to be able to respond to a collection of information; search data sources; complete and review the collection of information; and transmit or otherwise disclose the information. An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number. Send comments regarding the burden estimate, any other aspect of the collection of information, or suggestions for improving this form, including any suggestions which may increase or reduce this burden to: Director, OPPE Regulatory Information Division (2137), USEPA, 401 M Street, SW, Washington, D.C. 20460. Include the OMB control number of this form on any correspondence. Do not send the completed No Exposure Certification form to this address.

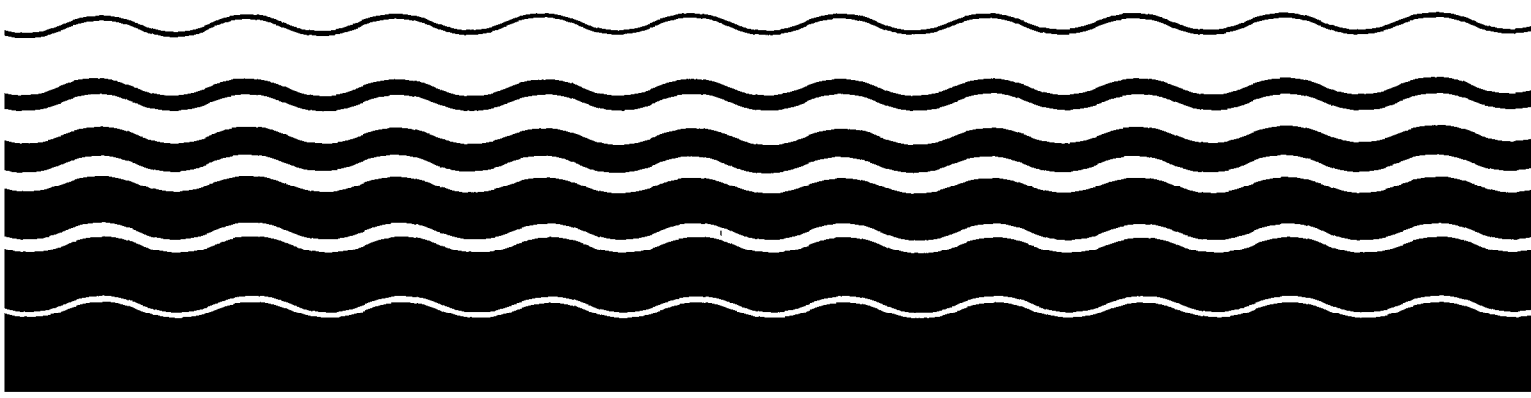
United States
Environmental Protection
Agency

Office Of Water
(EN-336)

EPA 833-B-92-00
July 1992

EPA

NPDES Storm Water Sampling Guidance Document



DISCLAIMER

This document was issued in support of EPA regulations and policy initiatives involving the development and implementation of a national storm water program. This document is agency guidance only. It does not establish or affect legal rights or obligations. Agency decisions in any particular case will be made applying the laws and regulations on the basis of specific facts when permits are issued or regulations promulgated. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

Pollutants in storm water discharges from many sources are largely uncontrolled. The National Water Quality Inventory, 1990 Report to Congress provides a general assessment of water quality based on biennial reports submitted by the States under Section 305(b) of the Clean Water Act. The report indicates that roughly 30% of identified cases of water quality impairment reported by the States are attributable to storm water discharges.

Sampling data from storm water discharges is an important tool which provides information on the types and amounts of pollutants present. This data can then be used to identify pollutant sources and to develop storm water pollution prevention plans and best management practices priorities to control these sources.

This manual is for operators of facilities that discharge storm water associated with industrial activity and operators of large and medium municipal separate storm sewer systems. This manual describes the basic sampling requirements for NPDES storm water discharge permit applications and provides procedural guidance on how to conduct sampling. Many of the concepts in this guidance may also be applicable to sampling requirements contained in NPDES storm water permits.

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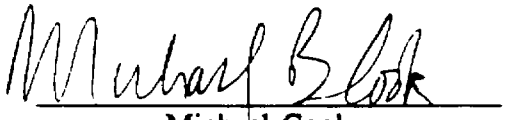
This document is expected to be revised periodically to reflect advances in this rapidly evolving area. Comments from users are welcomed. Send comments to the U.S. Environmental Protection Agency, Office of Wastewater Enforcement and Compliance, 401 M Street, SW, Mailcode EN-336, Washington, DC 20460.



Michael Cook,
Director
Office of Wastewater Enforcement
and Compliance

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1. INTRODUCTION

The 1972 Federal Water Pollution Control Act [(FWPCA), also referred to as the Clean Water Act (CWA)] prohibits the discharge of any pollutant to waters of the U.S. from a point source unless the discharge is authorized by a National Pollutant Discharge Elimination System (NPDES) permit. Efforts to improve water quality under the NPDES program have focused traditionally on reducing pollutants in industrial process wastewater discharges and from municipal sewage treatment plants. Past efforts to address storm water discharges, in particular through the NPDES program, have generally been limited to certain industrial categories, using effluent limitations for storm water as a permit condition.

Recognizing the need for more comprehensive control of storm waters discharges, Congress amended the CWA in 1987 and established a two-phase program. In Phase I, Congress required the U.S. Environmental Protection Agency (EPA) to establish NPDES requirements for certain classes of storm water discharges.

- A storm water discharge for which a permit has been issued prior to February 4, 1987
- A storm water discharge associated with industrial activity
- A storm water discharge from a municipal separate storm sewer system serving a population of 250,000 or more (large system)
- A storm water discharge from a municipal separate storm sewer system serving a population of 100,000 or more, but less than 250,000 (medium system)
- A discharge for which the Administrator or the State determines that the storm water discharge contributes to a violation of a water quality standard or is a significant contributor of pollutants to the waters of the United States.

To implement these requirements, EPA published on November 16, 1990 (55 Fed. Reg. 47990), permit application requirements that include storm water sampling. EPA and the States will subsequently issue NPDES storm water permits based on these applications, and many of these

permits will require storm water sampling. Congress intended for EPA to address all other point source discharges of storm water in Phase II of the program.

1.1 PURPOSE OF THIS MANUAL

This manual is for operators of facilities that discharge storm water associated with industrial activity and operators of large and medium municipal separate storm sewer systems. Storm water sampling is sometimes difficult due to the unpredictability of storm events and the variable nature of storm water discharges. This manual is primarily designed to assist operators/owners in planning for and fulfilling the NPDES storm water discharge sampling requirements for permit applications as well as for other storm water sampling needs.

It is assumed that applicants already have a basic understanding of the storm water permit application requirements. This document is designed to supplement existing storm water application guidance by focusing on the technical aspects of sampling. Since many industrial storm water permits and all municipal storm water permits will require regular storm water sampling, many of the concepts in this guidance may be applicable to sampling requirements contained in NPDES storm water permits.

The information in this manual pertains specifically to individual industrial storm water applications, group storm water applications (Part 2), and municipal Part 2 storm water permit applications for storm water discharges. For information on other storm water application requirements for industrial facilities and large and medium municipal separate storm sewer systems, see EPA's Guidance Manual for the Preparation of NPDES Permit Applications for Storm Water Discharges Associated with Industrial Activity (EPA-505/8-91-002, NTIS # PB-92-199058, April 1991), and EPA's Guidance Manual for the Preparation of Part 1 of the NPDES Permit Applications for Discharges from Municipal Separate Storm Sewer Systems (EPA-505/8-91-003A, NTIS # PB-92-114578, April 1991), respectively. These manuals can be requested by calling the National Technical Information Service (NTIS) **[(703) 487-4650]**. Additional background documents for further information are listed in Technical Appendix D.

1.2 ORGANIZATION OF THIS MANUAL

This manual explains the basic requirements of storm water sampling and provides procedural guidance on sampling for permit applications. Chapter 2 discusses background information (i.e., a

summary of permit application requirements, who must sample, when and where to sample, and staffing considerations). Chapter 3 presents the fundamentals of sampling (i.e., types of sampling, obtaining flow data, handling samples, and sending them to the laboratory). Chapter 4 presents analytical considerations, including the storm water pollutants that must be analyzed under the regulations. Chapter 5 discusses regulatory flexibility with respect to storm water sampling, and Chapter 6 includes health and safety considerations.

Technical Appendices provide information as follows:

- Technical Appendix A—Forms 2F and 1
- Technical Appendix B—NOAA Weather Radio Information
- Technical Appendix C—Required Containers, Preservation Techniques, Holding Times and 40 Code of Federal Regulations (CFR) Part 136
- Technical Appendix D—References
- Technical Appendix E—Glossary
- Technical Appendix F—Acronyms.

2. BACKGROUND FOR STORM WATER SAMPLING

This chapter presents background information, definitions, and a description of the fundamentals of sampling. Specifically, it covers the following areas:

- The benefits of sampling
- A summary of storm water application regulations
- Who must sample
- When sampling is required
- Where to sample
- Staffing considerations

In response to the 1987 Water Quality Act amendments to the CWA, EPA published the storm water final rule on November 16, 1990. In this rule, EPA established the initial scope of the storm water program by defining the phrase "storm water discharge associated with industrial activity" in terms of 11 categories of industrial activity and the phrase "large and medium municipal separate storm sewer systems" to include municipal systems serving a population greater than 100,000. These terms are discussed in greater detail in Section 2.6, "Who Must Sample."

In addition to defining the initial scope of the storm water program, the final rule established permit application requirements, including requirements for storm water sampling. Sampling data gathered for the application will be used to characterize storm water discharges, and will serve as a basis for establishing requirements in NPDES storm water permits. It is important to note that the applicant must report data that are representative of the storm water discharge, and that the intentional misrepresentation of discharge characteristics is unlawful.

2.1 BENEFITS OF SAMPLING

Data that characterize storm water discharges are valuable to permitting authorities and permittees for several reasons. First, storm water sampling provides a means for evaluating the environmental risk of the storm water discharge by identifying the types and amounts of pollutants present. Evaluating these data helps to determine the relative potential for the storm water discharge to contribute to water quality impacts or water quality standard violations. And, storm water sampling

data can be used to identify potential sources of pollutants. These sources can then be either eliminated or controlled more specifically by the permit.

2.2 INDUSTRIAL FACILITY APPLICATION REQUIREMENTS

The storm water permit application regulations provide operators of facilities (including those owned by the government) that have storm water discharges associated with industrial activity with three application options: (1) submit an individual application; (2) participate in a group application (a two-part application); or (3) submit a Notice of Intent (NOI) to be covered by a general permit where general permits are available. This guidance focuses on sampling requirements for individual applications and Part 2 of group applications. Sampling data generally will not be required for an NOI, however, the general permit may require sampling during the term of the permit. State permitting authorities may also require sampling information for an NOI at their discretion, and should, therefore, be consulted prior to submittal.

Industrial facilities submitting individual applications must submit sampling data on a completed application Form 2F (entitled "Application for Permit to Discharge Storm Water Discharges Associated with Industrial Activity"). Facilities selected to be part of the sampling subgroup for a group application must submit sampling data with Part 2 of the application. Members of the sampling subgroup must complete only the quantitative data portions of Form 2F, including Sections VII, VIII, IX, and the certification in Section X. Exhibit 2-1 details the types of information required for each section of Form 2F. Exhibit 2-2 describes what sampling information must be provided in Part 2 of the group application. It should be noted that States may require the use of different forms and submittal of additional documentation.

Form 1 must also be submitted with Form 2F by applicants submitting individual permit applications. General information about the facility is provided on Form 1 (i.e., addresses, operators, etc.); it does not request sampling data. Forms 1 and 2F are reproduced in Technical Appendix A.

Facilities with unpermitted combined discharges of storm water and process or nonprocess wastewater must submit Form 2C or 2E, respectively, in addition to Forms 1 and 2F. Facilities with storm water discharges combined with new sources or new discharges of process wastewater must submit Form 2D as well as Forms 1 and 2F.

EXHIBIT 2-1. FORM 2F APPLICATION REQUIREMENTS	
Section	**Requirement**
2F-I	Outfall location(s), including longitude and latitude and receiving water(s)
2F-II	Facility improvements which may affect the discharges described in the application
2F-III	Site drainage map
2F-IVA	Estimates of impervious area within each outfall drainage area
2F-IVB	A narrative description of pollutant sources (i.e., onsite materials which may come in contact with storm water runoff)
2F-IVC	Location and description of existing structural and nonstructural pollutant control measures
2F-VA	Certification that outfalls have been tested or evaluated for non-storm water discharges
2F-VB	Description of method used for testing/evaluating presence of non-storm water discharges
2F-VI	History of significant leaks or spills of toxic or hazardous pollutants at the facility within the last 3 years
2F-VII	Discharge characterization for all required pollutants
2F-VIII	Statement of whether biological testing for acute or chronic toxicity was performed and list of pollutants it was performed for
2F-IX	Information on contract laboratories or consulting firms
2F-X	Certification that information supplied is accurate and complete
Note: See Form 2F and the instructions for more detail on application requirements.	

2.3 MUNICIPALITIES' APPLICATION REQUIREMENTS

Operators of large and medium municipal separate storm sewer systems are required to submit a two-part application. Both parts contain sampling requirements: Part 1 requires information characterizing discharges from the separate storm sewer system, including field screening sample data for identifying illicit/illegal connections; Part 2 requires sampling at representative locations and estimates of pollutant loadings for those sites. These sampling data are to be used to design a long-term storm water monitoring plan that will be implemented during the term of the permit. The sampling data that must be submitted in Parts 1 and 2 of municipal applications are listed in Exhibit 2-3. There is no standard application form for municipalities.

EXHIBIT 2-2. PART 2 GROUP APPLICATION SAMPLING REQUIREMENTS

Quantitative Testing Data

- For groups with 4 to 20 members, 50 percent of the facilities must submit data; for groups with 21 to 99 members, a minimum of 10 dischargers must submit quantitative data; for groups with 100 to 1,000 members, a minimum of 10 percent of the facilities must submit data; for groups with greater than 1,000 members, no more than 100 facilities must submit data; there must be 2 dischargers from each precipitation zone in which 10 or more members of the group are located, or 1 discharger from each precipitation zone in which 9 or fewer members are located.
- Sampling and analysis requirements are described in 40 Code of Federal Regulations (CFR) 122.26(c)(1)(i)(E) and 40 CFR 122.21(g)(7). Pollutants to be analyzed depend on the type(s) of industries applying as a group.
- Sampling subgroup must provide all quantitative discharge information required in Form 2F Sections VII-IX plus the certification in Section X.
- The group application sampling subgroup must collect grab samples during the first 30 minutes of the storm event and flow-weighted composite samples as required in 40 CFR 122.21(g)(7).

2.4 APPLICATION SUBMITTAL DEADLINES

Deadlines for submitting permit applications and associated sampling requirements are presented in Exhibit 2-4 for individual and group industrial applications and for municipal applications.

2.5 WHERE TO SUBMIT APPLICATIONS

Storm water discharge permit applications are generally submitted directly to the permit-issuing authority. The appropriate authority is the State, where the State has been granted the authority to issue NPDES permits, or the EPA Regional office, where the State does not have NPDES authorization. Exhibit 2-5 indicates which States have approved NPDES permitting programs. It also provides contact names and addresses where applications should be submitted for each State or EPA Regional Office (depending on who the permitting authority is in each case). It should be noted, however, that both parts of a group application must instead be submitted to EPA Headquarters. Group applications must be sent to: Director, Office of Wastewater Enforcement and Compliance, Attention Mr. William Swietlik, U.S. EPA, EN-336, 401 M Street, SW, Washington, DC 20640.

EXHIBIT 2-3. MUNICIPAL APPLICATION SAMPLING REQUIREMENTS

Part 1

- Monthly mean rainfall and snowfall estimates
- Existing quantitative data on the depth and quality of storm water discharges
- A list of receiving water bodies and existing information concerning known water quality impacts
- Field screening analysis for illicit connections and illegal dumping
- Identification of representative outfalls for further sampling in Part 2

Part 2

- Quantitative data from 5 to 10 representative locations in approved sampling plans
- Estimates of the annual pollutant load and event mean concentration (EMC) of system discharges
- Proposed schedule to provide estimates of seasonal pollutant loads and the EMC for certain detected constituents in a representative storm event during the term of the permit
- Proposed monitoring program for representative data collection during the term of the permit

Applications submitted by industrial facilities must be certified by a responsible corporate officer as described in 40 CFR 122.22 (e.g., president, secretary, treasurer, vice president of the corporation in charge of a principal business function). Applications submitted by municipalities must be certified by a principal executive officer or ranking elected official as described in 40 CFR 122.22.

2.6 WHO MUST SAMPLE

Operators of facilities that have storm water discharges associated with industrial activity and operators of large and medium municipalities are required to conduct storm water sampling as part of their NPDES permit applications. Specifically, the following types of industries and municipalities must sample storm water discharges:

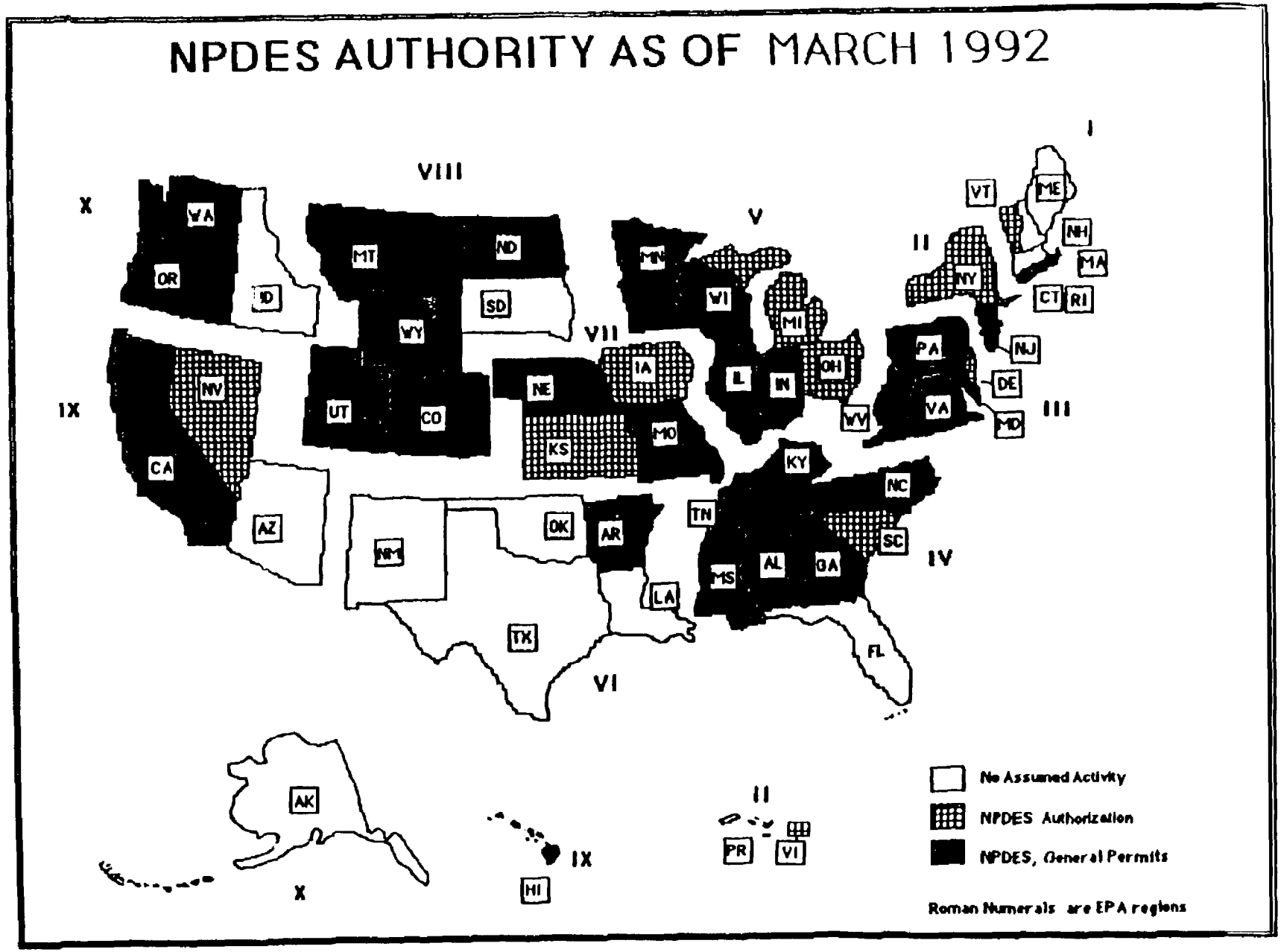
EXHIBIT 2-4 PERMIT APPLICATION SUBMISSION DEADLINES

	Date	Sampling Requirement
Industrial		
Individual	October 1, 1992	Sampling data due
Group		
• Part 1	September 30, 1991	Sampling subgroup identified
• Part 2	October 1, 1992	Sampling data due
Municipal		
Large Municipalities		
• Part 1	November 18, 1991	Illicit connection screening due and identification of sampling points
• Part 2	November 16, 1992	Effluent characterization due
		Monitoring management program identified
Medium Municipalities		
• Part 1	May 18, 1992	Illicit connection screening due and identification of sampling points
• Part 2	May 17, 1993	Effluent characterization due
		Monitoring management program identified

*NOI under a general permit is due on October 1, 1992 or the date specified in the permit, whichever comes first.

- Storm Water Discharges Associated With Industrial Activities - Under Phase I, the storm water permit application regulations identify, by Standard Industrial Classification (SIC) code and narrative description, 11 categories of facilities considered to be "engaging in industrial activity" for the purposes of storm water permit application requirements. Those facilities included in 40 CFR 122.26(b)(14)(i) through (xi) of the storm water permit application regulations with storm water point source discharges to waters of the U.S. or separate storm sewers and those designated under Section 402(p)(2)(E) of the CWA are required to apply for storm water permit coverage by October 1, 1992. Industrial facilities include those that are Federally, State, or municipally owned or operated. Exhibit 2-6 lists these industrial facilities. The Transportation Act of 1991 provides an exemption from storm water permitting requirements for certain industrial activities owned or operated by municipalities with a population of less than 100,000. Such municipalities must submit storm water discharge permit applications for only airports, power plants, and uncontrolled sanitary landfills that they own or operate, unless a permit is otherwise required by the permitting authority.

- Municipal Separate Storm Sewer Systems - Under Phase I, those municipalities with separate storm sewer systems serving 100,000 people or more are required to submit an application for discharges from the system. (Only the part of the population served by municipal separate storm sewers is to be included in the 100,000 count, not the part served by combined sewers.) Regulated municipalities are listed in Appendices F through I in the November 16, 1990, final rule or have been designated by their permitting authority.


NPDES AUTHORITY AS OF MARCH 1992
I
II
III
IV
V
VI
VII
VIII
IX
X
WA
OR
ID
MT
ND
SD
WY
NV
UT
CO
CA
AZ
NM
NE
KS
IA
MO
OK
AR
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LA
MN
WI
MI
IL
IN
OH
WV
KY
TN
MS
AL
GA
FL
SC
NC
VA
PA
NY
VT
ME
NH
MA
CT
RI
NJ
DE
MD
AK
HI
PR
VI
No Assumed Activity
NPDES Authorization
NPDES, General Permits
Roman Numerals are EPA regions

EXHIBIT 2-5. NPDES STORM WATER PROGRAM PERMITTING AUTHORITIES (Continued)

State	Permitting Authority	Contact
Alabama	yes	Aubrey White Water Division 1751 Dickinson Dr. Montgomery, AL 36130 (205) 271-7811
Arizona	no	Eugene Bromley U.S. EPA Region 9 75 Hawthorne St. W-5-1 San Francisco, CA 94105 (415) 744-1906
California	yes	Archie Matthews Storm Water Research Control Board Water Quality 901 P St. Sacramento, CA 95814 (916) 657-1110
Connecticut	yes	Dick Mason Dept. of Environmental Protection Water Management Bureau Water Discharge Management 165 Capitol Ave. Hartford, CT 06106 (203) 566-7167
Florida	no	Chris Thomas U.S. EPA Region 4 345 Courtland St. N.E. 4WM-FP Atlanta, GA 30365 (404) 347-3633
Hawaii	yes	Steve Chang Dept. of Health Clean Water Branch Five Water Front Plaza #500 Ala-Moana Blvd. Honolulu, HI 96813 (808) 586-4309
Illinois	yes	Tim Kluge EPA Water Pollution Control 2200 Churchill Rd. P.O. Box 19276 Springfield, IL 62794-9276 (217) 782-0610
Iowa	yes	Monica Wnuk Department of Natural Resources Wallace State Building 900 E. Grand St. Des Moines, IA 50319-0034 (515) 281-7017
Alaska	no	Steve Bubnick U.S. EPA Region 10 1200 6th Ave. WD-134 Seattle, WA 98101 (206) 553-8399
Arkansas	yes	Maryna Jastrzebski 8001 National Dr. P.O. Box 8913 Little Rock, AR 72219-8913 (501) 562-7444
Colorado	yes	Patricia Nelson Dept. of Health Water Quality Control 4210 E. 11th Ave. Denver, CO 80220 (303) 331-4590
Delaware	yes	Sarah Cooksey Dept. of Natural Resources Surface Water Management 89 Kings Highway P.O. Box 1401 Dover, DE 19903 (302) 739-5731
Georgia	yes	Mike Creason Dept. of Natural Resources Environmental Protection 205 Butler St. S.E. Room 1070 Atlanta, GA 30334 (404) 656-4887
Idaho	no	Steve Bubnick U.S. EPA Region 10 1200 6th Ave. WD-134 Seattle, WA 98101 (206) 553-8399
Indiana	yes	Lonnie Brumfield Dept. of Environmental Management NPDES Permits Group 105 S. Meridian St. P.O. Box 6015 Indianapolis, IN 46206 (317) 232-8705
Kansas	yes	Don Carlson Dept. of Environment Water Bureau Forbes Field, Building 740 Topeka, KS 66620 (913) 296-5555

EXHIBIT 2-5. NPDES STORM WATER PROGRAM PERMITTING AUTHORITIES (Continued)

State	Permitting Authority	Contact
Kentucky	yes	Douglas Allgeier Dept. of Environmental Protection Water Division 18 Reilly Road Frankfort, KY 40601 (502) 564-3410
Maine	no	Shelley Puleo U.S. EPA Region 1 U.S. EPA/JFK Building/WCP Boston, MA 02203 (617) 565-3525
Massachusetts	no	Shelley Puleo U.S. EPA Region 1 U.S. EPA/JFK Building/WCP Boston, MA 02203 (617) 565-3525
Minnesota	yes	Scott Thompson Pollution Control Agency 520 Lafayette Rd. St. Paul, MN 55155-3898 (612) 296-7203
Missouri	yes	Bob Hentges Dept. of Natural Resources Water Pollution Control Program 205 Jefferson St. P.O. Box 176 Jefferson City, MO 65102 (314) 751-6825
Nebraska	yes	Clark Smith Environmental Control Water Quality Division P.O. Box 98922 Lincoln, NE 68509 (402) 471-4239
New Hampshire	no	Shelley Puleo U.S. EPA Region 1 U.S. EPA/JFK Building/WCP Boston, MA 02203 (617) 565-3525
New Mexico	no	Brent Larson U.S. EPA Region 6 1445 Ross Ave. 6W-PM Dallas, TX 75202 (214) 655-7175
Louisiana	no	Brent Larson U.S. EPA Region 6 1455 Ross Ave. 6W-PM Dallas, TX 75202 (214) 655-7175
Maryland	yes	Edward Gertler MD Dept. of Environment Industrial Discharge Program 2500 Broening Highway Baltimore, MD 21224 (410) 631-3323
Michigan	yes	Gary Boersen Dept. of Natural Resources Surface Water Division P.O. Box 30028 Lansing, MI 48909 (517) 373-1982
Mississippi	yes	Jerry Cain Dept. of Environmental Quality Office of Pollution Control Industrial Waste Water Branch P.O. Box 10385 Jackson, MS 39289-0385 (601) 961-5171
Montana	yes	Fred Shewman Water Quality Bureau Cogswell Building Helena, MT 59620 (406) 444-2406
Nevada	yes	Rob Saunders Conservation and Natural Resources Environmental Protection 123 W. Nye Lane Carson City, NV 89710 (702) 687-4670
New Jersey	yes	Sandra Cohen NJ DEPE Office of Regulatory Policy CN029 Trenton, NJ 08625-0029 NJ Hotline: (609) 633-7021
New York	yes	Ken Stevens Wastewater Facilities Design NY State DEC 50 Wolf Road Albany, NY 12233 (518) 457-1157

EXHIBIT 2-5. NPDES STORM WATER PROGRAM PERMITTING AUTHORITIES (Continued)

State	Permitting Authority	Contact
North Carolina	yes	Coleen Sullins Environmental Management Water Quality Planning P.O. Box 29535 Raleigh, NC 27626-0535 (919) 733-5083
Ohio	yes	Bob Phelps OEPA Water Pollution Control P.O. Box 1049 1800 Watermark Columbus, OH 43266 (614) 644-2034
Oregon	yes	Ranei Nomura DEQ-Water Quality 811 SW 6th St. Portland, OR 97204 (503) 229-5256
Puerto Rico	no	José Rivera U.S. EPA Region 2 Water Permits & Compliance Branch 26 Federal Plaza, Room 845 New York, NY 10278 (212) 264-2911
South Carolina	yes	Birgot McDade Dept. of Health & Env. Ctrl. Industry and Agriculture Waste Water Division 2600 Bull St. Columbia, SC 29201 (803) 734-5241
Tennessee	yes	Robert Haley Dept. of Environment Water Pollution Control 150 9th Ave. N., 4th Floor Nashville, TN 37243-1534 (615) 741-3275
Utah	yes	Harry Campbell Dept. of Environmental Quality P.O. Box 16690 Salt Lake City, UT 84116 (801) 538-6146
North Dakota	yes	Sheila McClenathan Dept. of Health Water Quality Division 1200 Missouri Ave. P.O. Box 5520 Bismarck, ND 58502-5520 (701) 221-5210
Oklahoma	no	Brent Larson U.S. EPA Region 6 1445 Ross Ave. 6W-PM Dallas, TX 75202 (214) 655-7175
Pennsylvania	yes	R.B. Patel Environmental Resources Water Quality Management P.O. Box 2063 Harrisburg, PA 17120 (717) 787-8184
Rhode Island	yes	Angela Liberti Division of Water Resources 291 Promenade St. Providence, RI 02908 (401) 277-6519
South Dakota	no	Vern Berry U.S. EPA Region 8 999 18th St. 8-WM-C Denver, CO 80202-2466 (303) 293-1630
Texas	no	Brent Larson U.S. EPA Region 6 1445 Ross Ave. 6W-PM Dallas, TX 75202 (214) 655-7175
Vermont	yes	Brian Koiker Environmental Conservation Permits and Compliance 103 S. Main St. Annex Building Waterbury, VT 05671-0405 (802) 244-5674

EXHIBIT 2-5. NPDES STORM WATER PROGRAM PERMITTING AUTHORITIES (Continued)

State	Permitting Authority	Contact
Virgin Islands	yes	Marc Pacifico Dept. of Planning & Natural Resources 1118 Watergut Project Christiansted St. Croix, VI 00820-5065 (809) 773-0565
Washington	yes	Gary Kruger Dept. of Ecology Water Quality Division P.O. Box 47600 Olympia, WA 98504-7600 (206) 438-7529
West Virginia	yes	Jerry Ray Division of Water Resources 1201 Greenbriar St. Charleston, WV 25311 (304) 348-0375
Wyoming	yes	John Wagner Dept. of Environmental Quality Herschler Building, 4th Floor Cheyenne, WY 82002 (307) 777-7082
Virginia	yes	Burton Tuxford Water Control Board Permits Section P.O. Box 11143 Richmond, VA 23230-1143 (804) 527-5083
Washington D.C.	no	Kevin Magerr U.S. EPA Region 3 841 Chestnut Bldg. 3WM53 Philadelphia, PA 19107 (215) 597-1651
Wisconsin	yes	Anne Maul Dept. of Natural Resources Wastewater Management P.O. Box 7921 Madison, WI 53707 (608) 267-7364

2.7 WHEN SAMPLING IS REQUIRED

Industrial individual and group applicants must include sampling data from at least one representative storm event. Operators of large or medium municipal separate storm sewer systems must submit sampling data from three different representative storm events. How to determine "representativeness" and other considerations for when to sample are presented below.

2.7.1 STORM EVENT CRITERIA

Storm water discharge permit application requirements establish specific criteria for the type of storm event that must be sampled:

- The depth of the storm must be greater than 0.1 inch accumulation
- The storm must be preceded by at least 72 hours of dry weather
- Where feasible, the depth of rain and duration of the event should not vary by more than 50 percent from the average depth and duration.

EXHIBIT 2-6 INDUSTRIAL FACILITIES WHICH MUST SUBMIT APPLICATIONS FOR STORM WATER PERMITS

40 CFR 122.26(b)(14) Subpart	Description
(i)	Facilities subject to storm water effluent limitations guidelines, new source performance standards, or toxic pollutants effluent standards under 40 CFR, Subchapter N [except facilities which are exempt under category (xi)].
(ii)	Facilities classified as: SIC 24 (except 2434) . . . . . Lumber and Wood Products SIC 26 (except 265 and 267) . Paper and Allied Products SIC 28 (except 283 and 285) . Chemicals and Allied Products SIC 29 . . . . . Petroleum and Coal Products SIC 311 . . . . . Leather Tanning and Finishing SIC 32 (except 323) . . . . . Stone, Clay and Glass Products SIC 33 . . . . . Primary Metal Industries SIC 3441 . . . . . Fabricated Structural Metal SIC 373 . . . . . Ship and Boat Building and Repairing
(iii)	Facilities classified as SIC 10 through 14, including active or inactive mining operations and oil and gas exploration, production, processing, or treatment operations, or transmission facilities that discharge storm water contaminated by contact with, or that has come into contact with, any overburden, raw material, intermediate products, finished products, byproducts, or waste products located on the site of such operations. SIC 10 . . . . . Metal Mining SIC 11 . . . . . Anthracite Mining SIC 12 . . . . . Coal Mining SIC 13 . . . . . Oil and Gas Extraction SIC 14 . . . . . Nonmetallic Minerals, except Fuels
(iv)	Hazardous waste treatment, storage, or disposal facilities, including those that are operating under interim status or a permit under Subtitle C of the Resource Conservation and Recovery Act (RCRA).
(v)	Landfills, land application sites, and open dumps that receive or have received any industrial wastes including those that are subject to regulation under subtitle D or RCRA.
(vi)	Facilities involved in the recycling of material, including metal scrapyards, battery reclaimers, salvage yards, and automobile junkyards, including but limited to those classified as: SIC 5015 . . . . . Motor Vehicle Parts, Used SIC 5093 . . . . . Scrap and Waste Materials
(vii)	Steam electric power generating facilities, including coal handling sites.
(viii)	Transportation facilities which have vehicle maintenance shops, equipment cleaning operations, or airport de-icing operations. Only those portions of the facility that are either involved in vehicle maintenance (including vehicle rehabilitation, mechanical repairs, painting, fueling, and lubrication), equipment cleaning operations, or airport de-icing operations, or which are otherwise listed in another category, are included. SIC 40 . . . . . Railroad Transportation SIC 41 . . . . . Local and Suburban Transit SIC 42 (except 4221-25) . . . . Motor Freight and Warehousing SIC 43 . . . . . U.S. Postal Service SIC 44 . . . . . Water Transportation SIC 45 . . . . . Transportation by Air SIC 5171 . . . . . Petroleum Bulk Stations and Terminals

EXHIBIT 2-6. INDUSTRIAL FACILITIES WHICH MUST SUBMIT APPLICATIONS FOR STORM WATER PERMITS (Continued)

40 CFR 122.26(b)(14) Subpart	Description
(ix)	Treatment works treating domestic sewage or any other sewage sludge or wastewater treatment device or system, used in the storage, treatment, recycling, and reclamation of municipal or domestic sewage, including lands dedicated to the disposal of the sewage sludge that are located within the confines of the facility, with a design flow of 1.0 million gallons per day or more, or required to have an approved pretreatment program under 40 CFR Part 403. Not included are farm lands, domestic gardens, or lands used for sludge management where sludge is beneficially reused and which are not physically located in the confines of the facility, or areas that are in compliance with Section 405 of the CWA.
(x)	Construction activity including clearing, grading, and excavation activities except operations that result in the disturbance of less than 5 acres of total land area and those that are not part of a larger common plan of development or sale.*
(xi)	Facilities under the following SICs [which are not otherwise included in categories (ii)-(x)], including only storm water discharges where material handling equipment or activities, raw materials, intermediate products, final products, waste materials, byproducts, or industrial machinery are exposed to storm water.* SIC 20 . . . . . Food and Kindred Products SIC 21 . . . . . Tobacco Products SIC 22 . . . . . Textile Mill Products SIC 23 . . . . . Apparel and Other Textile Products SIC 2434 . . . . . Wood Kitchen Cabinets SIC 25 . . . . . Furniture and Fixtures SIC 265 . . . . . Paperboard Containers and Boxes SIC 267 . . . . . Converted Paper and Paper Board Products (except containers and boxes) SIC 27 . . . . . Printing and Publishing SIC 283 . . . . . Drugs SIC 285 . . . . . Paints, Varnishes, Lacquer, Enamels SIC 30 . . . . . Rubber and Misc. Plastics Products SIC 31 (except 311) . . . . . Leather and Leather Products SIC 323 . . . . . Products of Purchased Glass SIC 34 (except 3441) . . . . . Fabricated Metal Products SIC 35 . . . . . Industrial Machinery and Equipment, except Electrical SIC 36 . . . . . Electronic and Other Electric Equipment SIC 37 (except 373) . . . . . Transportation Equipment SIC 38 . . . . . Instruments and Related Products SIC 39 . . . . . Miscellaneous Manufacturing Industries SIC 4221 . . . . . Farm Products Warehousing and Storage SIC 4222 . . . . . Refrigerated Warehousing and Storage SIC 4225 . . . . . General Warehousing and Storage

Source: Federal Register, Vol. 55, No. 222, p. 48065, November 16, 1990.
*On June 11, 1992, the U.S. Court of Appeals for the Ninth Circuit remanded the exemption for construction sites of less than five acres in category (x) and for manufacturing facilities in category (xi) which do not have materials or activities exposed to storm water to the EPA for further rulemaking. (Nos. 90-70671 & 91-70200).

These criteria were established to: (1) ensure that adequate flow would be discharged; (2) allow some build-up of pollutants during the dry weather intervals; and (3) ensure that the storm would be "representative," (i.e., typical for the area in terms of intensity, depth, and duration).

Collection of samples during a storm event meeting these criteria ensures that the resulting data will accurately portray the most common conditions for each site. However, the permitting authority is authorized to approve modifications of this definition (especially for applicants in arid areas where there are few representative events). Section 5.1 of Chapter 5 discusses general protocol for requesting modifications to application requirements, including the definition of "representative storm."

In determining whether a storm is representative, there are two important steps to take. First, data on local weather patterns should be collected and analyzed to determine the range of representative storms for a particular area. Second, these results should be compared to measurements of duration, intensity, and depth to ensure that the storm to be sampled fits the representativeness criteria.

2.7.2 OBTAINING RAINFALL DATA

Several sources provide accurate local weather information for both: (1) determining what a representative storm event is for a particular area; and (2) assessing expected storm events to determine whether a predicted rainfall will be "representative," and thus, meet the requirements for storm water sampling. The National Oceanic and Atmospheric Administration (NOAA) National Climatic Data Center's (NCDC's) Climate Services Branch is responsible for collecting precipitation data. Data on hourly, daily, and monthly precipitation for each measuring station (with latitude and longitude) are available to the public on computer diskette, microfiche, or hard copy. Orders can be placed by calling (704) 259-0682, by fax at (704) 259-0876, or by writing to NCDC, Climate Services Branch, The Federal Building, Asheville, North Carolina 28071-2733.

The National Weather Service (NWS) of NOAA can also provide information on historic, current, and future weather conditions. Local NWS telephone numbers can be obtained from the NWS Public Affairs Office at (301) 713-0622. Telephone numbers are also usually in local phone directory listings under "National Weather Service" or "Weather." In addition, NOAA runs the NOAA NWS

Weather Radio, which provides continuous broadcasts of the most current weather information. This broadcast can be accessed with a radio that has a weather band feature. Approximately 90 percent of the United States population is within listening range of the 380 NWS stations. Technical Appendix B presents additional information on NOAA Weather Radio, including radio frequencies for specific locations and a listing of weather band radio manufacturers. Telephone recordings of weather conditions are also provided by most NWS offices.

Cable TV weather stations and local airports can also provide weather information. Weather information provided by the local newspaper or TV stations should be used only if more accurate data (as described above) are unavailable, since weather forecasts can change drastically within several hours.

Someone should be designated at the facility to follow current weather conditions by listening to NOAA Weather Radio, calling the local NWS offices, and watching cable TV weather news. Exhibit 2-7 presents a storm water sampling decision chart for mobilizing field personnel for a probable storm event.

Annual rainfall statistics can also be used to evaluate representativeness of storm events. For example, Exhibit 2-8 presents fifteen rain zones in the United States and related storm event statistics. (These rain zones are not those shown in 40 CFR Part 122 Appendix E.) To determine typical values of annual storm events for a particular facility, identify the zone in which the facility is located. The tabulated information lists the annual average number of storms and precipitation as well as the average duration, intensity, and depth of independent storm events for each zone. Care must be taken, however, in using annual rainfall statistics for determining representativeness of storm events, since the annual rainfall statistic may not be representative of seasonal rainfall events. If rainfall data is available at or close to a particular facility, it is preferable to use this data for determining average storm event statistics.

Rainfall data tabulated from NOAA precipitation data indicate for Alaska (not shown in Exhibit 2-8) that average storm events last from 14 to 24 hours in duration and are 0.6 to 1.05 inches in depth. Average storm event data for Hawaii are 9 to 11 hours in duration and from 0.6 to 1.6 inches in depth.

EXHIBIT 2-7 DECISION CHART FOR STORM WATER SAMPLING

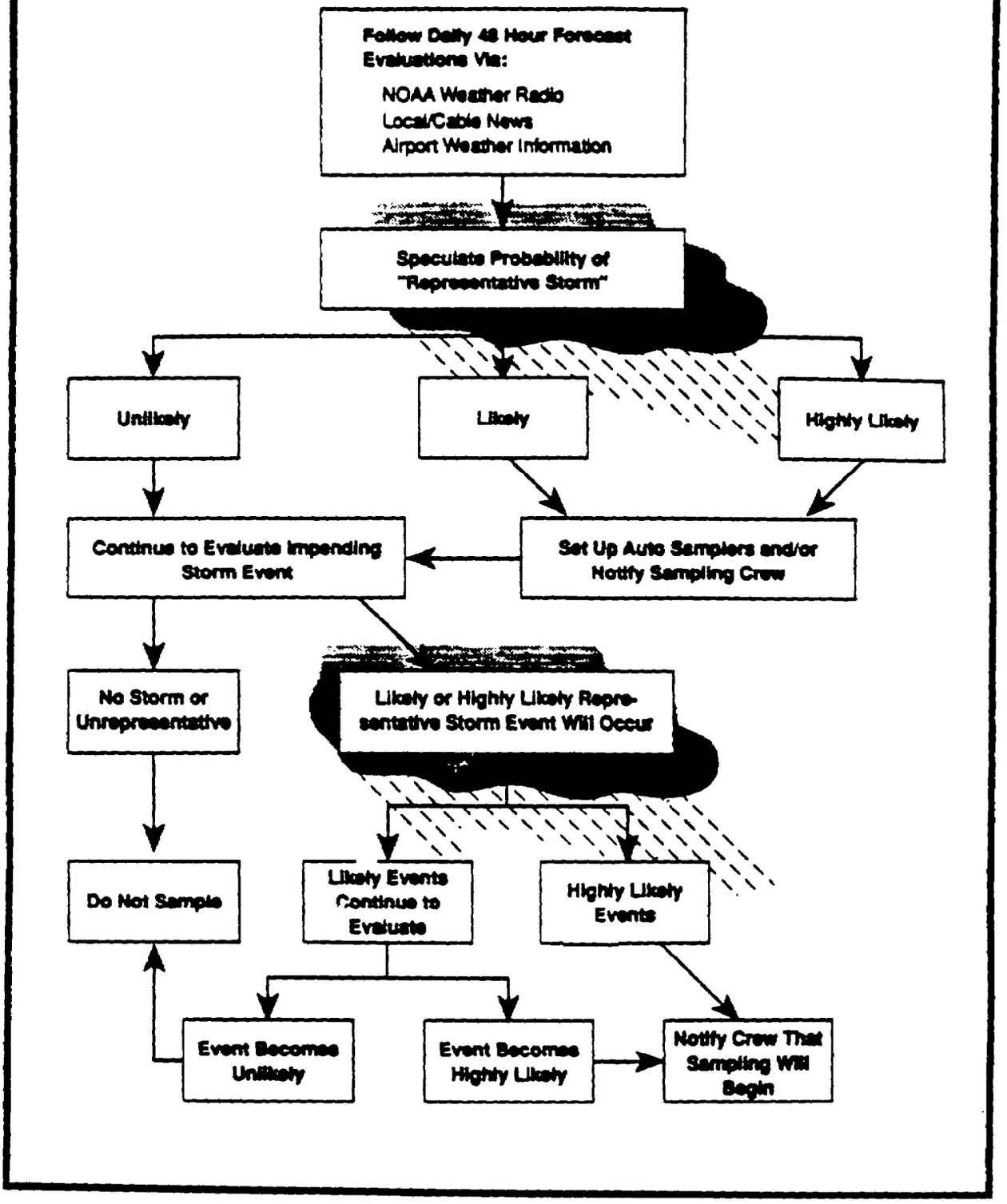
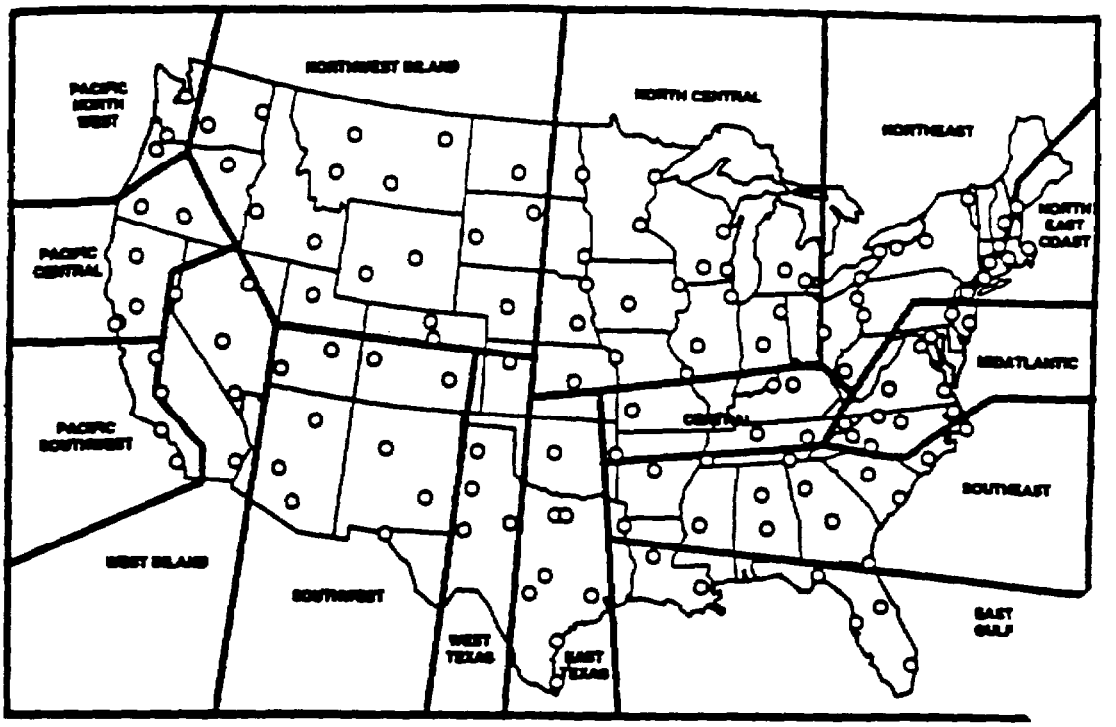


EXHIBIT 2-8. RAIN ZONES OF THE UNITED STATES



RAIN ZONE	Annual Statistics: No. of Storms Avg	No. of Storms COV	Precip. Avg (in)	Precip. COV	Independent Storm Event Statistics: Duration Avg (hr)	Duration COV	Intensity Avg (in/hr)	Intensity COV	Volume Avg (in)	Volume COV	DELTA Avg (hr)	DELTA COV
NORTH EAST	70	0.13	34.6	0.18	11.2	0.81	0.067	1.23	0.50	0.95	126	0.94
NORTH EAST—COASTAL	63	0.12	41.4	0.21	11.7	0.77	0.071	1.05	0.66	1.03	140	0.87
MIDATLANTIC	62	0.13	39.5	0.18	10.1	0.84	0.092	1.20	0.64	1.01	143	0.97
CENTRAL	68	0.14	41.9	0.19	9.2	0.85	0.097	1.09	0.62	1.00	133	0.99
NORTH CENTRAL	55	0.16	29.8	0.22	9.5	0.83	0.087	1.20	0.55	1.01	167	1.17
SOUTHEAST	65	0.15	49.0	0.20	8.7	0.92	0.122	1.09	0.75	1.10	136	1.03
EAST GULF	68	0.17	53.7	0.23	6.4	1.05	0.178	1.03	0.80	1.19	130	1.25
EAST TEXAS	41	0.22	31.2	0.29	8.0	0.97	0.137	1.08	0.76	1.18	213	1.28
WEST TEXAS	30	0.27	17.3	0.33	7.4	0.98	0.121	1.13	0.57	1.07	302	1.53
SOUTHWEST	20	0.30	7.4	0.37	7.8	0.88	0.079	1.16	0.37	0.88	473	1.46
WEST INLAND	14	0.38	4.9	0.43	9.4	0.75	0.055	1.06	0.36	0.87	786	1.54
PACIFIC SOUTH	19	0.36	10.2	0.42	11.6	0.78	0.054	0.76	0.54	0.98	476	2.09
NORTHWEST INLAND	31	0.23	11.5	0.29	10.4	0.82	0.057	1.20	0.37	0.93	304	1.43
PACIFIC CENTRAL	32	0.25	18.4	0.33	13.7	0.80	0.048	0.85	0.58	1.05	265	2.00
PACIFIC NORTHWEST	71	0.15	35.7	0.19	15.9	0.80	0.035	0.73	0.50	1.09	123	1.50

COV = Coefficient of Variation = Standard Deviation/Mean
DELTA = Interval Between Storm Midpoints
o = Rain Gauge Stations

Source: Urban Targeting and BMP Selection, U.S. EPA Region 5, November 1990.

The NWS should be consulted for proper procedures for collecting and interpolating rainfall data if the applicant elects to collect the data rather than use existing data.

2.7.3 DETERMINING REPRESENTATIVENESS

An example of how to determine whether a rainfall event varies by more than 50 percent (i.e., is not representative) is shown in Exhibit 2-9.

EXHIBIT 2-9. EXAMPLE OF 50 PERCENT VARIANCE FROM AVERAGE RAINFALL

Event Type	Duration (hrs.)	Depth (in.)
Average event	5.2	0.43
50 percent average event	2.6	0.22
150 percent average event	7.8	0.65

Once the information on an average duration and depth storm event is obtained for a specific location, multiply these numbers by 0.5 to get the 50 percent average event numbers and multiply by 1.5 to get the 150 percent average event numbers.

A representative storm in both duration and depth for a specific area will fall between the shaded numbers above (i.e., between 2.6 and 7.8 hours in duration and 0.22 and 0.65 inches in depth).

Snowmelt creates runoff which may result in point source discharges very similar to that from other storm events. Pollutants accumulate in snow, and when a thaw occurs, the pollutants will be discharged to receiving waters much like during a rain storm event. Snowmelt may be sampled as long as the applicant works closely with the permitting authority to determine the proper sampling strategy, i.e., sampling procedures, techniques, and pollutant analyses.

For snowmelt, the sampling strategy should be developed depending on the drainage area being monitored for storm flow. The strategy should consider (1) snow removal or clearing practices, e.g., direct dumping into water bodies, plowing, and the creation of snow mounds (whether in a line along a roadway or in piles on parking lots, etc.), and (2) the melting process.

It is also important to consider what happens to snowmounds as they melt and evaporate, which can alter the pollutant concentration in the resulting runoff. In addition, pollutants from the surrounding

air and pavement can build up on snow mound surfaces in a crust or cake-like manner eventually leaving a residue (including previously dissolved solids that become a remaining solids residue) which is later left to be washed off by rainfall, manual flushing or other mechanisms.

The sampling of snow mounds, undisturbed snow itself, and hard pack requires a carefully thought out strategy. Given the complexities associated with snowmelt sampling, applicants should have proposed sampling strategies reviewed by the permitting authority before attempting to conduct sampling.

2.7.4 LOGISTICAL PROBLEMS WITH WHEN TO SAMPLE

Applicants may encounter weather conditions that may not meet minimum "representative" storm criteria; these conditions may prevent adequate collection of storm water samples prior to application submission deadlines. For instance, sampling may be problematic in parts of the country that experience drought or near-drought conditions or areas that are under adverse weather conditions such as freezing and flooding. Events with false starts and events with stop/start rains can also cause problems. Solutions for sampling under these circumstances are discussed below.

Where the timing of storm event sampling poses a problem, it may be appropriate for the applicant to petition the permitting authority for a sampling protocol/procedure modification either prior to sampling or after sampling is conducted (if the storm event is not acceptable). When the applicant requests a sampling protocol/procedure modification, a narrative justification should be attached. This justification should be certified by a corporate official (for industrial facilities) or the principle executive officer or ranking official (for municipalities), as per 40 CFR 122.22. Section 5.1 of Chapter 5 discusses protocol/procedure modifications.

Arid Areas

For arid or drought-stricken areas where a storm event does not occur prior to the time the applicant must sample and submit data with the application form, the applicant should submit the application, complete to the extent possible, with a detailed explanation of why sampling data are not provided and an appraisal of when sampling will be conducted. This explanation must be certified by the appropriate party (as described above). The applicant should also contact the permitting authority

for further direction. Where the applicant can anticipate such problems, approval for an extension to submit sampling data should be acquired prior to the deadline.

Adverse Weather Conditions

The applicant should never conduct storm water sampling during unsafe conditions. It is likely that, in areas that experience flooding, lightening storms, high winds, etc., another representative storm event will occur for which sampling conditions will be much safer. (For further information on safety issues, see Chapter 6.) If no other storm event occurs, the applicant should submit a justification as to why the event was not sampled. This information should be certified by the appropriate official.

False Starts and Stop/Start Rains

False start and stop/start rains can also cause problems. False starts may occur when weather conditions are unpredictable and it appears that a storm event may be representative, collection begins, and then the rain stops before an adequate sample volume is obtained. (Necessary sample volumes are discussed in Section 3.6.) Some latitude may be given for the 0.1-inch rainfall requirement as long as the sample volume is adequate; the permitting authority may accept the results with applicant justification and certification. Again, see Chapter 5 for information on requesting protocol/procedure modifications to storm water sampling requirements.

During stop/start rains (those in which rainfall is intermittent), samples should be taken until an adequate sample volume is obtained. Exhibit 2-10 summarizes logistical problems of storm water sampling and presents solutions to the problems identified.

2.7.5 WHEN INDUSTRIAL FACILITIES MUST SAMPLE

Industrial applicants must generally collect two types of storm water samples: (1) grab samples collected during the first 30 minutes of discharge; and (2) flow-weighted composite samples collected during the first 3 hours of discharge (or the entire discharge, if it is less than 3 hours). Information from both types of samples is critical to fully evaluate the types and concentrations of pollutants present in the storm water discharge.

EXHIBIT 2-10. LOGISTICAL PROBLEMS OF STORM WATER SAMPLING	
Problem:	Arid/drought areas
Solution:	Submit a petition requesting a modification to the protocol if problems are anticipated and, if it is approved, submit the application without sampling data by the application due date with a certified explanation. Provide sampling data to the permitting authority as soon as possible.
Problem:	Adverse weather conditions such as freezing, flooding, winds, tornadoes, electrical storms, and gully washes
Solution:	Sample another, less hazardous event or submit a certified justification of why the event was not sampled. Provide sampling data to the permitting authority as soon as possible.
Problem:	False starts
Solution:	Discard the sample if the volume is inadequate. If the volume is adequate, submit the sampling data with a certified explanation that the sample is from a non-representative event. Continue to monitor weather conditions and attempt to resample as soon as possible.
Problem:	Stop/start rains
Solution:	Continue to sample in case the storm event turns out to be representative and adequate sample volumes are obtained. If sample volumes are inadequate, continue to monitor weather conditions and attempt to resample as soon as possible.

The grab samples taken during the first 30 minutes of a storm event will generally contain higher concentrations of pollutants, since they pick up pollutants that have accumulated on drainage surfaces since the last storm event.

Composite samples characterize the average quality of the entire storm water discharge. Flow-weighted composite samples provide for the most accurate determination of mass load. The flow-weighted composite sample must be taken for either the first 3 hours or for the entire discharge (if the event is less than 3 hours long). Additional information on how to collect grab and composite samples is presented in Sections 3.3 and 3.4, respectively.

Industrial applicants are required at a minimum to sample only one storm event. However, if samples from more than one storm are analyzed and the results are representative of the discharge, the data representing each event must be reported. The facility must provide a description of each storm event tested. The average of all values within the last year must be determined and the

concentration, mass, and total number of storm events sampled must be reported on Form 2F. Furthermore, sampling should be conducted during normal operating procedures (day or night), and, not when the facility has been closed for a period of time.

Industrial applicants must certify, as a separate requirement, that all outfalls have been tested or evaluated to determine whether non-storm water discharges are present (e.g., process wastewater, sanitary wastes, cooling water, or rinse water) or whether illegal/illicit connections are occurring in the system. This testing should be conducted during dry weather to avoid any flows of storm water through the conveyance.

A checklist that can be used to conduct dry weather evaluations is provided in Exhibit 2-11. A narrative description of the method used to conduct dry weather evaluations and the date and the drainage points must be included in Section V.A of Form 2F. This statement must be certified by the appropriate party as described in Section 2.7.4.

A dry weather visual inspection is the simplest way to screen for illicit discharges. If one or more of the items on the checklist in Exhibit 2-11 are answered affirmatively, or if there are other reasons to believe that illicit connections exist, more detailed investigations (such as dye tests, smoke tests, evaluation of piping designs, and TV line monitoring) may be necessary. Dye testing involves releasing fluorescent, nontoxic dye into the suspected source of non-storm water, (e.g., a drain, sink, toilet, or pipe) and checking to see whether the dye shows up in the storm water outfall. Smoke testing involves pumping smoke into a storm sewer and viewing the facility to see if smoke escapes through unknown openings or storm sewer inlets. The presence of smoke indicates that storm water may enter the sewer through these openings or inlets. However, smoke testing may prove ineffective at finding non-storm water discharges to separate storm sewers. Smoke passage may be blocked due to line traps that are intended to block sewer gas.

TV line monitoring is a technique whereby a small video camera is placed in the storm sewer and a video image of the sewer is viewed on a monitor at the surface to identify illicit connections. The camera can be moved through the sewer by remote control. For more information on smoke and dye testing and TV line monitoring, consult EPA's Guidance Manual for the Preparation of NPDES

EXHIBIT 2-11. CHECKLIST FOR CONDUCTING DRY WEATHER EVALUATIONS

1. Date of inspection:_____ 2. Facility name and address:_____
3. Date of last rain event:_____ _____
4. Inspector name:_____ _____
5. Type of outfall

☐ Concrete ☐ Pipe ☐ Grassed ☐ Rock ☐ Other _____
_____

6. Is there visible flow from the pipe? ☐ Yes ☐ No
If yes, check all that apply. If no, go to number 7.

☐ Colored water (describe)_____ ☐ Oily sheen
☐ Odor* (describe)_____ ☐ Sludge present
☐ Murky ☐ Clear water
☐ Floating objects (describe)_____ ☐ Stains on conveyance
☐ Absence of plant life surrounding conveyance ☐ Notable difference in plant life surrounding conveyance
☐ Scum ☐ Suds ☐ Other:_____

*e.g., rotten eggs, earthy, chemical, chlorine, soap, putrescence, gasoline, musty, etc.

Estimate the flow either visually or by describing the width, height, and shape of the conveyance and the approximate percentage of the conveyance where flow is present or the approximate depth of the flow. Describe your estimate.

7. Is there standing water present? ☐ Yes ☐ No
If yes, check all that apply. If no, go to number 8.

☐ Colored water (describe)_____ ☐ Oily sheen
☐ Odor* (describe)_____ ☐ Sludge present
☐ Murky ☐ Clear water
☐ Floating objects (describe)_____ ☐ Stains on conveyance
☐ Absence of plant life surrounding conveyance ☐ Notable difference in plant life surrounding conveyance
☐ Suds ☐ Scum ☐ Other:_____
☐ Absence of plant life surrounding conveyance

*e.g., rotten eggs, earthy, chemical, chlorine, soap, putrescence, gasoline, musty, etc.

8. From the inspection locations, can you see any unusual piping or ditches that drain to the storm water conveyance? ☐ Yes ☐ No
9. Is there any overland flow visible from the discharge location? ☐ Yes ☐ No
10. Are there dead animals present? ☐ Yes ☐ No

Signature:

Permit Applications for Storm Water Discharges Associated with Industrial Activity (EPA-505/8-91-002, April 1991).

A problem with the dry weather evaluation process is that the presence of a dry weather/non-storm water discharge may be caused by infiltration of ground or surface waters through cracks in the storm water drainage system. In this situation, all other possible sources of the non-storm water discharge should be examined and ruled out. If no sources are found, the physical structure of the conveyance system should be inspected for deterioration.

The applicant should make every attempt to halt non-storm water discharges to the storm sewer system unless the discharge is covered by an NPDES permit. If it is not feasible to halt the discharge of non-storm water to the storm sewer system, and the discharge is not authorized by a process wastewater or storm water permit, the applicant must submit either Form 2C (for a process water discharge) or Form 2E (for a nonprocess water discharge), and check with state officials to see if alternate forms are required.

2.7.6 WHEN MUNICIPAL FACILITIES MUST SAMPLE

Municipal applicants are required to conduct sampling for both Parts 1 and 2 of their applications. In Part 1, municipalities must conduct a field screening analysis to detect illicit connections and illegal dumping into their storm sewer system. Where flow is observed during dry weather, two grab samples must be collected during a 24-hour period with a minimum of 4 hours between samples. These samples must be analyzed for pH, total chlorine, total copper, total phenol, and detergents (surfactants). Note that these are dry weather samples, rather than storm water samples. EPA's Guidance Manual for the Preparation of Part 1 of the NPDES Permit Applications for Discharges from Municipal Separate Storm Sewer Systems presents a description of conducting field screening sampling and provides a data sheet.

For Part 2 of the application, municipalities must submit grab (for certain pollutants) and flow-weighted sampling data from selected sites (5 to 10 outfalls) for 3 representative storm events at least 1 month apart. The flow-weighted composite sample must be taken for either the entire discharge or the first 3 hours (if the event lasts longer than 3 hours). Municipal facilities are not required to collect grab samples within the first 30 minutes of a storm event.

In addition to submitting quantitative data for the application, municipalities must also develop programs for future sampling activities that specify sampling locations, frequency, pollutants to be analyzed, and sampling equipment. Where necessary (as determined by the municipality or if required by the permitting authority), responsibilities may also include monitoring industries connected to the municipality's storm sewers for compliance with their facility-specific NPDES permits. Refer to EPA's Guidance Manual for the Preparation of Part 1 of the NPDES Permit Applications for Discharges from Municipal Separate Storm Sewer Systems for information on how to develop municipal sampling programs.

2.7.7 USE OF HISTORICAL DATA

Data from storm water samples analyzed in the past can be submitted with applications in lieu of new sampling data if:

- All data requirements in Form 2F are met
- Sampling was performed no longer than 3 years prior to submission of the permit application
- All water quality data are representative of the present discharge.

The historical data may be unacceptable if there have been significant changes since the time of that storm event in production level, raw materials, processes, or final products. Significant changes which may also impact storm water runoff include construction or installation of treatment or sedimentation/erosion control devices, buildings, roadways, or parking lots. Applicants should assess any such changes to determine whether they altered storm water runoff since the time of the storm event chosen for use in the permit application. Historical data can be used only in applications. Historical data cannot be used for fulfilling permit requirements.

2.8 WHERE TO SAMPLE STORM WATER DISCHARGES

Storm water samples should be taken at a storm water point source. A "point source" is defined as any discernible, confined, and discrete conveyance, including (but not limited to) any pipe, ditch, channel, tunnel, conduit, well, discrete fissure, container, rolling stock, concentrated animal feeding operation, landfill leachate collection system, vessel, or other floating craft from which pollutants are or may be discharged (as per 40 CFR 122.2). Included in the definition of storm water "point

sources" is storm water from an industrial facility that enters, and is discharged through, a municipal separate storm sewer. In short, most storm water discharges can be defined as "point source" discharges, since they ultimately flow into some kind of conveyance (e.g., a channel or swale).

2.8.1 INDUSTRIAL FACILITIES

Industrial applicants submitting individual applications must collect and analyze a grab sample taken within the first 30 minutes of the storm event and flow-weighted composite samples from each of the industrial storm water "point source" outfalls identified on the site drainage map submitted for Section III of Form 2F. Applicants submitting quantitative data for Part 2 of the group application must also collect samples for each outfall discharging storm water associated with industrial activity. All outfalls should be sampled during the same representative storm event if possible. If this is not feasible, outfalls may be sampled during different representative storm events upon approval by the permitting authority. Descriptions of each storm event and which outfalls were sampled during each event must be included in the application. Storm water runoff from employee parking lots, administration buildings, and landscaped areas that is not mixed with storm water associated with industrial activity, or storm water discharges to municipal sanitary sewers, do not need to be sampled.

Outfalls With Substantially Identical Effluents—Industrial Facilities

If an applicant has two or more outfalls with "substantially identical effluents," the facility may petition the permitting authority to sample and analyze only one of the identical outfalls and submit the results as representative of the other. "Substantially identical effluents" are defined as discharges from drainage areas undergoing similar activities where the discharges are expected to be of similar quantity and quality, and indistinguishable in expected composition. Chapter 5 presents an example of a petition for substantially identical effluents and discusses this process in more detail.

2.8.2 MUNICIPALITIES

Large and medium municipalities are required to sample storm water discharges from 5 to 10 outfalls or field screening points that were proposed in Part 1 of the application. The final decision on the number and location of sampling points will be determined by the permitting authority and will

depend on site-specific conditions such as land use or drainage area and results of data collected during the field screening analysis process for Part 1 of the application.

2.8.3 LOGISTICS OF WHERE TO SAMPLE

The ideal sampling location would be the lowest point in the drainage area where a conveyance discharges storm water to waters of the U.S. or to a municipal separate storm sewer system. A sample point also should be easily accessible on foot in a location that will not cause hazardous sampling conditions. Ideally, the sampling site should be on the applicant's property or within the municipality's easement; if not, the field personnel should obtain permission from the owner of the property where the discharge outfall is located. Typical sampling locations may include the discharge at the end of a pipe, a ditch, or a channel.

However, logistical problems with sample locations may arise (e.g., nonpoint discharges, inaccessibility of discharge point, etc.). Logistical problems with sample locations and suggested solutions are described in Exhibit 2-12. In many cases, it may be necessary to locate a sampling point further upstream of the discharge point (e.g., in a manhole or inlet). If the storm water at a selected location is not representative of a facility's total runoff, the facility may have to sample at several locations to best characterize the total runoff from the site. In situations where discharge points are difficult to sample for various reasons, the applicant should take the best sample possible and explain the conditions in the application. A discussion on sampling at retention ponds appears in Section 3.1.2.

2.9 STAFFING CONSIDERATIONS

Staffing needs for sampling must be determined by the applicant. Factors in making the determination include the number of sample locations, the size of the area to be sampled, how far apart the locations are, the type of sampling required, the technique to be used, the number of samples to be taken (depending on how many parameters must be analyzed), and safety considerations.

Training sampling personnel is important to the success of storm water discharge characterization. Training can be done using this manual. Sampling conducted by untrained personnel may result in

EXHIBIT 2-12. SOLUTIONS TO SAMPLE LOCATION PROBLEMS	
Problem:	Sampling where storm water commingles with process or non-process water
Solution:	Attempt to sample the storm water discharge before it mixes with the non-storm water discharge. If this is impossible, sample the discharge both during dry and wet weather and present both sets of data to the permitting authority. This will provide an indication of the contribution of pollutants from each source.
Problem:	Numerous small point discharges
Solution:	Impound channel or join together flow by building a weir or digging a ditch to collect discharge at a low point for sampling purposes. This artificial collection point should be lined with plastic to prevent infiltration and/or high levels of sediment. Or, sample at several locations to represent total site runoff.
Problem:	Inaccessible discharge point [examples include underwater discharges or unreachable discharges (e.g., out of a cliff)]
Solution:	Go up the pipe to sample (i.e., to the nearest manhole or inspection point). If these are not available, tap into the pipe or sample at several locations to best represent total site runoff.
Problem:	Managing multiple sampling sites to collect grab samples during the first 30 minutes (industrial facilities only)
Solution:	Have a sampling crew ready for mobilization when forecasts indicate that a representative storm will occur or sample several different representative events. Also, for most parameters, automatic samplers may be used to collect samples within the first 30 minutes triggered by the amount of rainfall, the depth of flow, flow volume or time.
Problem:	Commingling of parking lot runoff with discharge associated with industrial activity
Solution:	The combined runoff must be sampled at the discharge point as near as possible to the receiving water or the parking lot drain inlet if there is one.
Problem:	Sampling in manholes
Solution:	Sample in manholes only when necessary. See Chapter 6 for safety information Sampling in manholes requires training on confined space entry.
Problem:	Runon from other property
Solution:	If possible, estimate the volume of offsite runon contributions and offsite runon sources of pollutants to perform a mass balance calculation. Include this information in the permit application. If this estimation is not possible, provide a narrative discussion of the upstream site (e.g., is it developed, if so the type of facility, the types of pollutants that may be present on the site, etc.).

data that is unrepresentative of the facility's storm water discharge. This data might be rejected by the permitting authority, who would then require another sampling effort.

3. FUNDAMENTALS OF SAMPLING

Because of the variable nature of storm water flows during a rainfall event and different analytical considerations for certain pollutants, the storm water regulations establish specific requirements for sample collection techniques. The quality of storm water discharges and logistical needs for sampling will be different for industrial applicants and municipal applicants. Therefore, specific sampling requirements vary. After a brief review of sampling fundamentals and special sampling requirements for storm water permit applications, the following sections are intended to teach applicants how to sample to meet these requirements.

The applicant should carefully plan his/her sampling strategy prior to the actual sampling event, e.g., walk the site to determine appropriate sampling locations, become familiarized with local rainfall patterns, train sampling staff in procedures and safety, consult with laboratory, and collect supplies.

3.1 TYPES AND TECHNIQUES OF SAMPLING

There are three basic aspects of sampling:

- Sample type (i.e., grab versus composite)
- Sample technique (i.e., manual versus automatic)
- Flow measurement methods.

These topics will be discussed in relation to requirements of an NPDES storm water discharge permit application. Once these aspects are addressed, step-by-step instructions on sampling procedures are presented. The sections below define and describe the types of storm water samples that must be collected and methods or techniques for collecting them. In addition, special sampling requirements for cert... pollutants are discussed.

3.1.1 SAMPLE TYPE VERSUS SAMPLE TECHNIQUE

It is important to understand the difference between sample type and technique. "Sample type" refers to the kind of sample that must be collected – either a grab or a composite. "Sample technique" refers to the method by which a grab or composite sample is actually collected – either manually or by automatic sampler. A generalized relationship between sample type and sample technique is presented in Exhibit 3-1. Sections 3.1.2 and 3.1.3 further explain the significance of these terms as they relate to storm water sampling requirements.

EXHIBIT 3-1. SAMPLE TYPE vs. SAMPLE TECHNIQUE

Sample Type	Sample Technique
Grab	Manual Automatic sampling system
Composite	Manual with manual compositing Automatic system or automatic sampling with manual compositing

3.1.2 SAMPLE TYPE: GRAB AND COMPOSITE SAMPLES

To comply with storm water application requirements, the sample type (grab or composite) must be collected in accordance with 40 CFR 122.21(g)(7) and 40 CFR Part 136. The storm water application requirements clearly specify which pollutants must be analyzed by grab sample, and which by composite sample. Although the requirements in 40 CFR 122.21(g)(7) do not explicitly specify either manual or automatic sampling techniques, the approved analytical methods contained in 40 CFR Part 136 direct that grab samples must be collected manually for certain pollutants. Sections 3.3 and 3.4 clarify which pollutants must be grabbed, which ones must be grabbed manually, and which ones must be flow-weighted composites.

The two types of storm water samples required by the regulations, grab and composite samples, are described below.

Grab Samples

A grab sample is a discrete, individual sample taken within a short period of time (usually less than 15 minutes). Analysis of grab samples characterizes the quality of a storm water discharge at a given time of the discharge.

Composite Samples

A composite sample is a mixed or combined sample that is formed by combining a series of individual and discrete samples of specific volumes at specified intervals. Although these intervals can be time-weighted or flow-weighted, the storm water regulations require the collection of flow-weighted composite samples. This means that discrete aliquots, or samples, are collected and combined in proportion to flow rather than time. Composite samples characterize the quality of a storm water discharge over a longer period of time, such as the duration of a storm event.

Application Requirements

Both types of samples must be collected and analyzed for storm water discharge permit applications. Grab samples must be collected for the following conditions:

- For storm water discharges associated with industrial activity, a grab sample must be obtained during the first 30 minutes of a discharge. This requirement is in addition to the composite sampling requirements. These samples are intended to characterize the maximum concentration of a pollutant that may occur in the discharge and/or may indicate intermingling of non-storm water discharges.

- For storm water discharges from large and medium municipal separate storm sewers, grab samples are required for Part 1 of the application if a discharge is noted during dry weather field screening. Two grab samples must be collected during a 24-hour period with a minimum of 4 hours between samples. These samples are intended to assist in the identification of illicit connections or illegal dumping. In Part 2, grab samples may be required for the analysis of certain pollutants for which municipalities are required to sample.

Flow-weighted composite samples must be collected during the first 3 hours of discharge or the entire discharge (if it is less than 3 hours) for both industrial and municipal applicants.

Pollutant-specific Requirements

The regulations at 40 CFR 122.21(g)(7) identify certain pollutants for which grab sampling is required:

- Monitoring by grab sample must be conducted for pH, temperature, cyanide, total phenols, residual chlorine, oil and grease (O&G), fecal coliform, and fecal streptococcus. Composite samples are not appropriate for these parameters due to their tendency to transform to different substances or change in concentration after a short period of time. Such transformations may be particularly likely in the presence of other reactive pollutants.

Sampling At Retention Ponds

Retention ponds with greater than a 24-hour holding time for a representative storm event may be sampled by grab sample. Composite sampling is not necessary. The rationale for this is that, because the water is held for at least 24 hours, a thorough mixing occurs within the pond. Therefore, a single grab sample of the effluent from the discharge point of the pond accurately represents a composite of the storm water contained in the pond. If the pond does not thoroughly mix the discharge, thereby compositing the sample, then a regular grab and composite sample should be taken at the inflow to the pond. Since each pond may vary in its capability to "composite" a sample, applicants must carefully evaluate whether the pond is thoroughly mixing the discharge. Such factors as pond design and maintenance are important in making this evaluation. Poor pond design, for example, where the outfall and inflow points are too closely situated, may cause short-circuiting and inadequate mixing. In addition, poor maintenance may lead to excessive re-suspension of any deposited silt and sediment during heavy inflows. Because of factors such as these, the applicant should determine the best location to sample the pond (e.g., at the outfall, at the outfall structure, in the pond) to ensure that a representative composite sample is taken. If adequate compositing is not occurring within the pond, the applicant should conduct routine grab and flow-weighted composite sampling.

A grab sample and a flow-weighted sample must be taken for storm water discharges collected in holding ponds with less than a 24-hour retention period. The applicant must sample the discharge in the same manner as for any storm water discharge [as described in 40 CFR 122.21(g)(7)]. In

effect, the applicant must take one grab sample within the first 30 minutes of discharge, or as soon as possible. The applicant must also collect a flow-weighted composite sample for at least the first 3 hours of the discharge, or for the event's entire duration (if it is less than 3 hours). The flow-weighted composite sample may be taken using a continuous sampler or as a combination of at least three sample aliquots taken during each hour of the discharge, with a minimum of 15 minutes between each aliquot. If the applicant does not know what retention period the pond is designed for, the design engineer of the pond should be consulted.

3.1.3 SAMPLE TECHNIQUE: MANUAL VERSUS AUTOMATIC SAMPLING

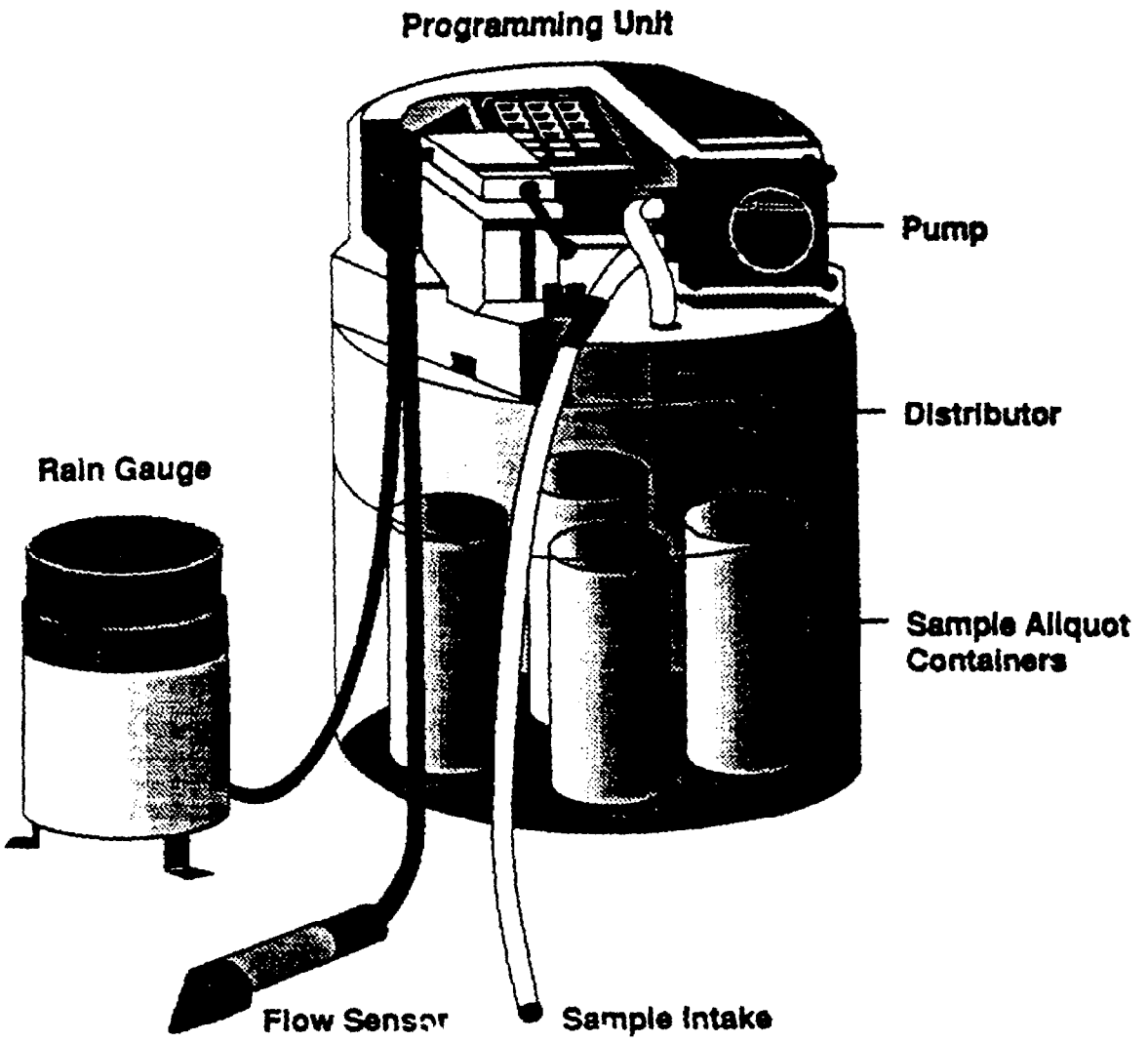
As previously discussed, manual and automatic sampling techniques are methods by which both grab and composite samples can be collected. Manual samples are simply samples collected by hand. Automatic samplers are powered devices that collect samples according to preprogrammed criteria. A typical automatic sampler configuration is shown in Exhibit 3-2.

For most pollutants, either manual or automatic sample collection will conform with 40 CFR Part 136. However, one case in which automatic samplers cannot be used is for the collection of volatile organic compound (VOC) samples because VOCs will likely volatilize as a result of agitation during automatic sampler collection. Samples collected for VOC analysis should be filled until a reverse meniscus is found over the top of the collection bottle and capped immediately to leave no air space. Automatic samplers do not perform this function. Special requirements for VOC sampling are discussed in Section 3.5.2.

Although both collection techniques are available, several other pollutants may not be amenable to collection by an automatic sampler, for example fecal streptococcus, fecal coliform and chlorine have very short holding times (i.e., 6 hours), pH and temperature need to be analyzed immediately and oil and grease requires teflon coated equipment to prevent adherence to the sampling equipment.

Other restrictions on sample collection techniques (such as container type and preservation) should be determined by consulting the approved analytical methods listed in 40 CFR Part 136. Section 3.5 and Technical Appendix C provide additional information on sample handling, holding times, and preservation methods.

EXHIBIT 3-2. AUTOMATIC SAMPLER



Manual and automatic techniques have advantages and disadvantages that the applicant should consider in relation to the sampling program. The main advantage of manual sampling is that it can be less costly than purchasing or renting automatic samplers. Automatic samplers, however, can be often more convenient. Exhibit 3-3 presents a matrix of advantages and disadvantages associated with each technique. Ultimately, the best technique to use will depend on each applicant's situation.

3.2 OBTAINING FLOW DATA

In addition to collecting samples of storm water discharges, applicants must collect data characterizing the flow rate and flow volume for each storm water discharge sampled. Flow rate is the quantity of storm water discharged from an outfall per unit of time. Total flow is a measure of the total volume of storm water runoff discharged during a rain event. Flow rates and volumes can either be measured specifically or can be estimated (based on rainfall measurements, velocities, and depth of flows). To collect flow-weighted composite samples, flow rate data is necessary to combine proportional volumes of individually collected aliquots. Applicants must also report the mass of pollutants contained in storm water discharges (see Section 3.2.5). To determine mass loadings of pollutants, applicants must measure both discharge flow rate and pollutant concentration. This section presents methods for obtaining flow data.

3.2.1 MEASURING FLOW RATES

Flow rates for storm water discharges are most accurately measured using either primary or secondary flow measurement devices. Facilities should use these devices to characterize their discharge as precisely as possible. Where flow measurement devices are not already installed, portable devices should be considered. There are many permanent and portable types of flow measurement devices available. This discussion is limited to the most common flow measurement devices. To purchase flow measurement devices and rain gauges, pertinent engineering journals can be consulted for equipment vendor listings. Proper analysis of site discharge conditions must be conducted prior to purchase and installment of flow measurement devices.

Primary Flow Measurement Devices

A primary flow measurement device is a man-made flow control structure which, when inserted into an open channel, creates a geometric relationship between the depth of the flow and the rate of the

EXHIBIT 3-3. COMPARISON OF MANUAL AND AUTOMATIC SAMPLING TECHNIQUES

Sample Method	Advantages	Disadvantages
Manual Grabs	• Appropriate for all pollutants • Minimum equipment required	• Labor-intensive • Environment possibly dangerous to field personnel • May be difficult to get personnel and equipment to the storm water outfall within the 30 minute requirement • Possible human error
Manual Flow-Weighted Composites (multiple grabs)	• Appropriate for all pollutants • Minimum equipment required	• Labor-intensive • Environment possibly dangerous to field personnel • Human error may have significant impact on sample representativeness • Requires flow measurements taken during sampling
Automatic Grabs	• Minimizes labor requirements • Low risk of human error • Reduced personnel exposure to unsafe conditions • Sampling may be triggered remotely or initiated according to present conditions	• Samples collected for O&G may not be representative • Automatic samplers cannot properly collect samples for VOCs analysis • Costly if numerous sampling sites require the purchase of equipment • Requires equipment installation and maintenance • Requires operator training • May not be appropriate for pH and temperature • May not be appropriate for parameters with short holding times (e.g., fecal streptococcus, fecal coliform, chlorine) • Cross-contamination of aliquot if tubing/bottles not washed
Automatic Flow-Weighted Composites	• Minimizes labor requirements • Low risk of human error • Reduced personnel exposure to unsafe conditions • May eliminate the need for manual compositing of aliquots • Sampling may be triggered remotely or initiated according to on-site conditions	• Not acceptable for VOCs sampling • Costly if numerous sampling sites require the purchase of equipment • Requires equipment installation and maintenance, may malfunction • Requires initial operator training • Requires accurate flow measurement equipment tied to sampler • Cross-contamination of aliquot if tubing/bottles not washed

flow. The depth of the flow, referred to as the head (H), can then be measured at the respective reference point/area with a ruler or other staff gauge. When substituted into a formula, which mathematically describes the relationship between depth and discharge for the primary devices, the head measurement can be used to calculate a flow rate (Q). The most common primary flow measurement devices are weirs and flumes. Weirs and flumes are flow structures designed to provide a known, repeatable relationship between flow and depth.

Weirs

Weirs consist of a crest located across the width of an open channel (at a right angle to the direction of the flow). The flow of water is impeded, causing water to overflow the crest. Diagrams and formulas of some typically found weirs are provided in Exhibit 3-4. Weirs are inexpensive and particularly valuable in measuring flow in natural or man-made swales because they are easily installed in irregularly shaped channels.

Weirs can only provide accurate flow measurements when head measurements are appropriately taken. When flow exceeds the capacity of the weir and water overtops the weir crest, flow depth actually diminishes as the water approaches the weir, as shown in Exhibit 3-5. Therefore, measuring the depth at the weir crest will result in an inaccurate measurement of the actual head. Under these circumstances, the head should be measured upstream, at a point determined by the type of weir and the estimated amount of flow. A staff gauge can be installed at a nonturbulent point upstream of the weir crest to provide accurate and convenient measurements.

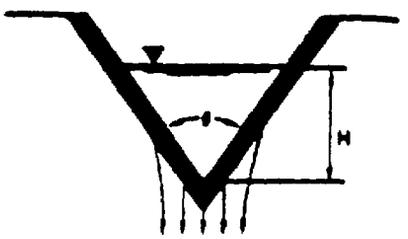
Flumes

Flumes are structures which force water through a narrow channel. They consist of a converging section, a throat, and a diverging section. Exhibit 3-6 portrays the most common type of flume, the Parshall flume, and also provides formulas for calculating appropriate flow rates.

Parshall flumes have fixed specifications relating to geometric shape. They vary only in throat width. Due to these geometric constraints, Parshall flumes may be expensive to install. They are typically used in permanent flow measurement points and are most commonly placed in concrete-lined channels. However, Parshall flumes can also be used in temporary points. Parshall flumes provide accurate measurements for a relatively wide range of flow rates. The flow rate through the Parshall flume (see Exhibit 3-6) is calculated from the depth (H_a) of flow measured in the converging

EXHIBIT 3-4. WEIRS

V-Notch

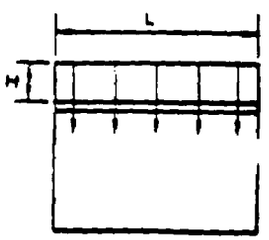


$Q = 2.5\ H^{2.5}$ (90°)
$Q = 1.443\ H^{2.5}$ (60°)
$Q = 1.035\ H^{2.5}$ (45°)
$Q = 0.676\ H^{2.5}$ (30°)
$Q = 0.497\ H^{2.5}$ (22½°)

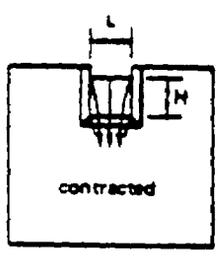
Q = Flow Rate

H = Depth of flow (Head)

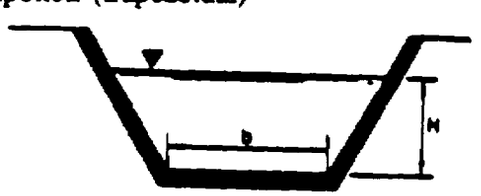
Rectangular (without contractions) $Q = 3.33\ L\ H^{1.5}$



Rectangular (with contractions) $Q = 3.33\ (L - 0.2\ H)^{1.5}$

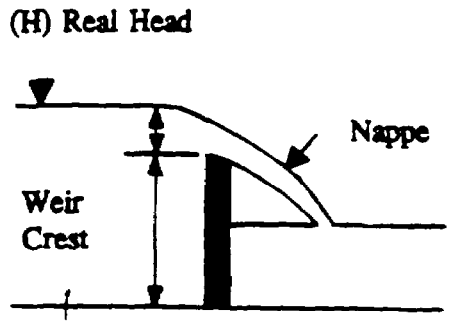


Cipolleti (trapezoidal) $Q = 3.367\ b\ H^{1.5}$



Source: Civil Engineering Reference Manual, 5th Edition, by Michael R. Lindeburg, PE, with permission from the publisher, Professional Publications, Inc., Belmont, California, 1989.

EXHIBIT 3-5. SUPPRESSED FLOW OVER THE WEIR CREST



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section of the flume. The exact location of the depth measurement depends on the specific design of the Parshall flume. Exhibit 3-6 indicates the equations used to calculate flow rate through a typical Parshall flume. These equations are accurate only when the submergence ratio (H_b/H_a) is greater than 0.7. The manufacturers' information should be consulted for the flow rate equation and measuring points for a specific Parshall flume.

Palmer-Bowlus flumes, shown in Exhibit 3-7, are also used at some facilities. Palmer-Bowlus flumes are designed to be installed in an existing circular channel (such as a manhole channel) and are available as portable measurement devices. While Palmer-Bowlus flumes are inexpensive, self cleaning, and easy to install, they can only measure flow rates accurately over a narrow range of flow.

The flow from a Palmer-Bowlus flume is calculated using the height between the floor of the flume portion and the water level, not the total head of the water level. Head measurements are taken at

EXHIBIT 3-6. FLUMES

Parshall Flume

$Q = 0.338\ H^{1.55}$ (1 inch)

$Q = 0.676\ H^{1.55}$ (2 inches)

$Q = 0.992\ H^{1.547}$ (3 inches)

$Q = 2.09\ H^{1.58}$ (6 inches)

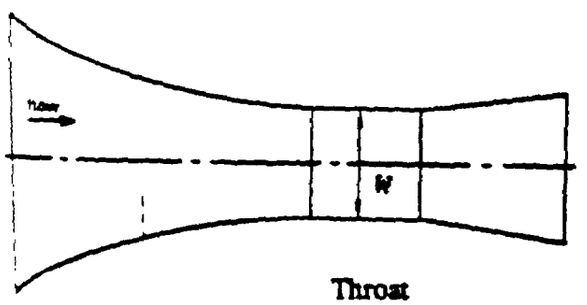
$Q = 3.07\ H^{1.53}$ (9 inches)

$Q = 4\ W\ H^{1.522}\ W^{0.26}$ (1-8 feet)

$Q = (3.6875\ W + 2.5)H^{1.6}$ (10-50 feet)

Q = Flow rate

H = Depth of flow (Head)

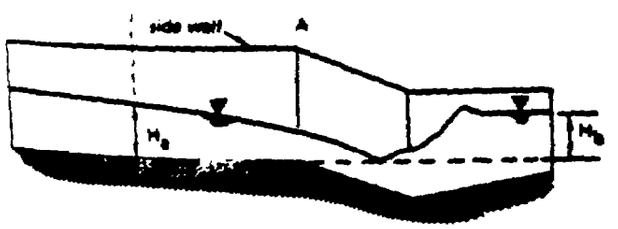


Top View

Throat

Converging Section

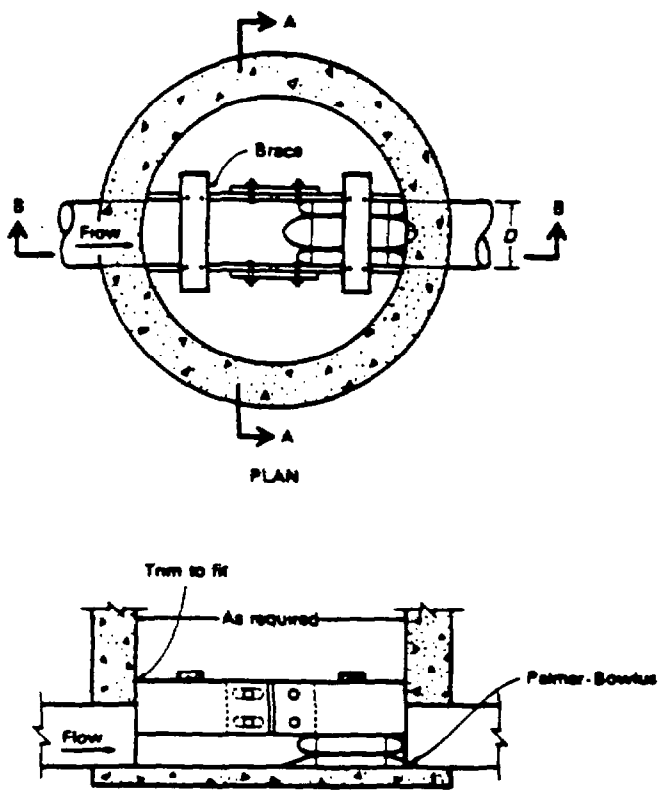
Diverging Section



Side View

Source: Civil Engineering Reference Manual, 5th Edition, by Michael R. Lindeburg, PE, with permission from the publisher, Professional Publications, Inc., Belmont, California, 1989.

EXHIBIT 3-7. PALMER-BOWLUS FLUME



Source: Wastewater Engineering: Treatment, Disposal, Reuse, 2nd Edition, Metcalf & Eddy, Inc., with permission from the publisher, McGraw-Hill Book Co., New York, 1979.

a distance from the throat equal to one half the width of the flume. The dimensions of a Palmer-Bowlus flume have been standardized in a generic sense, but the flume shape may vary. Therefore, there are no formulas that can be applied to all Palmer-Bowlus flumes. Device-specific head-flow relationships for each device should be obtained from the manufacturer.

There are a number of other, less common, flow measurement devices available which will not be discussed (see Appendix D for additional references).

Secondary Flow Measurement Devices

Secondary flow measurement devices are automated forms of flow rate and volume measurement. Typically, a secondary device is used in conjunction with a primary device to automatically measure the flow depth or head. This value is then processed, using established mathematical relationships to relate the depth measurement to a corresponding flow rate. The device also may have the capacity to convert this flow rate to a volume. Secondary flow measurement devices include floats, ultrasonic transducers, pressure transducers, and bubblers. The output of the secondary device is transmitted to a display, recorder, and/or totalizer to provide flow rate and volume information. The user manuals for these devices should be consulted for proper usage.

Evaluation of Flow Measurement Devices

To ensure accurate results, facilities should evaluate, via visual observation and routine checks, the design, installation, and operation of flow measurement devices. When evaluating design, select a device which:

- Is accurate over the entire range of expected flow rates
- Can be installed in the channel to be monitored
- Is appropriate to the sampling location (i.e., power setup, submersible, etc.).

When evaluating the installation of flow measurement devices, ensure that:

- There are no leaks and/or bypasses of flow around the measuring device
- The primary device is level and squarely installed
- The secondary device is calibrated.

When evaluating the operation of flow measurement devices, look for:

- Excessive flows which submerge the measuring device
- Flows outside the accuracy range of the device
- Leaks and/or bypasses around the measuring device

- Turbulent flow through the measuring device
- Corrosion, scaling, or solids accumulation within the measuring device
- Obstructions to the measuring device
- Use of the correct factor or formula to convert head readings to actual flow rate.

Other than ensuring appropriate design and installation, accuracy checks are difficult to accomplish for primary flow measurement devices. Secondary flow measurement devices, on the other hand, may require evaluation of design, installation, and calibration. Applicants should examine the secondary recording devices and their readouts after installation to ensure that they are operating properly. Unusual fluctuations or breaks in flow indicate operational or design flaws.

3.2.2 ESTIMATING FLOW RATES

There are a variety of techniques for estimating flow rates. These methods are not as accurate as the methods described in Section 3.2.1 above, but are suitable for those discharges where primary or secondary devices are not practical or economically feasible. Each of the following methods is suitable for certain types of flow situations, as indicated. For each, the procedure for collecting flow rate data will be given along with a sample calculation.

Float Methods

Float methods can be used for any discharge where the flow is exposed and/or easily accessible. It is particularly useful for overland flows, gutter flows, and open drain or channel flows. The flow rate is calculated in each of the float methods by estimating the velocity of the flow and the cross-sectional area of the discharge and using the standard flow rate equation:

$$\textit{Flow Rate } (cfm) = \textit{Velocity } (ft/min) \times \textit{Area } (ft^2)$$

The velocity is estimated by measuring the time it takes a float to travel between two points (point A and point B) along the flow path. For most accurate results, the two points should be at least 5 feet apart. The cross-sectional area is estimated by measuring the depth of the water and the width of the flow, and multiplying the depth by the width. This assumes a uniform cross-section in the flow path and a geometric cross-section shape. The float method can also be used for any accessible pipe or ditch where the movement of the float can be traced downstream for at least 5 feet. Subsurface storm water flows can be measured with the float method where there are two accessible manholes.

If the flow is overland, the water will need to be directed into a narrow channel or ditch so that the measurements can be taken. The initial preparation for this method requires that a shallow channel or ditch be dug that is 6 feet long or longer and 4 to 12 inches wide. The channel or ditch should be shallow enough to easily obtain flow depths but should be deep enough to carry the flow that will be diverted to it. Boards or other barriers should be placed on the ground above the channel (so that the flow is diverted into the channel) and along the edges of the channel or ditch (flush with the ground surface so that flow does not seep under them).

The procedure for measuring the flow rate by the float method involves measuring the length of the channel between chosen points A and B (which must be 5 feet apart or more). The depth of the water at point B, in the middle of the channel, must be determined, and the width of the water flow must be measured at point B. A float is then placed in the water and timed as it moves from point A to point B. Exhibit 3-8 provides an example of estimating the flow rate using the float method.

For runoff flows from many directions into a drain in a low or flat area where ponding is evident, the float method can also be used. The total flow rate is calculated by measuring flow rates for several points into the drain and adding these values together. Exhibit 3-9 provides an example of estimating the flow rate using the float method in this situation.

Bucket and Stopwatch Method

The bucket and stopwatch method of estimating flow rate is the easiest of all the flow rate estimation procedures. However, it can only be used under certain conditions. The flow or discharge to be measured must be flowing from a small pipe or ditch, and it must be free-flowing. In other words,

EXHIBIT 3-8. EXAMPLE CALCULATION OF FLOAT METHOD FOR UNIMPEDED OPEN CHANNEL FLOW

Step 1: When each sample or aliquot is taken, record the data for the time the sample was taken and the length between points A and B (at least 5 feet apart). See columns A, B, and C.

EXAMPLE DATA:

A	B	C	D	E	F	G
Sample Number	Time in Minutes	Distance Between Points A & B (feet)	Time of Travel (A to B) (min)	Depth of Water at Point B (feet)	Width of Flow at Point B (feet)	Calculated Flow Rate (cfm)
1	0	5.0	0.17	0.12	0.5	1.8
2	20	5.0	0.18	0.25	0.5	3.5
3	40	5.0	0.20	0.29	0.5	3.6
4	60	5.0	0.21	0.33	0.5	3.9
5	80	5.0	0.18	0.29	0.5	4.0
6	100	5.0	0.17	0.25	0.5	3.7
7	120	5.0	0.17	0.12	0.5	1.8
8	140	5.0	0.16	0.12	0.5	1.9
9	160	5.0	0.18	0.12	0.5	1.7

Step 2: Place a float in the water flow at point A and time it as it moves from point A to point B. Record the time in minutes. See column D.

Step 3: Measure the depth of the water and the width of the flow at point B. See columns E and F.

Step 4: Calculate the flow rate for each sample time using the common flow rate formula. See column G.

Formulas:

$$Velocity\ (V) = \frac{Length\ from\ A\ to\ B}{Time\ of\ Travel}$$

$$Area\ (A) = Water\ Depth \times Width\ of\ Flow$$

$$Flow\ Rate\ (Q) = (V) \times (A)$$

Example: For Sample 1

$$V = \frac{5.0\ ft}{0.17\ min} = 29.4\ ft/min$$

$$A = 0.12\ ft \times 0.5\ ft = 0.06\ ft^2$$

$$Q = 29.4\ ft/min \times 0.06\ ft^2 = 1.8\ cfm$$

EXHIBIT 3-9. EXAMPLE CALCULATION OF FLOAT METHOD FOR ESTIMATING DRAIN FLOW RATES

Step 1: When each sample or aliquot is taken, record the data for the time the sample was taken. Measure the outer perimeter or edge of the drain where the water flows in. See columns B and C.

Step 2: Designate three evenly spaced points surrounding the drain approximately 3 to 5 feet from the drain. These points will be referred to as points A, B, and C. Record the distance from each point to the edge of the drain. See column D.

EXAMPLE DATA: Assume the drain dimensions are 1 ft x 1 ft square, and flow surrounds drain.

A	B	C	D			E			F			G
Sample Number	Sample Time (min)	Drainage Perimeter (feet)	Distance of Point to Drain (feet)			Time of Travel to Drain (min)			Depth of Water (feet)			Calculated Flow Rate (cfm)
			Pt. A	Pt. B	Pt. C	Pt. A	Pt. B	Pt. C	Pt. A	Pt. B	Pt. C	
1	0	4	3	4	5	0.2	0.3	0.5	0.08	0.08	0.08	4 cfm
2	20	4	3	4	5	0.3	0.4	0.5	0.11	0.12	0.14	5 cfm
3	40	4	3	4	5	0.3	0.4	0.5	0.11	0.12	0.14	5 cfm
4	60	4	3	4	5	0.4	0.5	0.6	0.16	0.17	0.20	6 cfm
5	80	4	3	4	5	0.3	0.4	0.5	0.11	0.12	0.14	5 cfm
6	100	4	3	4	5	0.3	0.4	0.5	0.11	0.12	0.14	5 cfm
7	120	4	3	4	5	0.3	0.4	0.5	0.11	0.12	0.14	5 cfm
8	140	4	3	4	5	0.3	0.4	0.5	0.11	0.12	0.14	5 cfm
9	160	4	3	4	5	0.2	0.3	0.5	0.08	0.08	0.08	4 cfm

Step 3: Place a float at each of the three points and measure the time it takes to reach the drain. Record the times in minutes. See column E.

Step 4: Determine the depth of flow at each place where the float enters the drain from points A, B, and C. Record the depth in feet. See column F.

Step 5: Calculate the flow rate by adding the individual flow rates for points A, B, and C. Record the data in column G.

Formulas:

$$Velocity\ (V) = \frac{Distance\ of\ Point\ from\ Drain}{Time\ of\ Travel}$$

$$Area\ (A) = Water\ Depth \times Drainage\ Perimeter$$

$$Flow\ Rate\ (Q) = 1/n\Sigma A_n V_n,\ where\ n\ equals\ points\ A,\ B,\ and\ C$$

Example: For Sample 1

$$V_A = \frac{3\ Feet}{0.2\ Min} = 15\ ft/min$$

$$A_A = 0.08\ ft \times 4\ ft = 0.32\ ft^2$$

EXHIBIT 3-9. EXAMPLE CALCULATION OF FLOAT METHOD FOR ESTIMATING DRAIN FLOW RATES (Continued)

$$Q_{TOTAL} = \frac{1}{4}(V_A A_A + V_B A_B + V_C A_C)$$

$$= \frac{1}{4}[(15\ ft/min)(0.32\ ft^2) + (13\ ft/min)(0.32\ ft^2) + (10\ ft/min)(0.32\ ft^2)]$$

$$= 4\ cfm$$

the pipe or ditch must be raised above the ground. Also, the flow must be small enough to be captured by a bucket or other suitable container without overflowing. If these conditions are not present, another method must be used. The procedure involves recording the time that each sample is taken, the time it takes for the container to be filled, and the volume of discharge collected. The flow rate is then calculated in gallons per minute (gpm) or in cubic feet per minute (cfm). The basis for the bucket and stopwatch method is the collection of a measured amount of flow over a measured amount of time to determine flow per unit of time (or flow rate) as per the formula below.

$$Flow\ Rate\ Q\ (gpm) = \frac{Volume\ of\ Bucket\ (gal)}{Time\ to\ Fill\ (sec)} \times \frac{60\ sec}{1\ min}$$

Exhibit 3-10 provides an example of estimating flow rates with the bucket and stopwatch method.

Slope and Depth Method

The slope and depth method is also a relatively easy method for estimating flow rates in pipes and ditches. This procedure requires that the slope of the pipe or ditch be known. A survey or engineering design data such as sewer or grading plans may provide the slope or grade of the pipe or ditch. In addition, the flow or effluent to be measured should not fully fill the pipe or ditch from which it is flowing. To measure the depth of the flow at the center of the pipe or ditch at the outfall, the outfall should be accessible. If these conditions are not present, another method should be used. The procedure involves recording the time that each sample is taken and measuring the depth of the flow in the middle of the pipe or ditch. If the flow is coming from a pipe, the inside diameter of the pipe should be recorded. If the effluent is coming from a ditch, the width of the flow in the ditch should be measured. Also, the modified slope of the ditch should be calculated. The flow rate is calculated in cfm using the same formulas for both pipes and ditches. Exhibit 3-11 provides an example of estimating the flow rate with the slope and depth method.

EXHIBIT 3-10. EXAMPLE CALCULATION OF BUCKET AND STOPWATCH METHOD FOR ESTIMATING FLOWS

Step 1: When each sample or aliquot is taken, record the data for the time the sample was taken. See column B.

EXAMPLE DATA:

A	B	C	D	E	F
Sample Number	Time (minutes)	Time to Fill Bucket (seconds)	Volume of Bucket (gallons)	Calculated Flow Rate (gpm)	Calculated Flow Rate in (cfm)
1	0	40.0	2.0	3.0	0.4
2	20	26.0	2.0	4.6	0.6
3	40	24.0	2.0	5.0	0.7
4	60	32.0	2.0	3.7	0.5
5	80	45.0	2.0	2.7	0.4
6	100	31.0	2.0	3.9	0.5
7	120	50.0	2.0	2.4	0.3
8	140	21.0	2.0	5.7	0.8
9	160	28.0	2.0	4.3	0.6

Step 2: Put a bucket beneath the flow, while measuring with a stopwatch the time it takes to fill the bucket to a certain level. If the water spills over the sides, the process must be redone. Record the time it took to fill the volume of water. See columns C and D.

Step 3: Calculate the flow rate in gpm and cfm.

Formulas:

$$Flow\ Rate,\ Q(gpm) = \frac{Volume\ of\ bucket\ (gal)}{Time\ to\ fill\ (sec)} \times \frac{60\ sec}{1\ min}$$

$$Q(cfm) = Q(gpm) \times 0.1337\ ft^3/gal$$

Example: For Sample 1

$$Q\ (gpm) = \frac{2\ gal}{40.0\ sec} \times \frac{60\ sec}{1\ min} = 3.0\ gpm$$

$$Q\ (cfm) = 3.0\ gpm \times 0.1337\ ft^3/gal = 0.4\ cfm$$

EXHIBIT 3-11. EXAMPLE CALCULATION OF SLOPE AND DEPTH METHOD FOR ESTIMATING FLOW RATES

Step 1: Obtain the pipe or ditch channel percent slope from engineering data. Determine the inside diameter if the flow is from a pipe.

EXAMPLE DATA: For purposes of this example, a ditch with a 2 percent slope is assumed.

Step 2: When each sample or aliquot is taken, record the data for the time the sample was taken. See column B.

EXAMPLE DATA:

A	B	C	D	E	F	G
Sample Number	Time (minutes)	Depth of Water (in)	Width of Flow (ditch only) (feet)	"M" Modified Slope (ditch only)	Calculated Flow Rate (cfm Pipe only)	Calculated Flow Rate (cfm ditch only)
1	0	3.6	2.2	3.7	-	246.1
2	20	6.0	3.2	3.2	-	713.6
3	40	7.2	4.0	3.3	-	1,237.3
4	60	8.4	4.2	3.0	-	1,532.9
5	80	7.2	4.0	3.3	-	1,237.3
6	100	6.0	3.2	3.2	-	713.6
7	120	6.0	3.0	3.0	-	624.2
8	140	6.0	2.9	2.9	-	581.8
9	160	4.6	2.5	3.3	-	374.1

Step 3: Measure the depth of the water in the center of the pipe or ditch. Record the data in feet. See column C.

Step 4: Measure the width of the flow only if the flow is in a ditch. Record the data in feet. See column D.

Step 5: Calculate the modified side slope only if the flow is in a ditch (leave column E blank if the flow is in a pipe).

Formula:
$$\text{Modified slope } (M) = \frac{12.0 \text{ in/ft} \times \text{flow width (ft)}}{2.0 \times \text{water depth (in)}}$$

Example: Sample 1:
$$M = \frac{12.0 \text{ in/ft} \times 2.2 \text{ ft}}{2.0 \times 3.6 \text{ in}} = 3.7$$

Step 6: For pipes, calculate the flow rate and record the data in column F.

$$\text{Flow Rate } (Q) = 0.004 \times (I.D.)^{1.67} \times D \times \sqrt{S}$$

where Q = flow rate in pipe (cfm), $I.D.$ = inside diameter of pipe (in), D = water depth (in), S = pipe slope (%)

Step 7: For ditches or channels, calculate the flow rate in cfm. Record the flow rate in column G.

Formula:
$$\text{Flow Rate } (Q) = \frac{0.42M \times (M)^{1.67} \times (D)^{2.67} \times \sqrt{S}}{(M^2 + 1)^{0.33}}$$

where Q = flow rate in ditch (cfm), M = modified slope, D = water depth (in), S = ditch slope (%)

Example: For Sample 1:
$$Q_1 = \frac{0.42\,(3.7) \times (3.7)^{1.67} \times (3.6)^{2.67} \times \sqrt{2}}{[(3.7)^2 + 1]^{0.33}}$$

$$Q = 246.1 \text{ cfm}$$

Runoff Coefficient Methods

Runoff coefficient methods are the least accurate of all the flow rate estimation methods. These methods should only be used for composite flow-weighted samples if all of the other methods are inappropriate for the site. Although the least accurate, runoff coefficients are the simplest method of estimating runoff rates.

Runoff coefficients represent the fraction of total rainfall that will be transmitted as runoff from the drainage area that flows into the facility outfall. Runoff coefficients consider the ground surface or cover material and determine the amount of storm water flow which may infiltrate or runoff as a discharge. A simple estimate of runoff volume assumes that paved areas and other impervious structures such as roofs have a runoff coefficient of 0.90 (i.e., 90 percent of the rainfall leaves the area as runoff). For unpaved surfaces, a runoff coefficient of 0.50 is normally assumed. A more accurate estimate can be made by using more specific runoff coefficients for different areas of the facility, based on the specific type of ground cover. Commonly used runoff coefficients are listed in Exhibit 3-12.

The average runoff coefficient can be estimated for drainage areas that have both paved and unpaved areas by weighting the coefficients based on their proportion of the total area. An equation for this would be:

$$\textit{Estimated Average Runoff Coef.} = \frac{(\textit{Area A})(\textit{Runoff Coef. A}) + (\textit{Area B})(\textit{Runoff Coef. B})}{\textit{Area A} + \textit{Area B}}$$

The area of the drainage basin can generally be obtained from land surveys conducted at the time of facility purchase or site surveys taken from design documents developed as part of construction planning. If these are not available, the applicant may estimate the drainage areas from a topographic map of the area. The areas used in this calculation should include only those areas drained by the sampled outfall. When determining the basin area that drains through the outfall, some special considerations should be noted: (1) storm water from sources outside an industrial facility's property boundary may contribute to the discharge; and (2) storm water not associated with industrial activity may contribute to the flow volume. Where these conditions occur, the facility should accurately quantify and appropriately address these contributions.

EXHIBIT 3-12. TYPICAL "c" COEFFICIENTS FOR 5- TO 10-YEAR FREQUENCY DESIGN STORMS

Description of Area	Runoff Coefficients
Business	
• Downtown areas	0.70-0.95
• Neighborhood areas	0.50-0.70
Residential	
• Single-family areas	0.30-0.50
• Multiunits (detached)	0.40-0.60
• Multiunits (attached)	0.60-0.75
Residential (suburban)	0.25-0.40
Apartment dwelling areas	0.50-0.70
Industrial	
• Light areas	0.50-0.80
• Heavy areas	0.60-0.90
Parks and cemeteries	0.10-0.25
Playgrounds	0.20-0.35
Railroad yard areas	0.20-0.40
Unimproved areas	0.10-0.30
Streets	
• Asphalt	0.70-0.95
• Concrete	0.80-0.95
• Brick	0.70-0.85
Drives and walks	0.75-0.85
Roofs	0.75-0.95
Lawns - course textured soil (greater than 85 percent sand)	
• Slope: Flat (2 percent)	0.05-0.10
Average (2-7 percent)	0.10-0.15
Steep (7 percent)	0.15-0.20
Lawns - fine textured soil (greater than 40 percent clay)	
• Slope: Flat (2 percent)	0.13-0.17
Average (2-7 percent)	0.18-0.22
Steep (7 percent)	0.25-0.35

Source: *Design and Construction of Sanitary and Storm Sewers*, with permission from the publisher, American Society of Civil Engineers, *Manual of Practice*, page 37, New York, 1960.

There are two specific methods to estimate flow rate using runoff coefficients. The first method uses depth of flow in a pipe or ditch and an average runoff rate to estimate each of the sample flow rates where the slope/pitch of the pipe or ditch is unknown. Exhibit 3-13 provides an example calculation of estimating flow rates based on depth and runoff coefficients. The second method uses only rainfall accumulation and runoff coefficients to estimate a flow associated with the time the sample was taken. No actual flows or flow depths are measured. Exhibit 3-14 provides an example of estimating the flow rate based on rainfall depth and runoff coefficients.

3.2.3 MEASURING TOTAL FLOW VOLUMES FOR THE SAMPLED RAIN EVENT

Similar to measuring flow rates, flow volumes may be measured using automatic flowmeters or primary/secondary devices as discussed in Section 3.2.1. Measurement of flow volume with these devices provides a reasonably accurate determination of the total flow volume for the entire storm water discharge. In many cases, however, primary or secondary devices have not been installed for storm water flow measurement. Portable flow measurement devices are often expensive. Many of the automatic samplers that are currently on the market can measure flow volumes as well as perform sampling. Where available and when economically feasible, measuring devices should be used to generate data for calculating flow.

3.2.4 ESTIMATING TOTAL FLOW VOLUMES FOR THE SAMPLED RAIN EVENT

Since accurate measurement of total flow volumes is often impracticable due to lack of equipment, total flow volumes are more commonly estimated. The two methods provided in this section require only simple estimated measurements. The first method is based on rainfall depths and runoff coefficients and the second is based on flow rates that can be either measured or estimated.

Runoff Coefficients Methods

Discharge volumes are most easily estimated using the area of the drainage basin contributing to the outfall, the rainfall accumulation, and a runoff coefficient. The total volume of discharge can be estimated using a simple equation that relates the amount of rainfall to the volume of discharge that will leave the site as runoff. The equation is as follows:

EXHIBIT 3-13. EXAMPLE CALCULATION OF RUNOFF COEFFICIENT/FLOW DEPTH METHOD FOR ESTIMATING FLOW RATES

Step 1: Estimate the runoff coefficient for the drainage area that contributes flow to the sampled outfall (see Section 3.2.2).

EXAMPLE: Assume the drainage area to the outfall is 3 acres. Two of those acres are paved with a runoff coefficient of .90, and 1 is unpaved with a runoff coefficient of .50. Using the equation for estimated runoff coefficient from the text in Section 2.2.2.2:

$$Est.\ Run.\ Coef. = \frac{(2\ Ac)\ (0.90) + (1\ Ac)\ (0.50)}{2\ Ac + 1\ Ac} = 0.77$$

The runoff coefficient for the entire drainage area is 0.77.

Step 2: Measure the rainfall depth. Record the total rainfall of the storm or the rainfall that occurred in the first 3 hours (if it lasted more than 3 hours). Also record the duration of the rain event.

EXAMPLE: Assume the rainfall depth to be 1.0 inches in 3 hours.

Step 3: Calculate an average runoff rate.

Formula:

$$Average\ Runoff\ Rate = \frac{Drainage\ Area \times Runoff\ Coef. \times Rainfall\ Depth}{Rainfall\ Duration}$$

Example:

$$Average\ Runoff\ Rate = \frac{3\ Ac \times .77 \times 1\ in}{3\ hrs} \times \frac{43{,}560\ ft^2}{Ac} \times \frac{ft}{12\ in} \times \frac{hr}{60\ min} = 47\ cfm$$

When each sample or aliquot is taken, record the data for the time the samples were taken and the depth of the water in the center of the ditch or pipe. Record the data in columns B and C.

EXAMPLE DATA:

A	B	C	D	E
Sample Numbers	Time (minutes)	Channel or Ditch Water Depth (feet)	Calculated Depth-Weighted Flow Factor	Flow Rate (cfm)
1	0	1.0	0.82	39
2	20	1.1	0.90	42
3	40	1.2	0.98	46
4	60	1.25	1.02	48
5	80	1.3	1.06	50
6	100	1.25	1.02	48
7	120	1.2	0.98	46
8	140	1.7	1.39	65
9	160	1.0	0.82	39

Step 4: Sum up all the water depths for each sample taken as indicated above in column C.

Sum = 11.0 feet

EXHIBIT 3-13. EXAMPLE CALCULATION OF RUNOFF COEFFICIENT FLOW DEPTH METHOD FOR ESTIMATING FLOW RATES (Continued)

Step 5: Calculate a depth-weight flow factor and record the data in column D.

Formula:

$$Factor = \frac{Measured\ Water\ Depth \times Number\ of\ Flow\ Measurements}{Sum\ of\ all\ Water\ Depths}$$

Example: For Sample 1

$$Factor = \frac{(1\ ft) \times 9}{11.0} = 0.82$$

Step 6: Calculate the flow rate. Record the data in column E.

Formula:

$$Flow\ Rate,\ Q\ (cfm) = Average\ Runoff\ Rate \times Depth\ Factor$$

Example: For Sample 1

$$Q = 47\ cfm \times 0.82 = 39\ cfm$$

$$V_t = R_t \times [(A_{paved} \times C_{runoff}) + (A_{unpaved} \times C_{runoff})]$$

where: V_t = the total runoff volume in cubic feet
R_t = the total rainfall measured in feet
A_{paved} = the area (sq ft) within the drainage basin that is paved or roofed
$A_{unpaved}$ = the area (sq ft) within the drainage basin that is unpaved
C_{runoff} = a specific runoff coefficient (no units) for the drainage area ground cover

Exhibit 3-15 provides an example calculation of total runoff volume from rainfall data.

Discharge Volumes Estimated Based on Measured Flow Rates

Another method of estimating the total volume of a discharge uses a series of measured or estimated flow rates. The total volume of discharge can be estimated by first multiplying each of the flow rates by the time interval in between flow measurements. This time period represents the portion of the total storm duration that can be associated with the flow rate measurement. Adding all such partial volumes results in a total flow volume. A procedure for calculating the total runoff volume from a set of discrete measurements of flow depth and velocity in a ditch during a storm runoff event is presented in Exhibit 3-16.

EXHIBIT 3-14. EXAMPLE CALCULATION OF RUNOFF COEFFICIENT RAINFALL DEPTH METHOD FOR ESTIMATING FLOW RATES

Step 1: Estimate the runoff coefficient for the drainage area that contributes flows to the sampled outfall.

EXAMPLE: See Step 1 in Exhibit 3-14. The site for this example will be similar so a coefficient of .77 will be used for the same 3-acre drainage area.

Step 2: When each sample or aliquot is taken, record the data for the time the sample was taken. Record the data in column B.

EXAMPLE DATA:

A	B	C	D	E	F
Sample Number	Time (minutes)	Total Rainfall Depth (inches)	Time Since Last Sample	Incremental Rainfall (inches) per 20 minutes	Calculated Flow Rate (cfm)
1	0	0.0	0	0.0	–
2	20	0.2	20	0.2	84
3	40	0.3	20	0.1	42
4	60	0.5	20	0.2	84
5	80	0.6	20	0.1	42
6	100	0.8	20	0.2	84
7	120	0.9	20	0.1	42
8	140	1.0	20	0.1	42
9	160	1.1	20	0.1	42

Step 3: Using a rainfall gauge, measure the total rainfall depth (in inches) and record the data in column C.

EXAMPLE: See sample data above.

Step 4: Calculate the incremental time since the last flow measurement and record the data in column D.

EXAMPLE: Samples were taken 20 minutes apart so this increment will be 20 minutes for every sample.

Step 5: Calculate the additional or incremental rainfall that has occurred since the last measurement. Record the data in column E.

Formula:

$$Incremental\ Rainfall = Total\ Rainfall\ Sample\ 2 - Total\ Rainfall\ Sample\ 1$$

Example: For Sample 2

$$Incremental\ Rainfall = .2 - 0 = .2\ inches$$

Step 6: Calculate the flow rate. Record the data in column F

Formula:

$$Flow\ Rate\ (cfm) = \frac{(Drainage\ area)(Runoff\ coefficient)(Incremental\ rainfall)}{(Incremental\ time)}$$

Example:

$$Flow\ Rate = \frac{(3\ Ac)(0.77)(0.2\ in)}{20\ min} \times \frac{(43{,}560\ ft^2)}{Ac} \times \frac{1\ ft}{12\ in} = 84\ cfm$$

EXHIBIT 3-15. EXAMPLE CALCULATION OF TOTAL RUNOFF VOLUME FROM RAINFALL DATA

Step 1: Determine the area of drainage contributing to the runoff volume at the outfall and convert it to square feet.

Example: Using a land survey, a facility has determined its site encompasses 0.3 acres (13,068 square feet). The entire site is used for industrial activities, and therefore, any storm water discharges from the site will be associated with industrial activity. A berm surrounds the entire site limiting the drainage area to the site itself and preventing any dilution or contamination from other discharges. (Note: To convert acres to square feet, multiply the number of acres by 43,560, which is the conversion factor).

Step 2: Determine the rainfall depth during the event that was sampled to the nearest one-hundredth of an inch and convert it to feet.

Example: From the rain gauge, the rainfall accumulation is measured at 0.6 inches or 0.05 feet (ft). (Note: To convert inches to feet, divide the inches by 12, which is the conversion factor).

Step 3: Determine the runoff coefficients for each area.

Example: The facility has estimated that ⅓ of the site, or 4,356 square feet, is covered by impervious surfaces (i.e., roofs or paved roadways) and ⅔ of the site, or 8,712 square feet, is unpaved.

Step 4: Calculate the volume of flow using the following formula and convert the volume to liters.

Formula: ***Total runoff volume in cubic feet (cu ft) = total rainfall (ft) x [facility paved area (sq ft) x 0.90 + facility unpaved area (sq ft) x 0.50]***

Example: ***Total runoff volume (cu ft) = 0.05 x [4,356 x 0.90 + 8,712 x 0.50]***

Total runoff volume = 413.8 cu ft or 11,720 liters

(Note: To convert cubic feet to liters, multiply cubic feet by 28.32, which is the conversion factor).

EXHIBIT 3-16. EXAMPLE CALCULATION OF TOTAL RUNOFF VOLUME FROM FLOW RATE DATA

Step 1: Measure and tabulate flow depths and velocities every 20 minutes (at the same time that the sample is collected) during at least the first 3 hours of the runoff event.

EXAMPLE DATA:

A	B	C	D	E	F
Sample Number	**Time (minutes)**	**Flow Velocity (feet per minute)**	**Flow Depth (feet)**	**Width (feet)**	**Calculated Flow Rate (cfm)**
1	0	-	-	-	-
2	20	4	0.2	5	4
3	40	8	0.4	5	16
4	60	12	0.4	5	24
5	80	8	0.4	5	16
6	100	4	0.2	5	4
7	120	8	0.2	5	8
8	140	4	0.2	5	4
9	160	4	0.2	5	4

EXHIBIT 3.16. EXAMPLE CALCULATION OF TOTAL RUNOFF VOLUME FROM FLOW RATE DATA (Continued)

Step 2: Calculate and tabulate the cross-sectional area of flow for each of the flow depths measured. Calculate the flow rate for each discrete set of measurements.

Formula: $$\textit{Flow Rate } Q \textit{ (cfm)} = \textit{Velocity (ft/min)} \times \textit{Area (sq ft)}$$
$$\textit{Area} = \textit{Depth} \times \textit{Width}$$

Example: For Sample 1

$$\textit{Area} = 0.2 \textit{ ft} \times 5 \textit{ ft} = 1 \textit{ sq ft}$$
$$\textit{Flow Rate} = 4 \textit{ ft/min} \times 1 \textit{ sq ft} = 4 \textit{ cfm}$$

Step 3: Plot the flow rate, Q, versus time. Also, assume that flow drops uniformly from the last calculated flow rate (Q_9) to zero at the time when Q_{10} would have been taken.

Example: The flow rates calculated in Step 3 are plotted against the time between samples.

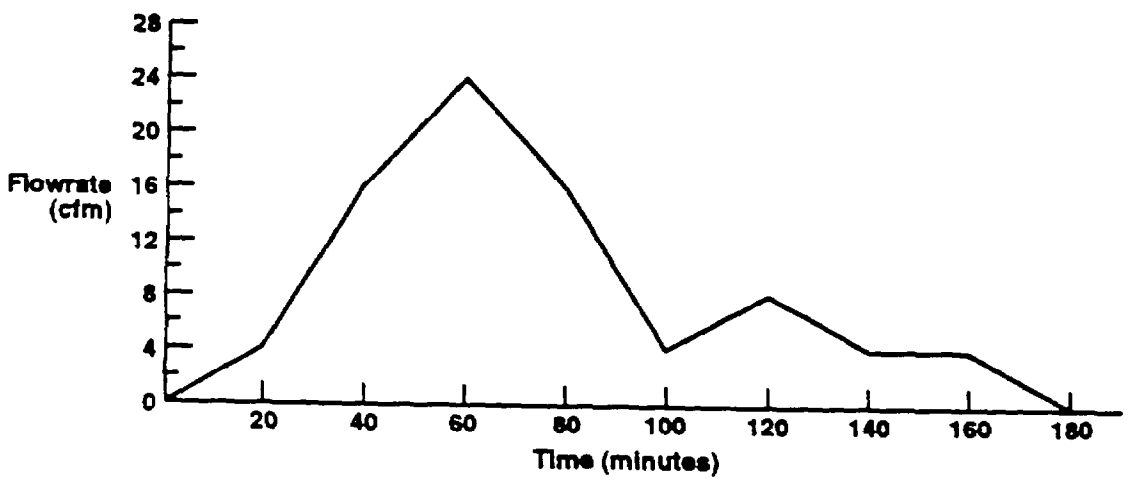
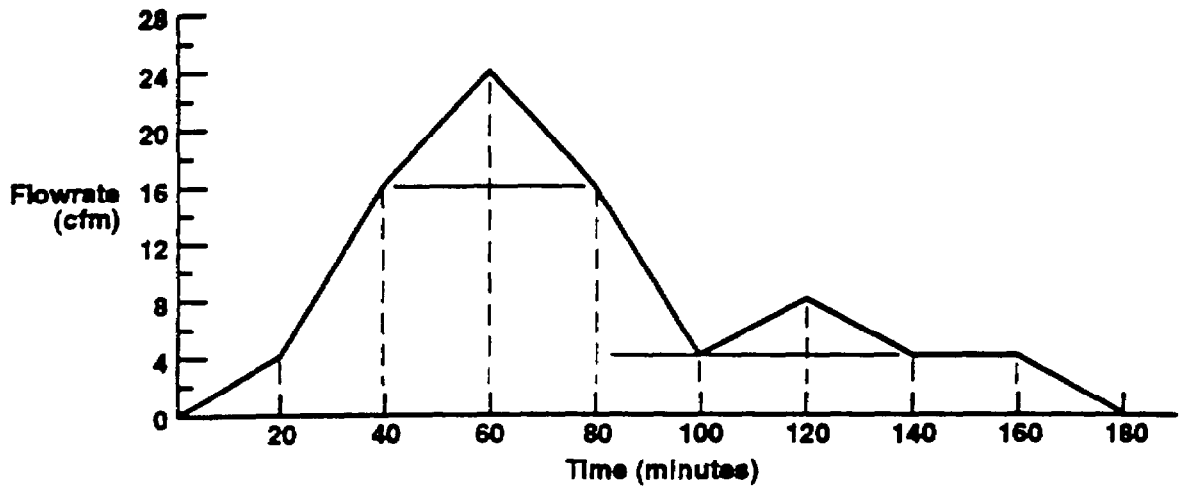


EXHIBIT 3-16. EXAMPLE CALCULATION OF TOTAL RUNOFF VOLUME FROM FLOW RATE DATA (Continued)

Step 4: The total flow volume (V_t) can be calculated by geometrically determining the area under the curve. The summation of the individual volumes per increment of time (V_1 through V_9) is the total flow volume of the event.

Example:



Step 5: Compute the flow volume associated with each observation (V_1, V_2, ..., V_9) by multiplying the measured flow rate by the duration (in this case, 20 minutes). Be sure the units are consistent. For example, if durations are in minutes and flow velocities are in cubic feet per second (cfs), convert the durations to seconds or the velocities to feet per minute.

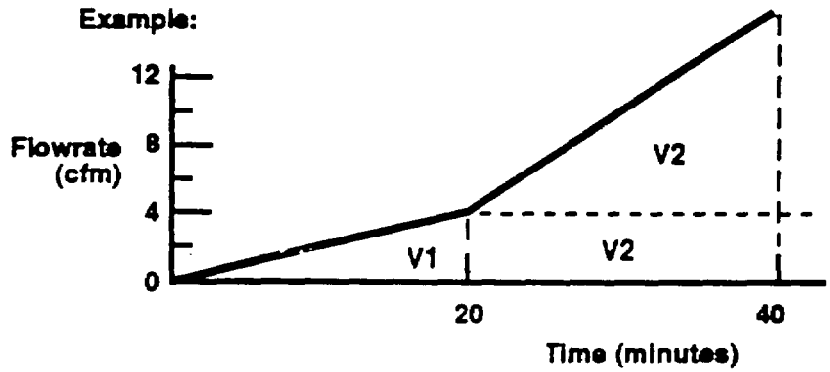


EXHIBIT 3-16. EXAMPLE CALCULATION OF TOTAL RUNOFF VOLUME FROM FLOW RATE DATA (Continued)

Formula: ***Volume (V) = Flow Rate (cfm) x Duration (minutes)***

Example:

$$V_1 = \frac{1}{2}(Q_1 - Q_0)(t_1 - t_0) = \frac{1}{2}(4-0)(20-0) = 40\ ft^3$$

$$V_2 = \frac{1}{2}(Q_2 - Q_1)(t_2 - t_1) + Q_1(t_2 - t_1)$$

$$= \frac{1}{2}(16-4)(40-20) + 4(20)$$

$$= 120 + 80 = 200\ ft^3$$

$V_1 = 40\ ft^3$
$V_2 = 200\ ft^3$
$V_3 = 400\ ft^3$
$V_4 = 400\ ft^3$
$V_5 = 200\ ft^3$
$V_6 = 120\ ft^3$
$V_7 = 120\ ft^3$
$V_8 = 80\ ft^3$
$V_9 = 40\ ft^3$

Step 6: Total the individual volumes calculated in Step 5 to obtain the total runoff volume.

Example:

Total Storm Runoff = 1,600 ft³

3.2.5 REPORTING STORM WATER DISCHARGE FLOW RATES AND VOLUMES

Form 2F requires applicants to provide quantitative data (reported both as concentration and as total mass) based on flow-weighted samples collected during storm events. In addition, applicants are required to provide flow estimates or flow measurements, as well as an estimate of the total volume of the discharge. The method of flow estimation or measurement must be described in the application. Although EPA only requires flow estimates in Form 2F, accurate flow measurement is necessary for collecting representative flow-weighted composite samples and reporting pollutant mass loadings.

3.2.6 MEASURING RAINFALL

Many types of instruments have been developed to measure the amount and intensity of precipitation. All forms of precipitation are measured on the basis of the depth of the water that would accumulate on a level surface if precipitation remained where it fell. There are two types of rain gauges -- standard and recording gauges. A standard rain gauge collects the rainfall so that the amount of rain can be easily measured. The standard gauge for the NWS has a collector which is 8 inches in diameter. Rain flows from the collector into a cylindrical measuring tube inside the overflow can. The measuring tube has a cross-sectional area one tenth the size of the collector so that 0.1 inch of rainfall will fill 1 inch of the measuring tube. While this standard gauge is both accurate and easy to use, any open receptacle with vertical sides can be an effective rain gauge. Standard rain gauges are simple and inexpensive; however, with a standard gauge, there is no way to record changes in the intensity of the rainfall without making frequent observations of the gauge during the storm.

The second type of gauge is the recording rain gauge, which provides a permanent record of the amount of rainfall which accumulates over time. Three common types of recording gauges are:

- Tipping Bucket Gauge - Water caught in a collector is funneled into a two-compartment bucket; a known quantity of rain fills one compartment, overbalancing the bucket and emptying it into a reservoir. This moves the second bucket into place beneath the funnel. The tipping of the bucket engages an electric circuit, which records the event.

- Weighing Type Gauge - Water is weighed when it falls into a bucket placed on the platform of a spring or lever balance. The weight of the contents is recorded on a chart, showing the accumulation of precipitation.

- Float Recording Gauge - Water is measured by the rise of a float that is placed in the receiver. These gauges may be self-siphoning, or may need to be emptied periodically by hand.

Recording rain gauges provide a permanent record of rainfall, and they can be used to determine variations in rainfall intensity over time without making frequent observations during the storm. But recording gauges are more complicated mechanically than standard gauges, making them more costly, less durable, and more difficult to operate.

Although all gauges are subject to error, most errors can be minimized. To minimize errors, the gauge should be placed on a level surface that is not windswept and is away from trees or buildings that might interfere with the path of rainfall. When taking measurements, other factors contributing to error should also be considered: mistakes in reading the scale, dents in the collector rim (which changes the receiving area), measuring sticks that may retain some of the water, and water lost to evaporation. In the case of tipping bucket gauges, water may not be collected while the bucket is still tipping. The most common source of inaccuracy is changes in data that are attributable to wind. It is possible to assess wind errors by comparing measurements of gauges that are protected from the wind with those that are not.

3.3 GRAB SAMPLE COLLECTION

Section 3.1.2 discussed both the parameters that must be monitored by grab sample and the conditions under which grab sampling is required. This section explains how to collect grab samples. The entire sample is collected at an uninterrupted interval (i.e., grabbed at one time). A grab sample provides information on the characterization of storm water at a given time and may be collected either manually or automatically as discussed below.

3.3.1 HOW TO MANUALLY COLLECT GRAB SAMPLES

A manual grab is collected by inserting a container under or downcurrent of a discharge with the container opening facing upstream. Generally, simplified equipment and procedures can be used. In most cases, the sample container itself may be used to collect the sample. Less accessible outfalls may require the use of poles and buckets to collect grab samples. To ensure that manual grab samples are representative of the storm water discharged, the procedures set forth in Exhibit 3-17 should be followed.

EXHIBIT 3-17. RECOMMENDED OPERATING PROCEDURES FOR TAKING GRAB SAMPLES

- Label sample containers before sampling event
- Take a cooler with ice to the sampling point
- Take the grab from the horizontal and vertical center of the channel
- Avoid stirring up bottom sediments in the channel
- Hold the container so the opening faces upstream
- Avoid touching the inside of the container to prevent contamination
- Keep the sample free from uncharacteristic floating debris
- Transfer samples into proper containers (e.g., from bucket to sample container), however, fecal coliform, fecal streptococcus, phenols and O&G should remain in original containers
- If taking numerous grabs, keep the samples separate and labelled clearly
- Use safety precautions (see Chapter 6)

Specialized equipment and procedures may be needed, particularly in situations where storm water discharges are inaccessible or where certain parameters are monitored. For example:

- When sampling for O&G and VOCs, equipment that safely and securely houses O&G bottles or VOC vials should be used. This may be necessary because: (1) O&G will adhere to containers and thus should not be transferred from one container to another; and (2) excessive aeration during sampling may result in the partial escape of VOCs.
- Since facilities sometimes use sample bottles that already contain preservatives (as provided by contract laboratories), extreme care should be taken when filling them to avoid spills, splatters, or washout of the preservatives.

All equipment and containers that come into contact with the sample must be clean to avoid contamination. Additionally, sample collection equipment and container materials should be totally unreactive to prevent leaching of pollutants. Cleaning procedures are discussed in detail in Section 3.5.

3.3.2 HOW TO COLLECT GRAB SAMPLES BY AUTOMATIC SAMPLER

Grab samples can also be collected using programmed automatic samplers. Automatic samplers come equipped with computers that can be programmed to collect grab samples. Programming for grabs is specific to the type of automatic sampler. Some samplers are portable and have been developed specifically to sample for storm water discharges. These samplers are frequently attached to a rain gauge and/or a flow sensor. Such samplers can be programmed to initiate sample collection by one or more of the following conditions: (1) depth of flow in a channel; (2) rainfall in inches; (3) flow rate; (4) time; (5) external signal; and (6) combinations of the first three conditions. For example, an automatic sampler could be used to collect a sample at 15-minute intervals after its sensors indicate that rainfall has begun.

When using an automatic sampler, planning is very important. First, all equipment must be properly cleaned, particularly the tubing and the sample containers. There are several different types of tubing available, including rubber and Tygon tubing. Tygon tubing is commonly used since it generally does not leach contaminants. Deionized water should be drawn through the sampler to remove any remaining pollutant residuals prior to taking samples. Tubing should also be replaced periodically to avoid algae or bacterial growth.

Sampling personnel should also use adequate and appropriate containers and ensure they are properly cleaned. Section 3.5 contains information on cleaning procedures which should be followed for all equipment. Additionally, the utilization of blanks (a control used to verify the accuracy of analytical results) is recommended to determine if cross-contamination of sampling equipment has occurred. Samplers should also be programmed, set up, and supplied with a source of power. Properly charged batteries should be readily available for portable samplers in advance of a storm event and, as a backup power supply in case of power failure. Finally, although automatic samplers may be useful in some situations, several parameters are not amenable to collection by automatic sampler. These pollutants include fecal streptococcus, fecal coliforms, oil and grease and VOCs which should be collected manually, not automatically, as discussed in Section 3.1.2.

3.4 FLOW-WEIGHTED COMPOSITE SAMPLE COLLECTION

Composite samples are samples simply comprised of a series of individual sample aliquots that have been combined to reflect average pollutant concentrations of the storm water discharge during the

sampling period. Composite samples can be developed based on time or flow rate. There are four different types of composite samples, as follows:

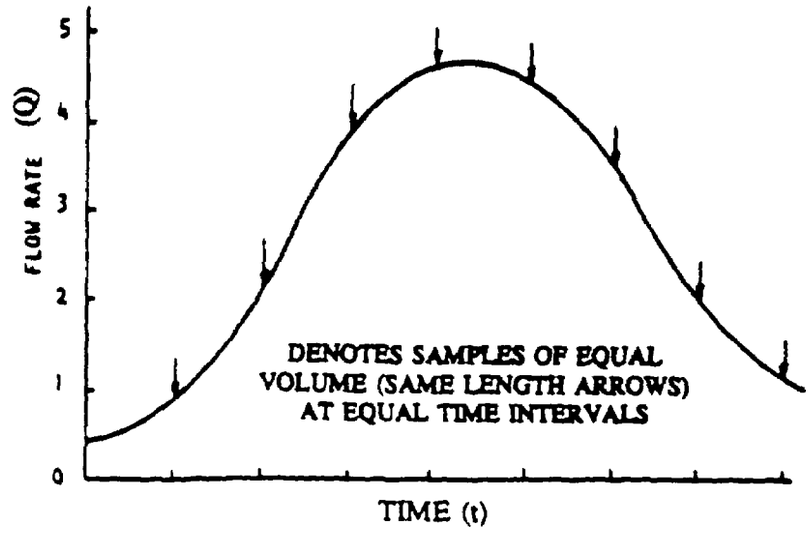
- Constant Time - Constant Volume - Samples of equal volume are taken at equal increments of time and composited to make an average sample (similar to Exhibit 3-18). This method is not acceptable for samples taken for compliance with the storm water permit application regulations.
- Constant Time - Volume Proportional to Flow Increment - Samples are taken at equal increments of time and are composited proportional to the volume of flow since the last sample was taken (see Exhibit 3-19).
- Constant Time - Volume Proportional to Flow Rate - Samples are taken at equal increments of time and are composited proportional to the flow rate at the time each sample was taken (see Exhibit 3-20).
- Constant Volume - Time Proportional to Flow Volume Increment - Samples of equal volume are taken at equal increments of flow volume and composited (see Exhibit 3-21).

Generally, flow-weighted composite samples must be collected for most parameters. The methods for generating flow-weighted composite samples are discussed in the following sections.

For storm water discharge permit applications, the aliquots for flow-weighted composite samples must be collected during a representative storm for the first 3 hours, or for the duration of the storm event if it is less than 3 hours long. The storm water application regulations allow for flow-weighted composite samples to be collected manually or automatically. For both methods, equal volume aliquots may be collected at the time of sampling and then flow-proportioned and composited in the laboratory, or the aliquot may be collected based on the flow rate at the time of sample collection and composited in the field. When composite samples are collected, the regulations require that each aliquot collection be separated by a minimum of 15 minutes and that a minimum of three sample aliquots be taken within each hour of the discharge. See Exhibit 3-22 for an example of how this requirement may be fulfilled.

The provisions set forth in 40 CFR 122.21(g)(7) for collecting flow-weighted composite samples establish specific requirements for minimum time duration between sample aliquots. Where these conditions cannot be met, the permitting authority may allow alternate protocols with respect to the time duration between sample aliquots (see Chapter 5). However, permission from the permitting

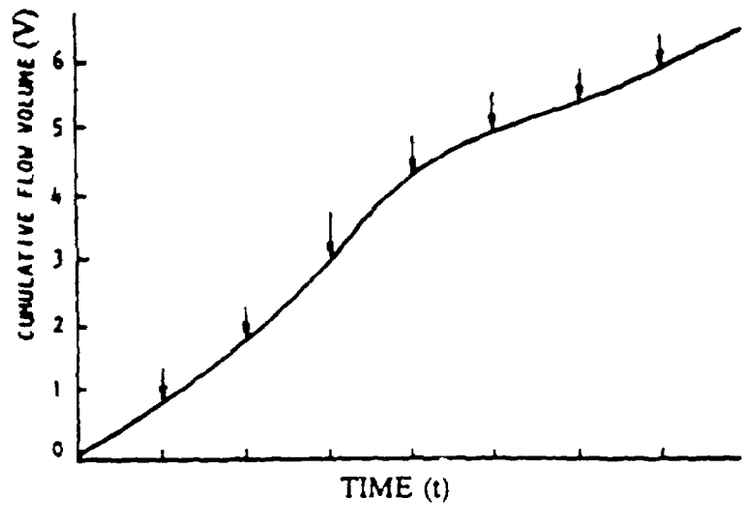
EXHIBIT 3-18. CONSTANT TIME - CONSTANT VOLUME



Method of compositing samples on a fixed volume-fixed time interval basis

Source: Methodology for the Study of Urban Storm Generated Pollution and Control, U.S. EPA 600/2-76-145, August 1976.

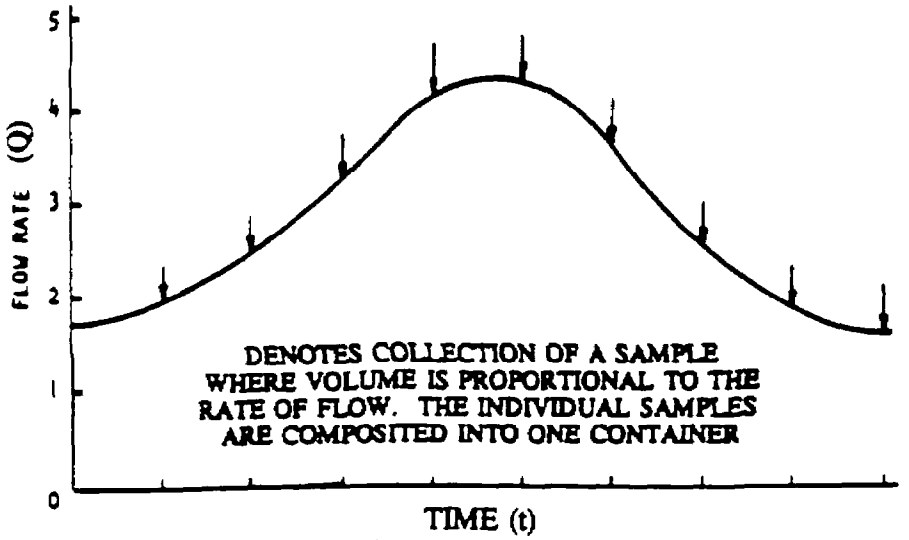
EXHIBIT 3-19. CONSTANT TIME - VOLUME PROPORTIONAL TO FLOW INCREMENT



Method of compositing samples proportional to flow volume at constant time interval

Source: Methodology for the Study of Urban Storm Generated Pollution and Control, U.S. EPA 600/2-76-145, August 1976.

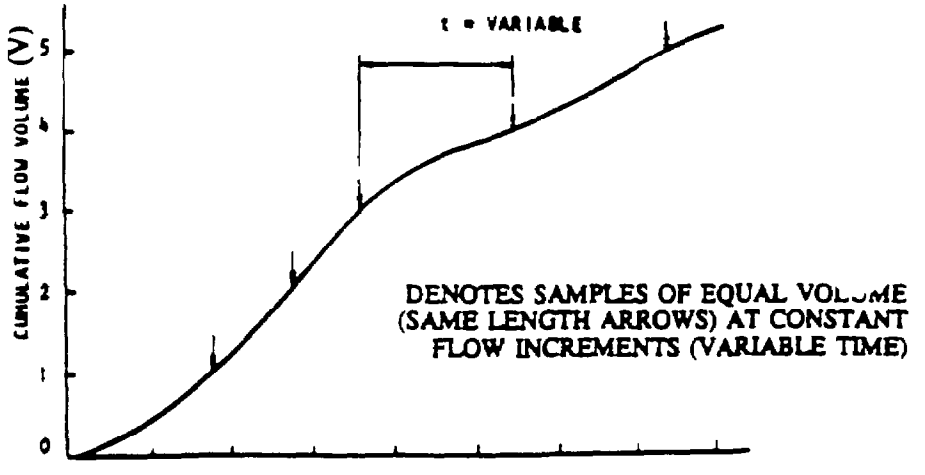
EXHIBIT 3-20. CONSTANT TIME - VOLUME PROPORTIONAL TO FLOW RATE



Method of compositing samples proportional to flow rate

Source: Methodology for the Study of Urban Storm Generated Pollution and Control, U.S. EPA 600/2-76-145, August 1976.

EXHIBIT 3-21. CONSTANT VOLUME - TIME PROPORTIONAL TO FLOW VOLUME INCREMENT

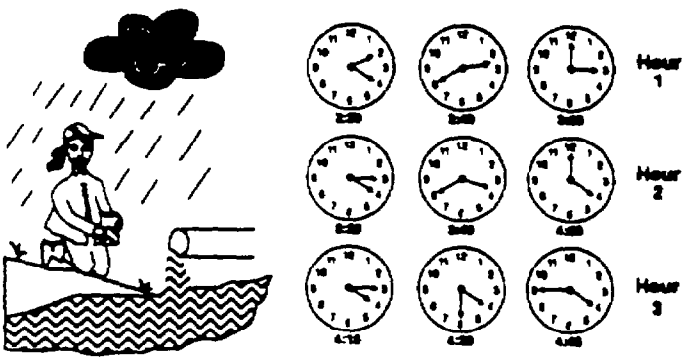


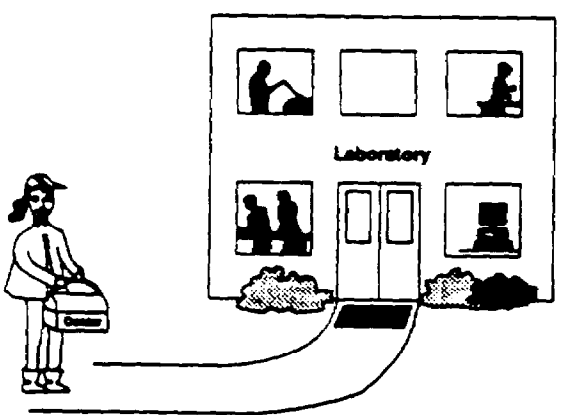
Method of compositing samples of equal volume at equal increments of flow

Source: Methodology for the Study of Urban Storm Generated Pollution and Control, U.S. EPA 600/2-76-145, August 1976.

EXHIBIT 3-22. EXAMPLE OF SAMPLING INTERVALS

Suppose that a storm water discharge began at 2:15 p.m. and lasted until 5:15 p.m. on a Friday. The field staff person wants to collect the samples at regular intervals, so s/he plans to collect an aliquot with a volume that is proportional to the flow every 20 minutes. After the third hour of collection, the field staff person must deliver the samples to the laboratory (which is 10 minutes away). The laboratory closes at 5:00 p.m. So, s/he should take the last sample at 4:45 p.m. One way of doing this would be to collect samples (in hour three) at 4:15, 4:30, and 4:45 p.m. This would comply with the three-sample minimum in hour three (4:15-5:15 p.m.) and the required 15-minute minimum interval between collections. It would also allow the field staff person to get the samples to the lab before it closes for the weekend. On the other hand, if s/he missed the sample collection at 4:15 p.m. and instead, collected the sample at 4:20 p.m., then s/he would have to collect the next sample at 4:35 p.m. and the last sample at 4:50 p.m., and the field staff person would not be able to deliver the sample until Monday (by which time the required maximum holding time would be exceeded), and the sampling would need to be repeated.





authority must be obtained before changes are initiated. Considerations applicable to the collection of flow-weighted composites by automatic and manual techniques are discussed in the following sections.

3.4.1 HOW TO MANUALLY COLLECT FLOW-WEIGHTED COMPOSITE SAMPLES

Manually collected, flow-weighted composite samples may be appropriate for a facility that prefers not to invest in automatic equipment. This technique is cost-effective for short-term monitoring programs and for facilities where few outfalls are being sampled. The fundamental requirement for facilities that use these methodologies is that they should have personnel available to perform the sampling when needed. Those facilities where VOCs analysis of storm water discharges are required should manually collect composite samples since these parameters may not be amenable to sampling by automatic samplers. Compositing of VOC samples should be conducted in the laboratory as discussed in Section 3.5.2.

The manual collection of a flow-weighted sample is performed in the same manner as taking manual grab samples (see Section 3.3.1). The only difference is that a series of samples (or aliquots) will be collected. As discussed in the previous section, there are two ways to manually collect and combine the aliquots for a flow-weighted sample:

- Collect sample aliquot volumes based on the flow at the time of sampling which can immediately be combined to make the composite sample in the field (see Exhibit 3-23)
- Collect equal volume sample aliquots at the time of sampling and then flow-proportion and composite the aliquots in the laboratory (see Exhibit 3-24).

When uniform time intervals are used between the collection of the sample aliquots, the volumes of each aliquot used in the composite sample can be determined based on either volumes of flow or the flow rate, as they will result in similar proportions. However, when there are different time intervals between the sample aliquots, the individual sample aliquot volumes should be based on the runoff volume (calculated from the individual flow rates and durations) associated with each sample aliquot.

Generally, 1,000 ml for each aliquot collected should provide enough sample volume, when composited, for pollutant analyses of the required parameters contained in Section VII.A of Form 2F (see Section 3.6). More aliquot volume may be required if sampling is conducted for additional parameters. The laboratory conducting the analyses should always be contacted prior to a sampling event to determine how much sample volume they will require.

EXHIBIT 3-23. EXAMPLE OF HOW TO COLLECT SAMPLE ALIQUOT VOLUMES BASED ON FLOW, AND PROPORTION AND COMPOSITE IN THE FIELD

Step 1: Determine the necessary volume for compositing purposes.

Example: To fulfill analyses for all parameters in Section VII.A of Form 2F for which composite samples are required [Biochemical Oxygen Demand (BOD_5), Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), Total Kjeldahl Nitrogen (TKN), nitrate plus nitrite, and phosphorous] a total composite sample volume of 5,000 ml is needed by the contract laboratory.

Step 2: Determine an appropriate interval for collection of samples.

Example: Manually collected flow-weighted composite samples must consist of at least three sample aliquots collected per hour and must be gathered at least 15 minutes apart. For this example, sample aliquots will be collected exactly 20 minutes apart.

Step 3: Estimate or measure the volume of discharge for each sampling event.

Example: A discharge flow volume of 4.8 cubic feet will be used here.

Step 4: Convert the discharge flow volume to liters.

Example: To convert cubic feet to liters, use the conversion factor of 28.32 liters per 1 cubic foot as set forth in the following formula:

$$Volume\ (liters) = Volume\ (cubic\ feet) \times \frac{28.32\ liters}{1\ cubic\ foot}$$

$$Volume = 4.8\ cubic\ feet \times \frac{28.32\ liters}{1\ cubic\ foot} = 136\ liters$$

Step 5: Using Steps 3 and 4, volumes that have been discharged between the collection of each aliquot can be calculated.

(Note that the discharge volumes provided for aliquot numbers 2-9 have already been given for the purposes of this exhibit.)

Example: The procedures set forth in Section 3.2 may be used to calculate discharge volumes. The following table presents aliquot numbers, time of aliquot collection, and discharge volumes.

Aliquot Number	Time of Aliquot Collection	Discharged Volume
1	2:15 p.m.	136 liters
2	2:35 p.m.	200 liters
3	2:55 p.m.	122 liters
4	3:15 p.m.	178 liters
5	3:35 p.m.	156 liters
6	3:55 p.m.	117 liters
7	4:15 p.m.	94 liters
8	4:30 p.m.	21 liters
9	4:45 p.m.	12 liters

EXHIBIT 3-23. EXAMPLE OF HOW TO COLLECT SAMPLE ALIQUOT VOLUMES BASED ON FLOW, AND PROPORTION AND COMPOSITE IN THE FIELD (Continued)

Step 6: Determine the appropriate minimum aliquot volume as the basis for collecting other aliquot samples which together will provide adequate volume to fulfill the analytic requirements.

Example: In Step 1, it was determined that at least 5,000 ml of sample were required for flow-weighted composite sample analytical testing. As discussed in Section 3.4.1, basing the sample collection on a minimum aliquot volume of 1,000 ml gathered every interval (i.e., every 15 minutes) should result in adequate sample volume.

Step 7: Calculate the volume of the sample aliquot which must be collected during each aliquot sample period using the following formula:

$$\textit{Aliquot volume (ml)} = \textit{Minimum aliquot volume (ml)} \times \frac{\textit{Aliquot's discharge volume (liters)}}{\textit{Initial discharge volume (liters)}}$$

Step 6 shows that the minimum aliquot volume is 1,000 ml.

$$\textit{Aliquot \#1 volume (ml)} = 1{,}000 \text{ ml} \times \frac{136 \text{ liters}}{136 \text{ liters}} = 1{,}000 \text{ ml}$$

$$\textit{Aliquot \#2 volume (ml)} = 1{,}000 \text{ ml} \times \frac{200 \text{ liters}}{136 \text{ liters}} = 1471 \text{ ml}$$

$$\textit{Aliquot \#3 volume (ml)} = 1{,}000 \text{ ml} \times \frac{122 \text{ liters}}{136 \text{ liters}} = 897 \text{ ml}$$

$$\textit{Aliquot \#4 volume (ml)} = 1{,}000 \text{ ml} \times \frac{178 \text{ liters}}{136 \text{ liters}} = 1{,}309 \text{ ml}$$

$$\textit{Aliquot \#5 volume (ml)} = 1{,}000 \text{ ml} \times \frac{156 \text{ liters}}{136 \text{ liters}} = 1{,}147 \text{ ml}$$

$$\textit{Aliquot \#6 volume (ml)} = 1{,}000 \text{ ml} \times \frac{117 \text{ liters}}{136 \text{ liters}} = 860 \text{ ml}$$

$$\textit{Aliquot \#7 volume (ml)} = 1{,}000 \text{ ml} \times \frac{94 \text{ liters}}{136 \text{ liters}} = 691 \text{ ml}$$

$$\textit{Aliquot \#8 volume (ml)} = 1{,}000 \text{ ml} \times \frac{21 \text{ liters}}{136 \text{ liters}} = 154 \text{ ml}$$

$$\textit{Aliquot \#9 volume (ml)} = 1{,}000 \text{ ml} \times \frac{12 \text{ liters}}{136 \text{ liters}} = 88 \text{ ml}$$

A table of these calculations follows:

Aliquot Number	Discharged Volume	Aliquot Volume
1	136 liters	1,000 ml
2	200 liters	1,471 ml
3	122 liters	897 ml
4	178 liters	1,309 ml
5	156 liters	1,147 ml
6	117 liters	860 ml
7	94 liters	691 ml
8	21 liters	154 ml
9	12 liters	88 ml

In conclusion, a combination of the above sample aliquots result in a composite of 7,617 ml.

EXHIBIT 3-24. EXAMPLE OF HOW TO MANUALLY COLLECT EQUAL SAMPLE ALIQUOTS WHICH ARE LATER FLOW-PROPORTIONED AND COMPOSITED IN THE LABORATORY

Step 1: Determine the necessary volume for compositing purposes.

Example: To fulfill analyses for all parameters in Section VII.A of Form 2F for which composite samples are required (BOD_5, COD, TSS, TKN, nitrate plus nitrite, and phosphorous) a total composite sample volume of 5,000 ml is needed by the contract laboratory.

Step 2: Determine an appropriate interval for collection of samples.

Example: Manually collected flow-weighted composite samples must consist of at least nine sample aliquots and must be gathered at least 15 minutes apart; only three or four samples per hour may be taken. For convenience, the minimum number of three is chosen. Sample aliquots will be collected every 20 minutes.

Step 3: Determine the aliquot which should be taken during each sampling event.

Example: At least 5,000 ml of sample is required for flow-weighted composite sample analytical testing. As discussed in Section 3.4.1, a minimum aliquot volume of 1,000 ml gathered every interval (i.e., every 15 minutes) should result in adequate sample volume to be used for later flow-weighted compositing.

Step 4: Estimate or measure the volume of discharge for each sampling event while collecting a discrete 1,000-ml aliquot, as discussed in Step 3, for later compositing.

Example: Section 3.2 discusses methods to calculate total discharge volumes. A discharge flow volume of 4.8 cubic feet will be used here.

Step 5: Convert the discharge flow volume to liters.

Example: To convert cubic feet to liters, use the conversion factor of 28.32 liters per 1 cubic foot as set forth in the following formula:

$$\textit{Volume (liters)} = \textit{Volume (cubic feet)} \times \frac{\textit{28.32 liters}}{\textit{1 cubic foot}}$$

$$\textit{Volume} = \textit{4.8 cubic feet} \times \frac{\textit{28.32 liters}}{\textit{1 cubic foot}} = \textit{136 liters}$$

EXHIBIT 3-24. EXAMPLE OF HOW TO MANUALLY COLLECT EQUAL SAMPLE ALIQUOTS WHICH ARE LATER FLOW-PROPORTIONED AND COMPOSITED IN THE LABORATORY (Continued)

Step 6: Using Steps 3 and 4, calculate the volumes that have been discharged between the collection of each aliquot.

Example: The procedures set forth in Section 3.2 may be used to calculate discharge volumes. The following table presents aliquot numbers, time of aliquot collection, and discharge volumes (note that the discharge volumes provided for aliquot numbers 2-9 were chosen for purposes of this exhibit).

Aliquot Number	Time of Aliquot Collection	Discharged Volume
1	2:15 p.m.	136 liters
2	2:35 p.m.	200 liters
3	2:55 p.m.	122 liters
4	3:15 p.m.	178 liters
5	3:35 p.m.	156 liters
6	3:55 p.m.	117 liters
7	4:15 p.m.	94 liters
8	4:30 p.m.	21 liters
9	4:45 p.m.	12 liters

Step 7: Determine the aliquot sample which is associated with the greatest discharge volume.

Example: Aliquot number 2 was taken when the volume was 200 liters. This is the largest discharge volume.

Step 8: Calculate the volume of sample aliquot which must be used subsequent to the sample event to comprise a flow-weighted composite sample. The following formula should be used:

$$\textit{Aliquot volume (ml)} = \textit{Minimum aliquot volume (ml)} \times \frac{\textit{Aliquot's discharge volume (liters)}}{\textit{Largest discharge volume (liters)}}$$

Step 3 shows that the minimum aliquot volume is 1,000 ml. Using this value and the data determined as part of Steps 6 and 7, the following can be calculated:

$$\textit{Aliquot \#1 volume (ml)} = 1{,}000 \text{ ml} \times \frac{136 \text{ liters}}{200 \text{ liters}} = 680 \text{ ml}$$

$$\textit{Aliquot \#2 volume (ml)} = 1{,}000 \text{ ml} \times \frac{200 \text{ liters}}{200 \text{ liters}} = 1{,}000 \text{ ml}$$

$$\textit{Aliquot \#3 volume (ml)} = 1{,}000 \text{ ml} \times \frac{122 \text{ liters}}{200 \text{ liters}} = 610 \text{ ml}$$

$$\textit{Aliquot \#4 volume (ml)} = 1{,}000 \text{ ml} \times \frac{178 \text{ liters}}{200 \text{ liters}} = 890 \text{ ml}$$

$$\textit{Aliquot \#5 volume (ml)} = 1{,}000 \text{ ml} \times \frac{156 \text{ liters}}{200 \text{ liters}} = 780 \text{ ml}$$

$$\textit{Aliquot \#6 volume (ml)} = 1{,}000 \text{ ml} \times \frac{117 \text{ liters}}{200 \text{ liters}} = 585 \text{ ml}$$

EXHIBIT 3-24. EXAMPLE OF HOW TO MANUALLY COLLECT EQUAL SAMPLE ALIQUOTS WHICH ARE LATER FLOW-PROPORTIONED AND COMPOSITED IN THE LABORATORY (Continued)

Aliquot #7 volume (ml) = 1,000 ml x $\frac{94 \text{ liters}}{200 \text{ liters}}$ = 470 ml

Aliquot #8 volume (ml) = 1,000 ml x $\frac{21 \text{ liters}}{200 \text{ liters}}$ = 105 ml

Aliquot #9 volume (ml) = 1,000 ml x $\frac{12 \text{ liters}}{200 \text{ liters}}$ = 60 ml

A table of these calculations follows

Aliquot Number	Discharged Volume	Aliquot Volume
1	136 liters	680 ml
2	200 liters	1,000 ml
3	122 liters	610 ml
4	178 liters	890 ml
5	156 liters	780 ml
6	117 liters	585 ml
7	94 liters	470 ml
8	21 liters	105 ml
9	12 liters	60 ml

In conclusion, a combination of the above sample aliquots results in a composite sample of **5,100 ml.**

Manually collected flow-weighted composite samples can also be prepared by collecting sample aliquots of equal volume where the collection times are related to the volume of discharge which has passed since the last sample aliquot collection. However, this method is subject to fluctuating flow rates and volumes which may dictate that samples be taken prior to the 15-minute interval required by the regulations. In that case, the alternative sampling protocol would have to be approved by the permitting authority.

3.4.2 HOW TO COLLECT FLOW-WEIGHTED COMPOSITE SAMPLES BY AUTOMATIC SAMPLER

The typical automatic sampler collects sample aliquots after a specific interval. These aliquots can be flow-weight composited by the automatic sampler; or by hand in the laboratory. The automatic

sampler may be programmed in one of three ways: (1) to collect a sample at equal time intervals and varying aliquot volumes commensurate with the flow (either rate or volume) that has passed; (2) to collect equal volume aliquots at varying time intervals commensurate with the flow volume that has passed; or (3) to collect equal volume aliquots of sample at equal time intervals.

The first two methods automatically composite the sample but require that the sampler be connected to a flow meter such that the sampler determines either the flow rate or the amount of volume that passes. Since these methods automatically composite samples, one main sample container may be used to receive all aliquots. The third method automatically collects the sample aliquots but does not automatically flow-weight composite the sample. As such, discrete sample containers must be used, and manual flow-weighted compositing must be conducted after the aliquots are collected. Exhibits 3-23 and 3-24 in Section 3.4.1 describe the manual compositing procedures that should be followed.

Manufacturers' instructions for the use of an automatic sampler provide the best explanation of programming options and should be consulted for information on programming samplers for storm water collection. Some of the points regarding automatic samplers discussed in Section 3.3.2 should also be considered.

3.5 SAMPLE HANDLING AND PRESERVATION

Samples must be handled and preserved in accordance with 40 CFR Part 136. This section describes acceptable analytical methods, including requirements regarding sample holding times, containers, sizes, and preservation requirements. For each pollutant or parameter that may have to be analyzed, 40 CFR Part 136 includes information on:

- Container types to be used to store the samples after collection
- Procedures to correctly preserve the samples
- The maximum holding time allowed for each parameter.

The following sections present a detailed discussion of preservation techniques and sample handling procedures. Technical Appendix C presents a matrix of required containers, preservation techniques,

and holding times for each parameter. Most laboratories can provide clean sample containers, preservatives, sealing, chain-of-custody forms and can advise further on sample handling and preservation.

3.5.1 DECONTAMINATION OF SAMPLE EQUIPMENT CONTAINERS

Storm water sample containers should be cleaned and prepared for field use according to the procedures set forth in 40 CFR Part 136. A summary of the procedures is presented below for plastic containers, any or all of which may be performed by the laboratory or container distributor:

- Nonphosphate detergent and tap water wash
- Tap water rinse
- 10 percent nitric acid rinse (only if the sample is to be analyzed for metals)
- Distilled/deionized water rinse
- Total air dry.

To clean glass containers, the same steps should be taken; but, after the distilled/deionized water rinse, the containers should be rinsed with solvent if appropriate prior to total air drying. After the decontamination procedures have been accomplished, the sample containers should be capped or sealed with foil, and the sampling device should be protected and kept clean. It is a good idea to label sample containers after cleaning. The laboratory should keep a record of the technician performing the cleaning procedure as well as the date and time. This begins the required chain-of-custody procedure for legal custody (see Section 3.10 for more information). A chain-of-custody record accompanies each sample to track all personnel handling the sample. This record is essential to trace the sample integrity in the event that quality control checks reveal problems. For this reason, as well as to avoid problems if contamination issues arise, it is suggested that the laboratory performing the analysis perform the cleaning.

3.5.2 SAMPLE PRESERVATION AND HOLDING TIMES

Preservation techniques ensure that the sample remains representative of the storm water discharge at the time of collection. Since many pollutants in the samples collected are unstable (at least to some extent), the sample should be analyzed immediately or preserved or fixed to minimize changes between the time of collection and analysis. Because immediate analysis is not always possible, most samples are preserved regardless of the time of analysis.

Problems may be encountered when flow-weighted composite samples are collected. Since sample deterioration can take place during the compositing process, it is necessary to preserve or stabilize the samples during compositing in addition to preserving aggregate samples before shipment to the laboratory. Preservation techniques vary depending on the pollutant parameter to be measured; therefore, familiarity with 40 CFR Part 136 (see Technical Appendix C) is essential to ensure effective preservation. It is important to verify that the preservation techniques for one parameter do not affect the analytical results of another in the same sample. If this is the case, two discrete samples should be collected and preserved accordingly.

Sample preservation techniques consist of refrigeration, pH adjustment, and chemical fixation. pH adjustment is necessary to stabilize the target analyte (e.g., addition of NaOH stabilizes cyanide); acidification of total metal samples ensures that metal salts do not precipitate. Refrigeration is the most widely used technique because it has no detrimental effect on the sample composition (i.e., it does not alter the chemistry of the sample), and it does not interfere with most analytical methods. Refrigeration requires the sample to be quickly chilled to a temperature of 4°C. This technique is used at the beginning of sample collection in the field, and is continued during sample shipment, and while the sample is in the laboratory. Even though samples taken for compositing purposes are taken over time each individual sample must be refrigerated. If taken manually, the samples can be placed in an ice box. If taken by a automatic sampler, the sampler unit should have refrigeration capabilities. The analytical laboratory may provide chemicals necessary for fixation, or may tell sampling personnel where they can be purchased.

In addition to preservation techniques, 40 CFR Part 136 indicates maximum holding times. A detailed list of holding times appears in Technical Appendix C. The holding time is the maximum

amount of time that samples may be held before analysis and still be considered valid. Samples exceeding these holding times are considered suspect and sample collection may have to be repeated.

Although Technical Appendix C provides required sample containers, preservation techniques, and holding times, some of the more commonly monitored parameters warrant additional discussion. The following provides a more detailed discussion of considerations pertaining to cyanide, VOCs, organics and pesticides, O&G, pH, total residual chlorine, fecal coliform, fecal streptococcus, and 5-day Biochemical Oxygen Demand (BOD_5).

Cyanide

Cyanide is very reactive and unstable. If the sample cannot be analyzed immediately, it must be preserved by pH adjustment after collection. However, prior to pH adjustment, procedures to eliminate residual chlorine and sulfides must be followed immediately.

Where chlorine has the possibility of being present, the sample should be tested for residual chlorine by using potassium iodide-starch test paper previously moistened with acetate buffer. If the sample contains residual chlorine (a blue color indicates the need for treatment), ascorbic acid must be added 0.6 gram (g) at a time until the tests produce a negative result; then, an additional 0.6 g of ascorbic acid should be added to the sample.

Samples containing sulfides may be removed, in which case the holding time is extended to 14 days. Sulfides must be removed as follows:

- Use lead acetate paper moistened with an acetic acid buffer solution to test for the presence of sulfide. Darkening of the lead acetate paper indicates sulfide is present in the sample.
- Add cadmium nitrate to be added to the sample in a manner similar to the ascorbic acid until the test is negative.
- Filter with a 0.45 micrometer (μm) filter and prefilter combination immediately after.

After chlorine and sulfide residuals have been eliminated, the pH must be adjusted to greater thar 12.0 standard units (s.u.) and chilled to 4°C.

If cyanide is suspected to be present, the sampling personnel should bring all materials mentioned above to the sampling location.

VOCs

Sampling for VOCs requires the use of a glass vial. The vial should contain a teflon-coated septum seal. Volatiles will escape from the water to the air if any air is entrapped in the container. Therefore, the sample should be collected so that there are no air bubbles in the container after the screw cap and septum seal are applied. To ensure that air bubbles are not trapped in the vial, the following procedures should be followed:

- Fill the vial until a reverse meniscus forms above the top of the vial
- Screw on the cap (the excess sample will overflow)
- Invert the vial to check for the presence of air bubbles
- If air bubbles are observed, the vial should be opened, emptied, then completely refilled, and the first three actions should be repeated.

VOC samples should not be composited in the field. To composite a sample, the sampling personnel would have to mix it thoroughly. This mixing action would aerate the sample and cause volatiles to be lost. Therefore, VOC samples should be sent to the laboratory where they can be immediately, and carefully, composited and analyzed with minimal volatilization as per method Nos. 502.1, 502.2, 524.1, and 524.2 as described at 40 CFR 141.24(f)(14)(iv) and (v). There are two ways flow-weighted compositing of VOCs can be accomplished—mathematical compositing or procedural compositing as discussed below.

Mathematical Compositing

In this method, the grab samples are analyzed separately. The sampling personnel collect the requisite number of samples and send them to the laboratory. The laboratory performs the individual analyses on each sample. Five ml (or 25 ml if greater sensitivity is required) of each grab sample are placed into the purge vessel of the GC or GC/MS for analysis. Special precautions must be made to maintain zero headspace in the syringe used to transfer the VOC sample into the purge vessel of the GC or GC/MS. These analytical results are mathematically flow-weight composited

using the calculation in Exhibit 3-24. The concentrations (C) should be adjusted by using the following formula:

$$\textit{Adjusted Concentration} = \frac{\textit{Individual Aliquot Volume}}{\textit{Total Composite Sample}} \times C$$

Each sample concentration should be adjusted, and all adjusted concentrations added, to obtain the flow-weighted VOC composite using this method.

Procedural Compositing

For the second method, sampling personnel collect the requisite number of samples and provide the laboratory with flow-weighted values for each sample using the calculation in Exhibit 3-24. The laboratory technician then draws the necessary volume from each aliquot into an adequately sized syringe, physically combining the samples to result in a flow-weighted composite sample for VOC analysis. Necessary volumes are drawn into the syringe with a volume control fitting. The samples are thus composited directly in the syringe and then placed in the purge vessel of the GC or GC/MS. The advantage of this procedure is that only one analysis on the GC or GC/MS has to be performed.

Although the applicant is required to report only flow-weighted composite concentrations, the mathematical compositing method may provide more information, as it will indicate the concentrations of each separate grab sample. For example, if the procedural compositing method is employed and one of the samples has a high concentration and the other three have non-detectable concentrations, the result will be an average which does not represent the concentration in any of the separate grab samples. In certain cases it may be important to know the concentration of each grab as well as the composite concentration. The mathematical compositing method would be the most appropriate compositing method in these cases.

Organics and Pesticides

The procedures affecting organics and pesticides [base/neutral/acids and pesticide polychlorinated biphenyls (PCBs)] are less complex than VOC procedures. Glass containers must be used for sample collection purposes, amber glass should be used to eliminate the potential for reactivity caused by light. These samples should be maintained at 4°C during storage and shipment. A preservative in

the form of 0.008 percent sodium thiosulfate ($Na_2S_2O_3$) must be added to organic samples if residual chlorine is present. To determine if chlorine is present, a small color indicator test kit can be used. Eighty ml of $Na_2S_2O_3$ per liter of sample must then be added and mixed well until chlorine tests indicate a negative result as per methods 604 and 625 of 40 CFR Part 136 Appendix A. The pH of pesticide samples must be adjusted to between 5 and 9 s.u.

Oil and Grease

O&G tends to adhere to the surfaces that it contacts. Therefore, it should not be transferred from one container to another; rather, a 1-liter container should be used to take the sample. The container used for O&G must be made of glass. A teflon insert should be included in the glass container's lid. However, if teflon is not available, aluminum foil extending out from under the lid may be used. Samples for O&G must be preserved by adding sulfuric acid (H_2SO_4) or hydrochloric acid (HC1) to a pH of less than 2 s.u. and then stored at 4°C.

Additional Considerations

Some pollutants have specific analysis requirements due to short holding times that the applicant must consider. For example:

- Requirements to analyze immediately (pH, total residual chlorine, temperature, sulfite, and dissolved oxygen)
- Requirements to preserve immediately and analyze within 6 hours (fecal coliform and fecal streptococcus)
- Requirements to analyze within 48 hours (BOD_5).

Because of these requirements, field testing equipment may need to be purchased, borrowed, or rented for those parameters that may require field analysis. If the laboratory is located nearby, analysis in the field may not be required.

Laboratories do not always operate in the evenings or on weekends. As a result, holding times for samples taken in the late afternoon or on a Friday may be exceeded. To prevent this from occurring, close coordination with laboratories is necessary. The latest date and time of delivery should be

established to avoid taking samples, only to discover they cannot be accepted by the laboratory and analyzed in accordance with 40 CFR Part 136 requirements.

3.6 SAMPLE VOLUMES

Exhibit 3-25 presents minimal suggested sample volumes for specific parameters. This exhibit should be consulted so that the proper volume is collected for analysis of each pollutant of concern. This exhibit may not include all parameters; if a particular parameter is not listed, refer to 40 CFR Part 136.

3.7 SAMPLE DOCUMENTATION

Information should be submitted to the laboratory with the sample to ensure proper handling by the laboratory. Exhibit 3-26 is an example form which can be used to document the following information.

- Unique Sample or Log Number - All samples should be assigned a unique identification number. If there is a serial number on the transportation case, the sampling personnel should add this number to the field records.

- Date and Time of Sample Collection - Date and time of sample collection (including notation of a.m. or p.m.) must be recorded. In the case of composite samples, the sequence of times and aliquot size should be noted.

- Source of Sample, Including Facility Name and Address - Use the outfall identification number from the site map with a narrative description; a diagram referring to the particular site where the sample was taken should be included.

- Name of Sampling Personnel - The names and initials of the persons taking the sample must be indicated. For a composite sample, the names of the persons installing the sampler and the names of the persons retrieving the sample should be included.

- Sample Type - Each sample should indicate whether it is a grab or composite sample. If the sample is a composite, the volume and frequency of individual aliquots should be noted.

- Preservation Used - Any preservatives (and the amount) added to the sample should be recorded. The method of preservation (e.g., refrigeration at 4°C) should be indicated.

- Analysis Required - All parameters for which the sample must be analyzed at the laboratory should be specified.

EXHIBIT 3-25. VOLUME OF SAMPLE REQUIRED FOR DETERMINATION OF THE VARIOUS CONSTITUENTS OF INDUSTRIAL WASTEWATER

Tests	Volume of Sample, ml*
Physical	
Color and odor**	100 to 500
Corrosivity**	flowing sample
Electrical conductivity**	100
pH, electrometric**	100
Radioactivity	100 to 1,000
Specific gravity**	100
Temperature**	flowing sample
Toxicity**	1,000 to 20,000
Turbidity**	100 to 1,000
Chemical	
VOCs	100
Dissolved Gases	
Ammonia,*** NH_3	500
Carbon Dioxide,*** free CO_2	200
Chlorine,*** free Cl_2	200
Hydrogen,*** H_2	1,000
Hydrogen sulfide,*** H_2S	500
Oxygen,*** O_2	500 to 1,000
Sulfur dioxide,*** free SO_2	100
Miscellaneous	
Acidity and alkalinity	100
Bacteria (fecal coliform)	500
Bacteria (fecal streptococcus)	100
Biochemical oxygen demand (BOD)	100 to 500
Carbon dioxide, total CO_2 (including CO_3^{--}, HCO_3^-, and free)	200
Chemical oxygen demand (dichromate)	50 to 100
Chlorine requirement	2,000 to 4,000
Chlorine, total residual Cl_2 (including OCl^-, HOCl, NH_2Cl, $NHCl_2$, and free)	200
Chloroform-extractable matter	1,000
Detergents	100 to 200
Hardness	50 to 100
Hydrazine	50 to 100

EXHIBIT 3-25. VOLUME OF SAMPLE REQUIRED FOR DETERMINATION OF THE VARIOUS CONSTITUENTS OF INDUSTRIAL WASTEWATER (Continued)

Tests	Volume of Sample, ml*
Miscellaneous (Continued)	
Micro-organisms	100 to 200
Volatile and filming amines	500 to 1,000
Oily matter	3,000 to 5,000
Organic nitrogen	500 to 1,000
Phenolic compounds	800 to 4,000
Polyphosphates	100 to 200
Silica	50 to 100
Solids, dissolved	100 to 20,000
Solids, suspended	50 to 1,000
Tannin and lignin	100 to 200
Cations	
Aluminum, Al+++	100 to 1,000
Ammonium,*** NH_4+	500
Antimony, Sb+++ to Sb+++++	100 to 1,000
Arsenic, As+++ to As+++++	100 to 1,000
Barium, Ba++	100 to 1,000
Cadmium, Cd++	100 to 1,000
Calcium, Ca++	100 to 1,000
Chromium, Cr+++ to Cr++++++	100 to 1,000
Copper, Cu++	200 to 4,000
Iron,*** Fe++ and Fe+++	100 to 1,000
Lead, Pb++	100 to 4,000
Magnesium, Mg++	100 to 1,000
Manganese, Mn++ to Mn+++++++	100 to 1,000
Mercury, Hg+ and Hg++	100 to 1,000
Potassium, Ni++	100 to 1,000
Nickel, Ni++	100 to 1,000
Silver, Ag+	100 to 1,000
Sodium, NA+	100 to 1,000
Strontium, Sr++	100 to 1,000
Tin, Sn++ and Sn++++	100 to 1,000
Zinc, Zn++	100 to 1,000

EXHIBIT 3-25. VOLUME OF SAMPLE REQUIRED FOR DETERMINATION OF THE VARIOUS CONSTITUENTS OF INDUSTRIAL WASTEWATER *(Continued)*

Tests	Volume of Sample, ml*
Anions	
Bicarbonate, HCO_3	100 to 200
Bromide, Br^-	100
Carbonate, CO_3^{--}	100 to 200
Chloride, Cl^-	25 to 100
Cyanide, Cn^-	25 to 100
Fluoride, Fl^-	200
Hydroxide, OH^-	50 to 100
Iodide, I^-	100
Nitrate, NO_3^-	10 to 100
Nitrite, NO_2^-	50 to 100
Phosphate, Ortho, PO_4^{--}, HPO_4^{--}, $H_2PO_4^-$	50 to 100
Sulfate, SO_4^{--}, HSO_4^-	100 to 1,000
Sulfide, S^{--}, HS^-	100 to 500
Sulfite, SO_3^{--}, HSO_3^-	50 to 100

*Volumes specified in this table should be considered as guides for the approximate quantity of sample necessary for a particular analysis. The exact quantity used should be consistent with the volume prescribed in the standard method of analysis, whenever a volume is specified.
**Aliquot may be used for other determinations.
***Samples for unstable constituents must be obtained in separate containers, preserved as prescribed, completely filled, and sealed against all exposure.

Source: Associated Water and Air Resource Engineers, Inc., 1973, *Handbook for Monitoring Industrial Wastewater*, EPA Technology Transfer.

- Flow - If flow is measured at the time of sampling, the measurement must be recorded and accompanied by a description of the flow measurement method and calculations.

- Date, Time, and Documentation of Sample Shipment - The shipment method (e.g., air, rail, or bus) as well as the shipping papers or manifest number should be noted.

- Comments - All relevant information pertaining to the sample or the sampling site should be recorded. Such comments could include the condition of the sample site, observed characteristics of the sample, environmental conditions that may affect the sample, and problems encountered during sampling.

EXHIBIT 3-26. FIELD SHEET FOR SAMPLE DOCUMENTATION

Sample Source	Sample ID #	Date: XX/XX/XX
Facility Name		Time: XX:XX a.m./p.m.
Address	Person Performing Sampling	
Outfall ID #	Signature	
Description	Preservation Method	
Diagram of Site	Comments	
Flow Description	Ship Via: Stable Shipping Paper/Manifest	
Flow Calculations	Analysis Required	

3.8 SAMPLE IDENTIFICATION AND LABELING

Prior to collection of the sample, a waterproof, gummed sample identification label or tag should be attached to the sample container. This label should contain relevant information for sample analysis, such as:

- Facility name
- Name of the sample collector
- Sample identification number
- Date and time of sample collection
- Type of analysis required
- Location of sample collection
- Preservatives used
- Type of sample (grab or composite).

Sample lids should be used to protect the sample's integrity from the time it is collected to the time it is opened in the laboratory. The lid should contain the collector's name, the date and time the sample was collected, and a sample identification number. Information on the seal must be identical to the information on the label. In addition, the lid should be taped shut so that the seal must be broken to open the sample container. Caution should be taken to ensure that glue from tape and label tag wires do not contaminate samples, particularly those containing volatile organics and metals. Also, waterproof ink should be used to avoid smearing on the label from melted ice used for cooling.

3.9 SAMPLE PACKAGING AND SHIPPING

If the samples are not hand-delivered to the laboratory or analyzed in an onsite laboratory, they should be placed in a transportation case (e.g., a cooler) along with the chain-of-custody record form, pertinent field records, and analysis request forms, and shipped to the laboratory. Glass bottles should be wrapped in foam rubber, plastic bubble wrap, or other material to prevent breakage during shipment. The wrapping can be secured around the bottle with tape. The container lid should also be sealed with tape. Samples should be placed in ice or a synthetic ice substitute that

will maintain the sample temperature at 4°C throughout shipment. Ice should be placed in double-wrapped watertight bags so the water will not leak from the shipping case. Metal or heavy plastic ice chests make good sample transportation cases. Filament tape wrapped around each end of the ice chest ensures that it will not open during transport. Sampling records (preferably laminated or waterproof) can be placed in a waterproof envelope and taped to the inside of the transportation case to avoid getting them wet in case a sample container or an ice bag leaks. Shipping containers should also be sealed to prevent tampering. A copy of all sampling records should be kept onsite in case they are requested by the permitting authority.

Most samples will not require any special transportation precautions except careful packaging to prevent breakage and/or spillage. If the sample is shipped by common carrier or sent through the U.S. mail, it must comply with Department of Transportation Hazardous Materials Regulations (49 CFR Parts 171-177). Air shipment of hazardous materials samples may also be covered by requirements of the International Air Transport Association (IATA). Before shipping a sample, the facility should be aware of, and follow, any special shipping requirements. Special packing and shipping rules apply to substances considered hazardous materials as defined by IATA rules. Storm water samples are not generally considered hazardous materials, but in the event of a spill, leakage, etc., at the collection site hazardous materials may be present in the samples. Be aware, before sampling, of what hazardous materials may be in the discharge drainage area. If the presence of hazardous materials is suspected, do not sample unless properly trained.

3.10 CHAIN-OF-CUSTODY PROCEDURES

Once samples have been obtained and collection procedures are properly documented, a written record of the chain of custody of that sample should be made. This is recommended so the applicant can be confident that the samples have not been tampered with and that the sample once analyzed is representative of the storm water discharge. "Chain-of-custody" refers to the documented account of changes in possession that occur for a particular sample or set of samples. The chain-of-custody record allows an accurate step-by-step recreation of the sampling path, from origin through analysis. Information necessary in chain-of-custody is:

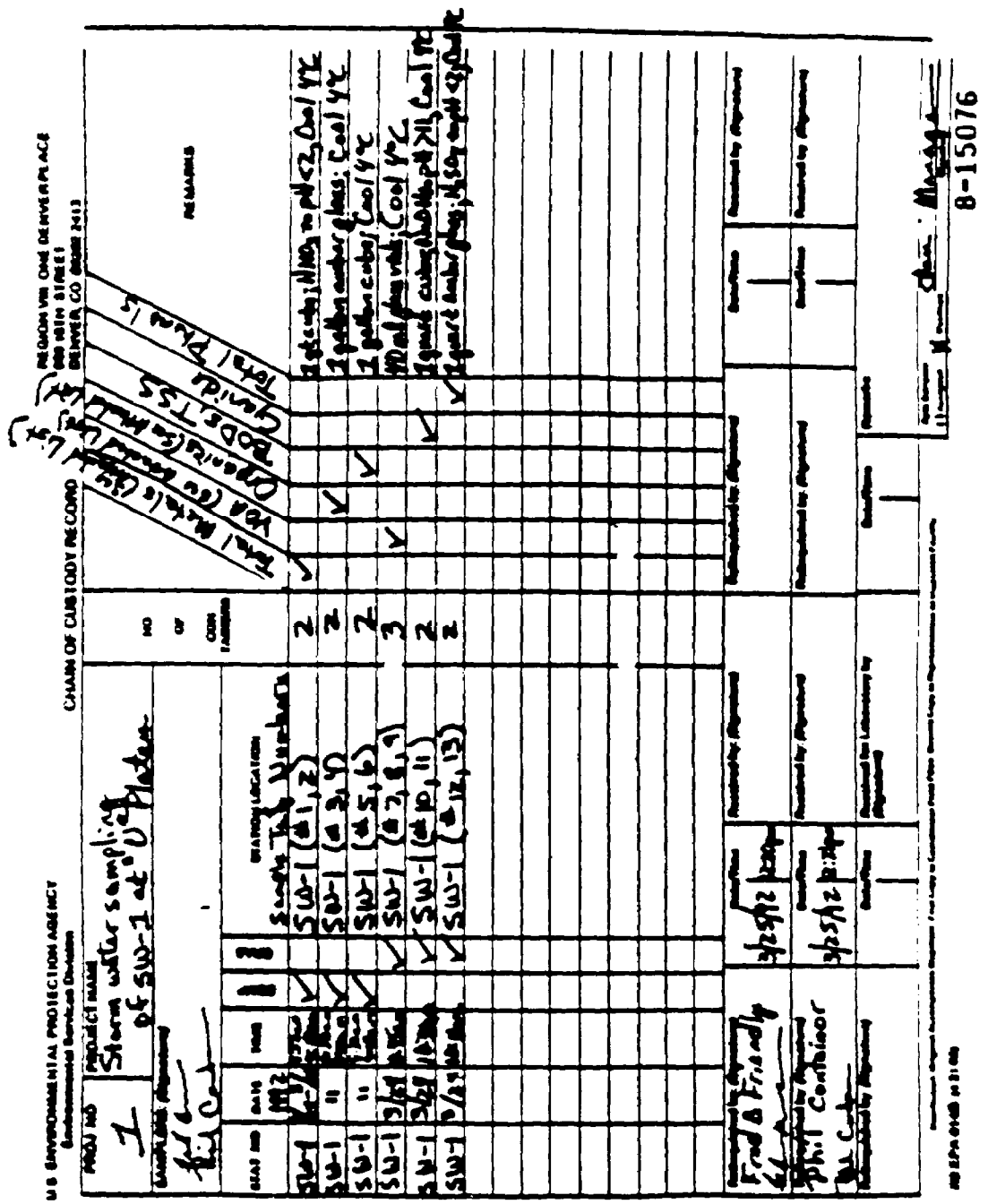
- Name of the persons collecting the sample
- Sample ID numbers

- Date and time of sample collection
- Location of sample collection
- Names and signatures of all persons handling the samples in the field and in the laboratory.

To ensure that all necessary information is documented, a chain-of-custody form should be developed. An example of such a form is found in Exhibit 3-27. Chain-of-custody forms should be printed on carbonless, multipart paper so all personnel handling the sample receive a copy. All sample shipments should be accompanied by the chain-of-custody record and a copy of these forms should be retained by the originator. In addition, all receipts associated with the shipment should be retained. Carriers typically will not sign for samples; therefore, seals must be used to verify that tampering has not occurred during shipment.

When transferring possession of samples, the transferee should sign and record the date and time on the chain-of-custody record. In general, custody transfers are made for each sample, although samples may be transferred as a group. Each person who takes custody should fill in the appropriate section of the chain-of-custody record.

EXHIBIT 3-27. EXAMPLE OF CHAIN-OF-CUSTODY FORM



Source: U.S. EPA, Region 8

4. ANALYTICAL CONSIDERATIONS

All storm water discharges must be sampled and analyzed in accordance with the test procedures provided in 40 CFR Part 136. This section discusses pollutant parameters which must be analyzed by storm water permit applicants. If the applicant wants to use an alternative test method, the facility must apply for approval (by submitting a description of the method to the permitting authority for approval) prior to application submission [see 40 CFR 136.4(d)(3)]. Section 5.4 elaborates on how to obtain approval for an analytical method for a parameter that is not included in 40 CFR Part 136. EPA-approved analytical methods at 40 CFR 136.3, Tables IB and IC are shown in Appendix C of this document.

When choosing the appropriate 40 CFR Part 136 analytical method, the applicant should consider sample interferences and potential field sampling error. Most method detection levels are established under ideal sample conditions (e.g., with little or no sample matrix interferences or sampling error). Thus, for storm water samples, the method chosen should account for sampling error and interferences.

4.1 INDUSTRIAL REQUIREMENTS

Industrial dischargers must provide information on the following parameters, as required in 40 CFR 122.26(c)(1)(i)(E):

- Any pollutant limited in an effluent guideline to which the facility is subject
- Any pollutant listed in the facility's NPDES permit for its process wastewater (if the facility has an existing NPDES permit)
- O&G, pH, BOD_5, COD, TSS, total phosphorus, TKN, and nitrate plus nitrite nitrogen
- Any pollutant known or believed to be present [as required in 40 CFR 122.21(g)(7)]
- Flow measurements or estimates of the flow rate, the total amount of discharge for the storm events sampled, and the method of flow measurement or estimation
- The date and duration (in hours) of the storm events sampled, rainfall measurements or estimates of the storm event (in inches) which generated the sampled runoff, and the time between the storm event sampled and the end of the previous measurable (greater than 0.1 inch rainfall) storm event (in hours).

4.1.1 INDIVIDUAL APPLICANTS

Industrial facilities submitting an individual permit application must provide sampling data in three parts of the Form 2F application form as discussed below. (Form 2F restates requirements listed in 40 CFR 122.21 and 122.26).

Section VII.A Parameters

Section VII.A of Form 2F requires the facility to sample (grab and flow-weighted samples) for O&G, BOD_5, COD, TSS, TKN, nitrate plus nitrite nitrogen, total phosphorus, and pH. These parameters are to be monitored by every facility applying for a storm water discharge permit, regardless of the type of operations that exist at the site. Sampling for additional parameters may be required, depending on the type of facility applying for the permit or the pollutants expected to be present in the discharge. These additional requirements are discussed in detail below.

Section VII.B Parameters

Section VII-B of Form 2F requires the applicant to identify all pollutants that are limited in an effluent guideline to which the facility is subject, as well as other toxic and nonconventional pollutants listed in the facility's NPDES permit for its process wastewater. EPA interprets that for pollutants listed in NPDES process wastewater permits, at a minimum, facilities must sample their storm water discharge for those pollutants specifically limited in their process wastewater permit. States can be more stringent, however, and may interpret this requirement to mean all pollutants listed in the permit. Once these parameters are identified, the applicant will be required to sample for these parameters by both grab and flow-weighted composite samples, except for the specified pollutants which must be grab sampled only. Form 2F requires the applicant to submit maximum values. The average values column is not compulsory, but should be completed if data are available. Applicable effluent guidelines appear in 40 CFR Parts 405-471. A listing of the Subchapter N—Effluent Guidelines and Standards by which the applicant may be regulated appears in Exhibit 4-1. The applicant must refer to the effluent guidelines and standards for the particular industry, and should determine which guidelines apply and which parameters should be listed in Section VII.B of Form 2F.

EXHIBIT 4-1. SUBCHAPTER N-EFFLUENT GUIDELINES AND STANDARDS

Part	Effluent Guidelines and Standards	Part	Effluent Guidelines and Standards
405	Dairy Products Processing Point Source Category	431	Builder's Paper and Board Mills Point Source Category
406	Grain Mils Point Source Category	432	Meat Products Point Source Category
407	Canned and Preserved Fruits and Vegetables Point Source Category	433	Metal Finishing Point Source Category
408	Canned and Preserved Seafood Point Source Category	434	Coal Mining Point Source Category
409	Sugar Processing Point Source Category	435	Oil and Gas Extraction Point Source Category
410	Textile Mills Point Source Category	436	Mineral Mining and Processing Point Source Category
411	Cement Manufacturing Point Source Category	439	Pharmaceutical Manufacturing Point Source Category
412	Feedlots Point Source Category	440	Ore Mining and Dressing Point Source Category
413	Electroplating Point Source Category	443	Paving and Roofing Point Source Category
414	Organic Chemicals, Plastics, and Synthetic Fibers Point Source Category	446	Paint Formulating Point Source Category
415	Inorganic Chemicals Manufacturing Point Source Category	447	Ink Formulating Point Source Category
416	(Reserved)	454	Gum and Wood Chemicals Manufacturing Point Source Category
417	Soap and Detergent Manufacturing Point Source Category	455	Pesticide Chemicals Manufacturing Point Source Category
418	Fertilizer Manufacturing Point Source Category	457	Explosives Manufacturing Point Source Category
419	Petroleum Refining Manufacturing Point Source Category	458	Carbon Black Manufacturing Point Source Category
420	Iron and Steel Manufacturing Point Source Category	459	Photographic Point Source Category
421	Nonferrous Metals Manufacturing Point Source Category	460	Hospital Point Source Category
422	Phosphate Manufacturing Point Source Category	461	Battery Manufacturing Point Source Category
423	Steam Electric Power Generating Point Source Category	463	Plastics Molding and Forming Point Source Category
424	Ferroalloy Manufacturing Point Source Category	464	Metal Molding and Casting Point Source Category
425	Leather Tanning and Finishing Point Source Category	465	Coil Coating Point Source Category
426	Glass Manufacturing Point Source Category	466	Porcelain Enameling Point Source Category
427	Asbestos Manufacturing Point Source Category	467	Aluminum Forming Point Source Category
428	Rubber Manufacturing Point Source Category	468	Copper Forming Point Source Category
429	Timber Products Processing Point Source Category	469	Electrical and Electronic Components Point Source Category
430	Pulp, Paper and Paperboard Point Source Category	471	Nonferrous Metals Forming and Metal Powders Point Source Category

Section VII.C Parameters

Section VII.C requires the applicant to list, for each outfall, each pollutant described in 40 CFR Part 122, Appendix D, Tables II, III, IV, and V (Tables 2F-2, 2F-3, and 2F-4 of application Form 2F) that it knows, or has reason to believe, may be present in the storm water discharge. These pollutants consist of conventional and nonconventional pollutants, toxic pollutants and total phenol, Gas Chromatography/Mass Spectrometry (GC/MS) fraction volatile compounds, acid compounds, base/neutral compounds, pesticides, and hazardous substances. These tables are also provided on the back of Form 2F. Tables II and III of 40 CFR Part 122 Appendix D have been combined in Table 2F-3 of application Form 2F. Table IV of 40 CFR Part 122 Appendix D is listed as Table 2F-2 of application Form 2F and Table V of 40 CFR Part 122 Appendix D is listed as Table 2F-4 of application Form 2F. There are specific requirements associated with each table. If pollutants in Table IV of 40 CFR Part 122 Appendix D (Table 2F-2 of application Form 2F), are directly or indirectly limited by an effluent guideline limitation, the applicant must analyze for it and report the data. For other pollutants listed in Table IV of 40 CFR Part 122 Appendix D (Table 2F-2 of the application form), the applicant must either report quantitative data, if available, or briefly describe the reasons the pollutant is expected to be in the discharge.

For every pollutant in Tables II and III of 40 CFR Part 122 Appendix D (Table 2F-3 of application Form 2F) expected to be discharged in concentrations of 10 parts per billion (ppb) or greater, the applicant must submit quantitative data. For acrolein, acrylonitrile, 2,4-dinitrophenol, and 2-methyl-4,6-dinitrophenol, the applicant must submit quantitative data if any of these four pollutants is expected to be discharged in concentrations of 100 ppb or greater. For every pollutant expected to be discharged with a concentration less than 10 ppb (or 100 ppb for the four parameters mentioned above), the applicant must either submit quantitative data or briefly explain why the pollutant is expected to be discharged.

For the parameters identified in Table V of 40 CFR Part 122 Appendix D (Table 2F-4 of application Form 2F) that the applicant believes to be present in the discharge, no sampling is required. If previous analyses of these parameters were conducted, the results must be reported. Otherwise, the applicant is required to explain why these pollutants are believed to be present.

Small Business Exemption

Small businesses are exempted from the reporting requirements for the organic toxic pollutants presented in 40 CFR Part 122, Table II of Appendix D. Applicants can claim a small business exemption if:

- The facility is a coal mine and the probable annual production is less than 100,000 tons per year. The applicant may submit past production data or estimate future production data instead of conducting analyses for the organic toxic pollutants listed in Table 2F-3 of application Form 2F.
- The facility is not a coal mine, and the gross total annual sales for the most recent 3 years is, on average, less than $100,000 per year (reflected in second quarter 1980 dollars). The applicant may submit sales data for those years instead of conducting analyses for the organic toxic pollutants listed in Table 2F-3 of application Form 2F.

Section VIII

Section VIII of Form 2F requires the applicant to provide biological toxicity testing data for storm water discharges associated with industrial activity. Applicants are required to perform biological toxicity testing for the storm water application if the facility's NPDES permit for its process wastewater lists biological toxicity (EPA interprets "listed" as limited). For example, if a facility's NPDES process wastewater permit has an acute toxicity limit of a lethal concentration (LC_{50}), equal to 75 percent effluent using ceriodaphnia, then that facility must also test its storm water discharges associated with industrial activity and report the results of the tests in Section VIII of Form 2F.

Until whole effluent toxicity methods are promulgated by EPA in 40 CFR Part 136, toxicity testing should be conducted using the most appropriate methods and species as determined by the permitting authority. In the absence of State acute toxicity testing protocols, EPA recommends using the methods described in Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Fresh Water and Marine Organisms. EPA/600/4-90-027 (Rev. September 1991)

4.1.2 GROUP APPLICANTS

Industrial facilities submitting a group application must also provide sampling data (from the sampling subgroup) which is required to be submitted in Sections VII, VIII, and IX along with the certification in Section X of Form 2F. At a minimum, these parameters include O&G, BOD_5, COD,

TSS, TKN, nitrate plus nitrite nitrogen, total phosphorous, and pH. Furthermore, all pollutants listed in an effluent guideline or limited in an NPDES permit applicable to the sampling facilities within the group must be sampled, as well as pollutants suspected of being present based on significant materials and industrial activities present onsite.

4.2 MUNICIPAL REQUIREMENTS

For Part 1 of the municipal permit application, municipalities must submit samples from the field screening effort for pH, total chlorine, total copper, phenol, and detergents (or surfactants). A narrative description of the color, odor, turbidity, and presence of oil sheen and surface scum must be included. For Part 2 of the permit application, municipalities must provide quantitative data for the organic pollutants listed in Table II of 40 CFR Part 122 Appendix D, and the pollutants listed in 40 CFR Part 122, Appendix D, Table III, as well as some additional pollutants. These pollutants are listed in Exhibit 4-2.

Furthermore, 40 CFR 122.26(d)(2)(iii)(A)(5) requires that estimates be provided of the annual pollutant load of the cumulative discharges to waters of the U.S. from all identified municipal outfalls, and the event mean concentration of the cumulative discharges to waters of the U.S. from all identified municipal outfalls during storm events for the parameters listed in Exhibit 4-2. Estimates of the parameters must be accompanied by a description of the procedures for estimating constituent loads and concentrations, including any modelling, data analysis, and calculation methods.

EXHIBIT 4.2. PARAMETERS WHICH MUST BE ANALYZED BY MUNICIPAL APPLICANTS

Pollutants Contained in Table III of 40 CFR Part 122, Appendix D				
Total antimony	Total cadmium	Total lead	Total selenium	Total zinc
Total arsenic	Total chromium	Total mercury	Total silver	Total cyanide
Total beryllium	Total copper	Total nickel	Total thallium	Total phenols
Pollutants Contained in Table II of 40 CFR Part 122, Appendix D				
Acrolein	Toluene	Benzo(a)pyrene	2,6-dinitrotoluene	Gamma-BHC
Acrylonitrile	1,2-trans-dichloroethylene	3,4-benzofluoranthene	Di-n-octyl phthalate	Delta-BHC
Benzene	1,1,1-trichloroethane	Benzo(ghi)perylene	1,2-diphenylhydrazine	Chlordane
Bromoform	1,1,2-trichloroethane	Benzo(k)fluoranthene	Fluoranthene	4,4'-DDT
Carbon Tetrachloride	Trichloroethylene	Bis(2-chloroethoxy)methane	Fluorene	4,4'-DDE
Chlorobenzene	Vinyl chloride	Bis(2-chloroethyl)ether	Hexachlorobenzene	4,4'-DDD
Chlorodibromo-methane	2-chlorophenol	Bis(2-chloroisopropyl)ether	Hexachlorobutadiene	Dieldrin
Chloroethane	2,4-dichlorophenol	Bis(2-ethylhexyl)phthalate	Hexachlorocyclopen-tadiene	Alpha-endosulfan
2-Chloroethylvinyl ether	2,4-dimethylphenol	4-bromophenyl phenyl ether	Hexachloroethane	Beta-endosulfan
Chloroform	4,6-dinitro-o-cresol	Butylbenzyl phthalate	Indeno(1,2,3-cd)pyrene	Endosulfan sulfate
Dichlorobromo-methane	2,4-dinitrophenol	2-chloronaphthalene	Isophorone	Endrin
1,1-dichloroethane	2-nitrophenol	4-chlorophenyl phenyl ether	Naphthalene	Endrin aldehyde
1,2-dichloroethane	4-nitrophenol	Chrysene	Nitrobenzene	Heptachlor
1,1-dichloroethylene	p-chloro-m-cresol	Dibenzo(a,h)anthracene	N-nitrosodimethylamine	Heptachlor epoxide
1,2-dichloropropane	Pentachlorophenol	1,2-dichlorobenzene	N-nitrosodi-n-propylamine	PCB-1242
1,3-dichloropropylene	Phenol	1,3-dichlorobenzene	N-nitrosodiphenylamine	PCB-1254
Ethylbenzene	2,4,6-trichlorophenol	1,4-dichlorobenzene	Phenanthrene	PCB-1221
Methyl bromide	Acenaphthene	3,3-dichlorobenzidine	Pyrene	PCB-1232
Methyl chloride	Acenaphthylene	Diethyl phthalate	1,2,4-trichlorobenzene	PCB-1248
Methylene chloride	Anthracene	Dimethyl phthalate	Aldrin	PCB-1260
1,1,2,2-tetrachloroethane	Benzidine	Di-n-butyl phthalate	Alpha-BHC	PCB-1016
Tetrachloroethylene	Benzo(a)anthracene	2,4-dinitrotoluene	Beta-BHC	Toxaphene

Additional Pollutants Which Must be Analyzed		
TSS	O&G	TKN
TDS	Fecal coliform	Nitrate plus nitrite nitrogen
COD	Fecal streptococcus	Total and dissolved phosphorus
BOD_5	pH	
	Total residual chlorine	

Source: 40 CFR Part 122, Appendix D

5. FLEXIBILITY IN SAMPLING

The requirements for storm water sampling for permit applications offer some flexibility by the permitting authority. The areas of flexibility are discussed below.

5.1 PROTOCOL MODIFICATIONS

The permitting authority may allow sampling protocol modifications for specific requirements on a case-by-case basis. For example, the permitting authority may accept application forms with incomplete sampling data if there was no rainfall at the applicant's facility prior to the submission deadline. However, the permitting authority will require that sampling data be submitted as soon as possible. The reason for not submitting data must be certified by a corporate official (for industrial facilities) or the principal executive officer or ranking official (for municipalities).

Another area where permitting authorities may allow flexibility in storm water sampling is acceptance of quantitative data from a storm event that does not meet the representative rainfall criteria of within 50 percent of the volume and duration for the average storm event for the area. The permitting authority may decide that the discharge data provided is better than no data at all.

In addition, the permitting authority may establish appropriate site-specific sampling procedures or requirements, including sampling locations; the season in which the sampling takes place; the minimum duration between the previous measurable storm event and the storm event sampled; the minimum or maximum level of precipitation required for an appropriate storm event; the form of precipitation sampled (snow melt or rainfall); protocols for collecting samples under 40 CFR Part 136; and additional time for submitting data on a case-by-case basis. The permitting authority should be contacted for preapproval of any necessary protocol modifications. In the case of group applications, EPA Headquarters should be contacted.

5.2 PETITION FOR SUBSTITUTING SUBSTANTIALLY IDENTICAL EFFLUENTS

As described at 40 CFR 122.21(g)(7), when an industrial applicant has two or more outfalls with substantially identical effluents, the permitting authority may allow the applicant to test only one outfall and to report that the quantitative data also apply to the substantially identical outfalls. In the case of group applications, the petition must be submitted to EPA Headquarters.

For facilities seeking to demonstrate that storm water outfalls are substantially identical, a variety of methods can be used as determined by the permitting authority. Three possible petition options are discussed here: (1) submission of a narrative description and a site map; (2) submission of matrices; or (3) submission of model matrices. Detailed guidance on each of the three options for demonstrating substantially identical outfalls is provided below. An owner/operator certification should be submitted with each option. See Section 5.2.3 for an example of this certification.

5.2.1 OPTION ONE: NARRATIVE DESCRIPTION/SITE MAP

Facilities demonstrating that storm water outfalls are substantially identical may submit a narrative description of the facility and a site map to the permitting authority . The narrative portion must include a description of why the outfalls are substantially identical. Petitioners may demonstrate that these outfalls contain storm water discharges associated with:

- Substantially identical industrial activities and processes;
- Substantially identical significant materials that may be exposed to storm water [including, but not limited to, raw materials, fuels, materials such as solvents, detergents, and plastic pellets; finished materials such as metallic products; raw materials used in food processing or production; hazardous substances designated under Section 101(14) of the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA); any chemical the facility is required to report pursuant to Section 313 of Title III of the Superfund Amendments and Reauthorization Act (SARA); fertilizers; pesticides; and waste products such as ashes, slag, and sludge that have the potential to be released with storm water discharges as per 40 CFR 122.26(b)(12)];
- Substantially identical storm water management practices (such as retention ponds, enclosed areas, diversion dikes, gutters, and swales) and material management practices (such as protective coverings and secondary containment); and
- Substantially identical flows, as determined by the estimated runoff coefficient and approximate drainage area at each outfall.

The site map should include an indication of the facility's topography; each of the drainage and discharge structures; the drainage area of each storm water outfall; paved areas and buildings within the drainage area for each storm water outfall; all past or present areas used for outdoor storage or disposal of significant materials; identification of the significant materials in each drainage area; and identification of each existing structural control measures used to reduce pollutants in storm water

runoff, materials loading and access areas, and areas where pesticides, herbicides, soil conditioners, and fertilizers are applied.

Exhibit 5-1 offers an example of a narrative description/site map petition that sufficiently demonstrates identical outfalls. A demonstration of how to determine runoff coefficient estimates was presented in Section 3.2.2. Exhibit 5-2 presents an example of a site map to be included with the narrative description.

5.2.2 OPTION TWO: USE OF MATRICES TO INDICATE IDENTICAL OUTFALLS

Facilities attempting to demonstrate that storm water outfalls are substantially identical may submit matrices and an owner/operator certification describing specific information associated with each outfall to the permitting authority. Matrix information is required only for those outfalls that the permit applicant is attempting to demonstrate are identical, not for all outfalls. Petitioners must demonstrate, using the matrices, that the outfalls have storm water discharges that meet the criteria listed in Section 5.2.1. Refer to Exhibit 5-3 for examples of matrices that demonstrate substantially identical outfalls and Section 3.2.2 for guidance on determining runoff coefficient estimates.

5.2.3 OPTION THREE: MODEL MATRICES

Facilities attempting to demonstrate that storm water outfalls are substantially identical may submit model matrices and an owner/operator certification to the permitting authority. This option is particularly appropriate for facilities with a large number of storm water outfalls and the potential for numerous groupings of identical outfalls. In addition, this option may be useful in group applications that have a large sampling subgroup.

Model matrices should contain information for one grouping of substantially identical outfalls. For example, if a facility has 150 outfalls and several groupings of identical outfalls, the facility would choose one of the groupings of identical outfalls to provide information in the model matrices. The petitioner must demonstrate, using these matrices, that all outfalls within this grouping have storm water discharges that meet the criteria listed in Section 5.2.1.

The facility should provide an owner certification that all other groupings of outfalls have been examined and certified as substantially identical outfalls according to the criteria established in the

EXHIBIT 5-1. PETITION TO SAMPLE SUBSTANTIALLY IDENTICAL OUTFALLS (NARRATIVE DESCRIPTION/SITE MAP)

Examples

I. The Pepper Company of Philadelphia, Pennsylvania, is primarily engaged in manufacturing paperboard, including paperboard coated on the paperboard machine (from wood pulp and other fiber pulp). This establishment is classified under SIC code 2631. Pursuant to the November 16, 1990, NPDES storm water permit application regulations, this facility is considered to be "engaging in industrial activity" for the purposes of storm water permit application requirements in 40 CFR 122.26(b)(14)(i) and (ii).

II. "When an applicant has two or more outfalls with substantially identical effluents, the Director may allow the applicant to test only one outfall and report that the quantitative data also apply to the substantially identical outfalls." [40 CFR 122.21(g)(7)]

In accordance with 40 CFR 122.21(g)(7) of the NPDES regulations, The Pepper Company hereby petitions the State of Pennsylvania (the permitting authority) for approval to sample certain representative storm water outfalls in groupings of storm water outfalls that are substantially identical. The Pepper Company will demonstrate that of the ten (10) outfalls discharging storm water from our paperboard manufacturing plant, there are two pairs of substantially identical outfalls. Outfalls 3 and 4 are substantially identical and should be grouped together. Outfalls 8 and 9 are substantially identical and should be grouped together. Outfalls 1, 2, 5, 6, 7, and 10 have distinct characteristics and, therefore, will not be grouped together with other outfalls for the purposes of storm water discharge sampling.

III. The Pepper Company will demonstrate that the substantially identical outfalls that have been grouped together contain storm water discharges associated with: (1) substantially identical industrial activities and processes that are occurring outdoors; (2) substantially identical significant materials (including raw materials, fuels, finished materials, waste products, and material handling equipment) that may be exposed to storm water; (3) substantially identical material management practices (such as runoff diversions, gutters and swales, protective coverings, and structural enclosures); and (4) substantially identical flows, as determined by the estimated runoff coefficient and approximate drainage area at each outfall.

EXHIBIT 5-1. PETITION TO SAMPLE SUBSTANTIALLY IDENTICAL OUTFALLS (NARRATIVE DESCRIPTION/SITE MAP) (Continued)

1. Industrial Activities

A. Description of Industrial Activities at the Pepper Company

The Pepper Company receives wastepaper in bales. This baled wastepaper is sent through a hydropulper and converted to pulp. The fiber material is concentrated, stored, and then drawn through refiners to the paper machines. Wires, plastics, and miscellaneous material are removed during the pulping.

Three systems are used to produce top liner, back paper, and filler. The highest quality fiber is used for the top liner, the medium quality is used for the back paper, and the poorest quality is used for the filler paper. Wireforming or conventional boxboard processes are employed to produce clay-coated boxboard, using a water-based clay-coating material. Additional materials may be used as binders. These are stored indoors and are not exposed to precipitation. Ammonia is used in the clay-coating process. Off-grade fiber and trim material are ground up and returned to the liquid process stream. Slime control agents, consisting of bactericides, are used in association with this process. These agents are organic materials used to prevent souring of mill operations. They are received in drums and stored indoors. Empty drums are returned to the supplier to reuse. In addition, the Pepper Company operates an onsite landfill for the disposal of miscellaneous waste materials removed during pulping and paper cuttings operations.

B. Demonstration of Why Outfalls Are Substantially Identical in Terms of Industrial Activities Conducted Outdoors.

Outfalls 3 and 4

Outfalls 3 and 4 are substantially identical in terms of industrial activities conducted outdoors. Both outfalls contain storm water discharges associated with the outdoor storage of baled wastepaper. The wastepaper, which consists of old corrugated containers, mixed paper, and other types of wastepaper, is received weekly and stored for up to 3 weeks in Storage Areas #1 and #2. These uncovered storage areas are enclosed by chain-link fencing.

Outfalls 8 and 9

Outfalls 8 and 9 drain storm water runoff from areas where all industrial activities occur indoors. The industrial activities occurring under roof cover at these two outfalls include hydropulping, storage of concentrated fiber material, refining, and paperboard production. These industrial processes have no potential for contact with precipitation.

EXHIBIT 5-1 PETITION TO SAMPLE SUBSTANTIALLY IDENTICAL OUTFALLS (NARRATIVE DESCRIPTION/SITE MAP) (Continued)

2. Significant Materials

A. Description of Significant Materials at the Pepper Company

The significant materials listed below are used by the Pepper Company to manufacture paperboard. These materials are stored indoors, unless otherwise indicated.

(i) Raw materials, including baled wastepaper (off-spec damaged paper stock or recycled paper) **[wastepaper is stored outdoors at Storage Areas #1 and #2]**; clays, ammonias, sizings, and slime control agents (chlorine dioxide); caustic; ammonia, which is stored in two tanks. **[See Storage Area #3]**.

(ii) Waste Materials, including miscellaneous materials removed during pulping and paper cuttings (such as staples, rubber bands, styrofoam, etc.). These waste materials are stored indoors in open dumpsters. However, prior to disposing of the waste in the onsite landfill, these dumpsters are moved outdoors where they are potentially exposed to precipitation for 12 hours or less. **[See Storage Area #3]**.

(iii) Finished Products, including paperboard and molded fiber products. These are always stored indoors.

(iv) Others, including wood pallets (which are used to transport and haul raw materials, waste materials, and finished products) are stored both indoors and outdoors. **[See Storage Area #3]**. The Pepper Company has an above-ground fuel tank with a pump. **[See Storage Area #3]**.

B. Demonstration of Why Outfalls are Substantially Identical in Terms of Significant Materials that Potentially May be Exposed to Storm Water

Outfalls 3 and 4

Outfalls 3 and 4 are substantially identical in terms of significant materials that may be exposed to storm water. Both outfalls contain storm water discharges associated with the outdoor storage of baled wastepaper. The wastepaper, which consists of old corrugated containers, mixed paper, and other types of wastepaper, is received weekly and stored for up to 3 weeks in Storage Areas #1 and #2. These uncovered storage areas are enclosed by chain-link fencing.

Outfalls 8 and 9

Outfalls 8 and 9 are substantially identical in terms of significant materials. Both outfalls contain storm water runoff from areas that have no significant materials potentially exposed to storm water. All industrial activities occurring in the areas drained by Outfalls 8 and 9 occur completely indoors.

EXHIBIT 5-1. PETITION TO SAMPLE SUBSTANTIALLY IDENTICAL OUTFALLS (NARRATIVE DESCRIPTION/SITE MAP) (Continued)

3. Material Management Practices

A. Description of Material Management Practices at the Pepper Company

The Pepper Company uses a wide range of storm water management practices and material management practices to limit the contact of significant materials with precipitation. Non-structural storm water management practices include employee training, spill reporting and clean-up, and spill prevention techniques. Structural storm water management practices include:

(i) Diversion Devices (both above-ground trenches and subterranean drains) are used to divert surface water from entering a potentially contaminated area.

(ii) Gutters/Swales (constructed of concrete or grass) channel storm water runoff to drainage systems leading to separate storm sewers.

(iv) Overland Flow (which is the flow of storm water over vegetative areas prior to entrance into a storm water conveyance) allows much of the storm water to infiltrate into the ground. The remainder is naturally filtered prior to reaching the storm water conveyance. This is not considered sheet flow since natural drainage channels may be carved out during a heavy storm event.

B. Demonstration of Why Outfalls Are Substantially Identical in Terms of Storm Water Management Practices Used

Outfalls 3 and 4

Outfalls 3 and 4 are substantially identical in terms of storm water management practices used. Both outfalls contain storm water discharges associated with the outdoor storage of baled wastepaper, located in Storage Areas #1 and #2. Concrete gutters at both sites channel storm water away from the storage areas down to the respective outfalls.

Outfalls 8 and 9

Outfalls 8 and 9 are substantially identical in terms of storm water management practices used. Both outfalls contain storm water runoff from areas that have no significant materials potentially exposed to storm water. All industrial activities occurring in the areas drained by Outfalls 8 and 9 occur completely indoors. Both outfalls receive overland flow storm water. From roof drains, the storm water in both drainage areas is then conveyed over similarly graded vegetative areas prior to entrance into the respective outfalls.

EXHIBIT 5-1 PETITION TO SAMPLE SUBSTANTIALLY IDENTICAL OUTFALLS (NARRATIVE DESCRIPTION/SITE MAP) (Continued)

4. **Flow Characteristics**

A. Demonstration of Why Outfalls Are Substantially Identical in Terms of Flow, as Determined by The Estimated Runoff Coefficient and Approximate Drainage Area at Each Outfall

Outfalls 3 and 4

Outfalls 3 and 4 are substantially identical in terms of flow. Both drainage areas have a 2 to 7 percent grade and contain fine textured soil (greater than 40 percent clay) with a vegetative cover. The estimated runoff coefficient for both outfalls is .2. The approximate drainage area for each outfall is similar. Outfall 3 has an approximate drainage area of 3,500 square feet. Outfall 4 has an approximate drainage area of 2,900 square feet.

Outfalls 8 and 9

Outfalls 8 and 9 are substantially identical in terms of flow. Both drainage areas have a 2 to 7 percent grade and contain fine textured soil (greater than 40 percent clay) with a vegetative cover. The estimated runoff coefficient for both outfalls is .2. The approximate drainage area for each outfall is similar. Outfall 8 has an approximate drainage area of 7,600 square feet. Outfall 9 has an approximate drainage area of 8,700 square feet.

EXHIBIT 5-2. SITE MAP

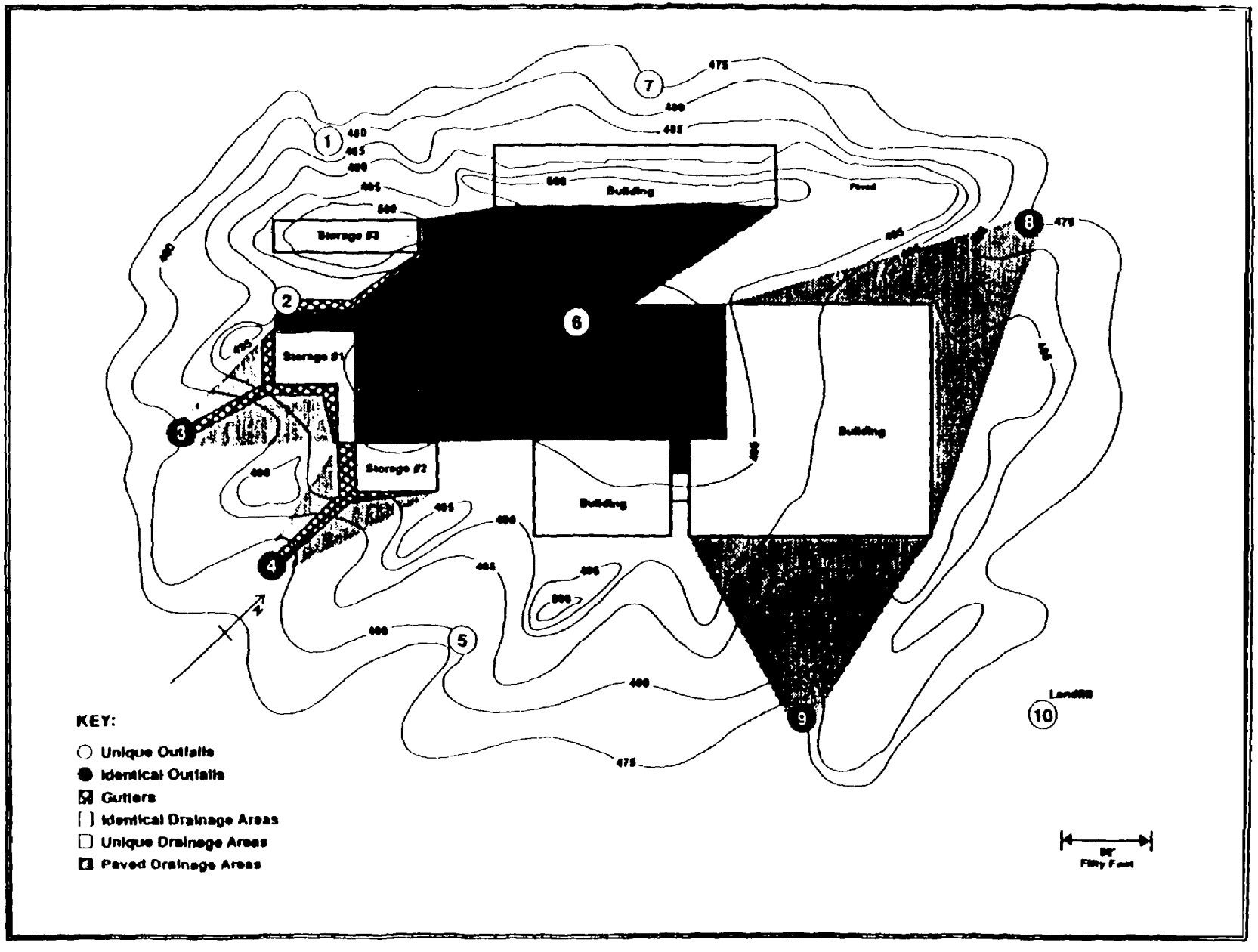


EXHIBIT 5.3 MATRICES DEMONSTRATING SUBSTANTIALLY IDENTICAL OUTFALLS

Industrial Activities

OUTFALL	A	B	C	D	E
3	X	–	–	X	–
4	X	–	–	X	–
8	–	–	–	–	–
9	–	–	–	–	–

Key:

A = Outdoor storage of raw materials and material-handling equipment
B = Fueling
C = Waste materials storage (dumpster)
D = Loading/unloading of raw materials, intermediate products, and final products
E = Landfill activity

Significant Materials That May Be Exposed to Storm Water

OUTFALL	A	B	C	D	E	F
3	-	–	–	–	X	–
4	–	–	–	–	X	–
8	–	–	–	–	–	–
9	–	–	–	–	–	–

Key:

A = Outdoor ammonia tank
B = Wood pallets
C = Above ground gas tank
D = Waste materials
E = Baled wastepaper
F = Finished products

EXHIBIT 5.3 MATRICES DEMONSTRATING SUBSTANTIALLY IDENTICAL OUTFALLS (Continued)

Storm Water Management Practices

OUTFALL	A	B	C
3	–	X	–
4	–	X	–
8	–	–	X
9	–	–	X

Key:

A = Runoff diversions
B = Gutters/swales
C = Overland flow (not sheet flow; flow through vegetative areas)

Flow Characteristics

OUTFALL	A	B
3	0.2	3,500
4	0.2	2,900
8	0.2	7,600
9	0.2	8,700

Key:

A = Estimated runoff coefficient
B = Approximate drainage area of outfall (square feet)

model matrices described in Exhibit 5-3. The owner/operator who signs documents in this section should include the following certification:

> "I certify under penalty of law that this document and all attachments were prepared under my direction or supervision in accordance with a system designed to assure that qualified personnel properly gather and evaluate the information submitted. Based on my inquiry of the person or persons who manage the system, or those persons directly responsible for gathering the information, the information submitted is, to the best of my knowledge and belief, true, accurate and complete. I am aware that there are significant penalties for submitting false information, including the possibility of fine and imprisonment for knowing violations" [as per 40 CFR 122.22(d)].

5.3 ALTERNATE 40 CFR PART 136 METHOD

As required in 40 CFR 136.4, the applicant must request the approval of an alternate test procedure in writing (in triplicate) prior to testing. The request must be submitted to the Regional Administrator through the Director of the State agency responsible for issuing NPDES permits. The applicant must:

- Provide the name and address of the responsible person or firm making the discharge (if not the applicant), the applicable identification number of the existing or pending permit, the issuing agency, the type of permit for which the alternate test procedure is requested, and the discharge serial number;
- Identify the pollutant or parameter for which approval of an alternate testing procedure is being requested;
- Provide justification for using testing procedures other than those specified in 40 CFR Part 136;
- Provide a detailed description of the proposed alternate test procedure, together with references to published studies of the applicability of the alternate test procedure to the effluents in question;
- Provide comparability data (for applicants applying for nation wide approval of an alternative test procedures).

The permitting authority will notify the applicant within 90 days regarding the approval of the alternate method.

5.4 LACK OF METHOD IN 40 CFR PART 136

If a specific pollutant that must be tested does not have a corresponding analytical method listed in 40 CFR Part 136, the applicant must submit information on an appropriate method to be used. The permitting authority must approve its use prior to collection and analysis of sampling data. The laboratory should be consulted for suggestions and information about analytical methods that can be used. All information justifying the alternative method should be sent to the permitting authority prior to use.

6. HEALTH AND SAFETY

Storm water sampling activities may occur when the sampling environment and/or storm water discharges create hazardous conditions. Hazardous conditions associated with sampling include:

- Hazardous weather conditions (e.g., wind, lightning, flooding, etc.)
- Sampling in confined spaces (e.g., manholes)
- Hazards associated with chemicals
- Biological hazards (e.g., rodents and snakes)
- Physical hazards (e.g., traffic, falling objects, sharp edges, slippery footing, and the potential for lifting injuries from opening or removing access panels and manhole covers, etc.)

It is essential that sampling personnel be aware of these hazards. Sampling personnel should be trained to evaluate potentially hazardous situations and develop ways for handling them. Since sampling hazards can be life threatening, safety must be the highest priority for all personnel. This chapter outlines general health and safety issues and concerns. Additional references discussed below should be consulted for more specific guidance to avoid adverse health and safety situations.

6.1 GENERAL TRAINING REQUIREMENTS

Preparation and training of all sampling personnel should be completed before beginning any sampling task. Extreme care should be taken to allow for safety precautions including proper equipment and appropriate operational techniques, sufficient time to accomplish the task, training on potential hazards, and emergency procedures. EPA's Order 1440.2 sets out the policy, responsibilities, and mandatory requirements for the safety of personnel who are involved in sampling activities. This order, which is found within the EPA NPDES Compliance Monitoring Inspector Training: Sampling manual, provides further guidance to applicants' storm water sampling personnel. Basic emergency precautions include having access to both local emergency phone numbers and communication equipment (i.e., phones or radios), and ensuring that personnel are trained in first aid and carry first aid equipment.

6.2 NECESSARY SAFETY EQUIPMENT

Exhibit 6-1 contains a list of safety equipment that may be appropriate depending on the characteristics of the sampling site.

EXHIBIT 6-1. LIST OF SAFETY EQUIPMENT	
Flashlight	18-inch traffic cones
Meters (for oxygen, explosivity, toxic gases)	Insect/rodent repellant
Ladder	Ventilation equipment
Safety harness	50 feet of 1/2-inch nylon rope
Hard hat	Safety shoes
Safety goggles	Rain wear
Coveralls	Gloves (rubber)
Respirator	First aid kit
Reflective vests	Self-contained breathing apparatus
Source: Adapted from NPDES Compliance Monitoring Inspector Training: Sampling, U.S. EPA, August 1990.	

6.3 HAZARDOUS WEATHER CONDITIONS

Common sense should dictate whether sampling be conducted during adverse weather conditions. No sampling personnel should place themselves in danger during high winds, lightning storms, or flooding conditions which might be unsafe. Under extreme conditions, a less hazardous storm event should be sampled.

6.4 SAMPLING IN CONFINED SPACES

Confined spaces encountered by storm water sampling personnel typically include manholes and deep, unventilated ditches. A confined space is generally defined as a space that is somewhat enclosed with limited access and inadequate ventilation.

The National Institute of Occupational Safety and Health (NIOSH) has developed a manual entitled "Working in Confined Spaces" which should be consulted prior to confined space entry. Also, several States have developed specific procedures which should also be consulted. Unless they have been trained for confined space entry, sampling personnel should avoid entry under all circumstances.

6.4.1 HAZARDOUS CONDITIONS IN CONFINED SPACES

Confined spaces pose a safety threat to sampling personnel because of low oxygen, explosivity, and toxic gases. When entering a confined space, a qualified person should ensure that the atmosphere is safe by sampling to test for oxygen levels, potential flammable hazards, and toxic materials known or suspected to be present. If atmospheric conditions are detected, the confined space should be ventilated or sampling personnel should use a self-contained air supply and wear a life line. At least one person should remain outside of the confined space in the event that problems arise. If atmospheric testing has not been properly conducted, the confined space should not be entered. Manholes can also pose a threat to safety because of the small confined area, slippery surfaces, sharp objects, unsafe ladders, etc.

6.4.2 SPECIAL TRAINING REQUIREMENTS

Personnel should not enter into a confined space unless trained in confined space entry techniques. Such training covers hazard recognition, the use of respiratory equipment and atmospheric testing devices, use of special equipment and tools, and emergency and rescue procedures. In addition, at least one member of the sampling crew should be certified in basic first aid and Cardiopulmonary Resuscitation (CPR). Sampling personnel should, on an annual basis, practice confined space rescues.

6.4.3 PERMIT SYSTEM

If entry into a confined space is necessary, an entry permit system should be developed which includes a written procedure. This permit should include, at a minimum:

- Description of type of work to be done
- Hazards that may be encountered

- Location and description of the confined space
- Information on atmospheric conditions at confined space
- Personnel training and emergency procedures
- Names of sampling personnel.

The manual developed by NIOSH discusses this permit system in more detail. Furthermore, the Occupational Safety and Health Administration (OSHA) proposed a rule on June 5, 1989 (54 FR 24080) that would implement a permit system. The rule is expected to be finalized and published late in 1992.

6.5 CHEMICAL HAZARDS

Sampling personnel can also be at risk of exposure to hazardous chemicals—either chemicals in the actual storm water discharge or the chemicals that have been placed in the sample collection containers for sample preservation. Therefore, direct contact with the preservatives and the storm water (if hazardous chemicals are suspected to be present) should be avoided. Sampling personnel should wear gloves and safety glasses to avoid skin and eye exposure to harmful chemicals. Sampling personnel should be trained to avoid exposure and instructed as to what to do if exposure occurs (e.g., flush the eyes, rinse the skin, ventilate the area, etc.).

6.6 BIOLOGICAL HAZARDS

Storm water sampling personnel may also encounter biological hazards such as rodents, snakes, and insects. The sampling crew should remain alert to these hazards. As mentioned in Section 6.2, necessary sampling equipment, for certain locations, should include insect/rodent repellant and a first aid kit.

6.7 PHYSICAL HAZARDS

The sampling crew should be aware of a number of physical hazards that could cause accidents at the sampling site. These hazards include traffic hazards, sharp edges, falling objects, slippery footing, and lifting injuries from removing manhole covers. Sampling personnel should pay close attention in order to prevent these safety hazards at all times.

If the sample point is in a manhole, a street gutter, or ditch near the street, particular attention must be given to marking off the work area to warn oncoming traffic of the presence of the sampling crew. Traffic cones, warning signs, and barricades should be placed in appropriate places around the sampling point.

TECHNICAL APPENDIX A

FORMS 2F AND 1

Please print or type in the unshaded areas only

EPA ID Number (copy from item I of Form 1)

Form Approved. OMB No. 2040-0086

Approval expires 5-31-92

Form 2F NPDES

EPA

United States Environmental Protection Agency
Washington, DC 20460

Application for Permit to Discharge Storm Water Discharges Associated with Industrial Activity

Paperwork Reduction Act Notice

Public reporting burden for this application is estimated to average 28.6 hours per application, including time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding the burden estimate, any other aspect of this collection of information, or suggestions for improving this form, including suggestions which may increase or reduce this burden to: Chief, Information Policy Branch, PM-223, U.S. Environmental Protection Agency, 401 M St., SW, Washington, DC 20460, or Director, Office of Information and Regulatory Affairs, Office of Management and Budget, Washington, DC 20503.

I. Outfall Location

For each outfall, list the latitude and longitude of its location to the nearest 15 seconds and the name of the receiving water.

A. Outfall Number (list)	B. Latitude	C. Longitude	D. Receiving Water (name)

II. Improvements

A. Are you now required by any Federal, State, or local authority to meet any implementation schedule for the construction, upgrading or operation of wastewater treatment equipment or practices or any other environmental programs which may affect the discharges described in this application? This includes, but is not limited to, permit conditions, administrative or enforcement orders, enforcement compliance schedule letters, stipulations, court orders, and grant or loan conditions.

1. Identification of Conditions, Agreements, Etc.	2. Affected Outfalls		3. Brief Description of Project	4. Final Compliance Date	
	number	source of discharge		a. req.	b. proj.

B. You may attach additional sheets describing any additional water pollution (or other environmental projects which may affect your discharges) you now have under way or which you plan. Indicate whether each program is now under way or planned, and indicate your actual or planned schedules for construction.

III. Site Drainage Map

Attach a site map showing topography (or indicating the outline of drainage areas served by the outfall(s) covered in the application if a topographic map is unavailable) depicting the facility including: each of its intake and discharge structures; the drainage area of each storm water outfall; paved areas and buildings within the drainage area of each storm water outfall, each known past or present areas used for outdoor storage or disposal of significant materials, each existing structural control measure to reduce pollutants in storm water runoff, materials loading and access areas, areas where pesticides, herbicides, soil conditioners and fertilizers are applied; each of its hazardous waste treatment, storage or disposal units (including each area not required to have a RCRA permit which is used for accumulating hazardous waste under 40 CFR 262.34); each well where fluids from the facility are injected underground, springs, and other surface water bodies which receive storm water discharges from the facility.

Continued from the Front

IV. Narrative Description of Pollutant Sources

A. For each outfall, provide an estimate of the area (include units) of impervious surfaces (including paved areas and building roofs) drained to the outfall, and an estimate of the total surface area drained by the outfall.

Outfall Number	Area of Impervious Surface (provide units)	Total Area Drained (provide units)	Outfall Number	Area of Impervious Surface (provide units)	Total Area Drained (provide units)

B. Provide a narrative description of significant materials that are currently or in the past three years have been treated, stored or disposed in a manner to allow exposure to storm water; method of treatment, storage, or disposal; past and present materials management practices employed to minimize contact by these materials with storm water runoff; materials loading and access areas; and the location, manner, and frequency in which pesticides, herbicides, soil conditioners, and fertilizers are applied.

C. For each outfall, provide the location and a description of existing structural and nonstructural control measures to reduce pollutants in storm water runoff; and a description of the treatment the storm water receives, including the schedule and type of maintenance for control and treatment measures and the ultimate disposal of any solid or fluid wastes other than by discharge.

Outfall Number	Treatment	List Codes from Table 2F-1

V. Nonstormwater Discharges

A. I certify under penalty of law that the outfall(s) covered by this application have been tested or evaluated for the presence of nonstormwater discharges, and that all nonstormwater discharges from these outfall(s) are identified in either an accompanying Form 2C or Form 2E application for the outfall.

Name and Official Title (type or print)	Signature	Date Signed

B. Provide a description of the method used, the date of any testing, and the onsite drainage points that were directly observed during a test.

VI. Significant Leaks or Spills

Provide existing information regarding the history of significant leaks or spills of toxic or hazardous pollutants at the facility in the last three years, including the approximate date and location of the spill or leak, and the type and amount of material released.

EPA ID Number (copy from item 1 of Form 1)

Continued from Page 2

VII. Discharge Information

A,B,C, & D: See instructions before proceeding. Complete one set of tables for each outfall. Annotate the outfall number in the space provided. Tables VII-A, VII-B, and VII-C are included on separate sheets numbered VII-1 and VII-2.

E. Potential discharges not covered by analyses - is any toxic pollutant listed in table 2F-2, 2F-3 or 2F-4, a substance or a component of a substance which you currently use or manufacture as an intermediate or final product or byproduct?

☐ Yes (list all such pollutants below) ☐ No (go to Section G)

VIII. Biological Toxicity Testing Data

Do you have any knowledge or reason to believe that any biological test for acute or chronic toxicity has been made on any of your discharges or on a receiving water in relation to your discharge within the last 3 years?

☐ Yes (list all such pollutants below) ☐ No (go to Section IX)

IX. Contract Analysis Information

Were any of the analyses reported in item VII performed by a contract laboratory or consulting firm?

☐ Yes (list the name, address, and telephone number of, and pollutants analyzed by, each such laboratory or firm below) ☐ No (go to Section X)

A. Name	B. Address	C. Area Code & Phone No.	D. Pollutants Analyzed

X. Certification

I certify under penalty of law that this document and all attachments were prepared under my direction or supervision in accordance with a system designed to assure that qualified personnel properly gather and evaluate the information submitted. Based on my inquiry of the person or persons who manage the system or those persons directly responsible for gathering the information, the information submitted is, to the best of my knowledge and belief, true, accurate, and complete. I am aware that there are significant penalties for submitting false information, including the possibility of fine and imprisonment for knowing violations.

A. Name & Official Title (type or print)	B. Area Code and Phone No.
C. Signature	D. Date Signed

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EPA ID Number (copy from Item 1 of Form 1)

Form Approved. OMB No. 2040-0086
Approval expires 5-31-92

VII. Discharge information *(Continued from page 3 of Form 2F)*

Part A - You must provide the results of at least one analysis for every pollutant in this table. Complete one table for each outfall. See instructions for additional details.

Pollutant and CAS Number (if available)	Maximum Values (include units)		Average Values (include units)		Number of Storm Events Sampled	Sources of Pollutants
	Grab Sample Taken During First 30 Minutes	Flow-weighted Composite	Grab Sample Taken During First 30 Minutes	Flow-weighted Composite		
Oil and Grease						
Biological Oxygen Demand (BOD5)						
Chemical Oxygen Demand (COD)						
Total Suspended Solids (TSS)						
Total Kjeldahl Nitrogen						
Nitrate plus Nitrite Nitrogen						
Total Phosphorus						
pH	Minimum	Maximum	Minimum	Maximum		

Part B - List each pollutant that is limited in an effluent guideline which the facility is subject to or any pollutant listed in the facility's NPDES permit for its process wastewater (if the facility is operating under an existing NPDES permit). Complete one table for each outfall. See the instructions for additional details and requirements.

Pollutant and CAS Number (if available)	Maximum Values (include units)		Average Values (include units)		Number of Storm Events Sampled	Sources of Pollutants
	Grab Sample Taken During First 30 Minutes	Flow-weighted Composite	Grab Sample Taken During First 30 Minutes	Flow-weighted Composite		

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Continued from the Front

Part C - List each pollutant shown in Tables 2F-2, 2F-3, and 2F-4 that you know or have reason to believe is present. See the instructions for additional details and requirements. Complete one table for each outfall.

Pollutant and CAS Number (if available)	Maximum Values (include units)		Average Values (include units)		Number of Storm Events Sampled	Sources of Pollutants
	Grab Sample Taken During First 30 Minutes	Flow-weighted Composite	Grab Sample Taken During First 30 Minutes	Flow-weighted Composite		

Part D - Provide data for the storm event(s) which resulted in the maximum values for the flow weighted composite sample.

1. Date of Storm Event	2. Duration of Storm Event (in minutes)	3. Total rainfall during storm event (in inches)	4. Number of hours between beginning of storm measured and end of previous measurable rain event	5. Maximum flow rate during rain event (gallons/minute or specify units)	6. Total flow from rain event (gallons or specify units)

7. Provide a description of the method of flow measurement or estimate.

Instructions - Form 2F
Application for Permit to Discharge Storm Water Associated with Industrial Activity

Who Must File Form 2F

Form 2F must be completed by operators of facilities which discharge storm water associated with industrial activity or by operators of storm water discharges that EPA is evaluating for designation as a significant contributor of pollutants to waters of the United States, or as contributing to a violation of a water quality standard.

Operators of discharges which are composed entirely of storm water must complete Form 2F (EPA Form 3510-2F) in conjunction with Form 1 (EPA Form 3510-1).

Operators of discharges of storm water which are combined with process wastewater (process wastewater is water that comes into direct contact with or results from the production or use of any raw material, intermediate product, finished product, byproduct, waste product, or wastewater) must complete and submit Form 2F, Form 1, and Form 2C (EPA Form 3510-2C).

Operators of discharges of storm water which are combined with nonprocess wastewater (nonprocess wastewater includes noncontact cooling water and sanitary wastes which are not regulated by effluent guidelines or a new source performance standard, except discharges by educational, medical, or commercial chemical laboratories) must complete Form 1, Form 2F, and Form 2E (EPA Form 3510-2E).

Operators of new sources or new discharges of storm water associated with industrial activity which will be combined with other nonstormwater new sources or new discharges must submit Form 1, Form 2F, and Form 2D (EPA Form 3510-2D).

Where to File Applications

The application forms should be sent to the EPA Regional Office which covers the State in which the facility is located. Form 2F must be used only when applying for permits in States where the NPDES permits program is administered by EPA. For facilities located in States which are approved to administer the NPDES permits program, the State environmental agency should be contacted for proper permit application forms and instructions.

Information on whether a particular program is administered by EPA or by a State agency can be obtained from your EPA Regional Office. Form 1, Table 1 of the "General Instructions" lists the addresses of EPA Regional Offices and the States within the jurisdiction of each Office.

Completeness

Your application will not be considered complete unless you answer every question on this form and on Form 1. If an item does not apply to you, enter "NA" (for not applicable) to show that you considered the question.

Public Availability of Submitted Information

You may not claim as confidential any information required by this form or Form 1, whether the information is reported on the forms or in an attachment. Section 402(j) of the Clean Water Act requires that all permit applications will be available to the public. This information will be made available to the public upon request.

Any information you submit to EPA which goes beyond that required by this form, Form 1, or Form 2C you may claim as confidential, but claims for information which are effluent data will be denied.

If you do not assert a claim of confidentiality at the time of submitting the information, EPA may make the information public without further notice to you. Claims of confidentiality will be handled in accordance with EPA's business confidentiality regulations at 40 CFR Part 2.

Definitions

All significant terms used in these instructions and in the form are defined in the glossary found in the General Instructions which accompany Form 1.

EPA ID Number

Enter your EPA Identification Number at the top of each odd-numbered page of Form 2F. You may copy this number directly from item I of Form 1.

Item I

You may use the map you provided for item XI of Form 1 to determine the latitude and longitude of each of your outfalls and the name of the receiving water.

Item II-A

If you check "yes" to this question, complete all parts of the chart, or attach a copy of any previous submission you have made to EPA containing the same information.

Item II-B

You are not required to submit a description of future pollution control projects if you do not wish to or if none is planned.

Item III

Attach a site map showing topography (or indicating the outline of drainage areas served by the outfall(s) covered in the application if a topographic map is unavailable) depicting the facility including:

each of its drainage and discharge structures;

the drainage area of each storm water outfall;

paved areas and building within the drainage area of each storm water outfall, each known past or present areas used for outdoor storage or disposal of significant materials, each existing structural control measure to reduce pollutants in storm water runoff, materials loading and access areas, areas where pesticides, herbicides, soil conditioners and fertilizers are applied;

each of its hazardous waste treatment, storage or disposal facilities (including each area not required to have a RCRA permit which is used for accumulating hazardous waste for less than 90 days under 40 CFR 262.34);

each well where fluids from the facility are injected underground; and

springs, and other surface water bodies which receive storm water discharges from the facility.

Item IV-A

For each outfall, provide an estimate of the area drained by the outfall which is covered by impervious surfaces. For the purpose of this application, impervious surfaces are surfaces where storm water runs off at rates that are *significantly higher than background rates* (e.g., *predevelopment levels*) and include paved areas, building roofs, parking lots, and roadways. Include an estimate of the total area (including all impervious and pervious areas) drained by each outfall. The site map required under item III can be used to estimate the total area drained by each outfall.

Item IV-B

Provide a narrative description of significant materials that are currently or in the past three years have been treated, stored, or disposed in a manner to allow exposure to storm water; method of treatment, storage or disposal of these materials; past and present materials management practices employed, in the last three years, to minimize contact by these materials with storm water runoff; materials loading and access areas and the location, manner, and frequency in which pesticides, herbicides, soil conditioners, and fertilizers are applied. Significant materials should be identified by chemical name; form (e.g., powder, liquid, etc.), and type of container or treatment unit. Indicate any materials treated, stored, or disposed of together. "Significant materials" includes, but is not limited to: raw materials; fuels; materials such as solvents, detergents, and plastic pellets; finished materials such as metallic products; raw materials used in food processing or production; hazardous substances designated under Section 101(14) of CERCLA; any chemical the facility is required to report pursuant to Section 313 of Title III of SARA; fertilizers; pesticides; and waste products such as ashes, slag and sludge that have the potential to be released with storm water discharges.

Item IV-C

For each outfall, structural controls include structures which enclose material handling or storage areas, covering materials, berms, dikes, or diversion ditches around manufacturing, production, storage or treatment units, retention ponds, etc. Nonstructural controls include practices such as spill prevention plans employee training, visual inspections, preventive maintenance, and housekeeping measures that are used to prevent or minimize the potential for releases of pollutants.

Item V

Provide a certification that all outfalls that should contain storm water discharges associated with industrial activity have been tested or evaluated for the presence of non-storm water discharges which are not covered by an NPDES permit. Tests for such non-storm water discharges may include smoke tests, fluorometric dye tests, analysis of accurate schematics, as well as other appropriate tests. Part B must include a description of the method used, the date of any testing, and the onsite drainage points that were directly observed during a test. All non-storm water discharges must be identified in a Form 2C or Form 2E which must accompany this application (see beginning of instructions under section titled "Who Must File Form 2F" for a description of when Form 2C and Form 2E must be submitted).

Item VI

Provide a description of existing information regarding the history of significant leaks or spills of toxic or hazardous pollutants at the facility in the last three years.

Item VII-A, B, and C

These items require you to collect and report data on the pollutants discharged for each of your outfalls. Each part of this item addresses a different set of pollutants and must be completed in accordance with the specific instructions for that part. The following general instructions apply to the entire item.

General Instructions

Part A requires you to report at least one analysis for each pollutant listed. Parts B and C require you to report analytical data in two ways. For some pollutants addressed in Parts B and C, if you know or have reason to know that the pollutant is present in your discharge, you may be required to list the pollutant and test (sample and analyze) and report the levels of the pollutants in your discharge. For all other pollutants addressed in Parts B and C, you must list the pollutant if you know or have reason to know that the pollutant is present in the discharge, and either report quantitative data for the pollutant or briefly describe the reasons the pollutant is expected to be discharged. (See specific instructions on the form and below for Parts A through C.) Base your determination that a pollutant is present in or absent from your discharge on your knowledge of your raw materials, material management practices, maintenance chemicals, history of spills and releases, intermediate and final products and byproducts, and any previous analyses known to you of your effluent or similar effluent.

A. Sampling: The collection of the samples for the reported analyses should be supervised by a person experienced in performing sampling of industrial wastewater or storm water discharges. You may contact EPA or your State permitting authority for detailed guidance on sampling techniques and for answers to specific questions. Any specific requirements contained in the applicable analytical methods should be followed for sample containers, sample preservation, holding times, the collection of duplicate samples, etc. The time when you sample should be representative, to the extent feasible, of your treatment system operating properly with no system upsets. Samples should be collected from the center of the flow channel, where turbulence is at a maximum, at a site specified in your present permit, or at any site adequate for the collection of a representative sample.

   For pH, temperature, cyanide, total phenols, residual chlorine, oil and grease, and fecal coliform, grab samples taken during the first 30 minutes (or as soon thereafter as practicable) of the discharge must be used (you are not required to analyze a flow-weighted composite for these parameters). For all other pollutants both a grab sample collected during the first 30 minutes (or as soon thereafter as practicable) of the discharge and a flow-weighted composite sample must be analyzed. However, a minimum of one grab sample may be taken for effluents from holding ponds or other impoundments with a retention period of greater than 24 hours.

   All samples shall be collected from the discharge resulting from a storm event that is greater than 0.1 inches and at least 72 hours from the previously measurable (greater than 0.1 inch rainfall) storm event. Where feasible, the variance in the duration of the event and the total rainfall of the event should not exceed 50 percent from the average or median rainfall event in that area.

   A grab sample shall be taken during the first thirty minutes of the discharge (or as soon thereafter as practicable), and a flow-weighted composite shall be taken for the entire event or for the first three hours of the event.

   Grab and composite samples are defined as follows:

Grab sample: An individual sample of at least 100 milliliters collected during the first thirty minutes (or as soon thereafter as practicable) of the discharge. This sample is to be analyzed separately from the composite sample.

Flow-Weighted Composite sample: A flow-weighted composite sample may be taken with a continuous sampler that proportions the amount of sample collected with the flow rate or as a combination of a minimum of three sample aliquots taken in each hour of discharge for the entire event or for the first three hours of the event, with each aliquot being at least 100 milliliters and collected with a minimum period of fifteen minutes between aliquot collections. The composite must be flow proportional; either the time interval between each aliquot or the volume of each aliquot must be proportional to either the stream flow at the time of sampling or the total stream flow since the collection of the previous aliquot. Aliquots may be collected manually or automatically. Where GC/MS Volatile Organic Analysis (VOA) is required, aliquots must be combined in the laboratory immediately before analysis. Only one analysis for the composite sample is required.

Data from samples taken in the past may be used, provided that:

All data requirements are met;

Sampling was done no more than three years before submission; and

All data are representative of the present discharge.

Among the factors which would cause the data to be unrepresentative are significant changes in production level, changes in raw materials, processes, or final products, and changes in storm water treatment. When the Agency promulgates new analytical methods in 40 CFR Part 136, EPA will provide information as to when you should use the new methods to generate data on your discharges. Of course, the Director may request additional information, including current quantitative data, if they determine it to be necessary to assess your discharges. The Director may allow or establish appropriate site-specific sampling procedures or requirements, including sampling locations, the season in which the sampling takes place, the minimum duration between the previous measurable storm event and the storm event sampled, the minimum or maximum level of precipitation required for an appropriate storm event, the form of precipitation sampled (snow melt or rainfall), protocols for collecting samples under 40 CFR Part 136, and additional time for submitting data on a case-by-case basis.

B. **Reporting:** All levels must be reported as concentration and mass (note: grab samples are reported in terms of concentration). You may report some or all of the required data by attaching separate sheets of paper instead of filling out pages VII-1 and VII-2 if the separate sheets contain all the required information in a format which is consistant with pages VII-1 and VII-2 in spacing and identification of pollutants and columns. Use the following abbreviations in the columns headed "Units."

Concentration		Mass	
ppm	parts per million	lbs	pounds
mg/1	milligrams per liter	ton	tons (English tons)
ppb	parts per billion	mg	milligrams
ug/1	micrograms per liter	g	grams
kg	kilograms	T	tonnes (metric tons)

All reporting of values for metals must be in terms of "total recoverable metal," unless:

(1) An applicable, promulgated effluent limitation or standard specifies the limitation for the metal in dissolved, valent, or total form; or

(2) All approved analytical methods for the metal inherently measure only its dissolved form (e.g., hexavalent chromium); or

(3) The permitting authority has determined that in establishing case-by-case limitations it is necessary to express the limitations on the metal in dissolved, valent, or total form to carry out the provisions of the CWA. If you measure only one grab sample and one flow-weighted composite sample for a given outfall, complete only the "Maximum Values" columns and insert "1" into the "Number of Storm Events Sampled" column. The permitting authority may require you to conduct additional analyses to further characterize your discharges.

If you measure more than one value for a grab sample or a flow-weighted composite sample for a given outfall and those values are representative of your discharge, you must report them. You must describe your method of testing and data analysis. You also must determine the average of all values within the last year and report the concentration and mass under the "Average Values" columns, and the total number of storm events sampled under the "Number of Storm Events Sampled " columns.

C. **Analysis:** You must use test methods promulgated in 40 CFR Part 136; however, if none has been promulgated for a particular pollutant, you may use any suitable method for measuring the level of the pollutant in your discharge provided that you submit a description of the method or a reference to a published method. Your description should include the sample holding time, preservation techniques, and the quality control measures which you used. If you have two or more substantially identical outfalls, you may request permission from your permitting authority to sample and analyze only one outfall and submit the results of the analysis for other substantially identical outfalls. If your request is granted by the permitting authority, on a separate sheet attached to the application form, identify which outfall you did test, and describe why the outfalls which you did not test are substantially identical to the outfall which you did test.

Part VII-A

Part VII-A must be completed by all applicants for all outfalls who must complete Form 2F.

Analyze a grab sample collected during the first thirty minutes (or as soon thereafter as practicable) of the discharge and flow-weighted composite samples for all pollutants in this Part, and report the results except use only grab samples for pH and oil and grease. See discussion in General Instructions to Item VII for definitions of grab sample collected during the first thirty minutes of discharge and flow-weighted composite sample. The "Average Values" column is not compulsory but should be filled out if data are available.

Part VII-B

List all pollutants that are limited in an effluent guideline which the facility is subject to (see 40 CFR Subchapter N to determine which pollutants are limited in effluent guidelines) or any pollutant listed in the facility's NPDES permit for its process wastewater (if the facility is operating under an existing NPDES permit). Complete one table for each outfall. See discussion in General instructions to item VII for definitions of grab sample collected during the first thirty minutes (or as soon thereafter as practicable) of discharge and flow-weighted composite sample. The "Average Values" column is not compulsory but should be filled out if data are available.

Analyze a grab sample collected during the first thirty minutes of the discharge and flow-weighted composite samples for all pollutants in this Part, and report the results, except as provided in the General Instructions

Part VII-C

Part VII-C must be completed by all applicants for all outfalls which discharge storm water associated with industrial activity, or that EPA is evaluating for designation as a significant contributor of pollutants to waters of the United States, or as contributing to a violation of a water quality standard. Use both a grab sample and a composite sample for all pollutants you analyze for in this part except use grab samples for residual chlorine and fecal coliform. The "Average Values" column is not compulsory but should be filled out if data are available. Part C requires you to address the pollutants in Table 2F-2, 2F-3, and 2F-4 for each outfall. Pollutants in each of these Tables are addressed differently.

Table 2F-2: For each outfall, list all pollutants in Table 2F-2 that you know or have reason to believe are discharged (except pollutants previously listed in Part VII-B). If a pollutant is limited in an effluent guideline limitation which the facility is subject to, the pollutant must be analyzed and reported in Part VII-B. If a pollutant in Table 2F-2 is indirectly limited by an effluent guideline limitation through an indicator (e.g., use of TSS as an indicator to control the discharge of iron and aluminum), you must analyze for it and report the data in Part VII-B. For other pollutants listed in Table 2F-2 (those not limited directly or indirectly by an effluent limitation guideline), that you know or have reason to believe are discharged, you must either report quantitative data or briefly describe the reasons the pollutant is expected to be discharged.

Table 2F-3: For each outfall, list all pollutants in Table 2F-3 that you know or have reason to believe are discharged. For every pollutant in Table 2F-3 expected to be discharged in concentrations of 10 ppb or greater, you must submit quantitative data. For acrolein, acrylonitrile, 2,4 dinitrophenol, and 2-methyl-4 6 dinitrophenol, you must submit quantitative data if any of these four pollutants is expected to be discharged

in concentrations of 100 ppb or greater. For every pollutant expected to be discharged in concentrations less than 10 ppb (or 100 ppb for the four pollutants listed above), then you must either submit quantitative data or briefly describe the reasons the pollutant is expected to be discharged.

Small Business Exemption - If you are a "small business," you are exempt from the reporting requirements for the organic toxic pollutants listed in Table 2F-3. There are two ways in which you can qualify as a "small business". If your facility is a coal mine, and if your probable total annual production is less than 100,000 tons per year, you may submit past production data or estimated future production (such as a schedule of estimated total production under 30 CFR 795.14(c)) instead of conducting analyses for the organic toxic pollutants. If your facility is not a coal mine, and if your gross total annual sales for the most recent three years average less than $100,000 per year (in second quarter 1980 dollars), you may submit sales data for those years instead of conducting analyses for the organic toxic pollutants. The production or sales data must be for the facility which is the source of the discharge. The data should not be limited to production or sales for the process or processes which contribute to the discharge, unless those are the only processes at your facility. For sales data, in situations involving intracorporate transfer of goods and services, the transfer price per unit should approximate market prices for those goods and services as closely as possible. Sales figures for years after 1980 should be indexed to the second quarter of 1980 by using the gross national product price deflator (second quarter of 1980 = 100). This index is available in National Income and Product Accounts of the United States (Department of Commerce, Bureau of Economic Analysis).

Table 2F-4: For each outfall, list any pollutant in Table 2F-4 that you know or believe to be present in the discharge and explain why you believe it to be present. No analysis is required, but if you have analytical data, you must report them. **Note:** Under 40 CFR 117.12(a)(2), certain discharges of hazardous substances (listed at 40 CFR 177.21 or 40 CFR 302.4) may be exempted from the requirements of section 311 of CWA, which establishes reporting requirements, civil penalties, and liability for cleanup costs for spills of oil and hazardous substances. A discharge of a particular substance may be exempted if the origin, source, and amount of the discharged substances are identified in the NPDES permit application or in the permit, if the permit contains a requirement for treatment of the discharge, and if the treatment is in place. To apply for an exclusion of the discharge of any hazardous substance from the requirements of section 311, attach additional sheets of paper to your form, setting forth the following information:

1. The substance and the amount of each substance which may be discharged.
2. The origin and source of the discharge of the substance.
3. The treatment which is to be provided for the discharge by:
    a. An onsite treatment system separate from any treatment system treating your normal discharge;
    b. A treatment system designed to treat your normal discharge and which is additionally capable of treating the amount of the substance identified under paragraph 1 above; or
    c. Any combination of the above.

See 40 CFR 117.12(a)(2) and (c), published on August 29, 1979, in 44 FR 50766, or contact your Regional Office (Table 1 on Form 1, Instructions), for further information on exclusions from section 311.

Part VII-D

If sampling is conducted during more than one storm event, you only need to report the information requested in Part VII-D for the storm event(s) which resulted in any maximum pollutant concentration reported in Part VII-A, VII-B, or VII-C.

Provide flow measurements or estimates of the flow rate, and the total amount of discharge for the storm event(s) sampled, the method of flow measurement, or estimation. Provide the data and duration of the storm event(s) sampled, rainfall measurements, or estimates of the storm event which generated the sampled runoff and the duration between the storm event sampled and the end of the previous measurable (greater than 0.1 inch rainfall) storm event.

Part VII-E

List any toxic pollutant listed in Tables 2F-2, 2F-3, or 2F-4 which you currently use or manufacture as an intermediate or final product or byproduct. In addition, if you know or have reason to believe that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is discharged or if you use or manufacture 2,4,5-trichlorophenoxy acetic

acid (2,4,5,-T); 2-(2,4,5-trichlorophenoxy) propanoic acid (Silvex, 2,4,5,-TP); 2-(2,4,5-trichlorophenoxy) ethyl 2,2-dichloropropionate (Erbon); O,O-dimethyl O-(2,4,5-trichlorphenyl) phosphorothioate (Ronnel); 2,4,5-trichlorophenol (TCP); or hexachlorophene (HCP); then list TCDD. The Director may waive or modify the requirement if you demonstrate that it would be unduly burdensome to identify each toxic pollutant and the Director has adequate information to issue your permit. You may not claim this information as confidential; however, you do not have to distinguish between use or production of the pollutants or list the amounts.

Item VIII

Self explanatory. The permitting authority may ask you to provide additional details after your application is received.

Item X

The Clean Water Act provides for severe penalties for submitting false information on this application form.

Section 309(c)(4) of the Clean Water Act provides that "Any person who knowingly makes any false material statement, representation, or certification in any application, . . . shall upon conviction, be punished by a fine of not more than $10,000 or by imprisonment for not more than 2 years, or by both. If a conviction of such person is for a violation committed after a first conviction of such person under this paragraph, punishment shall be by a fine of not more than $20,000 per day of violation, or by imprisonment of not more than 4 years, or by both." 40 CFR Part 122.22 requires the certification to be signed as follows:

(A) **For a corporation:** by a responsible corporate official. For purposes of this section, a responsible corporate official means (i) a president, secretary, treasurer, or vice-president of the corporation in charge of a principal business function, or any other person who performs similar policy- or decision-making functions for the corporation, or (ii) the manager of one or more manufacturing, production, or operating facilities employing more than 250 persons or having gross annual sales or expenditures exceeding $25,000,000 (in second-quarter 1980 dollars), if authority to sign documents has been assigned or delegated to the manager in accordance with corporate procedures.

Note: EPA does not require specific assignments or delegation of authority to responsible corporate officers identified in 122.22(a)(1)(i). The Agency will presume that these responsible corporate officers have the requisite authority to sign permit applications unless the corporation has notified the Director to the contrary. Corporate procedures governing authority to sign permit applications may provide for assignment or delegation to applicable corporate position under 122.22(a)(1)(ii) rather than to specific individuals.

(B) **For a partnership or sole proprietorship:** by a general partner or the proprietor, respectively, or

(C) **For a municipality, State, Federal, or other public agency:** by either a principal executive officer or ranking elected official. For purposes of this section, a principal executive officer of a Federal agency includes (i) the chief executive officer of the agency, or (ii) a senior executive officer having responsibility for the overall operations of a principal geographic unit of the agency (e.g., Regional Administrators of EPA).

Table 2F-1
Codes for Treatment Units

Physical Treatment Processes			
1-A	Ammonia Stripping	1-M	Grit Removal
1-B	Dialysis	1-N	Microstraining
1-C	Diatomaceous Earth Filtration	1-O	Mixing
1-D	Distillation	1-P	Moving Bed Filters
1-E	Electrodialysis	1-Q	Multimedia Filtration
1-F	Evaporation	1-R	Rapid Sand Filtration
1-G	Flocculation	1-S	Reverse Osmosis (Hyperfiltration)
1-H	Flotation	1-T	Screening
1-I	Foam Fractionation	1-U	Sedimentation (Settling)
1-J	Freezing	1-V	Slow Sand Filtration
1-K	Gas-Phase Separation	1-W	Solvent Extraction
1-L	Grinding (Comminutors)	1-X	Sorption
Chemical Treatment Processes			
2-A	Carbon Adsorption	2-G	Disinfection (Ozone)
2-B	Chemical Oxidation	2-H	Disinfection (Other)
2-C	Chemical Precipitation	2-I	Electrochemical Treatment
2-D	Coagulation	2-J	Ion Exchange
2-E	Dechlorination	2-K	Neutralization
2-F	Disinfection (Chlorine)	2-L	Reduction
Biological Treatment Processes			
3-A	Activated Sludge	3-E	Pre-Aeration
3-B	Aerated Lagoons	3-F	Spray Irrigation/Land Application
3-C	Anaerobic Treatment	3-G	Stabilization Ponds
3-D	Nitrification-Denitrification	3-H	Trickling Filtration
Other Processes			
4-A	Discharge to Surface Water	4-C	Reuse/Recycle of Treated Effluent
4-B	Ocean Discharge Through Outfall	4-D	Underground Injection
Sludge Treatment and Disposal Processes			
5-A	Aerobic Digestion	5-M	Heat Drying
5-B	Anaerobic Digestion	5-N	Heat Treatment
5-C	Belt Filtration	5-O	Incineration
5-D	Centrifugation	5-P	Land Application
5-E	Chemical Conditioning	5-Q	Landfill
5-F	Chlorine Treatment	5-R	Pressure Filtration
5-G	Composting	5-S	Pyrolysis
5-H	Drying Beds	5-T	Sludge Lagoons
5-I	Elutriation	5-U	Vacuum Filtration
5-J	Flotation Thickening	5-V	Vibration
5-K	Freezing	5-W	Wet Oxidation
5-L	Gravity Thickening		

Table 2F-2

Conventional and Nonconventional Pollutants

Bromide
Chlorine, Total Residual
Color
Fecal Coliform
Fluoride
Nitrate-Nitrite
Nitrogen, Total Organic
Oil and Grease
Phosphorus, Total
Radioactivity
Sulfate
Sulfite
Surfactants
Aluminum, Total
Barium, Total
Boron, Total
Cobalt, Total
Iron, Total
Magnesium, Total
Molybdenum, Total
Manganese, Total
Tin, Total
Titanium, Total

Table 2F-3

Toxic Pollutants

Toxic Pollutants and Total Phenol

Antimony, Total	Copper, Total	Silver, Total
Arsenic, Total	Lead, Total	Thallium, Total
Beryllium, Total	Mercury, Total	Zinc, Total
Cadmium, Total	Nickel, Total	Cyanide, Total
Chromium, Total	Selenium, Total	Phenols, Total

GC/MS Fraction Volatiles Compounds

Acrolein	Dichlorobromomethane	1,1,2,2,-Tetrachloroethane
Acrylonitrile	1,1-Dichloroethane	Tetrachloroethylene
Benzene	1,2-Dichloroethane	Toluene
Bromoform	1,1-Dichloroethylene	1,2-Trans-Dichloroethylene
Carbon Tetrachloride	1,2-Dichloropropane	1,1,1-Trichloroethane
Chlorobenzene	1,3-Dichloropropylene	1,1,2-Trichloroethane
Chlorodibromomethane	Ethylbenzene	Trichloroethylene
Chloroethane	Methyl Bromide	Vinyl Chloride
2-Chloroethylvinyl Ether	Methyl Chloride	
Chloroform	Methylene Chloride	

Acid Compounds

2-Chlorophenol	2,4-Dinitrophenol	Pentachlorophenol
2,4-Dichlorophenol	2-Nitrophenol	Phenol
2,4-Dimethylphenol	4-Nitrophenol	2,4,6-Trichlorophenol
4,6-Dinitro-O-Cresol	p-Chloro-M-Cresol	2-methyl-4,6 dinitrophenol

Base/Neutral

Acenaphthene	2-Chloronaphthalene	Fluoranthene
Acenaphthylene	4-Chlorophenyl Phenyl Ether	Fluorene
Anthracene	Chrysene	Hexachlorobenzene
Benzidine	Dibenzo(a,h)anthracene	Hexachlorobutadiene
Benzo(a)anthracene	1,2-Dichlorobenzene	Hexachloroethane
Benzo(a)pyrene	1,3-Dichlorobenzene	Indeno(1,2,3-cd)pyrene
3,4-Benzofluoranthene	1,4-Dichlorobenzene	Isophorone
Benzo(ghi)perylene	3,3'-Dichlorobenzidine	Naphthalene
Benzo(k)fluoranthene	Diethyl Phthalate	Nitrobenzene
Bis(2-chloroethoxy)methane	Dimethyl Phthalate	N-Nitrosodimethylamine
Bis(2-chloroethyl)ether	Di-N-Butyl Phthalate	N-Nitrosodi-N-Propylamine
Bis(2-chloroisopropyl)ether	2,4-Dinitrotoluene	N-Nitrosodiphenylamine
Bis(2-ethylhexyl)phthalate	2,6-Dinitrotoluene	Phenanthrene
4-Bromophenyl Phenyl Ether	Di-N-Octylphthalate	Pyrene
Butylbenzyl Phthalate	1,2-Diphenylhydrazine (as Azobenzene)	1,2,4-Trichlorobenzene

Pesticides

Aldrin	Dieldrin	PCB-1254
Alpha-BHC	Alpha-Endosulfan	PCB-1221
Beta-BHC	Beta-Endosulfan	PCB-1232
Gamma-BHC	Endosulfan Sulfate	PCB-1248
Delta-BHC	Endrin	PCB-1260
Chlordane	Endrin Aldehyde	PCB-1016
4,4'-DDT	Heptachlor	Toxaphene
4,4'-DDE	Heptachlor Epoxide	
4,4'-DDD	PCB-1242	

Table 2F-4
Hazardous Substances

Toxic Pollutant

Asbestos

Hazardous Substances

Acetaldehyde	Dinitrobenzene	Naphthenic acid
Allyl alcohol	Diquat	Nitrotoluene
Allyl chloride	Disulfoton	Parathion
Amyl acetate	Diuron	Phenolsulfonate
Aniline	Epichlorohydrin	Phosgene
Benzonitrile	Ethion	Propargite
Benzyl chloride	Ethylene diamine	Propylene oxide
Butyl acetate	Ethylene dibromide	Pyrethrins
Butylamine	Formaldehyde	Quinoline
Carbaryl	Furfural	Resorcinol
Carbofuran	Guthion	Strontium
Carbon disulfide	Isoprene	Strychnine
Chlorpyrifos	Isopropanolamine	Styrene
Coumaphos	Kelthane	2,4,5-T (2,4,5-Trichlorophenoxyacetic acid)
Cresol	Kepone	TDE (Tetrachlorodiphenyl ethane)
Crotonaldehyde	Malathion	2,4,5-TP [2-(2,4,5-Trichlorophenoxy) propanoic acid]
Cyclohexane	Mercaptodimethur	Trichlorofan
2,4-D (2,4-Dichlorophenoxyacetic acid)	Methoxychlor	Triethylamine
Diazinon	Methyl mercaptan	Trimethylamine
Dicamba	Methyl methacrylate	Uranium
Dichlobenil	Methyl parathion	Vanadium
Dichlone	Mevinphos	Vinyl acetate
2,2-Dichloropropionic acid	Mexacarbate	Xylene
Dichlorvos	Monoethyl amine	Xylenol
Diethyl amine	Monomethyl amine	Zirconium
Dimethyl amine	Naled	

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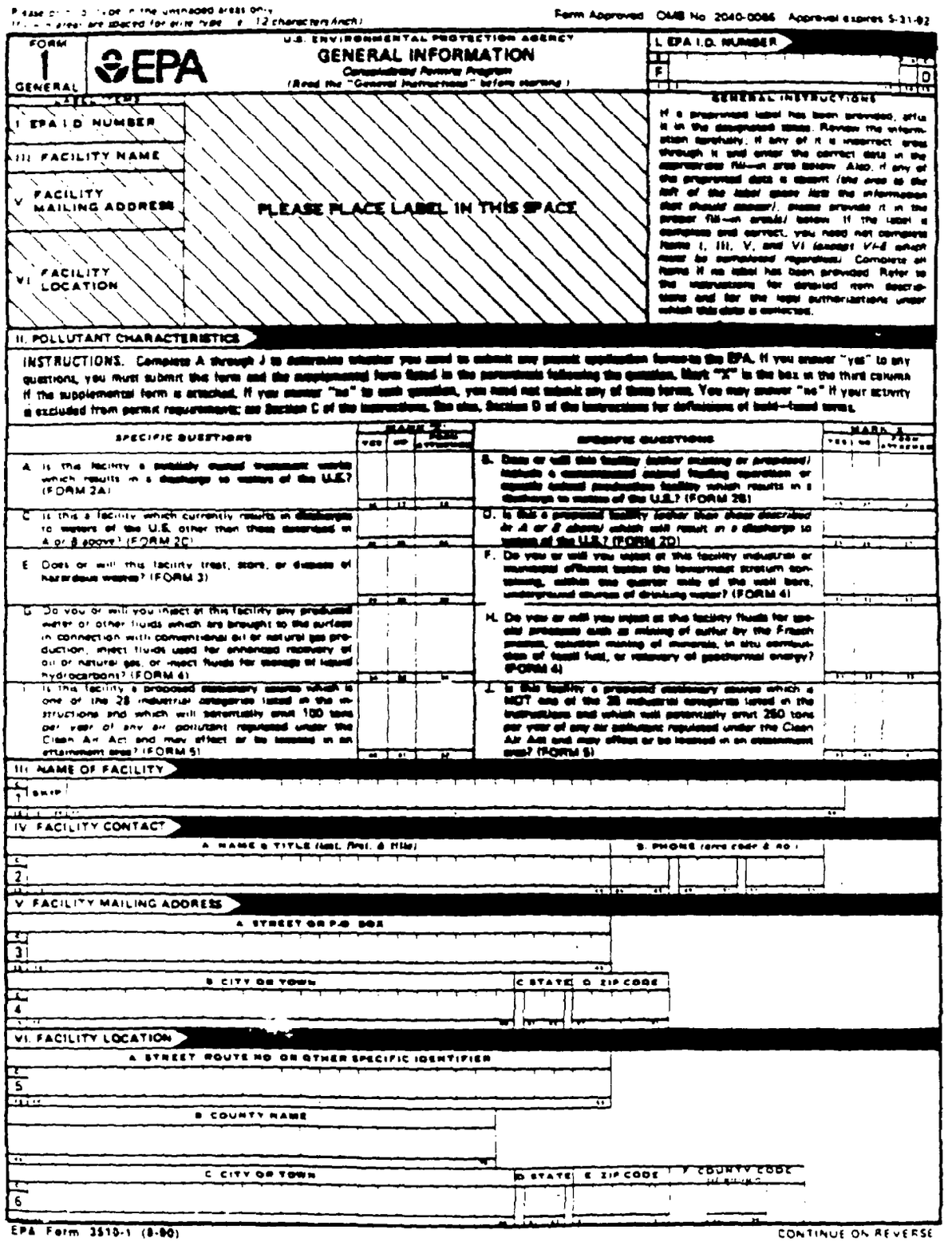

Please print or type in the unshaded areas only
(Fill-in areas are spaced for elite type, i.e., 12 characters/inch)

Form Approved. OMB No. 2040-0086. Approval expires 5-31-92

FORM 1 GENERAL

EPA

U.S. ENVIRONMENTAL PROTECTION AGENCY

GENERAL INFORMATION

Consolidated Permits Program

(Read the "General Instructions" before starting.)

I. EPA I.D. NUMBER

F | | | | | | | | | | | | D

GENERAL INSTRUCTIONS

If a preprinted label has been provided, affix it in the designated space. Review the information carefully; if any of it is incorrect, cross through it and enter the correct data in the appropriate fill-in area below. Also, if any of the preprinted data is absent (the area on the left of the label space lists the information that should appear), please provide it in the proper fill-in area(s) below. If the label is complete and correct, you need not complete Items I, III, V, and VI (except VI-B which must be completed regardless). Complete all items if no label has been provided. Refer to the instructions for detailed item descriptions and for the legal authorizations under which this data is collected.

I. EPA I.D. NUMBER

III. FACILITY NAME

V. FACILITY MAILING ADDRESS

VI. FACILITY LOCATION

PLEASE PLACE LABEL IN THIS SPACE

II. POLLUTANT CHARACTERISTICS

INSTRUCTIONS. Complete A through J to determine whether you need to submit any permit application forms to the EPA. If you answer "yes" to any questions, you must submit this form and the supplemental form listed in the parenthesis following the question. Mark "X" in the box in the third column if the supplemental form is attached. If you answer "no" to each question, you need not submit any of these forms. You may answer "no" if your activity is excluded from permit requirements; see Section C of the instructions. See also, Section D of the instructions for definitions of bold-faced terms.

SPECIFIC QUESTIONS	MARK 'X' YES	NO	ATTACHED	SPECIFIC QUESTIONS	MARK 'X' YES	NO	ATTACHED
A. Is this facility a publicly owned treatment works which results in a discharge to waters of the U.S.? (FORM 2A)				B. Does or will this facility (either existing or proposed) include a concentrated animal feeding operation or aquatic animal production facility which results in a discharge to waters of the U.S.? (FORM 2B)			
C. Is this a facility which currently results in discharges to waters of the U.S. other than those described in A or B above? (FORM 2C)				D. Is this a proposed facility (other than those described in A or B above) which will result in a discharge to waters of the U.S.? (FORM 2D)			
E. Does or will this facility treat, store, or dispose of hazardous wastes? (FORM 3)				F. Do you or will you inject at this facility industrial or municipal effluent below the lowermost stratum containing, within one quarter mile of the well bore, underground sources of drinking water? (FORM 4)			
G. Do you or will you inject at this facility any produced water or other fluids which are brought to the surface in connection with conventional oil or natural gas production, inject fluids used for enhanced recovery of oil or natural gas, or inject fluids for storage of liquid hydrocarbons? (FORM 4)				H. Do you or will you inject at this facility fluids for special processes such as mining of sulfur by the Frasch process, solution mining of minerals, in situ combustion of fossil fuel, or recovery of geothermal energy? (FORM 4)			
I. Is this facility a proposed stationary source which is one of the 28 industrial categories listed in the instructions and which will potentially emit 100 tons per year of any air pollutant regulated under the Clean Air Act and may affect or be located in an attainment area? (FORM 5)				J. Is this facility a proposed stationary source which is NOT one of the 28 industrial categories listed in the instructions and which will potentially emit 250 tons per year of any air pollutant regulated under the Clean Air Act and may affect or be located in an attainment area? (FORM 5)			

III. NAME OF FACILITY

1 SKIP

IV. FACILITY CONTACT

A. NAME & TITLE (last, first, & title) | B. PHONE (area code & no.)

2

V. FACILITY MAILING ADDRESS

A. STREET OR P.O. BOX

3

B. CITY OR TOWN | C. STATE | D. ZIP CODE

4

VI. FACILITY LOCATION

A. STREET, ROUTE NO. OR OTHER SPECIFIC IDENTIFIER

5

B. COUNTY NAME

C. CITY OR TOWN | D. STATE | E. ZIP CODE | F. COUNTY CODE

6

EPA Form 3510-1 (8-90)

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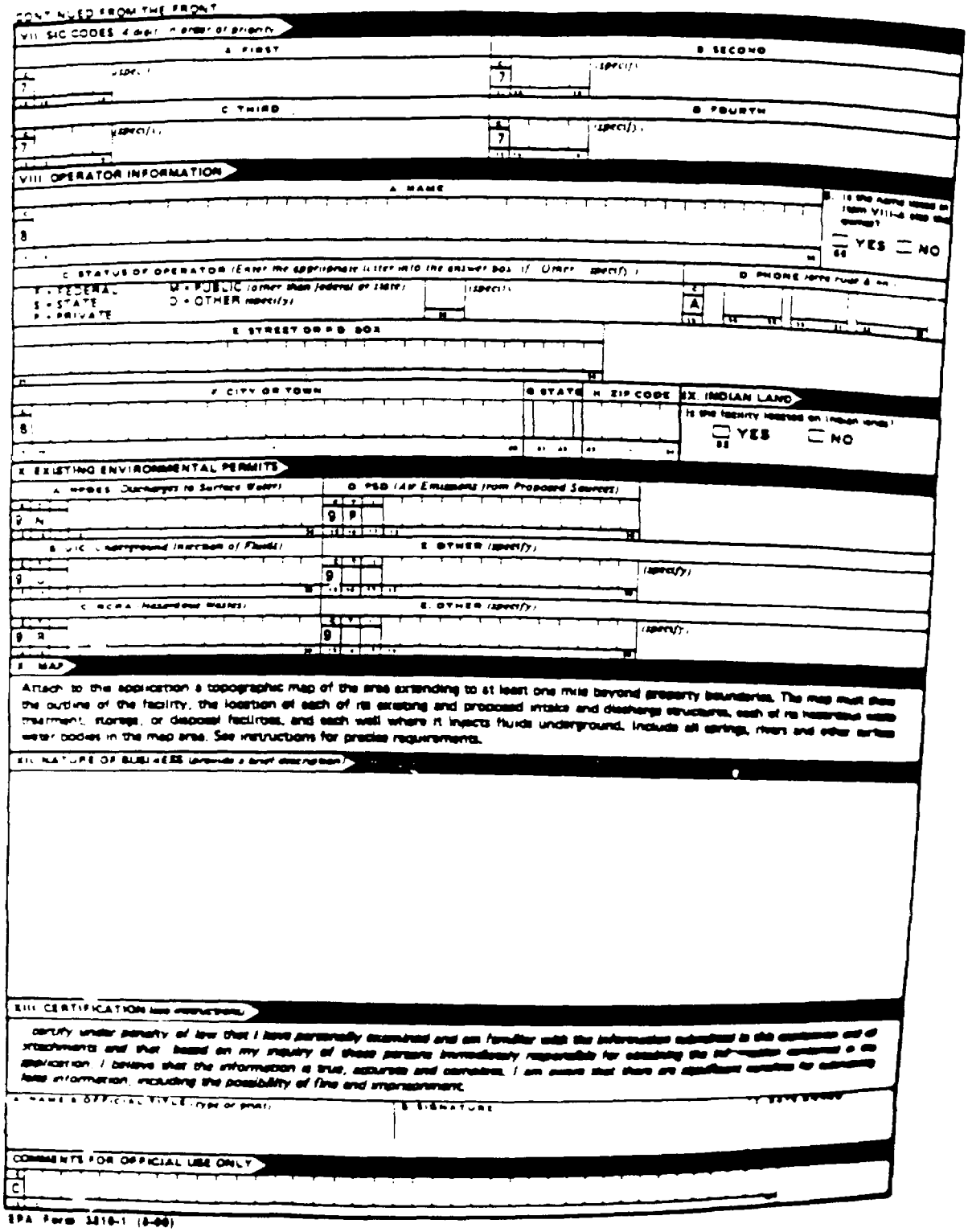

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VIII SIC CODES (4 digits, in order of priority)

A. FIRST

B. SECOND

C. THIRD

D. FOURTH

VIII OPERATOR INFORMATION

A. NAME

B. Is the name listed in Item VIII-A also the owner? YES NO

C. STATUS OF OPERATOR (Enter the appropriate letter into the answer box; if "Other", specify.)
F = FEDERAL
S = STATE
P = PRIVATE
M = PUBLIC (other than federal or state)
O = OTHER (specify)

D. PHONE (area code & no.)

E. STREET OR P.O. BOX

F. CITY OR TOWN

G. STATE

H. ZIP CODE

IX. INDIAN LAND

Is the facility located on Indian lands? YES NO

X. EXISTING ENVIRONMENTAL PERMITS

A. NPDES (Discharges to Surface Water)

B. UIC (Underground Injection of Fluids)

C. RCRA (Hazardous Wastes)

D. PSD (Air Emissions from Proposed Sources)

E. OTHER (specify)

XI. MAP

Attach to this application a topographic map of the area extending to at least one mile beyond property boundaries. The map must show the outline of the facility, the location of each of its existing and proposed intake and discharge structures, each of its hazardous waste treatment, storage, or disposal facilities, and each well where it injects fluids underground. Include all springs, rivers and other surface water bodies in the map area. See instructions for precise requirements.

XII. NATURE OF BUSINESS (provide a brief description)

XIII. CERTIFICATION (see instructions)

I certify under penalty of law that I have personally examined and am familiar with the information submitted in this application and all attachments and that, based on my inquiry of those persons immediately responsible for obtaining the information contained in the application, I believe that the information is true, accurate and complete. I am aware that there are significant penalties for submitting false information, including the possibility of fine and imprisonment.

A. NAME & OFFICIAL TITLE (type or print)

B. SIGNATURE

C. DATE SIGNED

COMMENTS FOR OFFICIAL USE ONLY

EPA Form 3510-1 (8-90)

APPENDIX B

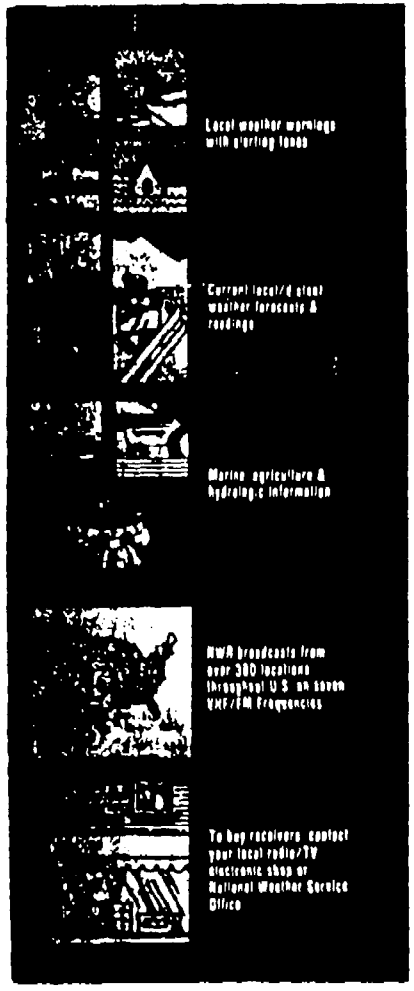
NOAA WEATHER RADIO INFORMATION

NOAA WEATHER RADIO MANUFACTURERS LIST	
RADIO SHACK Weather Radio 2617 West Seventh St. Fort Worth, TX 76107 (817) 390-3011	✓ ●
GENERAL ELECTRIC Model 7-2934 (800) 626-2000	✓ ●
UNIDEN BEARCAT Bearcat Weather Alert 6345 Castleway Court Indianapolis, IN 46250 (800) 722-6637	■
ELECTROLERT Weatheralert Forecaster 4949 South 25A Tipp City, OH 45371 (513) 667-2461	✓ ● ■
SPRINGFIELD INSTRUMENTS Talking Weather Center/Station 76 Paccaic St. Wood-Ridge, NJ 07075 (201) 777-2900	■
WOODSON ELECTRONICS Plectron 505 Lincoln St. Overton, NE 68863 (308) 987-2404	X
GORMAN - REDLICH MANUFACTURING James T. Gorman 257 West Union St. Athens, OH 45701 (617) 593-3150	X

PRICE RANGE:

- ✓ Under $50
- ■ $50 to $100
- X Over $100
- ● Features AM/FM model radios with weather band

PLEASE NOTE, THIS LIST IS NOT ALL-INCLUSIVE, AND INCLUSION ON THIS LIST DOES NOT CONSTITUTE ENDORSEMENT OF ANY COMPANY BY EPA OR THE U.S. GOVERNMENT.



NOAA WEATHER RADIO IS A SERVICE...

of the National Oceanic and Atmospheric Administration (NOAA) of the U.S. Department of Commerce. As the "Voice of the National Weather Service," it provides continuous broadcasts of the latest weather information directly from National Weather Service offices. Taped weather messages are repeated every four to six minutes and are routinely revised every one to three hours, or more frequently if needed. Most of the stations operate 24 hours daily.

During severe weather, National Weather Service forecasters can interrupt the routine weather broadcasts and substitute special warning messages. The forecasters can also activate specially designed warning receivers. Such receivers either sound an alarm indicating that an emergency exists, alerting the listener to turn the receiver up to an audible volume, or, when operated in a muted mode, are automatically turned on so that the warning message is heard. "Warning alarm" receivers are especially valuable for schools, hospitals, public-safety agencies, and news media offices.

Under a January 1975 White House policy statement, NOAA Weather Radio was designated the sole Government-operated radio system to provide direct warnings into private homes for both natural disasters and nuclear attack. This capability is to supplement warnings by sirens and by commercial radio and TV.

The broadcasts are tailored to weather information needs of people within the receiving area. For example, stations along the sea coasts and Great Lakes provide weather information for boaters, fishers, and others engaged in marine activities as well as general weather information.

NOAA Weather Radio broadcasts are made on one of seven high-band FM frequencies ranging from 162.40 to 162.55 megahertz (MHz). These frequencies are not found on the average home radio now in use. However, a number of radio manufacturers offer special weather radios to operate on these frequencies, with or without the emergency warning alarm. Also, there are now many radios on the market which offer standard AM/FM frequencies plus the so-called "weather band" as an added feature.

NOAA Weather Radio broadcasts can usually be heard as far as 40 miles from the antenna site, sometimes more. The effective range depends on many factors, particularly the height of the broadcasting antenna, terrain, quality of the receiver, and type of receiving antenna. As a general rule, listeners close to or perhaps beyond the 40 mile range should have a good quality receiver system if they expect reliable reception. Also, an outside antenna may be required in these fringe areas. If practicable, a receiver should be tried at its place of intended use before making a final purchase.

The National Weather Service operates about 380 stations. Approximately 80 percent of the Nation's population is within listening range of a NOAA Weather Radio broadcast.

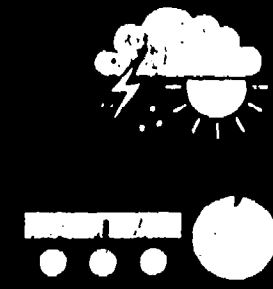
A similar network of about 15 stations using the same frequencies broadcasts continuous weather information across much of southern Canada.

If you have a question concerning NOAA Weather Radio or wish to receive a listing of NOAA Weather Radio receiver manufacturers, please contact your nearest National Weather Service Office, or write to National Weather Service (Attn: W/OM11), National Oceanic and Atmospheric Administration, Silver Spring, MD. 20910.

NOAA/PA 78015
Rev. July 1980

NOAA WEATHER RADIO

The Voice of the National Weather Service



NOAA WEATHER RADIO NETWORK

TECHNICAL APPENDIX C

REQUIRED CONTAINERS, PRESERVATION TECHNIQUES, HOLDING TIMES AND 40 CODE OF FEDERAL REGULATIONS (CFR) PART 136

REQUIRED CONTAINERS, PRESERVATION TECHNIQUES, AND HOLDING TIMES			
Parameter	**Container(1)**	**Preservative (2), (3)**	**Maximum Holding Time (4)**
Bacterial Tests			
Coliform, fecal and total	P, G	Cool, 4°C 0.008% $Na_2S_2O_3$ (5)	6 hours
Fecal streptococci	P, G	Cool, 4°C 0.008% $Na_2S_2O_3$ (5)	6 hours
Inorganic Tests			
Acidity	P, G	Cool, 4°C	14 days
Alkalinity	P, G	Cool, 4°C	14 days
Ammonia	P, G	Cool, 4°C H_2SO_4 to pH < 2	28 days
Biochemical oxygen demand	P, G	Cool, 4°C	48 hours
Biochemical oxygen demand, carbonaceous	P, G	Cool, 4°C	48 hours
Bromide	P, G	None required	28 days
Chemical oxygen demand	P, G	Cool, 4°C H_2SO_4 to pH < 2	28 days
Chloride	P, G	None required	28 days
Chlorine, total residual	P, G	None required	Analyze immediately
Color	P, G	Cool, 4°C	48 hours
Cyanide, total and amenable to chlorination	P, G	Cool, 4°C NaOH to pH > 12 0.6g ascorbic acid (5)	14 days (6)
Fluoride	P	None required	28 days
Hardness	P, G	HNO_3 to pH < 2 H_2SO_4 to pH < 2	6 months
Hydrogen ion (pH)	P, G	None required	Analyze immediately
Kjeldahl and organic Nitrogen	P, G	Cool, 4°C H_2SO_4 to pH < 2	28 days
Metals (7)			
Chromium VI	P, G	Cool, 4°C	28 hours
Mercury	P, G	HNO_3 to pH < 2	28 hours
Metals, except above	P, G	HNO_3 to pH < 2	6 months
Nitrate	P, G	Cool, 4°C	48 hours

REQUIRED CONTAINERS, PRESERVATION TECHNIQUES, AND HOLDING TIMES			
Parameter	**Container(1)**	**Preservative (2), (3)**	**Maximum Holding Time (4)**
Nitrate-nitrite	P, G	Cool, 4°C H_2SO_4 to pH<2	28 days
Nitrite	P, G	Cool, 4°C	48 hours
O&G	G	Cool, 4°C H_2SO_4 or HCl to pH<2	28 days
Organic carbon	P, G	Cool, 4°C HCl or H_2SO_4 to pH<2	28 days
Orthophosphate	P, G	Filter immediately Cool, 4°C	48 hours
Oxygen, Dissolved Probe	G bottle and top	None required	Analyze immediately
Dissolved oxygen, Winkler method	G bottle and top	Fix on site and store in dark	8 hours
Phenols	G only	Cool, 4°C H_2SO_4 to pH<2	28 days
Phosphorus (elemental)	G	Cool, 4°C	48 hours
Phosphorus, total	P, G	Cool, 4°C H_2SO_4 to pH<2	28 days
Residue, total	P, G	Cool, 4°C	7 days
Residue, filterable	P, G	Cool, 4°C	7 days
Residue, nonfilterable (TSS)	P, G	Cool, 4°C	7 days
Residue, settleable	P, G	Cool, 4°C	48 hours
Residue, volatile	P, G	Cool, 4°C	7 days
Silica	P	Cool, 4°C	28 days
Specific conductance	P, G	Cool, 4°C	28 days
Sulfate	P, G	Cool, 4°C	28 days
Sulfide	P, G	Cool, 4°C, add zinc acetate plus sodium hydroxide to pH>9	7 days
Sulfite	P, G	None required	Analyze immediately
Surfactants	P, G	Cool, 4°C	48 hours
Temperature	P, G	None required	Analyze
Turbidity	P, G	Cool, 4°C	48 hours

REQUIRED CONTAINERS, PRESERVATION TECHNIQUES, AND HOLDING TIMES			
Parameter	**Container(1)**	**Preservative (2), (3)**	**Maximum Holding Time (4)**
Organic Tests (8)			
Purgeable halocarbons	G, Teflon-lined septum	Cool, 4°C 0.008% $Na_2S_2O_3$ (5)	14 days
Purgeable aromatics	G, Teflon-lined septum	Cool, 4°C 0.008% $Na_2S_2O_3$ (5) HCl to pH<2 (9)	14 days
Acrolein and acrylonitrile	G, Teflon-lined septum	Cool, 4°C 0.008% $Na_2S_2O_3$ (5) Adjust pH to 4-5 (10)	14 days
Phenols (11)	G, Teflon-lined cap	Cool, 4°C 0.008% $Na_2S_2O_3$ (5)	7 days until extraction, 40 days after extraction
Benzidines (11)	G, Teflon-lined cap	Cool, 4°C 0.008% $Na_2S_2O_3$ (5)	7 days until extraction (13)
Phthalate esters (11)	G, Teflon-lined cap	Cool, 4°C	7 days until extraction, 40 days after extraction
Nitrosamines (11), (14)	G, Teflon-lined cap	Cool, 4°C store in dark 0.008% $Na_2S_2O_3$	7 days until extraction, 40 days after extraction
PCBs (11) acrylonitrite	G, Teflon-lined cap	Cool, 4°C	7 days until extraction, 40 days after extraction
Nitroaromatics and isophorone (11)	G, Teflon-lined cap	Cool, 4°C store in dark 0.008% $NA_2S_2O_3$ (5)	7 days until extraction, 40 days after extraction
Polynuclear aromatic hydrocarbons (11)	G, Teflon-lined cap	Cool, 4°C store in dark 0.008% $Na_2S_2O_3$ (5)	7 days until extraction, 40 days after extraction
Haloethers (11)	G, Teflon-lined cap	Cool, 4°C 0.008% $Na_2S_2O_3$ (5)	7 days until extraction, 40 days after extraction
Chlorinated hydrocarbons (11)	G, Teflon-lined cap	Cool, 4°C	7 days until extraction, 40 days after extraction
TCDD (11)	G, Teflon-lined cap	Cool, 4°C 0.008% $Na_2S_2O_3$ (5)	7 days until extraction, 40 days after extraction
Pesticides Tests			
Pesticides (11)	G, Teflon-lined cap	Cool, 4°C pH 5-9 (15)	7 days until extraction, 40 days after extraction
Radiological Tests			
Alpha, beta, and radium	P, G	HNO_3 to pH<2	6 months

40 CFR 136.3 TABLE II NOTES

(1) Polyethylene (P) or Glass (G).

(2) Sample preservation should be performed immediately upon sample collection. For composite chemical samples each aliquot should be preserved at the time of collection. When use of an automated sampler makes it impossible to preserve each aliquot, then chemical samples may be preserved by maintaining at 4°C until compositing and sample splitting is completed.

(3) When any sample is to be shipped by common carrier or sent through the United States Mails, it must comply with the Department of Transportation Hazardous Materials Regulations (49 CFR Part 172). The person offering such material for transportation is responsible for ensuring such compliance. For the preservation requirements of Table II, the Office of Hazardous Materials, Materials Transportation Bureau, Department of Transportation has determined that the Hazardous Materials Regulations do not apply to the following materials: Hydrochloric acid (HCl) in water solutions at concentrations of 0.04% by weight or less (pH about 1.96 or greater); Nitric acid (HNO_3) in water solutions at concentrations of 0.15% by weight or less (pH about 1.62 or greater); Sulfuric acid (H_2SO_4) in water solutions at concentrations of 0.35% by weight or less (pH about 1.15 or greater); and Sodium hydroxide (NaOH) in water solutions at concentrations of 0.080% by weights or less (pH about 12.30 or less).

(4) Samples should be analyzed as soon as possible after collection. The times listed are the maximum times that samples may be held before analysis and still be considered valid. Samples may be held for longer periods only if the permittee, or monitoring laboratory, has data on file to show that the specific types of samples under study are stable for the longer time, and has received a variance from the Regional Administrator under § 136.3(e). Some samples may not be stable for the maximum time period given in the table. A permittee, or monitoring laboratory, is obligated to hold the sample for a shorter time if knowledge exists to show that this is necessary to maintain sample stability. See § 136.3(e) for details.

(5) Should only be used in the presence of residual chlorine.

(6) Maximum holding time is 24 hours when sulfide is present. Optionally all samples may be tested with lead acetate paper before pH adjustments in order to determine if sulfide is present. If sulfide is present, it can be removed by the addition of cadmium nitrate powder until a negative spot test is obtained. The sample is filtered and then NaOH is added to pH 12.

(7) Samples should be filtered immediately on-site before adding preservative for dissolved metals.

(8) Guidance applies to samples to be analyzed by GC, LC, or GC/MS for specific compounds.

(9) Sample receiving no pH adjustment must be analyzed within seven days of sampling.

(10) The pH adjustment is not required if acrolein will not be measured. Samples for acrolein receiving no pH adjustment must be analyzed within 3 days of sampling.

40 CFR 136.3 TABLE II NOTES
(11) When the extractable analytes of concern fall within a single chemical category, the specified preservative and maximum holding times should be observed for optimum safeguard of sample integrity. When the analytes of concern fall within two or more chemical categories, the sample may be preserved by cooling to 4°C, reducing residual chlorine with 0.008% sodium thiosulfate, storing in the dark,and adjusting the pH to 6-9; samples preserved in this manner may be held for seven days before extraction and for forty days after extraction. Exceptions to this optional preservation and holding time procedure are noted in footnote 5 (re the requirement for thiosulfate reduction of residual chlorine), and footnotes 12, 13 (re the analysis of benzidine).
(12) If 1,2-diphenylhydrazine is likely to be present, adjust the pH of the sample to 4.0 $\pm$ 0.2 to prevent rearrangement to benzidine.
(13) Extracts may be stored up to 7 days before analysis if storage is conducted under an inert (oxidant-free) atmosphere.
(14) For the analysis of diphenylnitrosamine, add 0.008% $Na_2S_2O_3$ and adjust pH to 7-10 with NaOH within 24 hours of sampling.
(15) The pH adjustment may be performed upon receipt at the laboratory and may be omitted if the samples are extracted within 72 hours of collection. For the analysis of aldrin, add 0.008% $Na_2S_2O^3$.
Source: 40 CFR 136.3 Table II

TABLE IA—LIST OF APPROVED BIOLOGICAL TEST PROCEDURES

Parameter and units	Method [1]	EPA [2]	Reference (Method Number or Page)		
			Standard Methods 16th Ed.	ASTM	USGS
Bacteria:					
1. Coliform (fecal) number per 100 ml.	MPN, 5 tubes, 3 dilution; or, membrane filter (MF)[3]; single step.	Page 132.	909C		
2. Coliform (fecal) in presence of chlorine number per 100 ml.	MPN, 5 tubes, 3 dilution; or, MF [4]; single step.[4a]	p. 124	909		B-0050-77.
3. Coliform (total, number per 100 ml.	MPN, 5 tube, 3 dilution; or, MF [4] single step or two step.	p. 114	908A		
		p. 108	908A		B-0055-77.
4. Coliform (total) in presence of chlorine, number per 100 ml.	MPN, 5 tube, dilution; or MF [4] with enrichment.	p. 114	908A		
5. Fecal streptococci, number per 100 ml.	MPN, 5 tube, 3 dilution; MF [4]; or, plate count.	p. 111	908 (A+A.5c)		
		p. 139	910A		
	p. 136		910B		B0055-77.[5]
	p. 143		910C		

Table IA Notes

[1] The method used must be specified when results are reported.

[2] "Microbiological Methods for Monitoring the Environment, Water and Wastes, 1978", EPA-600/8-78-017, U.S. Environmental Protection Agency.

[3] Greeson, P.E., *et al.*, "Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples," U.S. Geological Survey, Techniques of Water-Resources Investigations, Book 5, Chapter A4, Laboratory Analysis, 1977.

[4] 0.45 μm membrane filter or other pore size certified by the manufacturer to fully retain organisms to be cultivated, and free of extractables which could interfere with their growth and development.

[4a] Since the membrane filter technique usually yields low and variable recovery from chlorinated wastewaters, the MPN method will be required to resolve any controversies.

[5] Approved only if dissolution of the KF Streptococcus Agar (Section 5.1, USGS Method B-0055-77) is made in a boiling water bath to avoid scorching of the medium.

TABLE IB—LIST OF APPROVED INORGANIC TEST PROCEDURES

Parameter, units and method	Reference (method No. or page)				
	EPA 1979 [24]	Std. methods 16th ED	ASTM	USGS [1]	Other
1. Acidity, as $CaCO_3$, mg/L: Electrometric end point or phenolphthalein end point.	305.1	402(4.a)	1067-82(E)		
2. Alkalinity, as $CaCO_3$, mg/L:					
Electrometric or colorimetric titration to pH 4.5, manual; or,	310.1	403	D1067-82(B)	I-1030-84	33.014[3].
Automated	310.2			I-2030-84	
3. Aluminum—Total,[6] mg/L: Digestion [8] followed by:					
AA direct aspiration	202.1	303C		I-3051-85	
AA furnace	202.2	304			
Inductively coupled plasma (ICP)					200.7.[4]
Direct current plasma (DCP) or					Note 33.
Colorimetric (Eriochrome cyanine R)		308B			
4. Ammonia (as N), mg/L: Manual distillation (at pH 9.5) [9] followed by:	350.2	417A			33.057 [9]
Nesslerization	350.2	417B	D1426-79(A)	I-3520-84	33.057 [9]
Titration	350.2	417D			
Electrode	350.3	417 E or F	D1426-79(D)		
Automated phenate or	350.1	417G	D1426-79(C)	I-4523-84	
Automated electrode					Note 6.
5. Antimony—Total [6], mg/L: Digestion [8] followed by:					
AA direct aspiration	204.1	303A			
AA furnace, or	204.2	304			
Inductively coupled plasma					200.7.[4]
6. Arsenic—Total [6], mg/L: Digestion [8] followed by	206.5				
AA gaseous hydride	206.3	303E	D2972-84(B)	I-3062-84	
AA furnace	206.2	304			
Inductively coupled plasma, or					200.7.[4]
Colorimetric (SDDC)	206.4	307B	D2972-84(A)	I-3060-84	
7. Barium—Total,[6] mg/L: Digestion [8] followed by:					
AA direct aspiration	208.1	303C		I-3084-85	
AA furnace	208.2	304			
ICP, or					200.7.[4]
DCP					Note 33.
8. Beryllium—Total,[6] mg/L: Digestion [8] followed by:					
AA direct aspiration	210.1	303C	D3645-84(A)	I-3095-85	
AA furnace	210.2	304			

TABLE IB—LIST OF APPROVED INORGANIC TEST PROCEDURES—Continued

Parameter, units and method	Reference (method No. or page)				
	EPA 1979 [44]	Std. methods 18th ED	ASTM	USGS [1]	Other
35. Mercury—Total [5], mg/L:					
Cold vapor, manual or	245.1	303F	D3223-80	I-3462-84	33.086 [1]
Automated	245.2				
36. Molybdenum—Total [5] mg/L. Digestion [6] followed by:					
AA direct aspiration	246.1	303C		I-3490-85	
AA furnace	246.2	304			
ICP, or					200.7 [4]
DCP					Note 33.
37. Nickel—Total [5] mg/L. Digestion [6] followed by:					
AA direct aspiration	249.1	303 A or B	D1886-84 (C or D).	I-3499-85	
AA furnace	249.2	304			
ICP					200.7 [4]
DCP, or					Note 33.
Colorimetric (Heteropoly)		321B			
38. Nitrate (as N), mg/L: Colorimetric (Brucine sulfate), or Nitrate-nitrite N minus Nitrite N (See parameters 39 and 40).	352.1		D992-71		33.063 [1], 4180 [10], p. 28. [9]
39. Nitrate-nitrite (as N), mg/L: Cadmium reduction, Manual or	353.3	418C	D3867-85(B)		
Automated, or	353.2	418F	D3867-85(A)	I-4545-84	
Automated hydrazine	353.1				
40. Nitrite (as N), mg/L: Spectrophotometric:					
Manual or	354.1	419	D1254-67		Note 24.
Automated (Diazotization)				I-4540-84	
41. Oil and grease—Total recoverable, mg/L: Gravimetric (extraction).	413.1	503A			
42. Organic carbon—Total (TOC), mg/L: Combustion or oxidation.	415.1	505	D2579-85 (A or B).		33.044 [1], p. 4 [11]
43. Organic nitrogen (as N) mg/L: Total Kjeldahl N (Parameter 31) minus ammonia N (Parameter 4).					
44. Orthophosphate (as P), mg/L: Ascorbic acid method:					
Automated or	365.1	424G		I-4601-84	33.118 [1]
Manual single reagent	365.2	424F	D515-62(A)		33.111. [1]
or Manual two reagent	365.3				
45. Osmium—Total [5], mg/L: Digestion [6] followed by:					
AA direct aspiration, or	252.1	303C			
AA furnace	252.2	304			
46. Oxygen dissolved, mg/L: Winkler (Azide modification), or	360.2	421B	D888-81(C)	I-1575-78 [7]	33.028. [1]
Electrode	360.1	421F		I-1576-78 [7]	
47. Palladium—Total [5] mg/L: Digestion [6] followed by:					
AA direct aspiration	253.1				p. 527. [9]
AA furnace	253.2				p. 528. [9]
DCP					Note 33.
48. Phenols, mg/L:					
Manual distillation [12]	420.1		D1783-80 (A or B).		Note 28.
Followed by:					
Colorimetric (4AAP) manual, or	420.1				Note 28.
Automated [13]	420.2				
49. Phosphorus (elemental) mg/L: Gas-liquid chromatography.					Note 27
50. Phosphorus—Total, mg/L: Persulfate digestion followed by	365.2	424C(III)			33.111. [1]
Manual or	365.2 or 365.3.	424F	D515-62(A)		
Automated ascorbic acid reduction, or	365.1	424G		I-4600-84	33.116. [1]
Semi-automated block digestor	365.4				
51. Platinum—Total [5] mg/L: Digestion [6] followed by:					
AA direct aspiration	255.1	303A			
AA furnace	255.2	304			
DCP					Note 33.
52. Potassium—total [5], mg/L: Digestion followed by:					
AA direct aspiration	258.1	303A		I-3630-84	33.103. [1]
Inductively coupled plasma					200.7 [4]
Flame photometric, or		322B	D1428-82(A)		
Colorimetric (Cobaltinitrite)					317B. [14]
53. Residue—Total, mg/L: Gravimetric, 103–105°C.	160.3	208A		I-3750-84	
54. Residue—filterable, mg/L: Gravimetric, 180°C.	160.1	208B		I-1750-84	
55. Residue—nonfilterable, (TSS), mg/L: Gravimetric, 103–105°C post washing of residue.	160.2	208C		I-3765-84	

TABLE IB—LIST OF APPROVED INORGANIC TEST PROCEDURES—Continued

Parameter, units and method	Reference (method No. or page)				
	EPA 1978 [44]	Std. methods 16th ED	ASTM	USGS [1]	Other
56. Residue—settleable, mg/L: Volumetric, (Imhoff cone) or gravimetric.	160.5	209E		1	
57. Residue—Volatile, mg/L: Gravimetric, 550°C.	160.4	209D		I-3753-84	
58. Rhodium—Total [6], mg/L: Digestion [5] followed by:					
AA direct aspiration, or	265.1	303A			
AA furnace	265.2	304			
59. Ruthenium—Total [6], mg/L: Digestion [5] followed by:					
AA direct aspiration, or	267.1	303A			
AA furnace	267.2	304			
60. Selenium—Total [6], mg/L: Digestion [5] followed by:					
AA furnace	270.2	304			
Inductively coupled plasma, or					200.7 [4]
AA gaseous hydride	270.3	303E	D3859-84(A)	I-3667-84	
61. Silica—Dissolved, mg/L: 0.45 micron filtration followed by:					
Colorimetric, Manual or	370.1	425C	D859-80(B)	I-1700-84	
Automated (Molybdosilicate), or				I-2700-84	
Inductively coupled plasma					200.7 [4]
62. Silver—Total,[66] mg/L: Digestion [5] followed by:					
AA direct aspiration	272.1	303 A or B		I-3720-85	33.088, [3] p. 37 [3]
AA furnace	272.2	304			
Colorimetric (Dithizone)					3196 [16]
ICP, or					200.7 [4]
DCP					Note 33.
63. Sodium—Total [9] mg/L: Digestion [5] followed by:					
AA direct aspiration	273.1	303A		I-3735-85	33.107 [3]
ICP					200.7 [4]
DCP, or					Note 33.
Flame photometric		325B	D1428-82(A)		
64. Specific conductance, micromhos/cm at 25°C. Wheatstone bridge	120.1	205	D1125-82(A)	I-1780-84	33.002 [3]
65. Sulfate (as SO_4), mg/L:					
Automated colorimetric (barium chloranilate).	375.1				
Gravimetric, or	375.3	426 A or B	D516-82(A)		33.124 [3]
Turbidimetric	375.4		D516-82(B)		426C [10]
66. Sulfide (as S), mg/L:					
Titrimetric (iodine) or	376.1	427D		I-3840-84	228A [10]
Colorimetric (methylene blue)	376.2	427C			
67. Sulfite (as SO_3), mg/L: Titrimetric (iodine-iodate).	377.1	428A	D1339-84(C)		
68. Surfactants, mg/L: Colorimetric (methylene blue).	425.1	512B	D2330-82(A)		
69. Temperature, °C.: Thermometric	170.1	212			Note 31.
70. Thallium—Total [6], mg/L: Digestion [5] followed by:					
AA direct aspiration	279.1	303A			
AA furnace, or	279.2	304			
Inductively coupled plasma					200.7 [4]
71. Tin—Total [6], mg/L: Digestion [5] followed by:					
AA direct aspiration, or	282.1	303A		I-3850-78 [7]	
AA furnace,	282.2	304			
72. Titanium—Total,[6] mg/L: Digestion [5] followed by:					
AA direct aspiration	283.1	303C			
AA furnace	283.2	304			
DCP					Note 33.
73. Turbidity, NTU: Nephelometric	180.1	214A	D1889-81	I-3860-84	
74. Vanadium—Total,[6] mg/L: Digestion [5] followed by:					
AA direct aspiration	286.1	303C			
AA furnace	286.2	304			
ICP					200.7 [4]
DCP, or					Note 33.
Colorimetric (Gallic acid)		327B	D3373-84(A)		
75. Zinc—Total,[6] mg/L: Digestion [5] followed by:					
AA direct aspiration	289.1	303 A or B	D1691-84 (C or D).	I-3900-85	33.089 [3] p. 37 [3]
AA furnace	289.2	304			
ICP					200.7 [4]
DCP, or					Note 33.
Colorimetric (Dithizone) or		329C			
(Zincon)					Note 32.

TABLE IB—LIST OF APPROVED INORGANIC TEST PROCEDURES—Continued

[1] "Methods for Analysis of Inorganic Substances in Water and Fluvial Sediments," U.S. Department of the Interior, U.S. Geological Survey, Open-File Report 85-495, 1986, unless otherwise stated.

[2] "Official Methods of Analysis of the Association of Official Analytical Chemists," methods manual, 14th ed. (1998).

[3] For the determination of total metals the sample is not filtered before processing. A digestion procedure is required to solubilize suspended material and to destroy possible organo-metal complexes. Two digestion procedures are given in "Methods for Chemical Analysis of Water and Wastes, 1979." One (section 4.1.3), is a vigorous digestion using nitric acid. A less vigorous digestion using nitric and hydrochloric acids (section 4.1.4) is preferred; however, the analyst should be cautioned that this mild digestion may not suffice for all sample types. Particularly, if a colorimetric procedure is to be employed, it is necessary to ensure that all organo-metallic bonds be broken so that the metal is in a reactive state. In those situations, the vigorous digestion is to be preferred making certain that at no time does the sample go to dryness. Samples containing large amounts of organic materials would also benefit by this vigorous digestion. Use of the graphite furnace technique, inductively coupled plasma, as well as determinations for certain elements such as arsenic, the noble metals, mercury, selenium, and titanium require a modified digestion and in all cases the method write-up should be consulted for specific instruction and/or cautions.

NOTE: If the digestion included in one of the other approved references is different than the above, the EPA procedure must be used.

Dissolved metals are defined as those constituents which will pass through a 0.45 micron membrane filter. Following filtration of the sample, the referenced procedure for total metals must be followed. Sample digestion for dissolved metals may be omitted for AA (direct aspiration or graphite furnace) and ICP analyses provided the sample solution to be analyzed meets the following criteria:

a. has a low COD (< 20).

b. is visibly transparent with a turbidity measurement of 1 NTU or less.

c. is colorless with no perceptable odor, and

d. is of one liquid phase and free of particulate or suspended matter following acidification.

[4] The full text of Method 200.7, "Inductively Coupled Plasma Atomic Emission Spectrometric Method for Trace Element Analysis of Water and Wastes," is given at Appendix C of this Part 136.

[5] Manual distillation is not required if comparability data on representative effluent samples are on company file to show that this preliminary distillation step is not necessary; however, manual distillation will be required to resolve any controversies.

[6] Ammonia, Automated Electrode Method, Industrial Method Number 379-75 WE, dated February 19, 1976, Technicon AutoAnalyzer II, Technicon Industrial Systems, Tarrytown, NY, 10591.

[7] The approved method is that cited in "Methods for Determination of Inorganic Substances in Water and Fluvial Sediments", USGS TWRI, Book 5, Chapter A1 (1979).

[8] American National Standard on Photographic Processing Effluents, Apr. 2, 1975. Available from ANSI, 1430 Broadway, New York, NY 10018.

[9] "Selected Analytical Methods Approved and Cited by the United States Environmental Protection Agency," Supplement to the Fifteenth Edition of *Standard Methods for the Examination of Water and Wastewater* (1981).

[10] The use of normal and differential pulse voltage ramps to increase sensitivity and resolution is acceptable.

[11] Carbonaceous biochemical oxygen demand ($CBOD_5$) must not be confused with the traditional BOD_5 test which measures "total BOD." The addition of the nitrification inhibitor is not a procedural option, but must be included to report the $CBOD_5$ parameter. A discharger whose permit requires reporting the traditional BOD_5 may not use a nitrification inhibitor in the procedure for reporting the results. Only when a discharger's permit specifically states $CBOD_5$ is required, can the permittee report data using the nitrification inhibitor.

[12] CIC Chemical Oxygen Demand Method, Oceanography International Corporation, 512 West Loop, P.O. Box 2980, College Station, TX 77840.

[13] Chemical Oxygen Demand, Method 8000, Hach Handbook of Water Analysis, 1979, Hach Chemical Company, P.O. Box 389, Loveland, CO 80537.

[14] The back titration method will be used to resolve controversy.

[15] Orion Research Instruction Manual, Residual Chlorine Electrode Model 97-70, 1977. Orion Research Incorporated, 840 Memorial Drive, Cambridge, MA 02138.

[16] The approved method is that cited in *Standard Methods for the Examination of Water and Wastewater*, 14th Edition, 1976.

[17] National Council of the Paper Industry for Air and Stream Improvement, (Inc.) Technical Bulletin 253, December 1971.

[18] Copper, Bicinchoninate Method, Method 8506, Hach Handbook of Water Analysis, 1979, Hach Chemical Company, P.O. Box 389, Loveland, CO 80537.

[19] After the manual distillation is completed, the autoanalyzer manifolds in EPA Methods 335.3 (cyanide) or 420.2 (phenols) are simplified by connecting the re-sample line directly to the sampler. When using the manifold setup shown in Method 335.3, the buffer 6.2 should be replaced with the buffer 7.6 found in Method 335.2.

[20] Hydrogen Ion (pH) Automated Electrode Method, Industrial Method Number 378-75WA, October 1976, Technicon AutoAnalyzer II, Technicon Industrial Systems, Tarrytown, NY 10591.

[21] Iron, 1,10-Phenanthroline Method, Method 8008, 1980, Hach Chemical Company, P.O. Box 389, Loveland, CO 80537.

[22] Manganese, Periodate Oxidation Method, Method 8034, Hach Handbook of Wastewater Analysis, 1979, pages 2-113 and 2-117, Hach Chemical Company, Loveland, CO 80537.

[23] Goerlitz, D., Brown, E., "Methods for Analysis of Organic Substances in Water," U.S. Geological Survey Techniques of Water-Resources Inv., book 5, ch. A3, page 4 (1972).

[24] Nitrogen, Nitrite, Method 8507, Hach Chemical Company, P.O. Box 389, Loveland, CO 80537.

[25] Just prior to distillate n, adjust the sulfuric-acid-preserved sample to pH 4 with 1 + 9 NaOH.

[26] The approved method is that cited in *Standard Methods for the Examination of Water and Wastewater*, 14th Edition. The colorimetric reaction is conducted at a pH of 10.0±0.2. The approved methods are given on pp. 576-81 of the 14th Edition: Method 510A for distillation, Method 510B for the manual colorimetric procedure, or Method 510C for the manual spectrophotometric procedure.

[27] R. F. Addison and R. G. Ackman, "Direct Determination of Elemental Phosphorus by Gas-Liquid Chromatography," *Journal of Chromatography*, vol. 47, No. 3, pp. 421-426, 1970.

[28] Approved methods for the analysis of silver in industrial wastewaters at concentrations of 1 mg/L and above are inadequate where silver exists as an inorganic halide. Silver halides such as the bromide and chloride are relatively insoluble in reagents such as nitric acid but are readily soluble in an aqueous buffer of sodium thiosulfate and sodium hydroxide to a pH of 12. Therefore, for levels of silver above 1 mg/L, 20 mL of sample should be diluted to 100 mL by adding 40 mL each of 2 M $Na_2S_2O_3$ and 2M NaOH. Standards should be prepared in the same manner. For levels of silver below 1 mg/L the approved method is satisfactory.

[29] The approved method is that cited in *Standard Methods for the Examination of Water and Wastewater*, 15th Edition.

[30] The approved method is that cited in *Standard Methods for the Examination of Water and Wastewater*, 13th Edition.

[31] Stevens, H. H., Ficke, J. F., and Smoot, G. F., "Water Temperature—Influential Factors, Field Measurement and Data Presentation," U.S. Geological Survey, Techniques of Water Resources Investigations, Book 1, Chapter D1, 1975.

[32] Zinc, Zincon Method, Method 8009, Hach Handbook of Water Analysis, 1979, pages 2-231 and 2-333, Hach Chemical Company, Loveland, CO 80537.

[33] "Direct Current Plasma (DCP) Optical Emission Spectrometric Method for Trace Elemental Analysis of Water and Wastes, Method AES0029," 1986, Applied Research Laboratories, Inc., 24911 Avenue Stanford, Valencia, CA 91355.

[34] Precision and recovery statements for the atomic absorption direct aspiration and graphite furnace methods, and for the spectrophotometric SDDC method for arsenic are provided in appendix D of this part titled, "Precision and Recovery Statements for Methods for Measuring Metals".

TABLE IC—LIST OF APPROVED TEST PROCEDURES FOR NON-PESTICIDE ORGANIC COMPOUNDS

Parameter [1]	EPA Method Number [2] [3]			Other
	GC	GC/MS	HPLC	
1. Acenaphthene	610	625, 1625	610	
2. Acenaphthylene	610	625, 1625	610	
3. Acrolein	603	[4]624, 1624		

TABLE IC—LIST OF APPROVED TEST PROCEDURES FOR NON-PESTICIDE ORGANIC COMPOUNDS—Continued

Parameter [1]	EPA Method Number [4, 7]			Other
	GC	GC/MS	HPLC	
4. Acrylonitrile	603	[3]624, 1624		
5. Anthracene	610	625, 1625	610	
6. Benzene	602	624, 1624		
7. Benzidine		[3]625, 1625	605	Note 3, p. 1;
8. Benzo(a)anthracene	610	625, 1625	610	
9. Benzo(a)pyrene	610	625, 1625	610	
10. Benzo(b)fluoranthene	610	625, 1625	610	
11. Benzo(g,h,i)perylene	610	625, 1625	610	
12. Benzo(k)fluoranthene	610	625, 1625	610	
13. Benzyl chloride				Note 3, p. 130; Note 6, p. 5102.
14. Benzyl butyl phthalate	606	625, 1625		
15. Bis(2-chloroethoxy) methane	611	625, 1625		
16. Bis(2-chloroethyl) ether	611	625, 1625		
17. Bis(2-ethylhexyl) phthalate	606	625, 1625		
18. Bromodichloromethane	601	624, 1624		
19. Bromoform	601	624, 1624		
20. Bromomethane	601	624, 1624		
21. 4-Bromophenylphenyl ether	611	625, 1625		
22. Carbon tetrachloride	601	624, 1624		Note 3, p. 130;
23. 4-Chloro-3-methylphenol	604	625, 1625		
24. Chlorobenzene	601, 602	624, 1624		Note 3, p. 130;
25. Chloroethane	601	624, 1624		
26. 2-Chloroethylvinyl ether	601	624, 1624		
27. Chloroform	601	624, 1624		Note 3, p. 130;
28. Chloromethane	601	624, 1624		
29. 2-Chloronaphthalene	612	625, 1625		
30. 2-Chlorophenol	604	625, 1625		
31. 4-Chlorophenylphenyl ether	611	625, 1625		
32. Chrysene	610	625, 1625	610	
33. Dibenzo(a,h)anthracene	610	625, 1625	610	
34. Dibromochloromethane	601	624, 1624		
35. 1,2-Dichlorobenzene	601, 602, 612	624, 625, 1625		
36. 1,3-Dichlorobenzene	601, 602, 612	624, 625, 1625		
37. 1,4-Dichlorobenzene	601, 602, 612	625, 1624, 1625		
38. 3,3 -Dichlorobenzidine		625, 1625	605	
39. Dichlorodifluoromethane	601			
40. 1,1-Dichloroethane	601	624, 1624		
41. 1,2-Dichloroethane	601	624, 1624		
42. 1,1-Dichloroethene	601	624, 1624		
43. trans-1,2-Dichloroethene	601	624, 1624		
44. 2,4-Dichlorophenol	604	625, 1625		
45. 1,2-Dichloropropane	601	624, 1624		
46. cis-1,3-Dichloropropene	601	624, 1624		
47. trans-1,3-Dichloropropene	601	624, 1624		
48. Diethyl phthalate	606	625, 1625		
49. 2,4-Dimethylphenol	604	625, 1625		
50. Dimethyl phthalate	606	625, 1625		
51. Di-n-butyl phthalate	606	625, 1625		
52. Di-n-octyl phthalate	606	625, 1625		
53. 2,4-Dinitrophenol	604	625, 1625		
54. 2,4-Dinitrotoluene	609	625, 1625		
55. 2,6-Dinitrotoluene	609	625, 1625		
56. Epichlorohydrin				Note 3, p. 130; Note 6, p. 5102.
57. Ethylbenzene	602	624, 1624		
58. Fluoranthene	610	625, 1625	610	
59. Fluorene	610	625, 1625	610	
60. Hexachlorobenzene	612	625, 1625		
61. Hexachlorobutadiene	612	625, 1625		
62. Hexachlorocyclopentadiene	612	[3]625, 1625		
63. Hexachloroethane	612	625, 1625		
64. Indeno(1,2,3-cd)pyrene	610	625, 1625	610	
65. Isophorone	609	625, 1625		
66. Methylene chloride	601	624, 1624		Note 3, p. 130;
67. 2-Methyl-4,6-dinitrophenol	604	6[illegible], 1625		
68. Naphthalene	610	625, 1625	610	
69. Nitrobenzene	609	625, 1625		
70. 2-Nitrophenol	604	625, 1625		
71. 4-Nitrophenol	604	625, 1625		
72. N-Nitrosodimethylamine	607	625, 1625		
73. N-Nitrosodi-n-propylamine	607	[3]625, 1625		
74. N-Nitrosodiphenylamine	607	[3]625, 1625		
75. 2,2'-Oxybis(1-chloropropane)	611	625, 1625		
76. PCB-1016	608	625		Note 3, p. 43;
77. PCB-1221	608	625		Note 3, p. 43;
78. PCB-1232	608	625		Note 3, p. 43;
79. PCB-1242	608	625		Note 3, p. 43;
80. PCB-1248	608	625		Note 3, p. 43;
81. PCB-1254	608	625		Note 3, p. 43;
82. PCB-1260	608	625		Note 3, p. 43;
83. Pentachlorophenol	604	625, 1625		Note 3, p. 140;
84. Phenanthrene	610	625, 1625	610	
85. Phenol	604	625, 1625		
86. Pyrene	610	625, 1625	610	

TABLE IC—LIST OF APPROVED TEST PROCEDURES FOR NON-PESTICIDE ORGANIC COMPOUNDS—Continued

Parameter [1]	EPA Method Number [2, 7]			Other
	GC	GC/MS	HPLC	
87. 2,3,7,8-Tetrachlorodibenzo-p-dioxin		[10] 613		
88. 1,1,2,2-Tetrachloroethane	601	624, 1624		Note 3, p. 130;
89. Tetrachloroethene	601	624, 1624		Note 3, p. 130;
90. Toluene	602	624, 1624		
91. 1,2,4-Trichlorobenzene	612	625, 1625		Note 3, p. 130;
92. 1,1,1-Trichloroethane	601	624, 1624		
93. 1,1,2-Trichloroethane	601	624, 1624		Note 3, p. 130;
94. Trichloroethene	601	624, 1624		
95. Trichlorofluoromethane	601	624		
96. 2,4,6-Trichlorophenol	604	625, 1625		
97. Vinyl chloride	601	624, 1624		

Table IC Notes

[1] All parameters are expressed in micrograms per liter (µg/L).

[2] The full test of Methods 601-613, 624, 625, 1624, and 1625, are given at Appendix A, "Test Procedures for Analysis of Organic Pollutants," of this Part 136. The standardized test procedure to be used to determine the method detection limit (MDL) for these test procedures is given at Appendix B, "Definition and Procedure for the Determination of the Method Detection Limit," of this Part 136.

[3] "Methods for Benzidine, Chlorinated Organic Compounds, Pentachlorophenol and Pesticides in Water and Wastewater," U.S. Environmental Protection Agency, September, 1978.

[4] Method 624 may be extended to screen samples for Acrolein and Acrylonitrile. However, when they are known to be present, the preferred method for these two compounds is Method 603 or Method 1624.

[5] Method 625 may be extended to include benzidine, hexachlorocyclopentadiene, N-nitrosodimethylamine, and N-nitrosodiphenylamine. However, when they are known to be present, Methods 605, 607, and 612, or Method 1625, are preferred methods for these compounds.

[6] 625, Screening only.

[7] "Selected Analytical Methods Approved and Cited by the United States Environmental Protection Agency," Supplement to the Fifteenth Edition of *Standard Methods for the Examination of Water and Wastewater* (1981).

[8] Each analyst must make an initial, one-time, demonstration of their ability to generate acceptable precision and accuracy with Methods 601-613, 624, 625, 1624, and 1625 (See Appendix A of this Part 136) in accordance with procedures each in section 8.2 of each of these Methods. Additionally, each laboratory, on an on-going basis must spike and analyze 10% (5% for Methods 624 and 625 and 100% for methods 1624, and 1625) of all samples to monitor and evaluate laboratory data quality in accordance with sections 8.3 and 8.4 of these Methods. When the recovery of any parameter falls outside the warning limits, the analytical results for that parameter in the unspiked sample are suspect and cannot be reported to demonstrate regulatory compliance.

NOTE: These warning limits are promulgated as an "interim final action with a request for comments."

TABLE ID—LIST OF APPROVED TEST PROCEDURES FOR PESTICIDES [1]

Parameter µg/L	Method	EPA [2,3]	Standard Methods 15th Ed	ASTM	Other
1. Aldrin	GC	608	508A	D3086	Note 3, p. 7; Note 4, p. 30.
	GC/MS	625			
2. Ametryn	GC				Note 3, p. 83; Note 6, p. 888.
3. Aminocarb	TLC				Note 3, p. 84; Note 6, p. 818.
4. Atraton	GC				Note 3, p. 83; Note 6, p. 888.
5. Atrazine	GC				Note 3, p. 83; Note 6, p. 888.
6. Azinphos methyl	GC				Note 3, p. 25; Note 6, p. 851.
7. Barban	TLC				Note 3, p. 104; Note 6, p. 884.
8. α-BHC	GC	608	508A	D3086	Note 3, p. 7.
	GC/MS	[4] 625			
9. β-BHC	GC	608		D3086	
	GC/MS	625			
10. δ-BHC	GC	608		D3086	
	GC/MS	[4] 625			
11. γ-BHC (Lindane)	GC	608	508A	D3086	Note 3, p. 7; Note 4, p. 30.
	GC/MS	625			
12. Captan	GC		508A		Note 3, p. 7.
13. Carbaryl	TLC				Note 3, p. 84; Note 6, p. 880.
14. Carbophenothion	GC				Note 4, p. 30; Note 6, p. 873.
15. Chlordane	GC	608	508A	D3086	Note 3, p. 7.
	GC-MS	625			
16. Chloropropham	TLC				Note 3, p. 104; Note 6, p. 884.
17. 2,4-D	GC		508B		Note 3, p. 115; Note 4, p. 36.
18. 4,4'-DDD	GC	608	508A	D3086	Note 3, p. 7; Note 4, p. 30.
	GC-MS	625			
19. 4,4'-DDE	GC	608	508A	D3086	Note 3, p. 7; Note 4, p. 30.
	GC/MS	625			
20. 4,4'-DDT	GC	608	508A	D3086	Note 3, p. 7; Note 4, p. 30.
	GC/MS	625			
21. Demeton-O	GC				Note 3, p. 25; Note 6, p. 851.
22. Demeton-S	GC				Note 3, p. 25; Note 6, p. 851.
23. Diazinon	GC				Note 3, p. 25; Note 4, p. 30; Note 6, p. 851.
24. Dicamba	GC				Note 3, p. 115.
25. Dichlofenthion	GC				Note 4, p. 30; Note 6, p. 873.
26. Dichloran	GC		508A		Note 3, p. 7.
27. Dicofol	[5]			D3086	
28. Dieldrin	GC	608	508A		Note 3, p. 7; Note 4, p. 30.
	GC/MS	625			
29. Dimethoate	GC				Note 4, p. 30; Note 6, p. 873.
30. Disulfoton	GC				Note 3, p. ; Note 6, p. 851.
31. Diuron	TLC				Note 3, p. 104; Note 6, p. 884.
32. Endosulfan I	GC	608	508A	D3C.J6	Note 3, p. 7.
	GC/MS	[4] 625			

TABLE ID—LIST OF APPROVED TEST PROCEDURES FOR PESTICIDES [1]—Continued

Parameter µg/L	Method	EPA [2,3]	Standard Methods 18th Ed	ASTM	Other
33. Endosulfan II	GC	608	6080A	D3086	Note 3, p. 7.
	GC/MS	* 625			
34. Endosulfan sulfate	GC	608			
	GC/MS	625			
35. Endrin	GC	608	6080A	D3086	Note 3, p. 7; Note 4, p. 30.
	GC/MS	* 625			
36. Endrin aldehyde	GC	608			
	GC/MS	625			
37. Ethion	GC				Note 4, p. 30; Note 6, p. 573.
38. Fenuron	TLC				Note 3, p. 104; Note 6, p. 864.
39. Fenuron-TCA	TLC				Note 3, p. 104; Note 6, p. 864.
40. Heptachlor	GC	608	6080A	D3086	Note 3, p. 7; Note 4, p. 30.
	GC/MS	625			
41. Heptachlor epoxide	GC	608	6080A	D3086	Note 3, p. 7; Note 4, p. 30; Note 6, p. 573.
	GC/MS	625			
42. Isodrin	GC				Note 4, p. 30; Note 6, p. 573.
43. Linuron	TLC				Note 3, p. 104; Note 6, p. 864.
44. Malathion	GC		6080A		Note 3, p. 29; Note 4, p. 30; Note 6, p. 551.
45. Methiocarb	TLC				Note 3, p. 94; Note 6, p. 860.
46. Methoxychlor	GC		6080A	D3086	Note 3, p. 7; Note 4, p. 30.
47. Mexacarbate	TLC				Note 3, p. 94; Note 6, p. 860.
48. Mirex	GC		6080A		Note 3, p. 7.
49. Monuron	TLC				Note 3, p. 104; Note 6, p. 864.
50. Monuron-TCA	TLC				Note 3, p. 104; Note 6, p. 864.
51. Neburon	TLC				Note 3, p. 104; Note 6, p. 864.
52. Parathion methyl	GC		6080A		Note 3, p. 29; Note 4, p. 30.
53. Parathion ethyl	GC		6080A		Note 3, p. 29.
54. PCNB	GC		6080A		Note 3, p. 7.
55. Perthane	GC			D3086	
56. Prometon	GC				Note 3, p. 83; Note 6, p. 568.
57. Prometryn	GC				Note 3, p. 83; Note 6, p. 568.
58. Propazine	GC				Note 3, p. 83; Note 6, p. 568.
59. Propham	TLC				Note 3, p. 104; Note 6, p. 864.
60. Propoxur	TLC				Note 3, p. 94; Note 6, p. 860.
61. Secbumeton	TLC				Note 3, p. 83; Note 6, p. 568.
62. Siduron	TLC				Note 3, p. 104; Note 6, p. 864.
63. Simazine	GC				Note 3, p. 83; Note 6, p. 568.
64. Strobane	GC		6080A		Note 3, p. 7.
65. Swep	TLC				Note 3, p. 104; Note 6, p. 864.
66. 2,4,5-T	GC		6080B		Note 3, p. 116; Note 4, p. 35.
67. 2,4,5-TP (Silvex)	GC		6080B		Note 3, p. 116.
68. Terbuthylazine	GC				Note 3, p. 83; Note 6, p. 568.
69. Toxaphene	GC	608	6080A	D3086	Note 3, p. 7; Note 4, p. 30.
	GC/MS	625			
70. Trifluralin	GC		6080A		Note 3, p. 7

Table ID Notes

[1] Pesticides are listed in this table by common name for the convenience of the reader. Additional pesticides may be found under Table IC, where entries are listed by chemical name.

[2] The full text of methods 608 and 625 are given at Appendix A, "Test Procedures for Analyses of Organic Pollutants," of this Part 136. The standardized test procedure to be used to determine the method detection limit (MDL) for these test procedures is given at Appendix B, "Definition and Procedure for the Determination of the Method Detection Limit", of this Part 136.

[3] "Methods for Benzidine, Chlorinated Organic Compounds, Pentachlorophenol and Pesticides in Water and Wastewater," U.S. Environmental Protection Agency, September, 1978. This EPA publication includes thin-layer chromatography (TLC) methods.

[4] "Methods for Analysis of Organic Substances in Water," U.S. Geological Survey, Techniques of Water-Resources Investigations, Book 5, Chapter A3 (1972).

[5] The method may be extended to include a-BHC, ß-BHC, endosulfan I, endosulfan II, and endrin. However, when they are known to exist, Method 608 is the preferred method.

[6] "Selected Analytical Methods Approved and Cited by the United States Environmental Protection Agency," Supplement to the Fifteenth Edition of *Standard Methods for the Examination of Water and Wastewater* (1981).

[7] Each analyst must make an initial, one-time, demonstration of their ability to generate acceptable precision and accuracy with Methods 608 and 625 (See Appendix A of this Part 136) in accordance with procedures given in section 8.2 of each of these methods. Additionally, each laboratory, on an on-going basis, must spike and analyze 10% of all samples analyzed with Method 608 or 5% of all samples analyzed with Method 625 to monitor and evaluate laboratory data quality in accordance with Sections 8.3 and 8.4 of these methods. When the recovery of any parameter falls outside the warning limits, the analytical results for that parameter in the unspiked sample are suspect and cannot be reported to demonstrate regulatory compliance.

NOTE: These warning limits are promulgated as an "interim final action with a request for comments."

TABLE IE—LIST OF APPROVED RADIOLOGICAL TEST PROCEDURES

Parameter and units	Methods	EPA [1]	Reference (method No. or page)		
			Standard methods 16th ed.	ASTM	USGS [2]
1. Alpha-Total, pCi per liter	Proportional or scintillation counter.	900.0	703	D1943-81	pp. 75 and 78. [3]
2. Alpha-Counting error, pCi per liter.	Proportional or scintillation counter.	Appendix B	703	D1943-81	p. 78.
3. Beta-Total, pCi per liter	Proportional counter	900.0	703	D1890-81	pp. 75 and 78. [3]
4. Beta-Counting error, pCi	Proportional counter	Appendix B	703	D1890-81	p. 78.
5. (a) Radium-Total, pCi per liter.	Proportional counter	903.0	705	D2460-70	
(b) ^{226}Ra, pCi per liter	Scintillation counter	903.1	706	D3454-79	p. 81.

TABLE IE NOTES:
[1] "Prescribed Procedures for Measurement of Radioactivity in Drinking Water," EPA-600/4-80-032 (1980 update), U.S. Environmental Protection Agency, August 1980.
[2] Fishman, M.J. and Brown, Eugene, "Selected Methods of the U.S. Geological Survey of Analyses of Wastewaters," U.S. Geological Survey, Open-File Report 76-177 (1976).
[3] The method found on p. 75 measures only the dissolved portion while the method on p. 78 measures only the suspended portion. Therefore, the two results must be added to obtain the "total."

TECHNICAL APPENDIX D

REFERENCES

REFERENCES

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U.S. EPA, Region V, "Urban Targeting and BMP Selection," November 1990.

Woodward-Clyde Consultants and Ted Friel Associates, "Guide for Industrial Storm Water Sampling," January 1992.

TECHNICAL APPENDIX E

GLOSSARY

GLOSSARY

Aliquot: A discrete sample used for analysis.

Biochemical Oxygen Demand (BOD): The quantity of oxygen consumed during the biochemical oxidation of matter over a specified period of time, usually 5 days (BOD_5).

Chain-of-Custody: Procedures used to minimize the possibility of tampering with samples.

Chemical Oxygen Demand (COD): Measurement of all the oxidizable matter found in a runoff sample, a portion of which could deplete dissolved oxygen in receiving waters.

Composite Sample: Used to determine "average" loadings or concentrations of pollutants, such samples are collected at regular time intervals, and pooled into one large sample, can be developed on time or flow rate.

Confined Space: Enclosed space that an employee can bodily enter and perform assigned work, that has limited means of exit and entry, that is not designed for continuous employee occupancy, and has one of the following characteristics:

- Contains or has a known potential to contain a hazardous atmosphere
- Contains a material with the potential for engulfment of an entrant
- Has an internal configuration such that an entrant could be trapped or asphyxiated by inwardly converging walls or a floor that slopes downward and tapers to a smaller cross section
- Contains any other recognized serious safety or health hazard.

Conveyance: A channel or passage which conducts or carries water including any pipe, ditch, channel, tunnel, conduit, well, or container.

Detention Ponds: A surface water impoundment constructed to hold and manage storm water runoff.

Discharge: Any addition of any pollutant to waters of the U.S. from any conveyance.

Effluent: Any discharge flowing from a conveyance.

Flumes: A specially shaped open channel flow section providing a change in the channel area and/or slope which results in an increased velocity and change in the level of the liquid flowing through the flume. A flume normally consists of three sections: (1) a converging section; (2) a throat section; and (3) a diverging section. The flow rate through the flume is a function of the liquid level at some point in the flume.

Flow-Weighted Composite Sample: Means a composite sample consisting of a mixture of aliquots collected at a constant time interval, where the volume of each aliquot is proportional to the flow rate of the discharge.

Flow-Proportional Composite Sample: Combines discrete aliquots of a sample collected over time, based on the flow of the wastestream being sampled. There are two methods used to collect this type of sample. One collects a constant sample volume at time intervals which vary based on stream flow. The other collects aliquots at varying volumes based on stream flow, at constant time intervals.

First Flush: Individual sample taken during the first 30 minutes of a storm event. The pollutants in this sample can often be used as a screen for non-storm water discharges since such pollutants are flushed out of the system during the initial portion of the discharge.

Grab Sample: A discrete sample which is taken from a wastestream on a one-time basis with no regard to flow or time; instantaneous sample that is analyzed separately.

Head of Liquid: Depth of flow.

Illicit Discharge: Any discharge to a municipal separate storm sewer that is not composed entirely of storm water except discharges pursuant to an NPDES permit and discharges from fire fighting activities.

Materials Management Practices: Practices used to limit the contact between significant materials and precipitation. These may include structural or nonstructural controls such as dikes, berms, sedimentation ponds, vegetation strips, spill response plans, etc.

Municipal Separate Storm Sewer Systems: A conveyance or system of conveyances including roads with drainage systems, storm drains, gutters, ditches under the jurisdiction of a city, town, borough, county, parish, or other public body.

Outfall: Point source where an effluent is discharged into receiving waters.

Point Source: Any discernible, confined, and discrete conveyance from which pollutants are or may be discharged. This term does not include return flows from irrigated agriculture or agricultural storm water runoff (see 40 CFR 122.3).

Reverse Meniscus: The curved upper surface of a liquid in a container.

Runoff Coefficient: Means the fraction of total rainfall that will appear at the conveyance as runoff.

Significant Materials: Include, but are not limited to, raw materials, fuels, solvents, detergents, metallic products, CERCLA hazardous substances, fertilizers, pesticides, and wastes such as ashes, slag, and sludge that have potential for release with storm water discharges [see 40 CFR 122.26(b)(12)].

Storm Water: Storm water runoff, snow melt runoff, and surface runoff, and drainage.

Storm Water Discharge Associated with Industrial Activity: Discharge from any conveyance which is used for collecting and conveying storm water which is directly related to manufacturing processing or raw materials storage areas at an industrial plant [see 40 CFR 122.26(b)(14)].

Time Composite Sample: Prepared by collecting fixed volume aliquots at specified time intervals and combining into a single sample for analysis.

Turbidity: Describes the capability of light to pass through water.

Weir: A device used to gauge the flow rate of liquid through a channel; is essentially a dam built across an open channel over which the liquid flows, usually through some type of notch.

TECHNICAL APPENDIX F

ACRONYMS

ACRONYMS

BOD_5	Biochemical Oxygen Demand (5-day)
CERCLA	Comprehensive Environmental Response Compensation and Liability Act
cfm	cubic feet per minute
CFR	Code of Federal Regulations
cfs	cubic feet per second
COD	Chemical Oxygen Demand
COV	Coefficient of Variation
CPR	Cardiopulmonary Resuscitation
CWA	Clean Water Act
DOT	Department of Transportation
ECD	Electron Capture Detector
EMC	Event Mean Concentration
EPA	Environmental Protection Agency
ESE	Environmental Science & Engineering, Inc.
FWPCA	Federal Water Pollution Control Act
FID	Flame Ionization Detector
FR	Federal Register
GC/MS	Gas Chromatography/Mass Spectometry
gpm	gallons per minute
H	Head
HCl	Hydrochloric Acid
HNO_3	Nitric Acid
HPLC	High Pressure Liquid Chromatography
H_2SO_4	Sulfuric Acid
IATA	International Air Transport Association
LC_{50}	Lethal Concentration
NaOH	Sodium Hydroxide
$Na_2S_2O_3$	Sodium Thiosulfate
NCDC	National Climate Data Center
NIOSH	National Institute of Occupational Safety and Health
NOAA	National Oceanic and Atmospheric Agency
NOI	Notice of Intent
NPDES	National Pollutant Discharge Elimination System
NWS	National Weather Service
O&G	Oil and Grease
OSHA	Occupational Safety and Health Administration
PCB	Polychlorinated Biphenyl
PE	Professional Engineer
ppb	parts per billion
Q	Flow Rate
RCRA	Resource Conservation and Recovery Act
SARA	Superfund Amendments and Reauthorization Act
SIC	Standard Industrial Classification
s.u.	standard units
TKN	Total Kjeldahl Nitrogen
TSS	Total Suspended Solids
VOC	Volatile Organic Compound

STREAM SYSTEMS TECHNOLOGY CENTER

STREAM NOTES

To Aid In Securing Favorable Conditions of Water Flows

Rocky Mountain Research Station

April 2004

Rapid Evaluation of Sediment Budgets

Researchers and land managers are increasingly interested in the response of erosion and sedimentation to changes occurring on watershed hillslopes or in stream channels. Managers need to predict how land use will alter erosion and sedimentation rates and the relative importance of different sediment sources in order to assign priorities for erosion control. They also must anticipate where sediment will be deposited, how long it will be stored, and how it will be re-mobilized. Sediment budgets are a useful tool for address these management problems.

Land managers and researchers often assume that sediment budgeting is a time consuming exercise suitable only for long-term studies. In the book ***Rapid Evaluation of Sediment Budgets,*** **Leslie Reid**, USDA Forest Service, Pacific Southwest Research Station, and Thomas Dunne, University of California at Santa Barbara, School of Environmental Sciences and Management, argue that sediment budgets can be constructed for a variety of applications using an approach that usually requires no longer than two months of fieldwork and analysis.

Rapid Evaluation of Sediment Budgets is intended as a guide for land managers who are contemplating the use of sediment budgets. It is not intended as a how-to manual for budget construction, although the book describes useful techniques that geomorphologists and hydrologists already involved in sediment budgeting might find useful. Although the methods of budget construction are relatively uncomplicated, they require that users have extensive backgrounds in geomorphology and hydrology if the methods are to be used appropriately.

A sediment budget is an accounting of the sources and disposition of sediment as it travels from its point of origin to its eventual exit from a drainage basin (figure 1). In its full form, a sediment budget accounts for rates and processes of erosion and sediment transport on hills and in channels; for temporary storage of sediment in bars, alluvial fans, and other sites; and for weathering and breakdown of sediments while in transport or storage. Although complete sediment budgets are of scientific interest, they are frequently more detailed than is necessary to address problems encountered in resource management.

STREAM NOTES is produced quarterly by the Stream Systems Technology Center, Rocky Mountain Research Station, *Fort Collins, Colorado.*
John Potyondy, Program Manager

The *PRIMARY AIM* is to exchange technical ideas and transfer technology among scientists working with wildland stream systems.

CONTRIBUTIONS are voluntary and will be accepted at any time. They should be typewritten, single-spaced, and limited to two pages. Graphics and tables are encouraged. *E-Mail: jpotyondy@ fs.fed.us*

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CORRESPONDENCE:
E-Mail: rmrs_stream@ fs.fed.us
Phone: (970) 295-5983
FAX: (970) 295-5988

Web Site:
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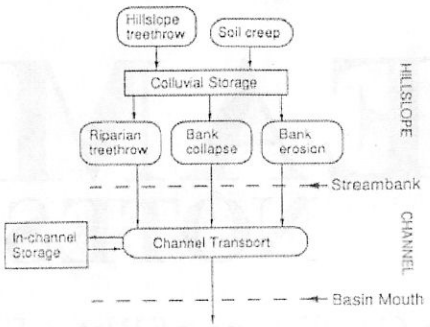


Figure 1. Simple flowchart of sediment transport on hillslopes and in channels. In this case, treethrow and soil creep intermittently transport sediment downslope to the channel bank, where it is eventually delivered to the channel by channel-side treethrow, bank collapse, and bank erosion. Sediment is then alternately stored and transported by the channel to the watershed mouth. Processes are noted as ovals, storage elements as rectangles, and transfers as arrows; the streambank appears as a dashed line.

Sediment Budget Construction Procedure

The construction of approximate sediment budgets proceeds most smoothly if it follows a consistent 7-step process:

Step 1: Carefully define the problem

Step 2: Acquire background information

Step 3: Subdivide the area

Step 4: Interpret aerial photographs

Step 5: Conduct fieldwork

Step 6: Analyze the data

Step 7: Check results.

For most applications, some combination of the following information is required:

- the type and location of major natural and management-related sources of sediment,
- the approximate amount of sediment contributed by each type of source,
- the grain-size distribution of sediment contributed from each source,
- the approximate volumes and grain sizes of sediment in storage along streams,
- the approximate transport rate of sediment through stream channels and valley floors.

The information that is relevant depends on the problem posed, and answers to the first three points often suffice.

Misconceptions

Reid and Dunne point out that there is a persistent misconception among both land managers and researchers that the construction of sediment budgets is a time-consuming, academic exercise that is impractical for addressing the goals of land-use planning or short-term research. Although sediment budgeting often uses long-term measurements, budgets can also be constructed using rapid measurements and estimates to provide results at a level of precision adequate for most management needs. Sediment budgets are mistakenly viewed as impractical for short-term analyses in part because the utility of approximate budgets is often overlooked.

A second misunderstanding arises because erosion and transport rates are difficult to measure precisely, accurately, and consistently. Erosion is perceived as being intractably variable and complex, and lengthy measurement periods are assumed to be necessary if monitoring is to produce a meaningful average erosion rate. However, it is possible to design simple sampling schemes that account for seasonal and local variations in process rates if the reasons for these variations are understood. In addition, most management applications require only that the order of magnitude or the relative importance of process rates be known.

A third misconception centers around the notion that sediment budgeting implies construction of detailed maps of erosion processes. Management problems usually involve areas that are too large to permit thorough examination either in the field or on aerial photos, so comprehensive mapping is impractical and sediment budgeting is thus assumed to be impossible. However, construction of budgets for large areas merely requires a modification of techniques. Large areas are divided ("stratified") into subunits of similar soils, bedrock, vegetation, topography, and land use, and each subunit is characterized by budgets constructed for representative areas within it.

Conclusions

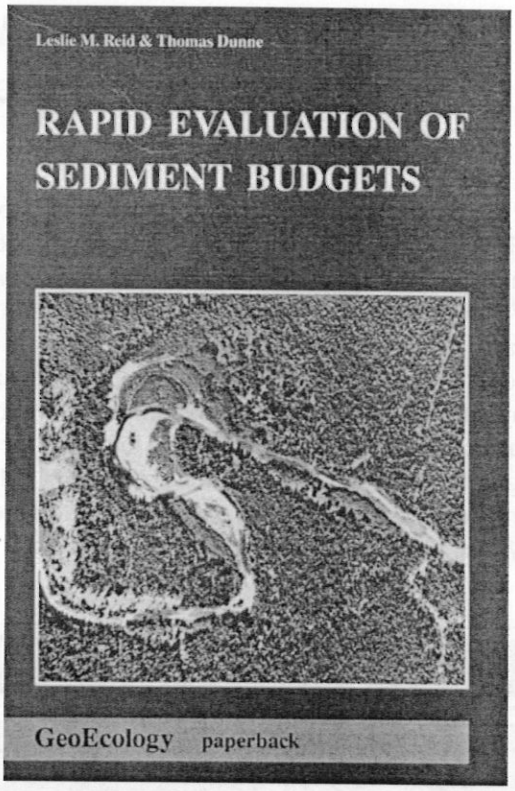
The 164 page book, ***Rapid Evaluation of Sediment Budgets,*** does not pretend to be a complete guide to sediment budget construction, but it provides a comprehensive overview of strategies and tools useful for understanding sediment production and transport in watersheds. The validity of a sediment budget then depends on how wisely these methods are employed.

Effective construction and interpretation of sediment budgets requires a sound understanding of erosion and sedimentation processes, experience in field mapping and in the measurement and analysis techniques to be used, and above all, good professional judgment. Each area represents its own difficulties and opportunities, so analysts must have a strong enough background in geomorphology and hydrology to take advantage of the peculiarities of the area to be evaluated, and they must be creative and open-minded in their approach.

An insufficient number of sediment budget studies exists to allow statistical evaluation of the accuracy and reproducibility of the general approach. However, Reid and Dunne have found that when several trained geomorphologists are asked to evaluate a process rate, results agree relatively closely, and certainly to well within an order of magnitude. Because many sediment budget applications require only approximate estimates, this level of accuracy is thought to be adequate.

Construction of sediment budgets is more difficult in some areas than others. At sites where sediment input is dominated by large, infrequent events, rates must be evaluated using as long a period of record as possible. In such cases, land use may cause small changes in process frequencies which can strongly affect long-term sediment yields, but which may not be observable over the time frame available for analysis.

The most difficult aspects of a sediment budget to quantify are those involving transport and storage of sediment in channels. In areas where these components are particularly important, sediment budgets can often reveal the process interactions that control channel response, the types of changes a channel may undergo, and the likely location of those changes, even if rates cannot be quantified.



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Several examples of rapidly constructed sediment budgets are provided by the authors. Anyone interested in constructing sediment budgets for management applications should find this book useful as a guide to specific analysis techniques and as a source of ideas for applying those techniques to management problems.

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Reid, Leslie M. and Thomas Dunne, 1996. ***Rapid Evaluation of Sediment Budgets.*** Reiskirchen: Germany, Catena Verlag (GeoEcology paperback), 164 p.

Available from CATENA VERLAG GMBH - GeoScience Publisher for $35.00 at (http://www.catena-verlag.de) or from their United States distributor: Humboldt State University Bookstore, Aracata, CA 95521;
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National Weather Service
Precipitation Frequency Data on the Web

The rainfall frequency atlases and technical papers published by the National Oceanic and Atmospheric Administration's (NOAA) National Weather Service (NWS) serve as de-facto national standards for rainfall intensity at specified frequencies and durations in the United States. Civil engineers and hydrologists have always used these probabilistic estimates of rainfall intensities for particular durations and locations for the design of a wide range of hydraulic structures. More recently their use has been extended to include a broad array of environmental management and analysis.

In 1953, the NWS began publishing general rainfall-intensity-frequency-duration values or "precipitation frequency estimates" (Weather Bureau Technical Paper 24, 1953). These estimates were produced by the NWS at the request of other Federal, state, and local agencies because the NWS is the primary Federal agency with the required meteorological and hydrometeorological expertise required to develop the estimates and NWS is an independent agency which does not regulate or design based on the estimates. The values have become de-facto national standards by inclusion or reference in the design and planning standards of a wide variety of

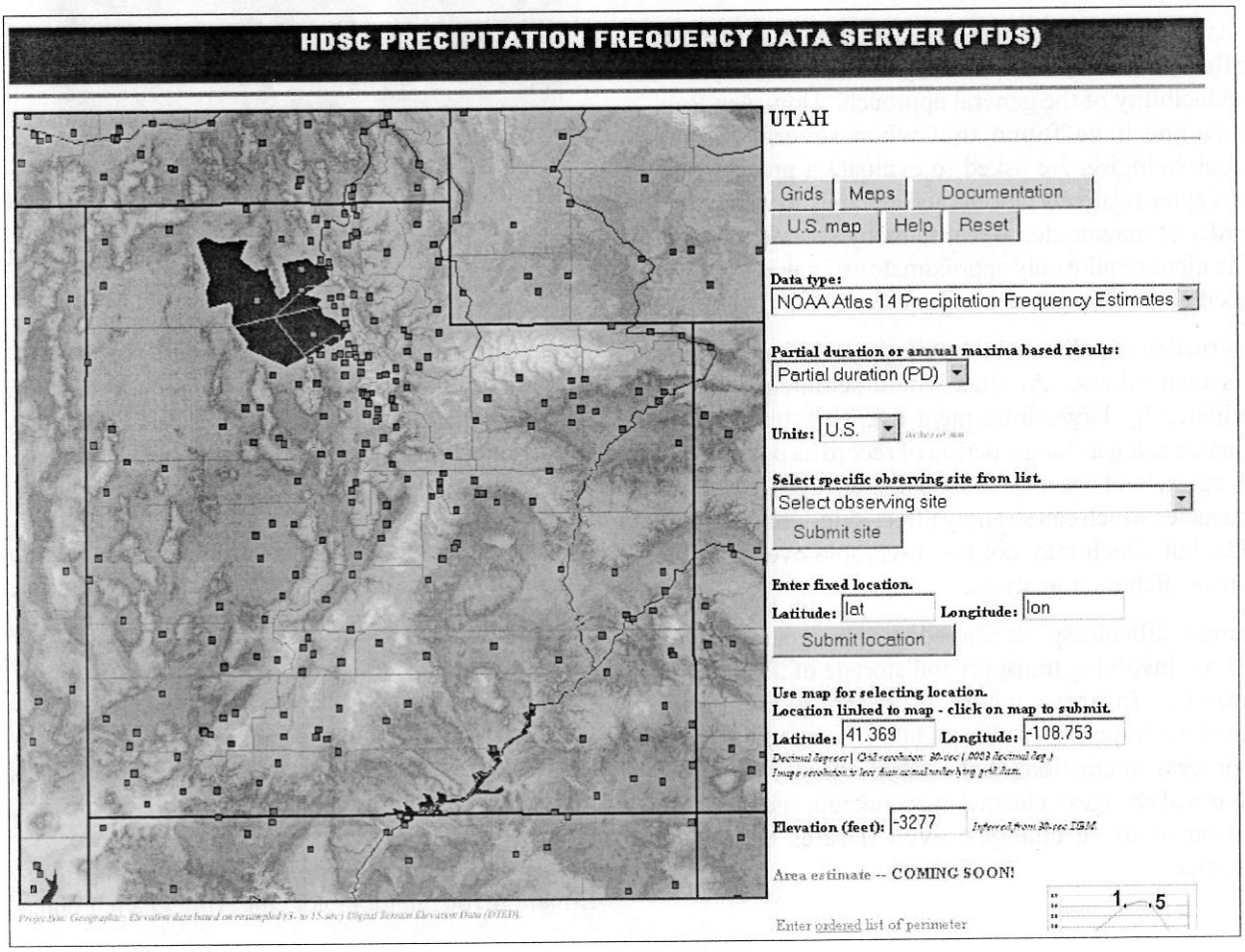

Figure 1. An example of the Precipitation Frequency Data Server screen for the State of Utah.

agencies at Federal, state, and local levels.

Precipitation frequency estimates are developed by using statistical hydrometeorological techniques that have evolved over time and which have been the subject of extensive research and discussion in peer reviewed, scientific literature. The general approach is to examine time series of annual maximum (or partial duration) values and determine appropriate underlying probability distribution functions that can be used to interpolate or extrapolate estimates at a variety of frequencies and durations. The resulting point estimates are then spatially interpolated to regular grids from which cartographic quality maps are derived.

NWS precipitation frequency estimates have traditionally been delivered in the form of Weather Bureau Technical Papers and Memoranda as well as NOAA Atlases, all hard copy documents. With the advent of the World Wide Web, these documents have been scanned and made available via web pages. The National Weather Service specifically developed the Precipitation Frequency Data Server (figure 1) as the primary web portal to precipitation frequency estimates and associated information. Recent updates to NWS precipitation frequency estimates are being delivered entirely in digital rather than hard copy form in order to make the estimates more widely available to the public and to provide the data in a broader and more accessible range of formats.

While the primary audience for precipitation frequency estimates consists of civil engineers, hydrologists, agriculture interests, environmental planners, and floodplain managers, the general public also maintains a general interest in the estimates. The presentation formats accessible through the Precipitation Frequency Data Server include:

- the Atlases and associated documentation of the underlying development methodology, including tables and maps, in Portable Document Format (PDF),
- downloadable tables and graphs of precipitation frequency estimates at any user-selected location (figure 2), and
- grids in a variety of formats including Federally mandated GIS formats.

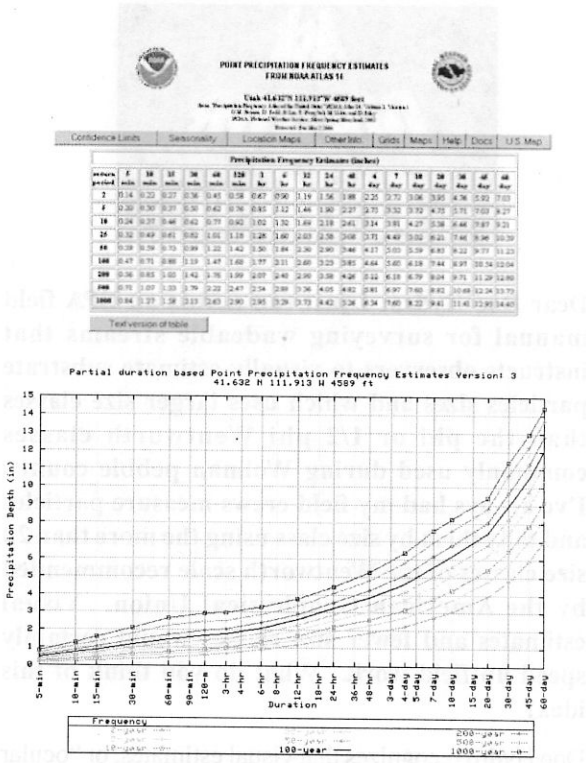

Figure 2. Example tabular and graphical displays of precipitation frequency estimates for a location in Utah.

The Precipitation Frequency Data Server is maintained by the NWS Hydrometeorological Design Studies Center in Silver Springs, Maryland. The Center's major function is to prepare national standards for Precipitation Frequency (PF) and Probable Maximum Precipitation (PMP).

The homepage for the Center is located at http://www.nws.noaa.gov/oh/hdsc/index.html and the Precipitation Frequency Data Server can be found at http://hdsc.nws.noaa.gov/hdsc/pfds/.

The site is fairly self-explanatory, but a user's guide for the Precipitation Frequency Data Server and other older precipitation frequency publications are also directly accessible from the server. Check it out and bookmark it for future reference when you may need rainfall frequency data.

Ask DOCTOR Hydro

Dear Doc Hydro: I just ran across an EPA field manual for surveying wadeable streams that instructs observers to visually estimate substrate particles sizes and which uses larger size classes than the phi or 1/2 phi Wentworth classes commonly used during Wolman pebble counts. I've always had my field crews measure particles and tally them by size class using the more than 20 size classes of the Wentworth scale recommended by the American Geophysical Union. Visual estimates and fewer size classes would certainly speed up field work. What do you think of this idea?

Doc Hydro recognizes that visual estimates, or "ocular assessments," have been widely used in the past by biologists to estimate the sizes of substrate particles. Typically, these estimates placed particles into broad size classes, for example, "1-to-3-inch gravel" thereby making it difficult to directly compare this data with more precise particle size distribution typically collected by geomorphologists and engineers based on the Wentworth scale. Despite the continued use of visual estimates, I'm unaware of any comprehensive published studies demonstrating that visual estimates are reproducible among different observers.

Since there's no substitute for real data, Doc Hydro decided to perform a little experiment comparing visual and measurement techniques on a 100 particle sample of river rocks. Although there are only 5 replications of each technique and statistically this is less rigorous than one might wish, the results provide valuable insight into differences among the various techniques.

The 100 particles were sampled along a grid located on a large river bar using the Wolman method. The round river rocks were placed into buckets and brought to the lab for easy measurement. In the lab, Doc Hydro carefully identified the intermediate axis of each rock and measured the diameter as precisely as possible using a caliper. The size distribution from this measurement is considered to represent the "true" size distribution of the particles. The caliper measured d_{16}, d_{50}, and d_{84} sizes were 20, 42, and 83 mm, respectively.

Five different observers, ranging from very experienced to inexperienced, were asked to visually estimate the size of each particle and tally them into one of the EPA EMAP size classes (table 1). Observers were provided with a marble and a tennis ball to help them calibrate their eyes.

Next each observer measured all of the particles using a FISP SA-97 Hand-held Particle-size Analyzer, commonly called a gravel-O-meter or gravel template, and tallied particles by standard 1/2 phi Wentworth size classes. The procedure was repeated a third time using a ruler and particles were again tallied by Wentworth size classes.

Figure 1 shows the plotted particle size distributions for all of the observers for each of the measurement techniques. Table 2 shows the average d_{16}, d_{50}, and d_{84} values of the 5 observers obtained from the plotted particles size distribution curves.

It's apparent from the plots that all of the observers had

EPA EMAP Class Name	EMAP Size Classes (mm)	EMAp Description
Fine Gravel	> 2 - 16	Lady bug to marble size
Course Gravel	>16 - 64	Marble to tennis ball size
Cobbles	>64 - 250	Tennis ball to basketball size
Boulders	>250 -4,000	Basketball to car size
Bedrock	>4,000	Rock bigger than a car

Table 1. Visual estimate size classes and descriptors. From: Peck, D.V., J.M. Lazorchak, and D.J. Klemm (editors). Unpublished draft. Environmental Monitoring and Assessment Program-Surface Waters: Western Pilot Study Field Operations Manual for Wadeable Streams. EPA/XXX/X-XX/XXXX. U.S. Environmental Protection Agency, Washington, D.C., April 2001.

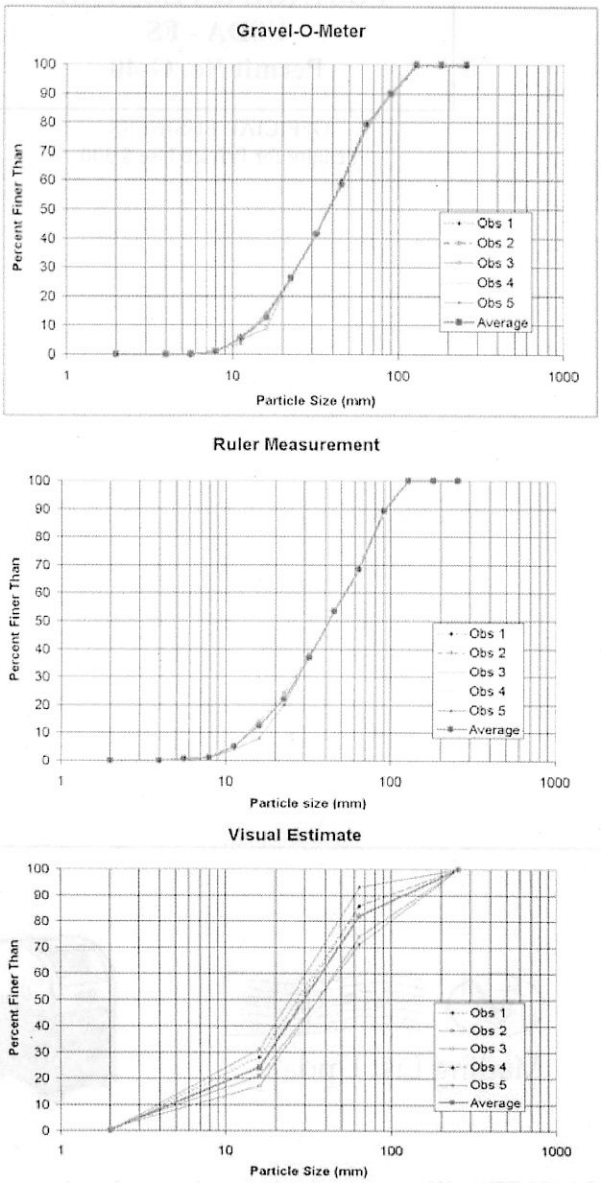

Figure 1. Plots of the particle size distributions for each of the 5 observers using the gravel-O-meter, a ruler measurement, and visual estimates. The size distributions from gravel-O-meter and ruler measurements are almost identical and tend to plot very tightly on top of each other. The particle size distributions for the visual estimates show considerably more scatter than the direct measurement techniques. For example, d_{50} values of visual estimates range from 31 to 44 mm for the 5 observers while the observers using the ruler and the gravel-O-meter consistently measured the d_{50} as 41 and 38 mm, respectively.

	d_{16}	d_{50}	d_{84}
Gravel-O--Meter	17.2 mm (3%)	38.0 mm (0%)	72.4 mm (1%)
Ruler Measured	17.6 mm (8%)	41.0 mm (0%)	81.2 mm (1%)
Visual Estimate	9.2 mm (34%)	30.4 mm (18%)	80 mm (33%)

Table 2. Average d_{16}, d_{50}, and d_{84} values from 5 observers using 3 different measurement techniques. Values in parenthesis are the coefficients of variation of the 5 observers.

very similar results using the gravel-O-meter and rulers since the particle size distribution curves plot almost directly on top of each other. In contrast, distribution curves from the visual estimates show considerable variability among observers.

Table 2 confirms this result with the gravel-O-meter and the ruler having almost identical d_{16} and d_{50} values and little variability (coefficients of variation zero to 8 percent). In contrast, visual estimated sizes are smaller and have coefficients of variation ranging from 18 to 34 percent indicating that different observers obtained a wide range of different size distribution statistics. Using a chi-square statistic to compare d_{16}, d_{50}, and d_{84} values to the caliper measured true values, only the visually estimated d_{16} size of 9.2 mm is statistically different from the others at the 95% confidence level.

Doc Hydro believes that the major expenditure of any field effort is getting to the field site. Once there, do the best technical job possible and take the time needed to do a quality job. Therefore, pick up the particles and measure them as accurately as you can and tally them using the standard Wentworth size classes so that the data are comparable among disciplines. Since different observers will most likely be collecting your data each year, it's especially important to reduce observer variability to the maximum extent possible. Doc Hydro recommends use of a gravel-O-meter because these measurements are less prone to observer error in identifying the intermediate axis and produce data that is comparable to sieve measurements.

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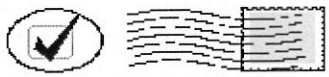

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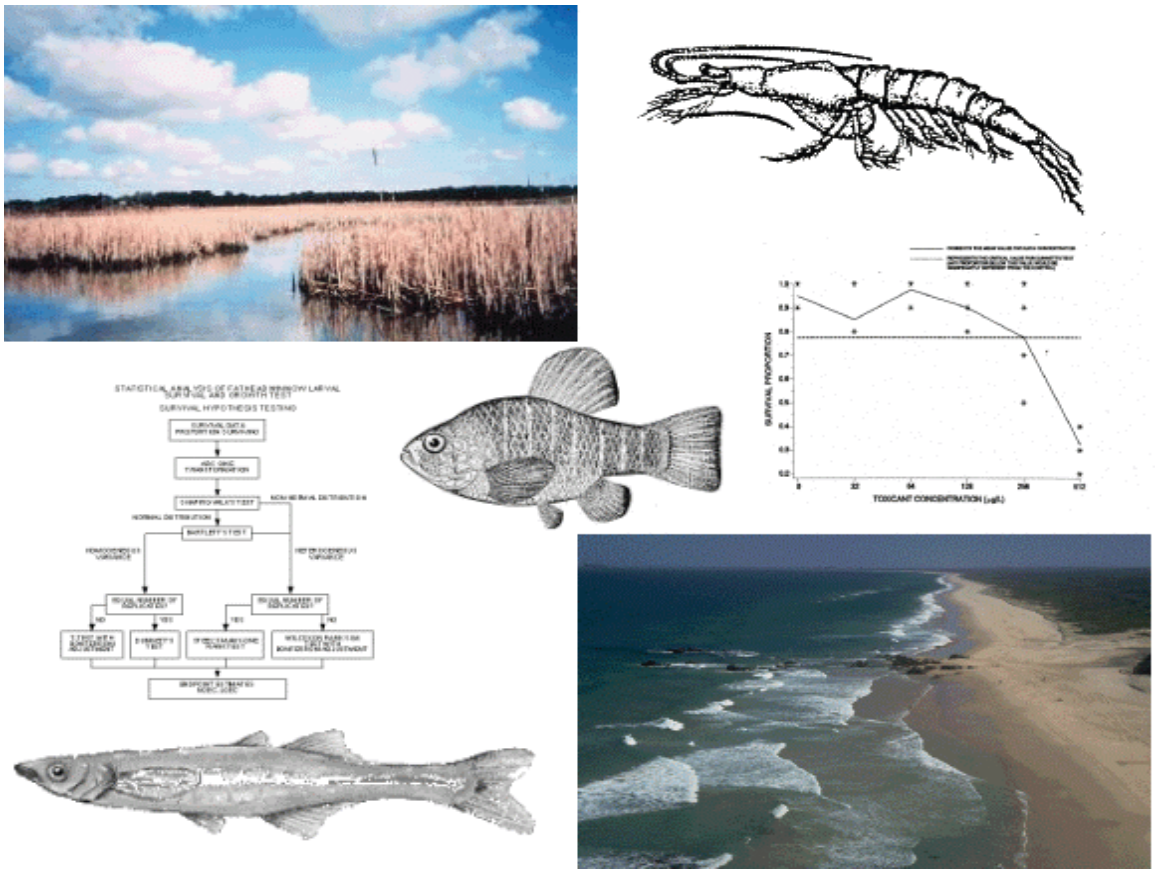
Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms

Third Edition

October 2002



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SECTION 1

INTRODUCTION

1.1 This manual describes chronic toxicity tests for use in the National Pollutant Discharge Elimination System (NPDES) Permits Program to identify effluents and receiving waters containing toxic materials in chronically toxic concentrations. With the exception of the Red Macroalga, *Champia parvula*, Reproduction Test Method 1009.0, the methods included in this manual are referenced in Table IA, 40 CFR Part 136 regulations and, therefore, constitute approved methods for chronic toxicity tests. They are also suitable for determining the toxicity of specific compounds contained in discharges. The tests may be conducted in a central laboratory or on-site, by the regulatory agency or the permittee. The Red Macroalga, *Champia parvula*, Reproduction Test Method 1009.0 is not listed at 40 CFR Part 136 for nationwide use.

1.2 The data are used for NPDES permits development and to determine compliance with permit toxicity limits. Data can also be used to predict potential acute and chronic toxicity in the receiving water, based on the LC50, NOEC, IC25, or IC50 (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis) and appropriate dilution, application, and persistence factors. The tests are performed as a part of self-monitoring permit requirements, compliance biomonitoring inspections, toxics sampling inspections, and special investigations. Data from chronic toxicity tests performed as part of permit requirements are evaluated during compliance evaluation inspections and performance audit inspections.

1.3 Modifications of these tests are also used in toxicity reduction evaluations and toxicity identification evaluations to identify the toxic components of an effluent, to aid in the development and implementation of toxicity reduction plans, and to compare and control the effectiveness of various treatment technologies for a given type of industry, irrespective of the receiving water (USEPA, 1988c; USEPA, 1989b; USEPA, 1989c; USEPA, 1989d; USEPA, 1989e; USEPA, 1991a; USEPA, 1991b; and USEPA, 1992).

1.4 This methods manual serves as a companion to the acute toxicity test methods for freshwater and marine organisms (USEPA, 2002a), the short-term chronic toxicity test methods for freshwater organisms (USEPA, 2002b), and the manual for evaluation of laboratories performing aquatic toxicity tests (USEPA, 1991c). In 2002, EPA revised previous editions of each of the three methods manuals (USEPA, 1993a; USEPA, 1994a; USEPA, 1994b).

1.5 Guidance for the implementation of toxicity tests in the NPDES program is provided in the Technical Support Document for Water Quality-Based Toxics Control (USEPA, 1991a).

1.6 These marine and estuarine short-term toxicity tests are similar to those developed for the freshwater organisms to evaluate the toxicity of effluents discharged to estuarine and coastal marine waters under the NPDES permit program. Methods are presented in this manual for five species from four phylogenetic groups. Five of the six methods were developed and extensively field tested by Environmental Research Laboratory-Narragansett (ERL-N). The methods vary in duration from one hour and 20 minutes to nine days.

1.7 The five species for which toxicity test methods are provided are: the sheepshead minnow, *Cyprinodon variegatus*; the inland silverside, *Menidia beryllina*; the mysid, *Mysidopsis bahia*; the sea urchin, *Arbacia punctulata*; and the red macroalga, *Champia parvula*.

1.7.1 Four of the methods incorporate the chronic endpoints of growth or reproduction (or both) in addition to lethality. The sheepshead minnow 9-day embryo-larval survival and teratogenicity test incorporates teratogenic effects in addition to lethality. The sea urchin sperm cell test uses fertilization as an endpoint and has the advantage of an extremely short exposure period (1 h and 20 min).

1.8 The validity of the marine/estuarine methods in predicting adverse ecological impacts of toxic discharges was

demonstrated in field studies (USEPA, 1986d).

1.9 The use of any test species or test conditions other than those described in the methods summary tables in this manual shall be subject to application and approval of alternate test procedures under 40 CFR 136.4 and 40 CFR 136.5.

1.10 These methods are restricted to use by or under the supervision of analysts experienced in the use or conduct of aquatic toxicity testing and the interpretation of data from aquatic toxicity testing. Each analyst must demonstrate the ability to generate acceptable test results with these methods using the procedures described in this methods manual.

1.11 The manual was prepared in the established EMSL-Cincinnati format (USEPA, 1983).

SECTION 2

SHORT-TERM METHODS FOR ESTIMATING CHRONIC TOXICITY

2.1 INTRODUCTION

2.1.1 The objective of aquatic toxicity tests with effluents or pure compounds is to estimate the "safe" or "no-effect" concentration of these substances, which is defined as the concentration which will permit normal propagation of fish and other aquatic life in the receiving waters. The endpoints that have been considered in tests to determine the adverse effects of toxicants include death and survival, decreased reproduction and growth, locomotor activity, gill ventilation rate, heart rate, blood chemistry, histopathology, enzyme activity, olfactory function, and terata. Since it is not feasible to detect and/or measure all of these (and other possible) effects of toxic substances on a routine basis, observations in toxicity tests generally have been limited to only a few effects, such as mortality, growth, and reproduction.

2.1.2 Acute lethality is an obvious and easily observed effect which accounts for its wide use in the early period of evaluation of the toxicity of pure compounds and complex effluents. The results of these tests were usually expressed as the concentration lethal to 50% of the test organisms (LC50) over relatively short exposure periods (one-to-four days).

2.1.3 As exposure periods of acute tests were lengthened, the LC50 and lethal threshold concentration were observed to decline for many compounds. By lengthening the tests to include one or more complete life cycles and observing the more subtle effects of the toxicants, such as a reduction in growth and reproduction, more accurate, direct, estimates of the threshold or safe concentration of the toxicant could be obtained. However, laboratory life cycle tests may not accurately estimate the "safe" concentration of toxicants because they are conducted with a limited number of species under highly controlled, steady state conditions, and the results do not include the effects of the stresses to which the organisms would ordinarily be exposed in the natural environment.

2.1.4 An early published account of a full life cycle, fish toxicity test was that of Mount and Stephan (1967). In this study, fathead minnows, *Pimephales promelas*, were exposed to a graded series of pesticide concentrations throughout their life cycle, and the effects of the toxicant on survival, growth, and reproduction were measured and evaluated. This work was soon followed by full life cycle tests using other toxicants and fish species.

2.1.5 McKim (1977) evaluated the data from 56 full life cycle tests, 32 of which used the fathead minnow, *Pimephales promelas*, and concluded that the embryo-larval and early juvenile life stages were the most sensitive stages. He proposed the use of partial life cycle toxicity tests with the early life stages (ELS) of fish to establish water quality criteria.

2.1.6 Macek and Sleight (1977) found that exposure of critical life stages of fish to toxicants provides estimates of chronically safe concentrations remarkably similar to those derived from full life cycle toxicity tests. They reported that "for a great majority of toxicants, the concentration which will not be acutely toxic to the most sensitive life stages is the chronically safe concentration for fish, and that the most sensitive life stages are the embryos and fry." Critical life stage exposure was considered to be exposure of the embryos during most, preferably all, of the embryogenic (incubation) period, and exposure of the fry for 30 days post-hatch for warm water fish with embryogenic periods ranging from one-to-fourteen days, and for 60 days post-hatch for fish with longer embryogenic periods. They concluded that in the majority of cases, the maximum acceptable toxicant concentration (MATC) could be estimated from the results of exposure of the embryos during incubation, and the larvae for 30 days post-hatch.

2.1.7 Because of the high cost of full life-cycle fish toxicity tests and the emerging consensus that the ELS test data usually would be adequate for estimating chronically safe concentrations, there was a rapid shift by aquatic toxicologists to 30- to 90-day ELS toxicity tests for estimating chronically safe concentrations in the late 1970s. In

1980, USEPA adopted the policy that ELS test data could be used in establishing water quality criteria if data from full life-cycle tests were not available (USEPA, 1980a).

2.1.8 Published reports of the results of ELS tests indicate that the relative sensitivity of growth and survival as endpoints may be species dependent, toxicant dependent, or both. Ward and Parrish (1980) examined the literature on ELS tests that used embryos and juveniles of the sheepshead minnow, *Cyprinodon variegatus*, and found that growth was not a statistically sensitive indicator of toxicity in 16 of 18 tests. They suggested that the ELS tests be shortened to 14 days posthatch and that growth be eliminated as an indicator of toxic effects.

2.1.9 In a review of the literature on 173 fish full life-cycle and ELS tests performed to determine the chronically safe concentrations of a wide variety of toxicants, such as metals, pesticides, organics, inorganics, detergents, and complex effluents, Woltering (1984) found that at the lowest effect concentration, significant reductions were observed in fry survival in 57%, fry growth in 36%, and egg hatchability in 19% of the tests. He also found that fry survival and growth were very often equally sensitive, and concluded that the growth response could be deleted from routine application of the ELS tests. The net result would be a significant reduction in the duration and cost of screening tests with no appreciable impact on estimating MATCs for chemical hazard assessments. Benoit et al. (1982), however, found larval growth to be the most significant measure of effect and survival to be equally or less sensitive than growth in early life-stage tests with four organic chemicals.

2.1.10 Efforts to further reduce the length of partial life-cycle toxicity tests for fish without compromising their predictive value have resulted in the development of an eight-day, embryo-larval survival and teratogenicity test for fish and other aquatic vertebrates (USEPA, 1981; Birge et al., 1985), and a seven-day larval survival and growth test (Norberg and Mount, 1985).

2.1.11 The similarity of estimates of chronically safe concentrations of toxicants derived from short-term, embryo-larval survival and teratogenicity tests to those derived from full life-cycle tests has been demonstrated by Birge et al. (1981), Birge and Cassidy (1983), and Birge et al. (1985).

2.1.12 Use of a seven-day, fathead minnow, *Pimephales promelas*, larval survival and growth test was first proposed by Norberg and Mount at the 1983 annual meeting of the Society for Environmental Toxicology and Chemistry (Norberg and Mount, 1983). This test was subsequently used by Mount and associates in field demonstrations at Lima, Ohio (USEPA, 1984), and at many other locations (USEPA, 1985c, USEPA, 1985d; USEPA, 1985e; USEPA, 1986a; USEPA, 1986b; USEPA, 1986c; USEPA, 1986d). Growth was frequently found to be more sensitive than survival in determining the effects of complex effluents.

2.1.13 Norberg and Mount (1985) performed three single toxicant fathead minnow larval growth tests with zinc, copper, and DURSBAN®, using dilution water from Lake Superior. The results were comparable to, and had confidence intervals that overlapped with, chronic values reported in the literature for both ELS and full life-cycle tests.

2.1.14 USEPA (1987b) and USEPA (1987c) adapted the fathead minnow larval growth and survival test for use with the sheepshead minnow and the inland silverside, respectively. When daily renewal 7-day sheepshead minnow larval growth and survival tests and 28-day ELS tests were performed with industrial and municipal effluents, growth was more sensitive than survival in seven out of 12 larval growth and survival tests, equally sensitive in four tests, and less sensitive in only one test. In four cases, the ELS test may have been three to 10 times more sensitive to effluents than the larval growth and survival test. In tests using copper, the No Observable Effect Concentrations (NOECs) were the same for both types of test, and growth was the most sensitive endpoint for both. In a four laboratory comparison, six of seven tests produced identical NOECs for survival and growth (USEPA, 1987a). Data indicate that the inland silverside is at least equally sensitive or more sensitive to effluents and single compounds than the sheepshead minnow, and can be tested over a wider salinity range, 5-30 ‰ (USEPA, 1987a).

2.1.15 Lussier et al. (1985) and USEPA (1987e) determined that survival and growth are often as sensitive as reproduction in 28-day life-cycle tests with the mysid, *Mysidopsis bahia*.

2.1.16 Nacci and Jackim (1985) and USEPA (1987g) compared the results from the sea urchin fertilization test, using organic compounds, with results from acute toxicity tests using the freshwater organisms, fathead minnows, *Pimphales promelas*, and *Daphnia magna*. The test was also compared to acute toxicity tests using Atlantic silverside, *Menidia menidia*, and the mysid, *Mysidopsis bahia*, and five metals. For six of the eight organic compounds, the results of the fertilization test and the acute toxicity test correlated well ($r^2 = 0.85$). However, the results of the fertilization test with the five metals did not correlate well with the results from the acute tests.

2.1.17 USEPA (1987f) evaluated two industrial effluents containing heavy metals, five industrial effluents containing organic chemicals (including dyes and pesticides), and 15 domestic wastewaters using the two-day red macroalga, *Champia parvula*, sexual reproduction test. Nine single compounds were used to compare the effects on sexual reproduction using a two-week exposure and a two-day exposure. For six of the nine compounds tested, the chronic values were the same for both tests.

2.1.18 The use of short-term toxicity tests in the NPDES Program is especially attractive because they provide a more direct estimate of the safe concentrations of effluents in receiving waters than was provided by acute toxicity tests, at an only slightly increased level of effort, compared to the fish full life-cycle chronic and 28-day ELS tests and the 28-day mysid life-cycle test.

2.2 TYPES OF TESTS

2.2.1 The selection of the test type will depend on the NPDES permit requirements, the objectives of the test, the available resources, the requirements of the test organisms, and effluent characteristics such as fluctuations in effluent toxicity.

2.2.2 Effluent chronic toxicity is generally measured using a multi-concentration, or definitive test, consisting of a control and a minimum of five effluent concentrations. The tests are designed to provide dose-response information, expressed as the percent effluent concentration that affects the hatchability, gross morphological abnormalities, survival, growth, and/or reproduction within the prescribed period of time (one hour and 20 minutes to nine days). The results of the tests are expressed in terms of either the highest concentration that has no statistically significant observed effect on those responses when compared to the controls or the estimated concentration that causes a specified percent reduction in responses versus the controls.

2.2.3 Use of pass/fail tests consisting of a single effluent concentration (e.g., the receiving water concentration or RWC) and a control **is not recommended**. If the NPDES permit has a whole effluent toxicity limit for acute toxicity at the RWC, it is prudent to use that permit limit as the midpoint of a series of five effluent concentrations. This will ensure that there is sufficient information on the dose-response relationship. For example, the effluent concentrations utilized in a test may be: (1) 100% effluent, (2) (RWC + 100)/2, (3) RWC, (4) RWC/2, and (5) RWC/4. More specifically, if the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

2.2.4 Receiving (ambient) water toxicity tests commonly employ two treatments, a control and the undiluted receiving water, but may also consist of a series of receiving water dilutions.

2.2.5 A negative result from a chronic toxicity test does not preclude the presence of toxicity. Also, because of the potential temporal variability in the toxicity of effluents, a negative test result with a particular sample does not preclude the possibility that samples collected at some other time might exhibit chronic toxicity.

2.2.6 The frequency with which chronic toxicity tests are conducted under a given NPDES permit is determined by the regulatory agency on the basis of factors such as the variability and degree of toxicity of the waste, production schedules, and process changes.

2.2.7 Tests recommended for use in this methods manual may be static non-renewal or static renewal. Individual methods specify which static type of test is to be conducted.

2.3 STATIC TESTS

2.3.1 Static non-renewal tests - The test organisms are exposed to the same test solution for the duration of the test.

2.3.2 Static-renewal tests - The test organisms are exposed to a fresh solution of the same concentration of sample every 24 h or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers.

2.4 ADVANTAGES AND DISADVANTAGES OF TOXICITY TEST TYPES

2.4.1 STATIC NON-RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Simple and inexpensive
2. Very cost effective in determining compliance with permit conditions.
3. Limited resources (space, manpower, equipment) required; would permit staff to perform many more tests in the same amount of time.
4. Smaller volume of effluent required than for static renewal or flow-through tests.

Disadvantages:

1. Dissolved oxygen (DO) depletion may result from high chemical oxygen demand (COD), biological oxygen demand (BOD), or metabolic wastes.
2. Possible loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Generally less sensitive than static renewal because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.4.2 STATIC RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Reduced possibility of DO depletion from high COD and/or BOD, or ill effects from metabolic wastes from organisms in the test solutions.
2. Reduced possibility of loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Test organisms that rapidly deplete energy reserves are fed when the test solutions are renewed, and are maintained in a healthier state.

Disadvantages:

1. Require greater volume of effluent than non-renewal tests.
2. Generally less chance of temporal variations in waste properties.

SECTION 3

HEALTH AND SAFETY

3.1 GENERAL PRECAUTIONS

3.1.1 Each laboratory should develop and maintain an effective health and safety program, requiring an ongoing commitment by the laboratory management and includes: (1) a safety officer with the responsibility and authority to develop and maintain a safety program; (2) the preparation of a formal, written, health and safety plan, which is provided to the laboratory staff; (3) an ongoing training program on laboratory safety; and (4) regularly scheduled, documented, safety inspections.

3.1.2 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to a lack of oxygen or the presence of noxious gases.

3.1.3 Prior to sample collection and laboratory work, personnel should determine that all necessary safety equipment and materials have been obtained and are in good condition.

3.1.4 Guidelines for the handling and disposal of hazardous materials must be strictly followed.

3.2 SAFETY EQUIPMENT

3.2.1 PERSONAL SAFETY GEAR

3.2.1.1 Personnel must use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes. Plastic netting on glass beakers, flasks and other glassware minimizes breakage and subsequent shattering of the glass.

3.2.2 LABORATORY SAFETY EQUIPMENT

3.2.2.1 Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, chemical spill clean-up kits, and eye fountains.

3.2.2.2 Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

3.3 GENERAL LABORATORY AND FIELD OPERATIONS

3.3.1 Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see safety manuals listed in Section 3, Health and Safety, Subsection 3.5). It is recommended that personnel collecting samples and performing toxicity tests should not work alone.

3.3.2 Because the chemical composition of effluents is usually only poorly known, they should be considered as potential health hazards, and exposure to them should be minimized. Fume and canopy hoods over the toxicity test areas must be used whenever possible.

3.3.3 It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

3.3.4 All containers should be adequately labeled to indicate their contents.

3.3.5 Staff should be familiar with safety guidelines on Material Safety Data Sheets for reagents and other chemicals purchased from suppliers. Incompatible materials should not be stored together. Good housekeeping contributes to safety and reliable results.

3.3.6 Strong acids and volatile organic solvents employed in glassware cleaning must be used in a fume hood or under an exhaust canopy over the work area.

3.3.7 Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.8 Mobile laboratories should be properly grounded to protect against electrical shock.

3.4 DISEASE PREVENTION

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, polio, and hepatitis B.

3.5 SAFETY MANUALS

3.5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general safety manuals, including USEPA (1986e), and Walters and Jameson (1984).

3.6 WASTE DISPOSAL

3.6.1 Wastes generated during toxicity testing must be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, state and Federal rules and regulations. It is extremely important that these rules and regulations be known, understood, and complied with by all persons responsible for, or otherwise involved in, performing toxicity testing activities. Local fire officials should be notified of any potentially hazardous conditions.

SECTION 4

QUALITY ASSURANCE

4.1 INTRODUCTION

4.1.1 Development and maintenance of a toxicity test laboratory quality assurance (QA) program (USEPA, 1991b) requires an ongoing commitment by laboratory management. Each toxicity test laboratory should (1) appoint a quality assurance officer with the responsibility and authority to develop and maintain a QA program, (2) prepare a quality assurance plan with stated data quality objectives (DQOs), (3) prepare written descriptions of laboratory standard operating procedures (SOPs) for culturing, toxicity testing, instrument calibration, sample chain-of-custody procedures, laboratory sample tracking system, glassware cleaning, etc., and (4) provide an adequate, qualified technical staff for culturing and toxicity testing the organisms, and suitable space and equipment to assure reliable data.

4.1.2 QA practices for toxicity testing laboratories must address all activities that affect the quality of the final effluent toxicity data, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation.

4.1.3 Quality control practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance and general guidance on good laboratory practices and laboratory evaluation related to toxicity testing, see FDA (1978); USEPA (1979d); USEPA (1980b); USEPA (1980c); USEPA (1991c); DeWoskin (1984); and Taylor (1987).

4.1.4 Guidelines for the evaluation of laboratory performing toxicity tests and laboratory evaluation criteria are found in USEPA (1991c).

4.2 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.2.1 Separate test organism culturing and toxicity testing areas should be provided to avoid possible loss of cultures due to cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation areas into organism culturing or testing areas, and from testing and sample preparation areas into culture rooms.

4.2.2 Laboratory and toxicity test temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 5, Facilities, Equipment, and Supplies; and specific toxicity test method).

4.3 TEST ORGANISMS

4.3.1 The test organisms used in the procedures described in this manual are the sheepshead minnow, *Cyprinodon variegatus*; the inland silverside, *Menidia beryllina*; the mysid, *Mysidopsis bahia*; the sea urchin, *Arbacia punctulata*; and the red macroalga, *Champia parvula*. The organisms used should be disease-free and appear healthy, behave normally, feed well, and have low mortality in cultures, during holding, and in test control. Test organisms should be positively identified to species (see Section 6, Test Organisms).

4.4 LABORATORY WATER USED FOR CULTURING AND TEST DILUTION WATER

4.4.1 The quality of water used for test organism culturing and for dilution water used in toxicity tests is extremely important. Water for these two uses should come from the same source. The dilution water used in effluent toxicity

tests will depend on the objectives of the study and logistical constraints, as discussed in Section 7, Dilution Water. The dilution water used in the toxicity tests may be natural seawater, hypersaline brine (100‰) prepared from natural seawater, or artificial seawater prepared from commercial sea salts, such as FORTY FATHOMS® or HW MARINEMIX®, if recommended in the method. GP2 synthetic seawater, made from reagent grade chemical salts (30‰) in conjunction with natural seawater, may also be used if recommended. Hypersaline brine and artificial seawater can be used with *Champia parvula* only if they are accompanied by at least 50% natural seawater. Types of water are discussed in Section 5, Facilities, Equipment, and Supplies. Water used for culturing and test dilution water should be analyzed for toxic metals and organics at least annually or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. The concentration of the metals, Al, As, Cr, Co, Cu, Fe, Pb, Ni, Zn, expressed as total metal, should not exceed 1 µg/L each, and Cd, Hg, and Ag, expressed as total metal, should not exceed 100 ng/L each. Total organochlorine pesticides plus PCBs should be less than 50 ng/L (APHA, 1992). Pesticide concentrations should not exceed USEPA's National Ambient Water Quality chronic criteria values where available.

4.5 EFFLUENT AND RECEIVING WATER SAMPLING AND HANDLING

4.5.1 Sample holding times and temperatures of effluent samples collected for on-site and off-site testing must conform to conditions described in Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

4.6 TEST CONDITIONS

4.6.1 Water temperature and salinity should be maintained within the limits specified for each test. The temperature of test solutions must be measured by placing the thermometer or probe directly into the test solutions, or by placing the thermometer in equivalent volumes of water in surrogate vessels positioned at appropriate locations among the test vessels. Temperature should be recorded continuously in at least one vessel during the duration of each test. Test solution temperatures should be maintained within the limits specified for each test. DO concentrations and pH should be checked at the beginning of the test and daily throughout the test period.

4.7 QUALITY OF TEST ORGANISMS

4.7.1 The health of test organisms is primarily assessed by the performance (survival, growth, and/or reproduction) of organisms in control treatments of individual tests. The health and sensitivity of test organisms is also assessed by reference toxicant testing. In addition to documenting the sensitivity and health of test organisms, reference toxicant testing is used to initially demonstrate acceptable laboratory performance (Subsection 4.15) and to document ongoing laboratory performance (Subsection 4.16).

4.7.2 Regardless of the source of test organisms (in-house cultures or purchased from external suppliers), the testing laboratory must perform at least one acceptable reference toxicant test per month for each toxicity test method conducted in that month (Subsection 4.16). If a test method is conducted only monthly, or less frequently, a reference toxicant test must be performed concurrently with each effluent toxicity test.

4.7.3 When acute or short-term chronic toxicity tests are performed with effluents or receiving waters using test organisms obtained from outside the test laboratory, concurrent toxicity tests of the same type must be performed with a reference toxicant, unless the test organism supplier provides control chart data from at least the last five monthly short-term chronic toxicity tests using the same reference toxicant and test conditions (see Section 6, Test Organisms).

4.7.4 The supplier should certify the species identification of the test organisms, and provide the taxonomic reference (citation and page) or name(s) of the taxonomic expert(s) consulted.

4.7.5 If a routine reference toxicant test fails to meet test acceptability criteria, then the reference toxicant test must be immediately repeated.

4.8 FOOD QUALITY

4.8.1 The nutritional quality of the food used in culturing and testing fish and invertebrates is an important factor in the quality of the toxicity test data. This is especially true for the unsaturated fatty acid content of brine shrimp nauplii, *Artemia*. Problems with the nutritional suitability of the food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. *Artemia* cysts and other foods must be obtained as described in Section 5, Facilities, Equipment, and Supplies.

4.8.2 Problems with the nutritional suitability of food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. If a batch of food is suspected to be defective, the performance of organisms fed with the new food can be compared with the performance of organisms fed with a food of known quality in side-by-side tests. If the food is used for culturing, its suitability should be determined using a short-term chronic test which will determine the affect of food quality on growth or reproduction of each of the relevant test species in culture, using four replicates with each food source. Where applicable, foods used only in chronic toxicity tests can be compared with a food of known quality in side-by-side, multi-concentration chronic tests, using the reference toxicant regularly employed in the laboratory QA program.

4.8.3 New batches of food used in culturing and testing should be analyzed for toxic organics and metals or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. If the concentration of total organochlorine pesticides exceeds 0.15 µg/g wet weight, or the concentration of total organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight, or toxic metals (Al, As, Cr, Cd, Cu, Pb, Ni, Zn, expressed as total metal) exceed 20 µg/g wet weight, the food should not be used (for analytical methods, see AOAC, 1990; and USDA, 1989).

4.84 For foods (e.g., YCT) which are used to culture and test organisms, the quality of the food should meet the requirements for the laboratory water used for culturing and test dilution water as described in Section 4.4 above.

4.9 ACCEPTABILITY OF CHRONIC TOXICITY TESTS

4.9.1 The results of the sheepshead minnow, *Cyprinodon variegatus*, inland silverside, *Menidia beryllina*, or mysid, *Mysidopsis bahia*, tests are acceptable if survival in the controls is 80% or greater. The sea urchin, *Arbacia punctulata*, test requires control egg fertilization equal to or exceeding 70%. However, greater than 90% fertilization may result in masking toxic responses. The red macroalga, *Champia parvula*, test is acceptable if survival is 100%, and the mean number of cystocarps per plant should equal or exceed 10. If the sheepshead minnow, *Cyprindon variegatus*, larval survival and growth test is begun with less-than-24-h old larvae, the mean dry weight of the surviving larvae in the control chambers at the end of the test must equal or exceed 0.60 mg, if the weights are determined immediately, or 0.50 mg if the larvae are preserved in a 4% formalin or 70% ethanol solution. If the inland silverside, *Menidia beryllina*, larval survival and growth test is begun with larvae seven days old, the mean dry weight of the surviving larvae in the control chambers at the end of the test must equal or exceed 0.50 mg, if the weights are determined immediately, or 0.43 mg if the larvae are preserved in a 4% formalin or 70% ethanol solution. The mean mysid dry weight of survivors must be at least 0.20 mg. Automatic or hourly feeding will generally provide control mysids with a dry weight of 0.30 mg. At least 50% of the females should bear eggs at the end of the test, but mysid fecundity is not a factor in test acceptability. However, fecundity must equal or exceed 50% to be used as an endpoint in the test. If these criteria are not met, the test must be repeated.

4.9.2 An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test conditions and test acceptability criteria summaries). The acceptability of the test will depend on the experience and professional judgment of the laboratory investigator and the reviewing staff of the regulatory authority. Any deviation from test specifications must be noted when reporting data from a test.

4.10 ANALYTICAL METHODS

4.10.1 Routine chemical and physical analyses for culture and dilution water, food, and test solutions must include established quality assurance practices outlined in USEPA methods manuals (USEPA, 1979a and USEPA, 1979b).

4.10.2 Reagent containers should be dated and catalogued when received from the supplier, and the shelf life should not be exceeded. Also, working solutions should be dated when prepared, and the recommended shelf life should be observed.

4.11 CALIBRATION AND STANDARDIZATION

4.11.1 Instruments used for routine measurements of chemical and physical parameters, such as pH, DO, temperature, conductivity, and salinity, must be calibrated and standardized according to instrument manufacturers procedures as indicated in the general section on quality assurance (see USEPA Methods 150.1, 360.1, 170.1, and 120.1 in USEPA, 1979b). Calibration data are recorded in a permanent log book.

4.11.2 Wet chemical methods used to measure hardness, alkalinity, and total residual chlorine, must be standardized prior to use each day according to the procedures for those specific USEPA methods (see USEPA Methods 130.2 and 310.1 in USEPA, 1979b).

4.12 REPLICATION AND TEST SENSITIVITY

4.12.1 The sensitivity of the tests will depend in part on the number of replicates per concentration, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data.

4.13 VARIABILITY IN TOXICITY TEST RESULTS

4.13.1 Factors which can affect test success and precision include: (1) the experience and skill of the laboratory analyst; (2) test organism age, condition, and sensitivity; (3) dilution water quality; (4) temperature control; (5) and the quality and quantity of food provided. The results will depend upon the species used and the strain or source of the test organisms, and test conditions, such as temperature, DO, food, and water quality. The repeatability or precision of toxicity tests is also a function of the number of test organisms used at each toxicant concentration. Jensen (1972) discussed the relationship between sample size (number of fish) and the standard error of the test, and considered 20 fish per concentration as optimum for Probit Analysis.

4.14 TEST PRECISION

4.14.1 The ability of the laboratory personnel to obtain consistent, precise results must be demonstrated with reference toxicants before they attempt to measure effluent toxicity. The single-laboratory precision of each type of test to be used in a laboratory should be determined by performing at least five or more tests with a reference toxicant.

4.14.2 Test precision can be estimated by using the same strain of organisms under the same test conditions, and employing a known toxicant, such as a reference toxicant.

4.14.3 Interlaboratory precision data from a 1991 study of chronic toxicity tests using two reference toxicants with the mysid, *Mysidopsis bahia*, and the inland silverside, *Menidia beryllina*, is listed in Table 1. Table 2 shows interlaboratory precision data from a study of three chronic toxicity test methods using effluent, receiving water, and reference toxicant sample types (USEPA, 2001a; USEPA, 2001b). For the *Mysidopsis bahia* and the *Cyprinodon variegatus* test methods, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample was bioassay-grade FORTY FATHOMS®

synthetic seawater spiked with KCl. For the *Menidia beryllina* test method, the effluent sample was an industrial wastewater spiked with $CuSO_4$, the receiving water sample was a natural seawater spiked with $CuSO_4$, and the reference toxicant sample was bioassay-grade FORTY FATHOMS® synthetic seawater spiked with $CuSO_4$. Additional precision data for each of the tests described in this manual are presented in the sections describing the individual test methods.

4.14.4 Additional information on toxicity test precision is provided in the Technical Support Document for Water Quality-based Toxic Control (see pp. 2-4, and 11-15 in USEPA, 1991a).

4.14.5 In cases where the test data are used in Probit Analysis or other point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis), precision can be described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated endpoints from the replicated tests. In cases where the test data are used in the Linear Interpolation Method, precision can be estimated by empirical confidence intervals derived by using the ICPIN Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). However, in cases where the results are reported in terms of the No-Observed-Effect-Concentration (NOEC) and Lowest-Observed-Effect-Concentration (LOEC) (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis), precision can only be described by listing the NOEC-LOEC interval for each test. It is not possible to express precision in terms of a commonly used statistic. However, when all tests of the same toxicant yield the same NOEC-LOEC interval, maximum precision has been attained. The "true" no effect concentration could fall anywhere within the interval, NOEC ± (LOEC minus NOEC).

4.14.6 It should be noted here that the dilution factor selected for a test determines the width of the NOEC-LOEC interval and the inherent maximum precision of the test. As the absolute value of the dilution factor decreases, the width of the NOEC-LOEC interval increases, and the inherent maximum precision of the test decreases. When a dilution factor of 0.3 is used, the NOEC could be considered to have a relative uncertainty as high as ± 300%. With a dilution factor of 0.5, the NOEC could be considered to have a relative variability of ± 100%. As a result of the variability of different dilution factors, **USEPA recommends the use of a ≥ 0.5 dilution factor**. Other factors which can affect test precision include: test organism age, condition, and sensitivity; temperature control; and feeding.

TABLE 1. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 1991: SUMMARY OF RESPONSES USING TWO REFERENCE TOXICANTS[1,2]

Organism	Endpoint	No. Labs	KCl(mg/L)[4]	SD	CV(%)[3]
Mysidopsis bahia	Survival, NOEC	34	NA	NA	NA
	Growth, IC25	26	480	3.47	28.9
	Growth, IC50	22	656	3.17	19.3
	Growth, NOEC	32	NA	NA	NA
	Fecundity, NOEC	25	NA	NA	NA
Organism	Endpoint	No. Labs	Cu(mg/L)[4]	SD	CV(%)[3]
Menidia beryllina	Survival, NOEC	19	NA	NA	NA
	Growth, IC25	13	0.144	1.56	43.5
	Growth, IC50	12	0.180	1.87	41.6
	Growth, NOEC	17	NA	NA	NA

1 From a national study of interlaboratory precision of toxicity test data performed in 1991 by the Environmental Monitoring Systems Laboratory-Cincinnati, U.S. Environmental Protection Agency, Cincinnati, OH 45268. Participants included federal, state, and private laboratories engaged in NPDES permit compliance monitoring.

2 Static renewal test, using 25 ‰ modified GP2 artificial seawater.

3 Percent coefficient of variation = (standard deviation X 100)/mean.

4 Expressed as mean.

TABLE 2. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 2000: PRECISION OF RESPONSES USING EFFLUENT, RECEIVING WATER, AND REFERENCE TOXICANT SAMPLE TYPES[1]

Organism	Endpoint	Number of Tests[2]	CV (%)[3]
Cyprinodon variegatus	Growth, IC25	21	10.5
Menidia beryllina	Growth, IC25	30	43.8
Mysidopsis bahia	Growth, IC25	36	41.3

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 Represents the number of valid tests (i.e., those that met test acceptability criteria) that were used in the analysis of precision. Invalid tests were not used.

3 CVs based on total interlaboratory variability (including both within-laboratory and between-laboratory components of variability) and averaged across sample types. IC25s or IC50s were pooled for all laboratories to calculate the CV for each sample type. The resulting CVs were then averaged across sample types.

4.15 DEMONSTRATING ACCEPTABLE LABORATORY PERFORMANCE

4.15.1 It is a laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicants before it performs toxicity tests with effluents for permit compliance purposes. To meet this requirement, the intralaboratory precision, expressed as percent coefficient of variation (CV%), of each type of test to be used in a laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (i.e., the same test duration, type of dilution water, age of test organisms, feeding, etc.), and same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations.

4.16 DOCUMENTING ONGOING LABORATORY PERFORMANCE

4.16.1 Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month with a reference toxicant for each toxicity test method conducted in the laboratory during that month. For a given test method, successive tests must be performed with the same reference toxicant, at the same concentrations, in the same dilution water, using the same data analysis methods. Precision may vary with the test species, reference toxicant, and type of test. Each laboratory's reference toxicity data will reflect conditions unique to that facility, including dilution water, culturing, and other variables; however, each laboratory's reference toxicity results should reflect good repeatability.

4.16.2 A control chart should be prepared for each combination of reference toxicant, test species, test conditions, and endpoints. Toxicity endpoints from five or six tests are adequate for establishing the control charts. Successive toxicity endpoints (NOECs, IC25s, LC50s, etc.) should be plotted and examined to determine if the results (X_1) are within prescribed limits (Figure 1). The chart should plot logarithm of concentration on the vertical axis against the date of the test or test number on the horizontal axis. The types of control charts illustrated (see USEPA, 1979a) are used to evaluate the cumulative trend of results from a series of samples, thus reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. For endpoints that are point estimates (LC50s and IC25s), the cumulative mean ($\bar{X}$) and upper and lower control limits (± 2S) are re-calculated with each successive test result. Endpoints from hypothesis tests (NOEC, NOAEC) from each test are plotted directly on the control chart. The control limits would consist of one concentration interval above and below the concentration representing the central tendency. After two years of data collection, or a minimum of 20 data points, the control chart should be maintained using only the 20 most recent data points.

4.16.3 Laboratories should compare the calculated CV (i.e., standard deviation / mean) of the IC25 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-2 in USEPA, 2000b). If the calculated CV exceeds the 75th percentile of CVs reported nationally, the laboratory should use the 75th and 90th percentiles to calculate warning and control limits, respectively, and the laboratory should investigate options for reducing variability. Note: Because NOECs can only be a fixed number of discrete values, the mean, standard deviation, and CV cannot be interpreted and applied in the same way that these descriptive statistics are interpreted and applied for continuous variables such as the IC25 or LC50.

4.16.4 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified. In the case of endpoints that are point estimates (LC50s and IC25s), at the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone. If more than one out of 20 reference toxicant tests fall outside the control limits, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Control limits for the NOECs will also be exceeded occasionally, regardless of how well a laboratory performs. In those instances when the laboratory can document the cause for the outlier (e.g., operator error, culture health or test system failure), the outlier should be excluded from the future calculations of the

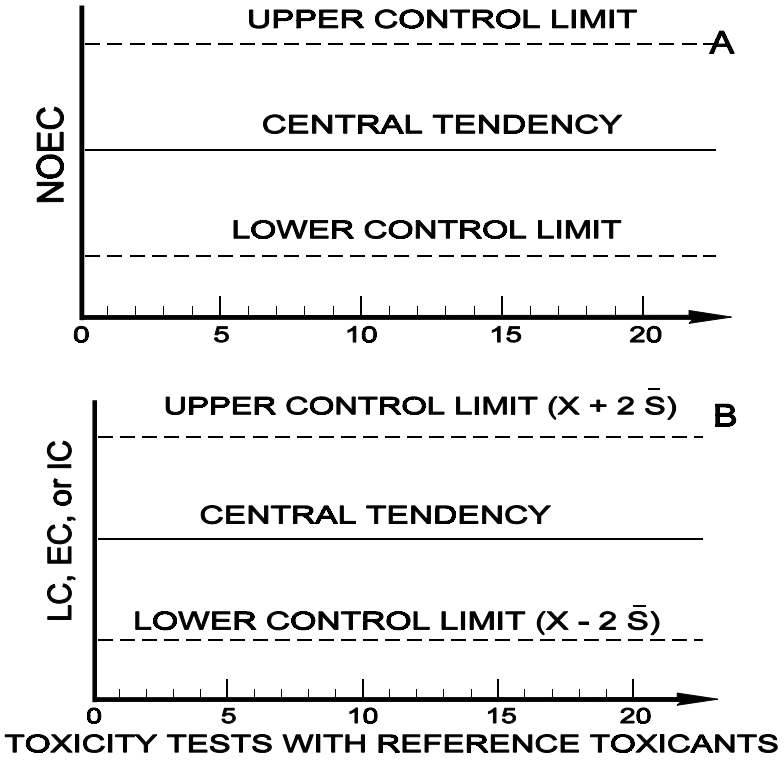
control limits. If two or more consecutive tests do not fall within the control limits, the results must be explained and the reference toxicant test must be immediately repeated. Actions taken to correct the problem must be reported.

4.16.5 If the toxicity value from a given test with a reference toxicant fall well outside the expected range for the test organisms when using the standard dilution water and other test conditions, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Performance should improve with experience, and the control limits for endpoints that are point estimates should gradually narrow. However, control limits of ± 2S will be exceeded 5% of the time by chance alone, regardless of how well a laboratory performs. Highly proficient laboratories which develop very narrow control limits may be unfairly penalized if a test result which falls just outside the control limits is rejected *de facto*. For this reason, the width of the control limits should be considered in determining whether or not a reference toxicant test result falls "well" outside the expected range. The width of the control limits may be evaluated by comparing the calculated CV (i.e., standard deviation / mean) of the IC25 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-2 in USEPA, 2000b). In determining whether or not a reference toxicant test result falls "well" outside the expected range, the result also may be compared with upper and lower bounds for ± 3S, as any result outside these control limits would be expected to occur by chance only 1 out of 100 tests (Environment Canada, 1990). When a result from a reference toxicant test is outside the 99% confidence intervals, the laboratory must conduct an immediate investigation to assess the possible causes for the outlier.

4.16.6 Reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. Reference toxicant testing is used for evaluating the health and sensitivity of organisms over time and for documenting initial and ongoing laboratory performance. While reference toxicant test results should not be used as a *de facto* criterion for test rejection, effluent and receiving water test results should be reviewed and interpreted in the light of reference toxicant test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increased test organism sensitivity or toward decreased test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test.

4.17 REFERENCE TOXICANTS

4.17.1 Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$), are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests. EMSL-Cincinnati plans to release USEPA-certified solutions of cadmium and copper for use as reference toxicants, through cooperative research and development agreements with commercial suppliers, and will continue to develop additional reference toxicants for future release. Standard reference materials can be obtained from commercial supply houses, or can be prepared inhouse using reagent grade chemicals. The regulatory agency should be consulted before reference toxicant(s) are selected and used.



$$\bar{X} = \frac{\sum_{i+1}^{n} X_i}{n}$$

$$S = \frac{\sqrt{\sum_{i=1}^{n} X_i^2 - \frac{(\sum_{i=1}^{n} X_i)^2}{n}}}{n-1}$$

Where: X_i = Successive toxicity values from toxicity tests.

n = Number of tests.

$\bar{X}$ = Mean toxicity value.

S = Standard deviation.

Figure 1. Control charts. (A) hypothesis testing results; (B) point estimates (LC, EC, or IC).

4.18 RECORD KEEPING

4.18.1 Proper record keeping is important. A complete file must be maintained for each individual toxicity test or group of tests on closely related samples. This file must contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the toxicity test(s); chemical analysis data on the sample(s); detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions employed; and results of reference toxicant tests. Laboratory data should be recorded on a real-time basis to prevent the loss of information or inadvertent introduction of errors into the record. Original data sheets should be signed and dated by the laboratory personnel performing the tests.

4.18.2 The regulatory authority should retain records pertaining to discharge permits. Permittees are required to retain records pertaining to permit applications and compliance for a minimum of 3 years [40 CFR 122.41(j)(2)].

SECTION 5

FACILITIES, EQUIPMENT, AND SUPPLIES

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities must include equipment for rearing and/or holding organisms. Culturing facilities for test organisms may be desirable in fixed laboratories which perform large numbers of tests. Temperature control can be achieved using circulating water baths, heat exchangers, or environmental chambers. Water used for rearing, holding, acclimating, and testing organisms may be natural seawater or water made up from hypersaline brine derived from natural seawater, or water made up from reagent grade chemicals (GP2) or commercial (FORTY FATHOMS® or HW MARINEMIX®) artificial sea salts when specifically recommended in the method. Air used for aeration must be free of oil and toxic vapors. Oil-free air pumps should be used where possible. Particulates can be removed from the air using BALSTON® Grade BX or equivalent filters, and oil and other organic vapors can be removed using activated carbon filters (BALSTON®, C-1 filter, or equivalent).

5.1.2 The facilities must be well ventilated and free of fumes. Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories and/or sample handling areas is not circulated to test organism culture rooms or toxicity test rooms, or that air from toxicity test rooms does not contaminate culture areas. Sample preparation, culturing, and toxicity testing areas should be separated to avoid cross-contamination of cultures or toxicity test solutions with toxic fumes. Air pressure differentials between such rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely-fitting doors. Organisms should be shielded from external disturbances.

5.1.3 Materials used for exposure chambers, tubing, etc., which come in contact with the effluent and dilution water, should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON®) should be used whenever possible to minimize sorption and leaching of toxic substances. These materials may be reused following decontamination. Containers made of plastics, such as polyethylene, polypropylene, polyvinyl chloride, TYGON®, etc., may be used as test chambers or to ship, store, and transfer effluents and receiving waters, but they should not be reused unless absolutely necessary, because they might carry over adsorbed toxicants from one test to another, if reused. However, these containers may be repeatedly reused for storing uncontaminated waters such as deionized or laboratory-prepared dilution waters and receiving waters. Glass or disposable polystyrene containers can be used as test chambers. The use of large ($\geq$20 L) glass carboys is discouraged for safety reasons.

5.1.4 New plastic products of a type not previously used should be tested for toxicity before initial use by exposing the test organisms in the test system where the material is used. Equipment (pumps, valves, etc.) which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below (see Section 5, Facilities, Equipment, and Supplies, Subsection 5.3.2). Fiberglass, in addition to the previously mentioned materials, can be used for holding, acclimating, and dilution water storage tanks, and in the water delivery system, but once contaminated with pollutants the fiberglass should not be reused. All material should be flushed or rinsed thoroughly with the test media before using in the test.

5.1.5 Copper, galvanized material, rubber, brass, and lead must not come in contact with culturing, holding, acclimation, or dilution water, or with effluent samples and test solutions. Some materials, such as several types of neoprene rubber (commonly used for stoppers) may be toxic and should be tested before use.

5.1.6 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

5.2 TEST CHAMBERS

5.2.1 Test chamber size and shape are varied according to size of the test organism. Requirements are specified in each toxicity test method.

5.3 CLEANING TEST CHAMBERS AND LABORATORY APPARATUS

5.3.1 New plasticware used for sample collection or organism exposure vessels generally does not require thorough cleaning before use. It is sufficient to rinse new sample containers once with dilution water before use. New, disposable, plastic test chambers may have to be rinsed with dilution water before use. New glassware must be soaked overnight in 10% acid (see below) and also should be rinsed well in deionized water and seawater.

5.3.2 All non-disposable sample containers, test vessels, pumps, tanks, and other equipment that has come in contact with effluent must be washed after use to remove surface contaminants, as described below.

1. Soak 15 minutes in tap water and scrub with detergent, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh dilute (10% V:V) hydrochloric acid or nitric acid to remove scale, metals and bases. To prepare a 10% solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with deionized water.

5.3.3 All test chambers and equipment must be thoroughly rinsed with the dilution water immediately prior to use in each test.

5.4 APPARATUS AND EQUIPMENT FOR CULTURING AND TOXICITY TESTS

5.4.1 Apparatus and equipment requirements for culturing and toxicity tests are specified in each toxicity test method. Also, see USEPA, 2002a.

5.4.2 WATER PURIFICATION SYSTEM

5.4.2.1 A good quality, laboratory grade deionized water, providing a resistance of 18 megaohm-cm, must be available in the laboratory and in sufficient quantity for laboratory needs. Deionized water may be obtained from MILLIPORE®, MILLI-Q®, MILLIPORE® QPAK™$_2$ or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade water deionizer with preconditioned water from a Culligan®, Continental®, or equivalent mixed-bed water treatment system.

5.5 REAGENTS AND CONSUMABLE MATERIALS

5.5.1 SOURCES OF FOOD FOR CULTURE AND TOXICITY TESTS

1. Brine Shrimp, *Artemia* sp. cysts -- Many commercial sources of brine shrimp cysts are available.
2. Frozen Adult Brine Shrimp, *Artemia* -- Available from most pet supply shops or other commercial sources.
3. Flake Food -- TETRAMIN® and BIORIL® or equivalent are available at most pet supply shops.
4. Feeding requirements and other specific foods are indicated in the specific toxicity test method.

5.5.1.1 All food should be tested for nutritional suitability and chemically analyzed for organochlorine pesticides, PCBs, and toxic metals (see Section 4, Quality Assurance).

5.5.2 Reagents and consumable materials are specified in each toxicity test method. Also, see Section 4, Quality Assurance.

5.6 TEST ORGANISMS

5.6.1 Test organisms are obtained from inhouse cultures or commercial suppliers (see specific toxicity test method; Sections 4, Quality Assurance and 6, Test Organisms).

5.7 SUPPLIES

5.7.1 See toxicity test methods (see Sections 11-16) for specific supplies.

SECTION 6

TEST ORGANISMS

6.1 TEST SPECIES

6.1.1 The species used in characterizing the chronic toxicity of effluents and/or receiving waters will depend on the requirements of the regulatory authority and the objectives of the test. It is essential that good quality test organisms be readily available throughout the year from inhouse or commercial sources to meet NPDES monitoring requirements. The organisms used in toxicity tests must be identified to species. If there is any doubt as to the identity of the test organisms, representative specimens should be sent to a taxonomic expert to confirm the identification.

6.1.2 Toxicity test conditions and culture methods for the species listed in Subsection 6.1.3 are provided in this manual (also, see USEPA, 2002a).

6.1.3 The organisms used in the short-term tests described in this manual are the sheepshead minnow, *Cyprinodon variegatus*; the inland silverside, *Menidia beryllina*; the mysid, *Mysidopsis bahia*; the sea urchin, *Arbacia punctulata*; and the red macroalga, *Champia parvula*.

6.1.4 Some states have developed culturing and testing methods for indigenous species that may be as sensitive or more sensitive, than the species recommended in Subsection 6.1.3. However, USEPA allows the use of indigenous species only where state regulations require their use or prohibit importation of the species in Subsection 6.1.3. Where state regulations prohibit importation of non-native fishes or use of the recommended test species, permission must be requested from the appropriate state agency prior to their use.

6.1.5 Where states have developed culturing and testing methods for indigenous species other than those recommended in this manual, data comparing the sensitivity of the substitute species and one or more of the recommended species must be obtained in side-by-side toxicity tests with reference toxicants and/or effluents, to ensure that the species selected are at least as sensitive as the recommended species. These data must be submitted to the permitting authority (State or Region) if required. USEPA acknowledges that reference toxicants prepared from pure chemicals may not always be representative of effluents. However, because of the observed and/or potential variability in the quality and toxicity of effluents, it is not possible to specify a representative effluent.

6.1.6 Guidance for the selection of test organisms where the salinity of the effluent and/or receiving water requires special consideration is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991a).

1. Where the salinity of the receiving water is < 1‰, freshwater organisms are used regardless of the salinity of the effluent.
2. Where the salinity of the receiving water is ≥1‰, the choice of organisms depends on state water quality standards and/or permit requirements.

6.2 SOURCES OF TEST ORGANISMS

6.2.1 The test organisms recommended in this manual can be cultured in the laboratory using culturing and handling methods for each organism described in the respective test method sections. Also, see USEPA (2002a).

6.2.2 Inhouse cultures should be established wherever it is cost effective. If inhouse cultures cannot be maintained or it is not cost effective, test organisms should be purchased from experienced commercial suppliers (see USEPA, 1993b).

6.2.3 Sheepshead minnows, inland silversides, mysids, and sea urchins may be purchased from commercial suppliers. However, some of these organisms (e.g., adult sheepshead minnows or adult inland silversides) may not always be available from commercial suppliers and may have to be collected in the field and brought back to the laboratory for spawning to obtain eggs and larvae.

6.2.4 If, because of their source, there is any uncertainty concerning the identity of the organisms, it is advisable to have them examined by a taxonomic specialist to confirm their identification. For detailed guidance on identification, see the individual toxicity test methods.

6.2.5 FERAL (NATURAL OCCURRING, WILD CAUGHT) ORGANISMS

6.2.5.1 The use of test organisms taken from the receiving water has strong appeal, and would seem to be the logical approach. However, it is generally impractical and not recommended for the following reasons:

1. Sensitive organisms may not be present in the receiving water because of previous exposure to the effluent or other pollutants.
2. It is often difficult to collect organisms of the required age and quality from the receiving water.
3. Most states require collection permits, which may be difficult to obtain. Therefore, it is usually more cost effective to culture the organisms in the laboratory or obtain them from private, state, or Federal sources. Fish such as sheepshead minnows and silversides, and invertebrates such as mysids, are easily reared in the laboratory or purchased.
4. The required QA/QC records, such as the single-laboratory precision data, would not be available.
5. Since it is mandatory that the identity of test organisms is known to the species level, it would be necessary to examine each organism caught in the wild to confirm its identity, which would usually be impractical or, at the least, very stressful to the organisms.
6. Test organisms obtained from the wild must be observed in the laboratory for a minimum of one week prior to use, to ensure that they are free of signs of parasitic or bacterial infections and other adverse effects. Fish captured by electroshocking must not be used in toxicity testing.

6.2.5.2 Guidelines for collection of natural occurring organisms are provided in USEPA (1973); USEPA (1990a); and USEPA (1993b).

6.2.6 Regardless of their source, test organisms should be carefully observed to ensure that they are free of signs of stress and disease, and in good physical condition. Some species of test organisms, such as trout, can be obtained from stocks certified as "disease-free."

6.3 LIFE STAGE

6.3.1 Young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as juvenile mysids and larval fish, is required for all tests. In a given test, all organisms should be approximately the same age and should be taken from the same source. Since age may affect the results of the tests, it would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility.

6.4 LABORATORY CULTURING

6.4.1 Instructions for culturing and/or holding the recommended test organisms are included in specified test methods (also, see USEPA, 2002a).

6.5 HOLDING AND HANDLING TEST ORGANISMS

6.5.1 Test organisms should not be subjected to changes of more than 3°C in water temperature or 3‰ in salinity in any 12 h period.

6.5.2 Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that are dropped or touch dry surfaces or are injured during handling must be discarded. Dipnets are best for handling larger organisms. These nets are commercially available or can be made from small-mesh nylon netting, silk bolting cloth, plankton netting, or similar material. Wide-bore, smooth glass tubes (4 to 8 mm ID) with rubber bulbs or pipettors (such as a PROPIPETTE® or other pipettor) should be used for transferring smaller organisms such as mysids, and larval fish.

6.5.3 Holding tanks for fish are supplied with a good quality water (see Section 5, Facilities, Equipment, and Supplies) with a flow-through rate of at least two tank-volumes per day. Otherwise, use a recirculation system where the water flows through an activated carbon or undergravel filter to remove dissolved metabolites. Culture water can also be piped through high intensity ultraviolet light sources for disinfection, and to photo-degrade dissolved organics.

6.5.4 Crowding should be avoided because it will stress the organisms and lower the DO concentrations to unacceptable levels. The DO must be maintained at a minimum of 4.0 mg/L. The solubility of oxygen depends on temperature, salinity, and altitude. Aerate gently if necessary.

6.5.5 The organisms should be observed carefully each day for signs of disease, stress, physical damage, or mortality. Dead and abnormal organisms should be removed as soon as observed. It is not uncommon for some fish mortality (5-10%) to occur during the first 48 h in a holding tank because of individuals that refuse to feed on artificial food and die of starvation. Organisms in the holding tanks should generally be fed as in the cultures (see culturing methods in the respective methods).

6.5.6 Fish should be fed as much as they will eat at least once a day with live brine shrimp nauplii, *Artemia*, or frozen adult brine shrimp or dry food (frozen food should be completely thawed before use). Adult brine shrimp can be supplemented with commercially prepared food such as TETRAMIN® or BIORIL® flake food, or equivalent. Excess food and fecal material should be removed from the bottom of the tanks at least twice a week by siphoning.

6.5.7 A daily record of feeding, behavioral observations, and mortality should be maintained.

6.6 TRANSPORTATION TO THE TEST SITE

6.6.1 Organisms are transported from the base or supply laboratory to a remote test site in culture water or standard dilution water in plastic bags or large-mouth screw-cap (500 mL) plastic bottles in styrofoam coolers. Adequate DO is maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags. Another method commonly used to maintain sufficient DO during shipment is to aerate with an airstone which is supplied from a portable pump. The DO concentration must not fall below 4.0 mg/L.

6.6.2 Upon arrival at the test site, organisms are transferred to receiving water if receiving water is to be used as the test dilution water. All but a small volume of the holding water (approximately 5%) is removed by siphoning, and replaced slowly over a 10 to 15 minute period with dilution water. If receiving water is used as dilution water, caution must be exercised in exposing the test organisms to it, because of the possibility that it might be toxic. For this reason, it is recommended that only approximately 10% of the test organisms be exposed initially to the dilution water. If this group does not show excessive mortality or obvious signs of stress in a few hours, the remainder of the test organisms are transferred to the dilution water.

6.6.3 A group of organisms must not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed, or if mortality appears to exceed 10% preceding the test. If the organisms fail to meet these criteria, the entire group must be discarded and a new group obtained. The mortality may be due to the presence of toxicity, if receiving

water is used as dilution water, rather than a diseased condition of the test organisms. If the acclimation process is repeated with a new group of test organisms and excessive mortality occurs, it is recommended that an alternative source of dilution water be used.

6.6.4 The marine organisms can be used at all concentrations of effluent by adjusting the salinity of the effluent to salinities specified for the appropriate species test condition or to the salinity approximating that of the receiving water, by adding sufficient dry ocean salts, such as FORTY FATHOMS®, or equivalent, GP2, or hypersaline brine.

6.6.5 Saline dilution water can be prepared with deionized water or a freshwater such as well water or a suitable surface water. If dry ocean salts are used, care must be taken to ensure that the added salts are completely dissolved and the solution is aerated 24 h before the test organisms are placed in the solutions. The test organisms should be acclimated in synthetic saline water prepared with the dry salts. **Caution**: addition of dry ocean salts to dilution water may result in an increase in pH. (The pH of estuarine and coastal saline waters is normally 7.5-8.3).

6.6.6 All effluent concentrations and the control(s) used in a test should have the same salinity. The change in salinity upon acclimation at the desired test dilution should not exceed 6‰. The required salinities for culturing and toxicity tests with estuarine and marine species are listed in the test method sections.

6.7 TEST ORGANISM DISPOSAL

6.7.1 When the toxicity test(s) is concluded, all test organisms (including controls) should be humanely destroyed and disposed of in an appropriate manner.

SECTION 7

DILUTION WATER

7.1 TYPES OF DILUTION WATER

7.1.1 The type of dilution water used in effluent toxicity tests will depend largely on the objectives of the study.

7.1.1.1 If the objective of the test is to estimate the absolute chronic toxicity of the effluent, a synthetic (standard) dilution water is used. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.2 If the objective of the test is to estimate the chronic toxicity of the effluent in uncontaminated receiving water, the test may be conducted using dilution water consisting of a single grab sample of receiving water (if non-toxic), collected outside the influence of the outfall, or with other uncontaminated natural water (surface water) or standard dilution water having approximately the same salinity as the receiving water. Seasonal variations in the quality of receiving waters may affect effluent toxicity. Therefore, the salinity of saline receiving water samples should be determined before each use. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.3 If the objective of the test is to determine the additive or mitigating effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected outside the influence of the outfall. A second set of controls, using culture water, should be included in the test.

7.1.2 An acceptable dilution water is one which is appropriate for the objectives of the test; supports adequate performance of the test organisms with respect to survival, growth, reproduction, or other responses that may be measured in the test (i.e., consistently meets test acceptability criteria for control responses); is consistent in quality; and does not contain contaminants that could produce toxicity. Receiving waters, synthetic waters, or synthetic waters adjusted to approximate receiving water characteristics may be used for dilution provided that the water meets the above listed qualifications for an acceptable dilution water. USEPA (2000a) provides additional guidance on selecting appropriate dilution waters.

7.1.3 When dual controls (one control using culture water and one control using dilution water) are used (see Subsections 7.1.1.1 - 7.1.1.3 above), the dilution water control should be used to determine test acceptability. It is also the dilution water control that should be compared to effluent treatments in the calculation and reporting of test results. The culture water control should be used to evaluate the appropriateness of the dilution water source. Significant differences between organism responses in culture water and dilution water controls could indicate toxicity in the dilution water and may suggest an alternative dilution water source. USEPA (2000a) provides additional guidance on dual controls.

7.2 STANDARD, SYNTHETIC DILUTION WATER

7.2.1 Standard, synthetic, dilution water is prepared with deionized water and reagent grade chemicals (GP2) or commercial sea salts (FORTY FATHOMS®, HW MARINEMIX®) (Table 3). The source water for the deionizer can be ground water or tap water.

7.2.2 DEIONIZED WATER USED TO PREPARE STANDARD, SYNTHETIC, DILUTION WATER

7.2.2.1 Deionized water is obtained from a MILLIPORE MILLI-Q®, MILLIPORE® QPAK™$_2$ or equivalent system. It is advisable to provide a preconditioned (deionized) feed water by using a Culligan®, Continental®, or equivalent system in front of the MILLI-Q® System to extend the life of the MILLI-Q® cartridges (see Section 5, Facilities, Equipment, and Supplies).

7.2.2.2 The recommended order of the cartridges in a four-cartridge deionizer (i.e., MILLI-Q® System or equivalent) is: (1) ion exchange, (2) ion exchange, (3) carbon, and (4) organic cleanup (such as ORGANEX-Q®, or equivalent), followed by a final bacteria filter. The QPAK™$_2$ water system is a sealed system which does not allow for the rearranging of the cartridges. However, the final cartridge is an ORGANEX-Q® filter, followed by a final bacteria filter. Commercial laboratories using this system have not experienced any difficulty in using the water for culturing or testing. Reference to the MILLI-Q® systems throughout the remainder of the manual includes all MILLIPORE® or equivalent systems.

7.2.3 STANDARD, SYNTHETIC SEAWATER

7.2.3.1 To prepare 20 L of a standard, synthetic, reconstituted seawater (modified GP2), using reagent grade chemicals (Table 3), with a salinity of 31‰, follow the instructions below. Other salinities can be prepared by making the appropriate dilutions. Larger or smaller volumes of modified GP2 can be prepared by using proportionately larger or smaller amounts of salts and dilution water.

1. Place 20 L of MILLI-Q® or equivalent deionized water in a properly cleaned plastic carboy.
2. Weigh reagent grade salts listed in Table 3 and add, one at a time, to the deionized water. Stir well after adding each salt.
3. Aerate the final solution at a rate of 1 L/h for 24 h.
4. Check the pH and salinity.

7.2.3.2 Synthetic seawater can also be prepared by adding commercial sea salts, such as FORTY FATHOMS®, HW MARINEMIX®, or equivalent, to deionized water. For example, thirty-one parts per thousand (31‰) FORTY FATHOMS® can be prepared by dissolving 31 g of sea salts per liter of deionized water. The salinity of the resulting solutions should be checked with a refractometer.

7.2.4 Artificial seawater is to be used only if specified in the method. EMSL-Cincinnati has found FORTY FATHOMS® artificial sea salts suitable for maintaining and spawning the sheepshead minnow, *Cyprinodon variegatus*, and for its use in the sheepshead minnow larval survival and growth test, suitable for maintaining and spawning the inland silverside, *Menidia beryllina*, and for its use in the inland silverside larval survival and growth test, suitable for culturing and maintaining mysid shrimp, *Mysidopsis bahia*, and its use in the mysid shrimp survival, growth, and fecundity test, and suitable for maintaining sea urchins, *Arbacia punctulata*, and for its use in the sea urchin fertilization test. The USEPA Region 6 Houston Laboratory has successfully used HW MARINEMIX® sea salts to maintain and spawn sheepshead minnows, and perform the larval survival and growth test and the embryo-larval survival and teratogenicity test. Also, HW MARINEMIX® sea salts has been used successfully to culture and maintain the mysid brood stock and perform the mysid survival, growth, fecundity test. An artificial seawater formulation, GP2 (Spotte et al., 1984), Table 3, has been used by the Environmental Research Laboratory-Narragansett, RI for all but the embryo-larval survival and teratogenicity test. The suitability of GP2 as a medium for culturing organisms has not been determined.

TABLE 3. PREPARATION OF GP2 ARTIFICIAL SEAWATER USING REAGENT GRADE CHEMICALS[1,2,3]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
KCl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

[1] Modified GP2 from Spotte et al. (1984).
[2] The constituent salts and concentrations were taken from USEPA (2002a). The salinity is 30.89 g/L.
[3] GP2 can be diluted with deionized (DI) water to the desired test salinity.

7.3 USE OF RECEIVING WATER AS DILUTION WATER

7.3.1 If the objectives of the test require the use of uncontaminated receiving water as dilution water, and the receiving water is uncontaminated, it may be possible to collect a sample of the receiving water close to the outfall, but should be away from or beyond the influence of the effluent. However, if the receiving water is contaminated, it may be necessary to collect the sample in an area "remote" from the discharge site, matching as closely as possible the physical and chemical characteristics of the receiving water near the outfall.

7.3.2 The sample should be collected immediately prior to the test, but never more than 96 h before the test begins. Except where it is used within 24 h, or in the case where large volumes are required for flow through tests, the sample should be chilled to 0-6°C during or immediately following collection, and maintained at that temperature prior to use in the test.

7.3.3 The investigator should collect uncontaminated water having a salinity as near as possible to the salinity of the receiving water at the discharge site. Water should be collected at slack high tide, or within one hour after high tide. If there is reason to suspect contamination of the water in the estuary, it is advisable to collect uncontaminated water from an adjacent estuary. At times it may be necessary to collect water at a location closer to the open sea, where the salinity is relatively high. In such cases, deionized water or uncontaminated freshwater is added to the saline water to dilute it to the required test salinity. Where necessary, the salinity of a surface water can be increased by the addition of artificial sea salts, such as FORTY FATHOMS®, HW MARINEMIX®, or equivalent, GP2, a

natural seawater of higher salinity, or hypersaline brine. Instructions for the preparation of hypersaline brine by concentrating natural seawater are provided below.

7.3.4 Receiving water containing debris or indigenous organisms, that may be confused with or attack the test organisms, should be filtered through a sieve having 60 µm mesh openings prior to use.

7.3.5 HYPERSALINE BRINE

7.3.5.1 Hypersaline brine (HSB) has several advantages that make it desirable for use in toxicity testing. It can be made from any high quality, filtered seawater by evaporation, and can be added to deionized water to prepare dilution water, or to effluents or surface waters to increase their salinity.

7.3.5.2 The ideal container for making HSB from natural seawater is one that (l) has a high surface to volume ratio, (2) is made of a noncorrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is used, use only oil-free air compressors to prevent contamination.

7.3.5.3 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several thorough deionized water rinses. High quality (and preferably high salinity) seawater should be filtered to at least 10 mm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

7.3.5.4 The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

7.3.5.5 After the required salinity is attained, the HSB should be filtered a second time through a l-µm filter and poured directly into portable containers (20-L CUBITAINERS® or polycarbonate water cooler jugs are suitable). The containers should be capped and labelled with the date the brine was generated and its salinity. Containers of HSB should be stored in the dark and maintained under room temperature until used.

7.3.5.6 If a source of HSB is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and brine before mixing in the effluent.

7.3.5.7 Divide the salinity of the HSB by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 25‰, 100‰ divided by 25‰ = 4.0. The proportion of brine is 1 part in 4 (one part brine to three parts deionized water).

7.3.5.8 To make 1 L of seawater at 25‰ salinity from a hypersaline brine of 100‰, 250 mL of brine and 750 mL of deionized water are required.

7.4 USE OF TAP WATER AS DILUTION WATER

7.4.1 The use of tap water in the reconstituting of synthetic (artificial) seawater as dilution water is discouraged unless it is dechlorinated and fully treated. Tap water can be dechlorinated by deionization, carbon filtration, or the use of sodium thiosulfate. Use of 3.6 mg/L (anhydrous) sodium thiosulfate will reduce 1.0 mg chlorine/L (APHA, 1992). Following dechlorination, total residual chlorine should not exceed 0.01 mg/L. Because of the possible

toxicity of thiosulfate to test organisms, a control lacking thiosulfate should be included in toxicity tests utilizing thiosulfate-dechlorinated water.

7.4.2 To be adequate for general laboratory use following dechlorination, the tap water is passed through a deionizer and carbon filter to remove toxic metals and organics, and to control hardness and alkalinity.

7.5 DILUTION WATER HOLDING

7.5.1 A given batch of dilution water should not be used for more than 14 days following preparation because of the possible build up of bacterial, fungal, or algal slime growth and the problems associated with it. The container should be kept covered and the contents should be protected from light.

SECTION 8

EFFLUENT AND RECEIVING WATER SAMPLING, SAMPLE HANDLING, AND SAMPLE PREPARATION FOR TOXICITY TESTS

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point should be the same as that specified in the NPDES discharge permit (USEPA, l988b). Conditions for exception would be: (l) better access to a sampling point between the final treatment and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to municipal waste treatment plants or separate wastewater streams in industrial facilities prior to their being combined with other wastewater streams or non-contact cooling water, additional sampling points may be chosen.

8.1.2 The decision on whether to collect grab or composite samples is based on the objectives of the test and an understanding of the short and long-term operations and schedules of the discharger. If the effluent quality varies considerably with time, which can occur where holding times are short, grab samples may seem preferable because of the ease of collection and the potential of observing peaks (spikes) in toxicity. However, the sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitively large number of separate samples and tests. Collection of a 24-h composite sample, however, may dilute toxicity spikes, and average the quality of the effluent over the sampling period. Sampling recommendations are provided below (also see USEPA, 2002a).

8.1.3 Aeration during collection and transfer of effluents should be minimized to reduce the loss of volatile chemicals.

8.1.4 Details of date, time, location, duration, and procedures used for effluent sample and dilution water collection should be recorded.

8.2 EFFLUENT SAMPLE TYPES

8.2.1 The advantages and disadvantages of effluent grab and composite samples are listed below:

8.2.1.1 GRAB SAMPLES

Advantages:

1. Easy to collect; require a minimum of equipment and on-site time.
2. Provide a measure of instantaneous toxicity. Toxicity spikes are not masked by dilution.

Disadvantages:

1. Samples are collected over a very short period of time and on a relatively infrequent basis. The chances of detecting a spike in toxicity would depend on the frequency of sampling, and the probability of missing spikes is high.

8.2.1.2 COMPOSITE SAMPLES:

Advantages:

1. A single effluent sample is collected over a 24-h period.
2. The sample is collected over a much longer period of time than grab samples and contains all toxicity spikes.

Disadvantages:

1. Sampling equipment is more sophisticated and expensive, and must be placed on-site for at least 24 h.
2. Toxicity spikes may not be detected because they are masked by dilution with less toxic wastes.

8.3 EFFLUENT SAMPLING RECOMMENDATIONS

8.3.1 When tests are conducted on-site, test solutions can be renewed daily with freshly collected samples.

8.3.2 When tests are conducted off-site, a minimum of three samples are collected. If these samples are collected on Test Days 1, 3, and 5, the first sample would be used for test initiation, and for test solution renewal on Day 2. The second sample would be used for test solution renewal on Days 3 and 4. The third sample would be used for test solution renewal on Days 5, 6, and 7.

8.3.3 Sufficient sample must be collected to perform the required toxicity and chemical tests. A 4-L (1-gal) CUBITAINER® will provide sufficient sample volume for most tests.

8.3.4 THE FOLLOWING EFFLUENT SAMPLING METHODS ARE RECOMMENDED:

8.3.4.1 Continuous Discharges

8.3.4.1.1 If the facility discharge is continuous, a single 24-h composite sample is to be taken.

8.3.4.2 Intermittent Discharges

8.3.4.2.1 If the facility discharge is intermittent, a composite sample is to be collected for the duration of the discharge but not more than 24 hours.

8.4 RECEIVING WATER SAMPLING

8.4.1 Logistical problems and difficulty in securing sampling equipment generally preclude the collection of composite receiving water samples for toxicity tests. Therefore, based on the requirements of the test, a single grab sample or daily grab samples of receiving water is collected for use in the test.

8.4.2 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples should be collected at mid-depth.

8.4.3 To determine the extent of the zone of toxicity in the receiving water at estuarine and marine effluent sites, receiving water samples are collected at several distances away from the discharge. The time required for the effluent-receiving-water mixture to travel to sampling points away from the effluent, and the rate and degree of mixing, may be difficult to ascertain. Therefore, it may not be possible to correlate receiving water toxicity with effluent toxicity at the discharge point unless a dye study is performed. The toxicity of receiving water samples from five stations in the discharge plume can be evaluated using the same number of test vessels and test organisms as used in one effluent toxicity test with five effluent dilutions.

8.5 EFFLUENT AND RECEIVING WATER SAMPLE HANDLING, PRESERVATION, AND SHIPPING

8.5.1 Unless the samples are used in an on-site toxicity test the day of collection (or hand delivered to the testing laboratory for use on the day of collection), it is recommended that they be held at 0-6°C until used to inhibit microbial degradation, chemical transformations, and loss of highly volatile toxic substances.

8.5.2 Composite samples should be chilled as they are collected. Grab samples should be chilled immediately following collection.

8.5.3 If the effluent has been chlorinated, total residual chlorine must be measured immediately following sample collection.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time (holding time) from sample collection to first use of each grab or composite sample must not exceed 36 h. EPA believes that 36 h is adequate time to deliver the sample to the laboratories performing the test in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the USEPA Regional Administrator under 40 CFR 136.3(e), should include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond more than 36 h. However, in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, each grab or composite sample may also be used to prepare test solutions for renewal at 24 h and/or 48 h after first use, if stored at 0-6°C, with minimum head space, as described in Subsection 8.5. If shipping problems (e.g., unsuccessful Saturday delivery) are encountered with renewal samples after a test has been initiated, the permitting authority may allow the continued use of the most recently used sample for test renewal. Guidance for determining the persistence of the sample is provided in Subsection 8.7.

8.5.5 To minimize the loss of toxicity due to volatilization of toxic constituents, all sample containers should be "completely" filled, leaving no air space between the contents and the lid.

8.5.6 SAMPLES USED IN ON-SITE TESTS

8.5.6.1 Samples collected for on-site tests should be used within 24 h.

8.5.7 SAMPLES SHIPPED TO OFF SITE FACILITIES

8.5.7.1 Samples collected for off site toxicity testing are to be chilled to 0-6°C during or immediately after collection, and shipped iced to the performing laboratory. Sufficient ice should be placed with the sample in the shipping container to ensure that ice will still be present when the sample arrives at the laboratory and is unpacked. Insulating material should not be placed between the ice and the sample in the shipping container unless required to prevent breakage of glass sample containers.

8.5.7.2 Samples may be shipped in one or more 4-L (l-gal) CUBITAINERS® or new plastic "milk" jugs. All sample containers should be rinsed with source water before being filled with sample. After use with receiving water or effluents, CUBITAINERS® and plastic jugs are punctured to prevent reuse.

8.5.7.3 Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. Saturday and Sunday shipping and receiving schedules of private carriers vary with the carrier.

8.6 SAMPLE RECEIVING

8.6.1 Upon arrival at the laboratory, samples are logged in and the temperature is measured and recorded. If the samples are not immediately prepared for testing, they are stored at 0-6°C until used.

8.6.2 Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory, and the sample holding time should not exceed 36 h unless a variance has been granted by the NPDES permitting authority.

8.7 PERSISTENCE OF EFFLUENT TOXICITY DURING SAMPLE SHIPMENT AND HOLDING

8.7.1 The persistence of the toxicity of an effluent prior to its use in a toxicity test is of interest in assessing the validity of toxicity test data, and in determining the possible effects of allowing an extension of the holding time. Where a variance in holding time (> 36 h, but ≤ 72 h) is requested by a permittee (See Subsection 8.5.4), information on the effects of the extension in holding time on the toxicity of the samples must be obtained by comparing the results of multi-concentration chronic toxicity tests performed on effluent samples held 36 h with toxicity test results using the same samples after they were held for the requested, longer period. The portion of the sample set aside for the second test must be held under the same conditions as during shipment and holding.

8.8 PREPARATION OF EFFLUENT AND RECEIVING WATER SAMPLES FOR TOXICITY TESTS

8.8.1 Adjust the sample salinity to the level appropriate for objectives of the study using hypersaline brine or artificial sea salts.

8.8.2 When aliquots are removed from the sample container, the head space above the remaining sample should be held to a minimum. Air which enters a container upon removal of sample should be expelled by compressing the container before reclosing, if possible (i.e., where a CUBITAINER® used), or by using an appropriate discharge valve (spigot).

8.8.3 It may be necessary to first coarse-filter samples through a NYLON® sieve having 2 to 4 mm mesh openings to remove debris and/or break up large floating or suspended solids. If samples contain indigenous organisms that may attack or be confused with the test organisms, the samples should be filtered through a sieve with 60-µm mesh openings. Since filtering may increase the dissolved oxygen (DO) in an effluent, the DO should be checked both before and after filtering. Low dissolved oxygen concentrations will indicate a potential problem in performing the test. **Caution**: filtration may remove some toxicity.

8.8.4 If the samples must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved oxygen and nitrogen may become a problem. To avoid this problem, samples may be warmed slowly in open test containers. If DO is still above 100% saturation, based on temperature and salinity (Table 4), after warming to test temperature, samples should be aerated moderately (approximately 500 mL/min) for a few minutes using an airstone. If DO is below 4.0 mg/L, the solutions must be aerated moderately (approximately 500 mL/min) for a few minutes, using an airstone, until the DO is within the prescribed range (≥ 4.0 mg/L). **Caution**: avoid excessive aeration.

8.8.4.1 Aeration during the test may alter the results and should be used only as a last resort to maintain the required DO. Aeration can reduce the apparent toxicity of the test solutions by stripping them of highly volatile toxic substances, or increase their toxicity by altering the pH. However, the DO in the test solution should not be permitted to fall below 4.0 mg/L.

8.8.4.2 In static tests (non-renewal or renewal) low DOs may commonly occur in the higher concentrations of wastewater. Aeration is accomplished by bubbling air through a pipet at the rate of 100 bubbles/min. If aeration is necessary, all test solutions must be aerated. It is advisable to monitor the DO closely during the first few hours of the test. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8 h after the test

is started. Unless aeration is initiated during the first 8 h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test.

8.8.5 At a minimum, pH, conductivity or salinity, and total residual chlorine are measured in the undiluted effluent or receiving water, and pH and conductivity are measured in the dilution water.

8.8.5.1 It is recommended that total alkalinity and total hardness also be measured in the undiluted effluent test water and the dilution water.

8.8.6 Total ammonia is measured in effluent and receiving water samples where toxicity may be contributed by unionized ammonia (i.e., where total ammonia ≥ 5 mg/L). The concentration (mg/L) of unionized (free) ammonia in a sample is a function of temperature and pH, and is calculated using the percentage value obtained from Table 5, under the appropriate pH and temperature, and multiplying it by the concentration (mg/L) of total ammonia in the sample.

8.8.7 Effluents and receiving waters can be dechlorinated using 6.7 mg/L anhydrous sodium thiosulfate to reduce 1 mg/L chlorine (APHA, 1992). Note that the amount of thiosulfate required to dechlorinate effluents is greater than the amount needed to dechlorinate tap water, (see Section 7, Dilution Water). Since thiosulfate may contribute to sample toxicity, a thiosulfate control should be used in the test in addition to the normal dilution water control.

8.8.8 The DO concentration in the samples should be near saturation prior to use. Aeration will bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH. However, aeration during collection, transfer, and preparation of samples should be minimized to reduce the loss of volatile chemicals.

8.8.9 Mortality or impairment of growth or reproduction due to pH alone may occur if the pH of the sample falls outside the range of 6.0 - 9.0. Thus, the presence of other forms of toxicity (metals and organics) in the sample may be masked by the toxic effects of low or high pH. The question about the presence of other toxicants can be answered only by performing two parallel tests, one with an adjusted pH, and one without an adjusted pH. Freshwater samples are adjusted to pH 7.0, and marine samples are adjusted to pH 8.0, by adding 1N NaOH or 1N HCl dropwise, as required, being careful to avoid overadjustment.

TABLE 4. OXYGEN SOLUBILITY (MG/L) IN WATER AT EQUILIBRIUM WITH AIR AT 760 MM HG (AFTER Richards and Corwin, 1956)

TEMP	SALINITY (‰)									
(C°)	0	5	10	15	20	25	30	35	40	43
0	14.2	13.8	13.4	12.9	12.5	12.1	11.7	11.2	10.8	10.6
1	13.8	13.4	13.0	12.6	12.2	11.8	11.4	11.0	10.6	10.3
2	13.4	13.0	12.6	12.2	11.9	11.5	11.1	10.7	10.3	10.0
3	13.1	12.7	12.3	11.9	11.6	11.2	10.8	10.4	10.0	9.8
4	12.7	12.3	12.0	11.6	11.3	10.9	10.5	10.1	9.8	9.5
5	12.4	12.0	11.7	11.3	11.0	10.6	0.2	9.8	9.5	9.3
6	12.1	11.7	11.4	11.0	10.7	10.3	0.0	9.6	9.3	9.1
8	11.5	11.2	10.8	10.5	10.2	9.8	9.5	9.2	8.9	8.7
10	10.9	10.7	10.3	10.0	9.7	9.4	9.1	8.8	8.5	8.3
12	10.5	10.2	9.9	9.6	9.3	9.0	8.7	8.4	8.1	7.9
14	10.0	9.7	9.5	9.2	8.9	8.6	8.3	8.1	7.8	7.6
16	9.6	9.3	9.1	8.8	8.5	8.3	8.0	7.7	7.5	7.3
18	9.2	9.0	8.7	8.5	8.2	8.0	7.7	7.5	7.2	7.1
20	8.9	8.6	8.4	8.1	7.9	7.7	7.4	7.2	6.9	6.8
22	8.6	8.4	8.1	7.9	7.6	7.4	7.2	6.9	6.7	6.6
24	8.3	8.1	7.8	7.6	7.4	7.2	6.9	6.7	6.5	6.4
26	8.1	7.8	7.6	7.4	7.2	7.0	6.7	6.5	6.3	6.1
28	7.8	7.6	7.4	7.2	7.0	6.8	6.5	6.3	6.1	6.0
30	7.6	7.4	7.1	6.9	6.7	6.5	6.3	6.1	5.9	5.8
32	7.3	7.1	6.9	6.7	6.5	6.3	6.1	5.9	5.7	5.6

TABLE 5. PERCENT UNIONIZED NH_3 IN AQUEOUS AMMONIA SOLUTIONS: TEMPERATURE 15-26°C AND pH 6.0-8.9[1]

pH	TEMPERATURE (°C)											
	15	16	17	18	19	20	21	22	23	24	25	26
6.0	0.0274	0.0295	0.0318	0.0343	0.0369	0.0397	0.0427	0.0459	0.0493	0.0530	0.0568	0.0610
6.1	0.0345	0.0372	0.0400	0.0431	0.0464	0.0500	0.0537	0.0578	0.0621	0.0667	0.0716	0.0768
6.2	0.0434	0.0468	0.0504	0.0543	0.0584	0.0629	0.0676	0.0727	0.0781	0.0901	0.0901	0.0966
6.3	0.0546	0.0589	0.0634	0.0683	0.0736	0.0792	0.0851	0.0915	0.0983	0.1134	0.1134	0.1216
6.4	0.0687	0.0741	0.0799	0.0860	0.0926	0.0996	0.107	0.115	0.124	0.133	0.143	0.153
6.5	0.0865	0.0933	0.1005	0.1083	0.1166	0.1254	0.135	0.145	0.156	0.167	0.180	0.19
6.6	0.109	0.117	0.127	0.136	0.147	0.158	0.170	0.182	0.196	0.210	0.226	0.242
6.7	0.137	0.148	0.159	0.171	0.185	0.199	0.214	0.230	0.247	0.265	0.284	0.305
6.8	0.172	0.186	0.200	0.216	0.232	0.250	0.269	0.289	0.310	0.333	0.358	0.384
6.9	0.217	0.234	0.252	0.271	0.292	0.314	0.338	0.363	0.390	0.419	0.450	0.482
7.0	0.273	0.294	0.317	0.342	0.368	0.396	0.425	0.457	0.491	0.527	0.566	0.607
7.1	0.343	0.370	0.399	0.430	0.462	0.497	0.535	0.575	0.617	0.663	0.711	0.762
7.2	0.432	0.466	0.502	0.540	0.581	0.625	0.672	0.722	0.776	0.833	0.893	0.958
7.3	0.543	0.586	0.631	0.679	0.731	0.786	0.845	0.908	0.975	1.05	1.12	1.20
7.4	0.683	0.736	0.793	0.854	0.918	0.988	1.061	1.140	1.224	1.31	1.41	1.51
7.5	0.858	0.925	0.996	1.07	1.15	1.24	1.33	1.43	1.54	1.65	1.77	1.89
7.6	1.08	1.16	1.25	1.35	1.45	1.56	1.67	1.80	1.93	2.07	2.21	2.37
7.7	1.35	1.46	1.57	1.69	1.82	1.95	2.10	2.25	2.41	2.59	2.77	2.97
7.8	1.70	1.83	1.97	2.12	2.28	2.44	2.62	2.82	3.02	3.24	3.46	3.71
7.9	2.13	2.29	2.46	2.65	2.85	3.06	3.28	3.52	3.77	4.04	4.32	4.62
8.0	2.66	2.87	3.08	3.31	3.56	3.82	4.10	4.39	4.70	5.03	5.38	5.75
8.1	3.33	3.58	3.85	4.14	4.44	4.76	5.10	5.46	5.85	6.25	6.68	7.14
8.2	4.16	4.47	4.80	5.15	5.52	5.92	6.34	6.78	7.25	7.75	8.27	8.82
8.3	5.18	5.56	5.97	6.40	6.86	7.34	7.85	8.39	8.96	9.56	10.2	10.9
8.4	6.43	6.90	7.40	7.93	8.48	9.07	9.69	10.3	11.0	11.7	12.5	13.3
8.5	7.97	8.54	9.14	9.78	10.45	11.16	11.90	12.7	13.5	14.4	15.2	16.2
8.6	9.83	10.5	11.2	12.0	12.8	13.6	14.5	15.5	16.4	17.4	18.5	19.5
8.7	12.07	12.9	13.8	14.7	15.6	16.6	17.6	18.7	19.8	21.0	22.2	23.4
8.8	14.7	15.7	16.7	17.8	18.9	20.0	21.2	22.5	23.7	25.1	26.4	27.8
8.9	17.9	19.0	20.2	21.4	22.7	24.0	25.3	26.7	28.2	29.6	31.1	32.6

[1] Table provided by Teresa Norberg-King, Duluth, Minnesota. Also see Emerson et al. (1975), Thurston et al. (1974), and USEPA (1985a).

8.9 PRELIMINARY TOXICITY RANGE-FINDING TESTS

8.9.1 USEPA Regional and State personnel generally have observed that it is not necessary to conduct a toxicity range-finding test prior to initiating a static, chronic, definitive toxicity test. However, when preparing to perform a static test with a sample of completely unknown quality, or before initiating a flow-through test, it is advisable to conduct a preliminary toxicity range-finding test.

8.9.2 A toxicity range-finding test ordinarily consists of a down-scaled, abbreviated static acute test in which groups of five organisms are exposed to several widely-spaced sample dilutions in a logarithmic series, such as 100%, 10.0%, 1.00%, and 0.100%, and a control, for 8-24 h. **Caution**: if the sample must also be used for the full-scale definitive test, the 36-h limit on holding time (see Subsection 8.5.4) must not be exceeded before the definitive test is initiated.

8.9.3 It should be noted that the toxicity (LC50) of a sample observed in a range-finding test may be significantly different from the toxicity observed in the follow-up, chronic, definitive test because: (1) the definitive test is longer; and (2) the test may be performed with a sample collected at a different time, and possibly differing significantly in the level of toxicity.

8.10 MULTICONCENTRATION (DEFINITIVE) EFFLUENT TOXICITY TESTS

8.10.1 The tests recommended for use in determining discharge permit compliance in the NPDES program are multiconcentration, or definitive, tests which provide (1) a point estimate of effluent toxicity in terms of an IC25, IC50, or LC50, or (2) a no-observed-effect-concentration (NOEC) defined in terms of mortality, growth, reproduction, and/or teratogenicity and obtained by hypothesis testing. The tests may be static renewal or static non-renewal.

8.10.2 The tests consist of a control and a minimum of five effluent concentrations. USEPA recommends the use of a $\geq$0.5 dilution factor for selecting effluent test concentrations. Effluent test concentrations of 6.25%, 12.5%, 25%, 50%, and 100% are commonly used, however, test concentrations should be selected independently for each test based on the objective of the study, the expected range of toxicity, the receiving water concentration, and any available historical testing information on the effluent. USEPA (2000a) provides additional guidance on choosing appropriate test concentrations.

8.10.3 When these tests are used in determining compliance with permit limits, effluent test concentrations should be selected to bracket the receiving water concentration. This may be achieved by selecting effluent test concentrations in the following manner: (1) 100% effluent, (2) [RWC + 100]/2, (3) RWC, (4) RWC/2, and (5) RWC/4. For example, where the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

8.10.4 If acute/chronic ratios are to be determined by simultaneous acute and short-term chronic tests with a single species, using the same sample, both types of tests must use the same test conditions, i.e., pH, temperature, water hardness, salinity, etc.

8.11 RECEIVING WATER TESTS

8.11.1 Receiving water toxicity tests generally consist of 100% receiving water and a control. The total salinity of the control should be comparable to the receiving water.

8.11.2 The data from the two treatments are analyzed by hypothesis testing to determine if test organism survival in the receiving water differs significantly from the control. Four replicates and 10 organisms per replicate are required for each treatment (see Summary of Test Conditions and Test Acceptability Criteria in the specific test method).

8.11.3 In cases where the objective of the test is to estimate the degree of toxicity of the receiving water, a definitive, multiconcentration test is performed by preparing dilutions of the receiving water, using a ≥0.5 dilution series, with a suitable control water.

SECTION 9

CHRONIC TOXICITY TEST ENDPOINTS AND DATA ANALYSIS

9.1 ENDPOINTS

9.1.1 The objective of chronic aquatic toxicity tests with effluents and pure compounds is to estimate the highest "safe" or "no-effect concentration" of these substances. For practical reasons, the responses observed in these tests are usually limited to hatchability, gross morphological abnormalities, survival, growth, and reproduction, and the results of the tests are usually expressed in terms of the highest toxicant concentration that has no statistically significant observed effect on these responses, when compared to the controls. The terms currently used to define the endpoints employed in the rapid, chronic and sub-chronic toxicity tests have been derived from the terms previously used for full life-cycle tests. As shorter chronic tests were developed, it became common practice to apply the same terminology to the endpoints. The terms used in this manual are as follows:

9.1.1.1 Safe Concentration - The highest concentration of toxicant that will permit normal propagation of fish and other aquatic life in receiving waters. The concept of a "safe concentration" is a biological concept, whereas the "no-observed-effect concentration" (below) is a statistically defined concentration.

9.1.1.2 No-Observed-Effect-Concentration (NOEC) - The highest concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle (short-term) test, that causes no observable adverse effects on the test organisms (i.e., the highest concentration of toxicant in which the values for the observed responses are not statistically significantly different from the controls). This value is used, along with other factors, to determine toxicity limits in permits.

9.1.1.3 Lowest-Observed-Effect-Concentration (LOEC) - The lowest concentration of toxicant to which organisms are exposed in a life-cycle or partial life-cycle (short-term) test, which causes adverse effects on the test organisms (i.e., where the values for the observed responses are statistically significantly different from the controls).

9.1.1.4 Effective Concentration (EC) - A point estimate of the toxicant concentration that would cause an observable adverse affect on a quantal, "all or nothing," response (such as death, immobilization, or serious incapacitation) in a given percent of the test organisms, calculated by point estimation techniques. If the observable effect is death or immobility, the term, Lethal Concentration (LC), should be used (see Subsection 9.1.1.5). A certain EC or LC value might be judged from a biological standpoint to represent a threshold concentration, or lowest concentration that would cause an adverse effect on the observed response.

9.1.1.5 Lethal Concentration (LC) - The toxicant concentration that would cause death in a given percent of the test population. Identical to EC when the observable adverse effect is death. For example, the LC50 is the concentration of toxicant that would cause death in 50% of the test population.

9.1.1.6 Inhibition Concentration (IC) - The toxicant concentration that would cause a given percent reduction in a nonquantal biological measurement for the test population. For example, the IC25 is the concentration of toxicant that would cause a 25% reduction in mean young per female or in growth for the test population, and the IC50 is the concentration of toxicant that would cause a 50% reduction in the mean population responses.

9.2 RELATIONSHIP BETWEEN ENDPOINTS DETERMINED BY HYPOTHESIS TESTING AND POINT ESTIMATION TECHNIQUES

9.2.1 If the objective of chronic aquatic toxicity tests with effluents and pure compounds is to estimate the highest "safe or no-effect concentration" of these substances, it is imperative to understand how the statistical endpoints of these tests are related to the "safe" or "no-effect" concentration. NOECs and LOECs are determined by hypothesis testing (Dunnett's Test, a t test with the Bonferroni adjustment, Steel's Many-One Rank Test, or the Wilcoxon Rank

Sum Test with Bonferroni adjustment), whereas LCs, ICs, and ECs are determined by point estimation techniques (Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, the Graphical Method or Linear Interpolation Method). There are inherent differences between the use of a NOEC or LOEC derived from hypothesis testing to estimate a "safe" concentration, and the use of a LC, IC, EC, or other point estimates derived from curve fitting, interpolation, etc.

9.2.2 Most point estimates, such as the LC, IC, or EC are derived from a mathematical model that assumes a continuous dose-response relationship. By definition, any LC, IC, or EC value is an estimate of some amount of adverse effect. Thus the assessment of a "safe" concentration must be made from a biological standpoint rather than with a statistical test. In this instance, the biologist must determine some amount of adverse effect that is deemed to be "safe," in the sense that from a practical biological viewpoint it will not affect the normal propagation of fish and other aquatic life in receiving waters.

9.2.3 The use of NOECs and LOECs, on the other hand, assumes either (1) a continuous dose-response relationship, or (2) a non-continuous (threshold) model of the dose-response relationship.

9.2.3.1 In the case of a continuous dose-response relationship, it is also assumed that adverse effects that are not "statistically observable" are also not important from a biological standpoint, since they are not pronounced enough to test as statistically significant against some measure of the natural variability of the responses.

9.2.3.2 In the case of non-continuous dose-response relationships, it is assumed that there exists a true threshold, or concentration below which there is no adverse effect on aquatic life, and above which there is an adverse effect. The purpose of the statistical analysis in this case is to estimate as closely as possible where that threshold lies.

9.2.3.3 In either case, it is important to realize that the amount of adverse effect that is statistically observable (LOEC) or not observable (NOEC) is highly dependent on all aspects of the experimental design, such as the number of concentrations of toxicant, number of replicates per concentration, number of organisms per replicate, and use of randomization. Other factors that affect the sensitivity of the test include the choice of statistical analysis, the choice of an alpha level, and the amount of variability between responses at a given concentration.

9.2.3.4 Where the assumption of a continuous dose-response relationship is made, by definition some amount of adverse effect might be present at the NOEC, but is not great enough to be detected by hypothesis testing.

9.2.3.5 Where the assumption of a noncontinuous dose-response relationship is made, the NOEC would indeed be an estimate of a "safe" or "no-effect" concentration if the amount of adverse effect that appears at the threshold is great enough to test as statistically significantly different from the controls in the face of all aspects of the experimental design mentioned above. If, however, the amount of adverse effect at the threshold were not great enough to test as statistically different, some amount of adverse effect might be present at the NOEC. In any case, the estimate of the NOEC with hypothesis testing is always dependent on the aspects of the experimental design mentioned above. For this reason, the reporting and examination of some measure of the sensitivity of the test (either the minimum significant difference or the percent change from the control that this minimum difference represents) is extremely important.

9.2.4 In summary, the assessment of a "safe" or "no-effect" concentration cannot be made from the results of statistical analysis alone, unless (1) the assumptions of a strict threshold model are accepted, and (2) it is assumed that the amount of adverse effect present at the threshold is statistically detectable by hypothesis testing. In this case, estimates obtained from a statistical analysis are indeed estimates of a "no-effect" concentration. If the assumptions are not deemed tenable, then estimates from a statistical analysis can only be used in conjunction with an assessment from a biological standpoint of what magnitude of adverse effect constitutes a "safe" concentration. In this instance, a "safe" concentration is not necessarily a truly "no-effect" concentration, but rather a concentration at which the effects are judged to be of no biological significance.

9.2.5 A better understanding of the relationship between endpoints derived by hypothesis testing (NOECs) and point estimation techniques (LCs, ICs, and ECs) would be very helpful in choosing methods of data analysis. Norberg-King (1991) reported that the IC25s were comparable to the NOECs for 23 effluent and reference toxicant data sets analyzed. The data sets included short-term chronic toxicity tests for the sea urchin, *Arbacia punctulata*, the sheepshead minnow, *Cyprinodon variegatus*, and the red macroalga, *Champia parvula*. Birge et al. (1985) reported that LC1s derived from Probit Analyses of data from short-term embryo-larval tests with reference toxicants were comparable to NOECs for several organisms. Similarly, USEPA (1988d) reported that the IC25s were comparable to the NOECs for a set of daphnia, *Ceriodaphnia dubia* chronic tests with a single reference toxicant. However, the scope of these comparisons was very limited, and sufficient information is not yet available to establish an overall relationship between these two types of endpoints, especially when derived from effluent toxicity test data.

9.3 PRECISION

9.3.1 HYPOTHESIS TESTS

9.3.1.1 When hypothesis tests are used to analyze toxicity test data, it is not possible to express precision in terms of a commonly used statistic. The results of the test are given in terms of two endpoints, the No-Observed-Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC). The NOEC and LOEC are limited to the concentrations selected for the test. The width of the NOEC-LOEC interval is a function of the dilution series, and differs greatly depending on whether a dilution factor of 0.3 or 0.5 is used in the test design. Therefore, **USEPA recommends the use of the ≥ 0.5 dilution factor** (see Section 4, Quality Assurance). It is not possible to place confidence limits on the NOEC and LOEC derived from a given test, and it is difficult to quantify the precision of the NOEC-LOEC endpoints between tests. If the data from a series of tests performed with the same toxicant, toxicant concentrations, and test species, were analyzed with hypothesis tests, precision could only be assessed by a qualitative comparison of the NOEC-LOEC intervals, with the understanding that maximum precision would be attained if all tests yielded the same NOEC-LOEC interval. In practice, the precision of results of repetitive chronic tests is considered acceptable if the NOECs vary by no more than one concentration interval above or below a central tendency. Using these guidelines, the "normal" range of NOECs from toxicity tests using a 0.5 dilution factor (two-fold difference between adjacent concentrations), would be four-fold.

9.3.2 POINT ESTIMATION TECHNIQUES

9.3.2.1 Point estimation techniques have the advantage of providing a point estimate of the toxicant concentration causing a given amount of adverse (inhibiting) effect, the precision of which can be quantitatively assessed (1) within tests by calculation of 95% confidence limits, and (2) across tests by calculating a standard deviation and coefficient of variation.

9.3.2.2 It should be noted that software used to calculate point estimates occasionally may not provide associated 95% confidence intervals. This situation may arise when test data do not meet specific assumptions required by the statistical methods, when point estimates are outside of the test concentration range, and when specific limitations imposed by the software are encountered. USEPA (2000a) provides guidance on confidence intervals under these circumstances.

9.4 DATA ANALYSIS

9.4.1 ROLE OF THE STATISTICIAN

9.4.1.1 The use of the statistical methods described in this manual for routine data analysis does not require the assistance of a statistician. However, the interpretation of the results of the analysis of the data from any of the toxicity tests described in this manual can become problematic because of the inherent variability and sometimes unavoidable anomalies in biological data. If the data appear unusual in any way, or fail to meet the necessary

assumptions, a statistician should be consulted. Analysts who are not proficient in statistics are strongly advised to seek the assistance of a statistician before selecting the method of analysis and using any of the results.

9.4.1.2 The statistical methods recommended in this manual are not the only possible methods of statistical analysis. Many other methods have been proposed and considered. Certainly there are other reasonable and defensible methods of statistical analysis for this kind of toxicity data. Among alternative hypothesis tests some, like Williams' Test, require additional assumptions, while others, like the bootstrap methods, require computer-intensive computations. Alternative point estimations approaches most probably would require the services of a statistician to determine the appropriateness of the model (goodness of fit), higher order linear or nonlinear models, confidence intervals for estimates generated by inverse regression, etc. In addition, point estimation or regression approaches would require the specification by biologists or toxicologists of some low level of adverse effect that would be deemed acceptable or safe. The statistical methods contained in this manual have been chosen because they are (1) applicable to most of the different toxicity test data sets for which they are recommended, (2) powerful statistical tests, (3) hopefully "easily" understood by nonstatisticians, and (4) amenable to use without a computer, if necessary.

9.4.2 PLOTTING THE DATA

9.4.2.1 The data should be plotted, both as a preliminary step to help detect problems and unsuspected trends or patterns in the responses, and as an aid in interpretation of the results. Further discussion and plotted sets of data are included in the methods and the Appendices.

9.4.3 DATA TRANSFORMATIONS

9.4.3.1 Transformations of the data, (e.g., arc sine square root and logs), are used where necessary to meet assumptions of the proposed analyses, such as the requirement for normally distributed data.

9.4.4 INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

9.4.4.1 Statistical independence among observations is a critical assumption in all statistical analysis of toxicity data. One of the best ways to ensure independence is to properly follow rigorous randomization procedures. Randomization techniques should be employed at the start of the test, including the randomization of the placement of test organisms in the test chambers and randomization of the test chamber location within the array of chambers. Discussions of statistical independence, outliers and randomization, and a sample randomization scheme, are included in Appendix A.

9.4.5 REPLICATION AND SENSITIVITY

9.4.5.1 The number of replicates employed for each toxicant concentration is an important factor in determining the sensitivity of chronic toxicity tests. Test sensitivity generally increases as the number of replicates is increased, but the point of diminishing returns in sensitivity may be reached rather quickly. The level of sensitivity required by a hypothesis test or the confidence interval for a point estimate will determine the number of replicates, and should be based on the objectives for obtaining the toxicity data.

9.4.5.2 In a statistical analysis of toxicity data, the choice of a particular analysis and the ability to detect departures from the assumptions of the analysis, such as the normal distribution of the data and homogeneity of variance, is also dependent on the number of replicates. More than the minimum number of replicates may be required in situations where it is imperative to obtain optimal statistical results, such as with tests used in enforcement cases or when it is not possible to repeat the tests. For example, when the data are analyzed by hypothesis testing, the nonparametric alternatives cannot be used unless there are at least four replicates at each toxicant concentration.

9.4.6 RECOMMENDED ALPHA LEVELS

9.4.6.1 The data analysis examples included in the manual specify an alpha level of 0.01 for testing the assumptions of hypothesis tests and an alpha level of 0.05 for the hypothesis tests themselves. These levels are common and well accepted levels for this type of analysis and are presented as a recommended minimum significance level for toxicity data analysis.

9.5 CHOICE OF ANALYSIS

9.5.1 The recommended statistical analysis of most data from chronic toxicity tests with aquatic organisms follows a decision process illustrated in the flowchart in Figure 2. An initial decision is made to use point estimation techniques (the Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, the Graphical Method, or Linear Interpolation Method) and/or to use hypothesis testing (Dunnett's Test, the t test with the Bonferroni adjustment, Steel's Many-one Rank Test, or Wilcoxon Rank Sum Test with the Bonferroni adjustment). **NOTE: For the NPDES Permit Program, the point estimation techniques are the preferred statistical methods in calculating end points for effluent toxicity tests.** If hypothesis testing is chosen, subsequent decisions are made on the appropriate procedure for a given set of data, depending on the results of tests of assumptions, as illustrated in the flowchart. A specific flow chart is included in the analysis section for each test.

9.5.2 Since a single chronic toxicity test might yield information on more than one parameter (such as survival, growth, and reproduction), the lowest estimate of a “no-observed-effect concentration” for any of the responses would be used as the “no observed effect concentration” for each test. It follows logically that in the statistical analysis of the data, concentrations that had a significant toxic effect on one of the observed responses would not be subsequently tested for an effect on some other response. This is one reason for excluding concentrations that have shown a statistically significant reduction in survival from a subsequent hypothesis test for effects on another parameter such as reproduction. A second reason is that the exclusion of such concentrations usually results in a more powerful and appropriate statistical analysis. In performing the point estimation techniques recommended in this manual, an all-data approach is used. For example, data from concentrations above the NOEC for survival are included in determining ICp estimates using the Linear Interpolation Method.

9.5.3 ANALYSIS OF GROWTH AND REPRODUCTION DATA

9.5.3.1 Growth data from the sheepshead minnow, *Cyprinodon variegatus*, and inland silverside, *Menidia beryllina*, larval survival and growth tests, and the mysid, *Mysidopsis bahia*, survival, growth, and fecundity test, are analyzed using hypothesis testing according to the flowchart in Figure 2. The above mentioned growth data may also be analyzed by generating a point estimate with the Linear Interpolation Method. Data from effluent concentrations that have tested significantly different from the control for survival are excluded from further hypothesis tests concerning growth effects. Growth is defined as the change in dry weight of the orginal number of test organisms when group weights are obtained. When analyzing the data using point estimating techniques, data from all concentrations are included in the analysis.

9.5.3.2 Fecundity data from the mysid, *Mysidopsis bahia*, test may be analyzed using hypothesis testing after an arc sine transformation according to the flowchart in Figure 2. The fecundity data from the mysid test may also be analyzed by generating a point estimate with the Linear Interpolation Method.

9.5.3.3 Reproduction data from the red macroalga, *Champia parvula*, test are analyzed using hypothesis testing as illustrated in Figure 2. The reproduction data from the red macroalga test may also be analyzed by generating a point estimate with the Linear Interpolation Method.

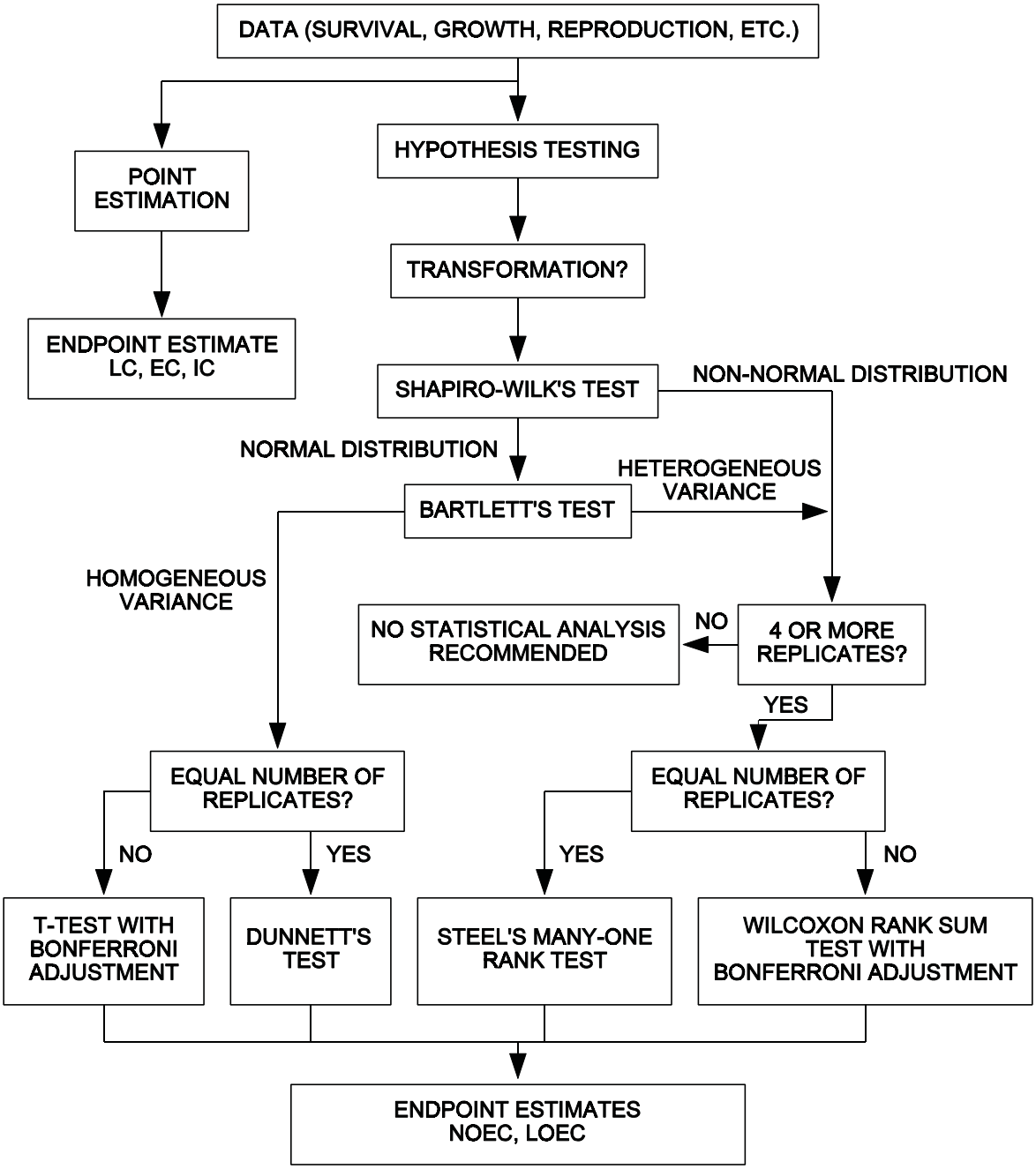


Figure 2. Flowchart for statistical analysis of test data

9.5.4 ANALYSIS OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION DATA

9.5.4.1 Data from the sea urchin, *Arbacia punctulata*, fertilization test may be analyzed by hypothesis testing after an arc sine transformation according to the flowchart in Figure 2. The fertilization data from the sea urchin test may also be analyzed by generating a point estimate with the Linear Interpolation Method.

9.5.5 ANALYSIS OF MORTALITY DATA

9.5.5.1 Mortality data are analyzed by Probit Analysis, if appropriate, or other point estimation techniques, (i.e., the Spearman-Karber Method, the Trimmed Spearman-Karber Method, or the Graphical Method) (see Appendices H-K) (see discussion below). The mortality data can also be analyzed by hypothesis testing, after an arc sine square root transformation (see Appendices B-F), according to the flowchart in Figure 2.

9.6 HYPOTHESIS TESTS

9.6.1 DUNNETT'S PROCEDURE

9.6.1.1 Dunnett's Procedure is used to determine the NOEC. The procedure consists of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison procedure for comparing each of the treatment means with the control mean, in a series of paired tests (see Appendix C). Use of Dunnett's Procedure requires at least three replicates per treatment to check the assumptions of the test. In cases where the numbers of data points (replicates) for each concentration are not equal, a t test may be performed with Bonferroni's adjustment for multiple comparisons (see Appendix D), instead of using Dunnett's Procedure.

9.6.1.2 The assumptions upon which the use of Dunnett's Procedure is contingent are that the observations within treatments are normally distributed, with homogeneity of variance. Before analyzing the data, these assumptions must be tested using the procedures provided in Appendix B.

9.6.1.3 If, after suitable transformations have been carried out, the normality assumptions have not been met, Steel's Many-one Rank Test should be used if there are four or more data points (replicates) per toxicant concentration. If the numbers of data points for each toxicant concentration are not equal, the Wilcoxon Rank Sum Test with Bonferroni's adjustment should be used (see Appendix F).

9.6.1.4 Some indication of the sensitivity of the analysis should be provided by calculating (1) the minimum difference between means that can be detected as statistically significant, and (2) the percent change from the control mean that this minimum difference represents for a given test.

9.6.1.5 A step-by-step example of the use of Dunnett's Procedure is provided in Appendix C.

9.6.2 T TEST WITH THE BONFERRONI ADJUSTMENT

9.6.2.1 The t test with the Bonferroni adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus, Dunnett's Procedure is a more powerful test.

9.6.2.2 The assumptions upon which the use of the t test with the Bonferroni adjustment is contingent are that the observations within treatments are normally distributed, with homogeneity of variance. These assumptions must be tested using the procedures provided in Appendix B.

9.6.2.3 The estimate of the safe concentration derived from this test is reported in terms of the NOEC. A step-by-step example of the use of a t-test with the Bonferroni adjustment is provided in Appendix D.

9.6.3 STEEL'S MANY-ONE RANK TEST

9.6.3.1 Steel's Many-one Rank Test is a multiple comparison procedure for comparing several treatments with a control. This method is similar to Dunnett's procedure, except that it is not necessary to meet the assumption of normality. The data are ranked, and the analysis is performed on the ranks rather than on the data themselves. If the data are normally or nearly normally distributed, Dunnett's Procedure would be more sensitive (would detect smaller differences between the treatments and control). For data that are not normally distributed, Steel's Many-one Rank Test can be much more efficient (Hodges and Lehmann, 1956).

9.6.3.2 It is necessary to have at least four replicates per toxicant concentration to use Steel's test. Unlike Dunnett's procedure, the sensitivity of this test cannot be stated in terms of the minimum difference between treatment means and the control mean that can be detected as statistically significant.

9.6.3.3 The estimate of the safe concentration is reported as the NOEC. A step-by-step example of the use of Steel's Many-One Rank Test is provided in Appendix E.

9.6.4 WILCOXON RANK SUM TEST WITH THE BONFERRONI ADJUSTMENT

9.6.4.1 The Wilcoxon Rank Sum Test is a nonparametric test for comparing a treatment with a control. The data are ranked and the analysis proceeds exactly as in Steel's Test except that Bonferroni's adjustment for multiple comparisons is used instead of Steel's tables. When Steel's test can be used (i.e., when there are equal numbers of data points per toxicant concentration), it will be more powerful (able to detect smaller differences as statistically significant) than the Wilcoxon Rank Sum Test with Bonferroni's adjustment.

9.6.4.2 The estimate of the safe concentration is reported as the NOEC. A step-by-step example of the use of the Wilcoxon Rank Sum Test with Bonferroni adjustment is provided in Appendix F.

9.6.5 A CAUTION IN THE USE OF HYPOTHESIS TESTING

9.6.5.1 If in the calculation of an NOEC by hypothesis testing, two tested concentrations cause statistically significant adverse effects, but an intermediate concentration did not cause statistically significant effects, the results should be used with extreme caution.

9.7 POINT ESTIMATION TECHNIQUES

9.7.1 PROBIT ANALYSIS

9.7.1.1 Probit Analysis is used to estimate an LC1, LC50, EC1, or EC50 and the associated 95% confidence interval. The analysis consists of adjusting the data for mortality in the control, and then using a maximum likelihood technique to estimate the parameters of the underlying log tolerance distribution, which is assumed to have a particular shape.

9.7.1.2 The assumption upon which the use of Probit Analysis is contingent is a normal distribution of log tolerances. If the normality assumption is not met, and at least two partial mortalities are not obtained, Probit Analysis should not be used. It is important to check the results of Probit Analysis to determine if use of the analysis is appropriate. The chi-square test for heterogeneity provides a good test of appropriateness of the analysis. The computer program (see discussion, Appendix H) checks the chi-square statistic calculated for the data set against the tabular value, and provides an error message if the calculated value exceeds the tabular value.

9.7.1.3 A discussion of Probit Analysis, and examples of computer program input and output, are found in Appendix H.

9.7.1.4 In cases where Probit Analysis is not appropriate, the LC50 and confidence interval may be estimated by the Spearman-Karber Method (Appendix I) or the Trimmed Spearman-Karber Method (Appendix J). If a test results in 100% survival and 100% mortality in adjacent treatments (all or nothing effect), the LC50 may be estimated using the Graphical Method (Appendix K).

9.7.2 LINEAR INTERPOLATION METHOD

9.7.2.1 The Linear Interpolation Method (see Appendix L) is a procedure to calculate a point estimate of the effluent or other toxicant concentration [Inhibition Concentration, (IC)] that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction, growth, fertilization, or fecundity of the test organisms. The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests.

9.7.2.2 Use of the Linear Interpolation Method is based on the assumptions that the responses (1) are monotonically non-increasing (the mean response for each higher concentration is less than or equal to the mean response for the previous concentration), (2) follow a piece-wise linear response function, and (3) are from a random, independent, and representative sample of test data. The assumption for piece-wise linear response cannot be tested statistically, and no defined statistical procedure is provided to test the assumption for monotonicity. Where the observed means are not strictly monotonic by examination, they are adjusted by smoothing. In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean.

9.7.2.3 The inability to test the monotonicity and piece wise linear response assumptions for this method makes it difficult to assess when the method is, or is not, producing reliable results. Therefore, the method should be used with caution when the results of a toxicity test approach an "all or nothing" response from one concentration to the next in the concentration series, and when it appears that there is a large deviation from monotonicity. See Appendix L for a more detailed discussion of the use of this method and a computer program available for performing calculations.

SECTION 10

REPORT PREPARATION AND TEST REVIEW

10.1 REPORT PREPARATION

The toxicity data are reported, together with other appropriate data. The following general format and content are recommended for the report:

10.1.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contract Laboratory (if the test was performed under contract)
   a. Name of firm
   b. Phone number
   c. Address
6. Objective of test

10.1.2 PLANT OPERATIONS

1. Product(s)
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Volume of waste flow (MGD, CFS, GPM)
8. Design flow of treatment facility at time of sampling

10.1.3 SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER

1. Effluent Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Mean daily discharge on sample collection date
   f. Lapsed time from sample collection to delivery
   g. Sample temperature when received at the laboratory

2. Receiving Water Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Tide stages
   f. Sample temperature when received at the laboratory
   g. Lapsed time from sample collection to delivery

3. Dilution Water Samples
   a. Source
   b. Collection date and time
   c. Pretreatment
   d. Physical and chemical characteristics

10.1.4 TEST METHODS

1. Toxicity test method used (title, number, source)
2. Endpoint(s) of test
3. Deviation(s) from reference method, if any, and the reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type of volume and test chambers
7. Volume of solution used per chamber
8. Number of organisms used per test chamber
9. Number of replicate test chambers per treatment
10. Acclimation of test organisms (temperature and salinity mean and range)
11. Test temperature (mean and range)
12. Specify if aeration was needed
13. Feeding frequency, and amount and type of food
14. Test salinity (mean and range)
15. Specify if (and how) pH control measures were implemented

10.1.5 TEST ORGANISMS

1. Scientific name and how determined
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)
7. Taxonomic key used for species identification

10.1.6 QUALITY ASSURANCE

1. Reference toxicant used routinely; source
2. Date and time of most recent reference toxicant test; test results and current control (cusum) chart
3. Dilution water used in reference toxicant test
4. Results (NOEC or, where applicable, LOEC, LC50, EC50, IC25 and/or IC50); report percent minimum significant difference (PMSD) calculated for sublethal endpoints determined by hypothesis testing in reference toxicant test
5. Physical and chemical methods used

10.1.7 RESULTS

1. Provide raw toxicity data in tabular form, including daily records of affected organisms in each concentration (including controls) and replicate, and in graphical form (plots of toxicity data)
2. Provide table of LC50s, NOECs, IC25, IC50, etc. (as required in the applicable NPDES permit)
3. Indicate statistical methods to calculate endpoints
4. Provide summary table of physical and chemical data
5. Tabulate QA data
6. Provide percent minimum significant difference (PMSD) calculated for sublethal endpoints

10.1.8 CONCLUSIONS AND RECOMMENDATIONS

1. Relationship between test endpoints and permit limits.
2. Action to be taken.

10.2 TEST REVIEW

10.2.1 Test review is an important part of an overall quality assurance program (Section 4) and is necessary for ensuring that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the regulatory authority.

10.2.2 SAMPLING AND HANDLING

10.2.2.1 The collection and handling of samples are reviewed to verify that the sampling and handling procedures given in Section 8 were followed. Chain-of-custody forms are reviewed to verify that samples were tested within allowable sample holding times (Subsection 8.5.4). Any deviations from the procedures given in Section 8 should be documented and described in the data report (Subsection 10.1).

10.2.3 TEST ACCEPTABILITY CRITERIA

10.2.3.1 Test data are reviewed to verify that test acceptability criteria (TAC) requirements for a valid test have been met. Any test not meeting the minimum test acceptability criteria is considered invalid. All invalid tests must be repeated with a newly collected sample.

10.2.4 TEST CONDITIONS

10.2.4.1 Test conditions are reviewed and compared to the specifications listed in the summary of test condition tables provided for each method. Physical and chemical measurements taken during the test (e.g., temperature, pH, and DO) also are reviewed and compared to specified ranges. Any deviations from specifications should be documented and described in the data report (Subsection 10.1).

10.2.4.2 The summary of test condition tables presented for each method identify test conditions as required or recommended. For WET test data submitted under NPDES permits, all required test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample. Deviations from recommended test conditions must be evaluated on a case-by-case basis to determine the validity of test results. Deviations from recommended test conditions may or may not invalidate a test result depending on the degree of the departure and the objective of the test. The reviewer should consider the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or accepting a test result as valid. For example, if dissolved oxygen is measured below 4.0 mg/L in one test chamber, the reviewer should consider whether any observed mortality in that test chamber corresponded with the drop in dissolved oxygen.

10.2.4.3 Whereas slight deviations in test conditions may not invalidate an individual test result, test condition deviations that continue to occur frequently in a given laboratory may indicate the need for improved quality control in that laboratory.

10.2.5 STATISTICAL METHODS

10.2.5.1 The statistical methods used for analyzing test data are reviewed to verify that the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report. Statistical methods other than those recommended in the statistical flowcharts may be appropriate (see Subsection 9.4.1.2), however, the laboratory must document the use of and provide the rationale for the use of any alternate statistical method. In all cases (flowchart recommended

methods or alternate methods), reviewers should verify that the necessary assumptions are met for the statistical method used.

10.2.6 CONCENTRATION-RESPONSE RELATIONSHIPS

10.2.6.1 The concept of a concentration-response, or more classically, a dose-response relationship is "the most fundamental and pervasive one in toxicology" (Casarett and Doull, 1975). This concept assumes that there is a causal relationship between the dose of a toxicant (or concentration for toxicants in solution) and a measured response. A response may be any measurable biochemical or biological parameter that is correlated with exposure to the toxicant. The classical concentration-response relationship is depicted as a sigmoidal shaped curve, however, the particular shape of the concentration-response curve may differ for each coupled toxicant and response pair. In general, more severe responses (such as acute effects) occur at higher concentrations of the toxicant, and less severe responses (such as chronic effects) occur at lower concentrations. A single toxicant also may produce multiple responses, each characterized by a concentration-response relationship. A corollary of the concentration-response concept is that every toxicant should exhibit a concentration-response relationship, given that the appropriate response is measured and given that the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent possesses toxicity and in identifying anomalous test results.

10.2.6.2 The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. USEPA (2000a) provides guidance on evaluating concentration-response relationships to assist in determining the validity of WET test results. All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to USEPA guidance on the evaluation of concentration-response relationships (USEPA, 2000a). This guidance provides review steps for 10 different concentration-response patterns that may be encountered in WET test data. Based on the review, the guidance provides one of three determinations: that calculated effect concentrations are reliable and should be reported, that calculated effect concentrations are anomalous and should be explained, or that the test was inconclusive and the test should be repeated with a newly collected sample. It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Data from some tests may suggest consultation with professional toxicologists and/or regulatory officials. Tests that exhibit unexpected concentration-response relationships also may indicate a need for further investigation and possible retesting.

10.2.7 REFERENCE TOXICANT TESTING

10.2.7.1 Test review of a given effluent or receiving water test should include review of the associated reference toxicant test and current control chart. Reference toxicant testing and control charting is required for documenting the quality of test organisms (Subsection 4.7) and ongoing laboratory performance (Subsection 4.16). The reviewer should verify that a quality control reference toxicant test was conducted according to the specified frequency required by the permitting authority or recommended by the method (e.g., monthly). The test acceptability criteria, test conditions, concentration-response relationship, and test sensitivity of the reference toxicant test are reviewed to verify that the reference toxicant test conducted was a valid test. The results of the reference toxicant test are then plotted on a control chart (see Subsection 4.16) and compared to the current control chart limits (± 2 standard deviations).

10.2.7.2 Reference toxicant tests that fall outside of recommended control chart limits are evaluated to determine the validity of associated effluent and receiving water tests (see Subsection 4.16). An out of control reference toxicant test result does not necessarily invalidate associated test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increasing test organism sensitivity or toward decreasing test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test. More frequent and/or concurrent reference toxicant testing may be advantageous if recent problems (e.g., invalid tests, reference toxicant test results outside of control chart limits, reduced health of organism cultures, or increased within-test variability) have been identified in testing.

10.2.8 TEST VARIABILITY

10.2.8.1 The within-test variability of individual tests should be reviewed. Excessive within-test variability may invalidate a test result and warrant retesting. For evaluating within-test variability, reviewers should consult EPA guidance on upper and lower percent minimum significant difference (PMSD) bounds (USEPA, 2000b).

10.2.8.2 When NPDES permits require sublethal hypothesis testing endpoints from Methods 1006.0 or 1007.0 (e.g., growth NOECs and LOECs), within-test variability must be reviewed and variability criteria must be applied as described in this section (10.2.8.2). When the methods are used for non-regulatory purposes, the variability criteria herein are recommended but are not required, and their use (or the use of alternative variability criteria) may depend upon the intended uses of the test results and the requirements of any applicable data quality objectives and quality assurance plan.

10.2.8.2.1 To measure test variability, calculate the percent minimum significant difference (PMSD) achieved in the test. The PMSD is the smallest percentage decrease in growth or reproduction from the control that could be determined as statistically significant in the test. The PMSD is calculated as 100 times the minimum significant difference (MSD) divided by the control mean. The equation and examples of MSD calculations are shown in Appendix C. PMSD may be calculated legitimately as a descriptive statistic for within-test variability, even when the hypothesis test is conducted using a non-parametric method. The PMSD bounds were based on a representative set of tests, including tests for which a non-parametric method was required for determining the NOEC or LOEC. The conduct of hypothesis testing to determine test results should follow the statistical flow charts provided for each method. That is, when test data fail to meet assumptions of normality or heterogeneity of variance, a non-parametric method (determined following the statistical flowchart for the method) should be used to calculate test results, but the PMSD may be calculated as described above (using parametric methods) to provide a measure of test variability.

10.2.8.2.2 Compare the PMSD measured in the test with the upper PMSD bound variability criterion listed in Table 6. When the test PMSD exceeds the upper bound, the variability among replicates is unusually large for the test method. Such a test should be considered insufficiently sensitive to detect toxic effects on growth or reproduction of substantial magnitude. A finding of toxicity at a particular concentration may be regarded as trustworthy, but a finding of "no toxicity" or "no statistically significant toxicity" at a particular concentration should not be regarded as a reliable indication that there is no substantial toxic effect on growth or reproduction at that concentration.

10.2.8.2.3 If the PMSD measured for the test is less than or equal to the upper PMSD bound variability criterion in Table 6, then the test's variability measure lies within normal bounds and the effect concentration estimate (e.g., NOEC or LOEC) would normally be accepted unless other test review steps raise serious doubts about its validity.

10.2.8.2.4 If the PMSD measured for the test exceeds the upper PMSD bound variability criterion in Table 6, then one of the following two cases applies (10.2.8.2.4.1, 10.2.8.2.4.2).

10.2.8.2.4.1 If toxicity is found at the permitted receiving water concentration (RWC) based upon the value of the effect concentration estimate (NOEC or LOEC), then the test shall be accepted and the effect concentration estimate may be reported, unless other test review steps raise serious doubts about its validity.

10.2.8.2.4.2 If toxicity is not found at the permitted RWC based upon the value of the effect concentration estimate (NOEC or LOEC) and the PMSD measured for the test exceeds the upper PMSD bound, then the test shall not be accepted, and a new test must be conducted promptly on a newly collected sample.

10.2.8.2.5 To avoid penalizing laboratories that achieve unusually high precision, lower PMSD bounds shall also be applied when a hypothesis test result (e.g., NOEC or LOEC) is reported. Lower PMSD bounds, which are based on the 10^{th} percentiles of national PMSD data, are presented in Table 6. The 10^{th} percentile PMSD represents a practical limit to the sensitivity of the test method because few laboratories are able to achieve such precision on a

regular basis and most do not achieve it even occasionally. In determining hypothesis test results (e.g., NOEC or LOEC), a test concentration shall not be considered toxic (i.e., significantly different from the control) if the relative difference from the control is less than the lower PMSD bounds in Table 6. See USEPA, 2000b for specific examples of implementing lower PMSD bounds.

10.2.8.3 To assist in reviewing within-test variability, EPA recommends maintaining control charts of PMSDs calculated for successive effluent tests (USEPA, 2000b). A control chart of PMSD values characterizes the range of variability observed within a given laboratory, and allows comparison of individual test PMSDs with the laboratory's typical range of variability. Control charts of other variability and test performance measures, such as the MSD, standard deviation or CV of control responses, or average control response, also may be useful for reviewing tests and minimizing variability. The log of PMSD will provide an approximately normal variate useful for control charting.

TABLE 6. VARIABILITY CRITERIA (UPPER AND LOWER PMSD BOUNDS) FOR SUBLETHAL HYPOTHESIS TESTING ENDPOINTS SUBMITTED UNDER NPDES PERMITS.[1]

Test Method	Endpoint	Lower PMSD Bound	Upper PMSD Bound
Method 1006.0, Inland Silverside Larval Survival and Growth Test	growth	11	28
Method 1007.0, *Mysidopsis bahia* Survival, Growth, and Fecundity Test	growth	11	37

[1] Lower and upper PMSD bounds were determined from the 10th and 90th percentile, respectively, of PMSD data from EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2000b).

SECTION 11

TEST METHOD

SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS* LARVAL SURVIVAL AND GROWTH TEST METHOD 1004.0

11.1 SCOPE AND APPLICATION

11.1.1 This method, adapted in part from USEPA (1987b), estimates the chronic toxicity of effluents and receiving waters to the sheepshead minnow, *Cyprinodon variegatus*, using newly hatched larvae in a seven-day, static-renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test species.

11.1.2 Daily observations on mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

11.1.3 Detection limits of the toxicity of an effluent or chemical are organism dependent.

11.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly volatile and highly degradable toxicants present in the source may not be detected in the test.

11.1.5 This method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

11.2 SUMMARY OF METHOD

11.2.1 Sheepshead minnow, *Cyprinodon variegatus*, larvae (preferably less than 24-h old) are exposed in a static renewal system for seven days to different concentrations of effluent or to receiving water. Test results are based on the survival and weight of the larvae.

11.3 INTERFERENCES

11.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

11.3.2 Adverse effects of low dissolved oxygen concentrations (DO), high concentrations of suspended and/or dissolved solids, and extremes of pH, may mask the effects of toxic substances.

11.3.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.3.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

11.3.5 Food added during the test may sequester metals and other toxic substances and reduce the apparent toxicity of the test substance. However, in a growth test the nutritional needs of the organisms must be satisfied, even if feeding has the potential to confound test results.

11.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 11.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 11.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

11.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 11.3.6.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample after adjusting the sample salinity for use in marine testing.

11.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ±0.3 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ± 0.3 pH units in pH-controlled tests (USEPA, 1996).

11.3.6.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

11.3.6.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 11.3.6.1.1).

11.3.6.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 11.3.6.2) is applied routinely to subsequent testing of the effluent.

11.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.9). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992); or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior

experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the sample after adjusting the sample salinity for use in marine testing. USEPA (1996) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

11.4 SAFETY

11.4.1 See Section 3, Health and Safety.

11.5 APPARATUS AND EQUIPMENT

11.5.1 Facilities for holding and acclimating test organisms.

11.5.2 Brine shrimp, *Artemia*, culture unit -- see Subsection 11.6.14 below and Section 4, Quality Assurance.

11.5.3 Sheepshead minnow culture unit -- see Subsection 11.6.15 below. The maximum number of larvae required per test will range from a maximum of 360, if 15 larvae are used in each of four replicates, to a minimum of 240 per test, if 10 larvae are used in each of four replicates. It is preferable to obtain the test organisms from an in-house culture unit. If it is not feasible to culture fish in-house, embryos or newly hatched larvae can be obtained from other sources if shipped in well oxygenated saline water in insulated containers.

11.5.4 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L.

11.5.5 Environmental chamber or equivalent facility with temperature control (25 ± 1°C).

11.5.6 Water purification system -- Millipore Milli-Q®, deionized water (DI) or equivalent.

11.5.7 Balance -- Analytical, capable of accurately weighing to 0.00001 g.

11.5.8 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus fish.

11.5.9 Drying oven -- 50-105°C range, for drying larvae.

11.5.10 Air pump -- for oil-free air supply.

11.5.11 Air lines, and air stones -- for aerating water containing embryos or larvae, or for supplying air to test solutions with low DO.

11.5.12 Meters, pH and DO -- for routine physical and chemical measurements.

11.5.13 Standard or micro-Winkler apparatus -- for determining DO (optional).

11.5.14 Dissecting microscope -- for checking embryo viability.

11.5.15 Desiccator -- for holding dried larvae.

11.5.16 Light box -- for counting and observing larvae.

11.5.17 Refractometer -- for determining salinity.

11.5.18 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

11.5.19 Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.

11.5.20 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, 1979b) -- to calibrate laboratory thermometers.

11.5.21 Test chambers -- four for each concentration and control. Borosilicate glass 1000 mL beakers or modified Norberg and Mount (1985) glass chambers used in the short-term inland silverside test may be used. It is recommended that each chamber contain a minimum of 50 mL/larvae and allow adequate depth of test solution (5.0 cm). To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic (6 mm thick).

11.5.22 Beakers -- six Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

11.5.23 Wash bottles -- for deionized water, for washing embryos from substrates and containers, and for rinsing small glassware and instrument electrodes and probes.

11.5.24 Crystallization dishes, beakers, culture dishes (1 L), or equivalent -- for incubating embryos.

11.5.25 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

11.5.26 Separatory funnels, 2-L -- two to four for culturing *Artemia* nauplii.

11.5.27 Pipets, volumetric -- Class A, 1-100 mL.

11.5.28 Pipets, automatic -- adjustable, 1-100 mL.

11.5.29 Pipets, serological -- 1-10 mL, graduated.

11.5.30 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

11.5.31 Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring larvae.

11.5.32 Siphon with bulb and clamp -- for cleaning test chambers.

11.5.33 Forceps -- for transferring dead larvae to weighing boats.

11.5.34 NITEX® or stainless steel mesh sieves (≤ 150 µm, 500 µm, 3 to 5 mm) -- for collecting *Artemia* nauplii and fish embryos, and for spawning baskets, respectively.

11.6 REAGENTS AND CONSUMABLE MATERIALS

11.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.6.2 Data sheets (one set per test) -- for data recording.

11.6.3 Vials, marked -- 24 per test, containing 4% formalin or 70% ethanol, to preserve larvae (optional).

11.6.4 Weighing pans, aluminum -- 24 per test.

11.6.5 Tape, colored -- for labeling test chambers.

11.6.6 Markers, waterproof -- for marking containers, etc.

11.6.7 Buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979b).

11.6.8 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.l, USEPA, 1979b), or reagents -- for modified Winkler analysis.

11.6.9 Laboratory quality control samples and standards -- for calibration of the above methods.

11.6.10 Reference toxicant solutions -- see Section 4, Quality Assurance.

11.6.11 Ethanol (70%) or formalin (4%) -- for use as a preservative for the fish larvae.

11.6.12 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.

11.6.13 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

11.6.13.1 Saline test and dilution water -- The salinity of the test water must be in the range of 20 to 32‰. The salinity should vary by no more than ± 2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar. This test is not recommended for salinities less than 20‰.

11.6.13.2 The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Exposure of sheepshead minnow larvae to these effluents will require adjustments in the salinity of the test solutions. It is important to maintain a constant salinity across all treatments. In addition, it may be desirable to match the test salinity with that of the receiving water. Two methods are available to adjust salinities -- a hypersaline brine derived from natural seawater or artificial sea salts.

11.6.13.3 Hypersaline brine (HSB): HSB has several advantages that make it desirable for use in toxicity testing. It can be made from any high quality, filtered seawater by evaporation, and can be added to the effluent or to deionized water to increase the salinity. HSB derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and may be stored for prolonged periods without any apparent degradation. However, if 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ salinity and 70% at 30‰ salinity.

11.6.13.3.1 The ideal container for making brine from natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is used, use only oil-free air compressors to prevent contamination.

11.6.13.3.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough deionized water rinses.

11.6.13.3.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

11.6.13.3.4 The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

11.6.13.3.5 After the required salinity is attained, the HSB should be filtered a second time through a l µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The containers should be capped and labelled with the date the HSB was generated and its salinity. Containers of HSB should be stored in the dark and maintained at room temperature until used.

11.6.13.3.6 If a source of HSB is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and HSB before adding the effluent.

11.6.13.3.7 Divide the salinity of the HSB by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 20‰, 100‰ divided by 20‰ = 5.0. The proportion of brine is 1 part in 5 (one part brine to four parts deionized water). To make 1 L of seawater at 20‰ salinity from a HSB of 100‰, divide 1 L (1000 mL) by 5.0. The result, 200 mL, is the quantity of brine needed to make 1 L of seawater. The difference, 800 mL, is the quantity of deionized water required.

11.6.13.4 Artificial sea salts: FORTY FATHOMS® brand sea salts have been used successfully at the EMSL-Cincinnati to maintain and spawn sheephead minnows and perform the larval survival and growth test (see Section 7, Dilution Water). HW MARINEMIX® sea salts have been used successfully at the USEPA Region 6 Houston Laboratory to maintain and spawn sheephead minnows and perform the larval growth and survival test and the embryo-larval survival and teratogenicity test. In addition, a slightly modified version of the GP2 medium (Spotte et al., 1984) has been successfully used to perform the sheepshead minnow survival and growth test (Table 1). Artifical sea salts may be used for culturing sheepshead minnows and for the larval survival and growth test if the criteria for acceptability of test data are satisfied (see Subsection 11.12).

11.6.13.4.1 Synthetic sea salts are packaged in plastic bags and mixed with deionized water or equivalent. The instructions on the package of sea salts should be followed carefully, and the salts should be mixed in a separate container -- not in the culture tank. The deionized water used in hydration should be in the temperature range of 21-26°C. Seawater made from artificial sea salts is conditioned (Spotte, 1973; Spotte et al., 1984; Bower, 1983) before it is used for culturing or testing. After adding the water, place an air stone in the container, cover, and aerate the solution mildly for 24 h before use.

11.6.13.4.2 The GP2 reagent grade chemicals (Table 1) should be mixed with deionized (DI) water or its equivalent in a container other than the culture or testing tanks. The deionized water used for hydration should be

between 21-26°C. The artificial seawater must be conditioned (aerated) for 24 h before use as the testing medium. If the solution is to be autoclaved, sodium bicarbonate is added after the solution has cooled. A stock solution of sodium bicarbonate is made up by dissolving 33.6 g $NaHCO_3$ in 500 mL of deionized water. Add 2.5 mL of this stock solution for each liter of the GP2 artificial seawater.

TABLE 1. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, TOXICITY TEST[1,2,3]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
KCl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

[1] Modified GP2 from Spotte et al. (1984).
[2] The constituent salts and concentrations were taken from USEPA (l990b). The salinity is 30.89 g/L.
[3] GP2 can be diluted with deionized (DI) water to the desired test salinity.

11.6.14 BRINE SHRIMP, *ARTEMIA*, NAUPLII -- for feeding cultures and test organisms

11.6.14.1 Newly-hatched *Artemia* nauplii (see USEPA, 2002a) are used as food for sheepshead minnow larvae in toxicity tests and in the maintenance of continuous stock cultures. Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are currently preferred because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size.

11.6.14.2 Each new batch of *Artemia* cysts must be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (Leger et al., 1985, and Leger et al., 1986) against known suitable reference cysts by performing a side-by-side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be a previously tested and acceptable batch of cysts. A sample of newly-hatched *Artemia* nauplii from each new batch of cysts should be chemically analyzed. The *Artemia* cysts should not be used if the concentration of total organochlorine pesticides exceeds 0.15 µg/g wet weight or the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight. (For analytical methods see USEPA, 1982.)

11.6.14.3 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or a solution prepared by adding 35.0 g uniodized salt (NaCl) or artificial sea salts to 1 L of deionized water, to a 2-L separatory funnel, or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24 h at 27°C. (Hatching time varies

with incubation temperature and the geographic strain of *Artemia* used (USEPA, 1985a; USEPA, 2002a; ASTM, 1993).

3. After 24 h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic, and will concentrate at the bottom of the funnel if it is covered for 5-10 minutes. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 min without aeration.
4. Drain the nauplii into a beaker or funnel fitted with a ≤ 150 µm NITEX® or stainless steel screen, and rinse with seawater or equivalent before use.

11.6.14.4 Testing *Artemia* nauplii as food for toxicity test organisms.

11.6.14.4.1 The primary criterion for acceptability of each new supply of brine shrimp cysts is the ability of the nauplii to support good survival and growth of the sheepshead minnow larvae (see Subsection 11.12). The larvae used to evaluate the suitability of the brine shrimp nauplii must be of the same geographical origin, species, and stage of development as those used routinely in the toxicity tests. Sufficient data to detect differences in survival and growth should be obtained by using three replicate test vessels, each containing a minimum of 15 larvae, for each type of food.

11.6.14.4.2 The feeding rate and frequency, test vessels, volume of control water, duration of the test, and age of the nauplii at the start of the test, should be the same as used for the routine toxicity tests.

11.6.14.4.3 Results of the brine shrimp nutrition assay, where there are only two treatments, can be evaluated statistically by use of a t test. The "new" food is acceptable if there are no statistically significant differences in the survival and growth of the larvae fed the two sources of nauplii.

11.6.15 TEST ORGANISMS, SHEEPSHEAD MINNOWS, *CYPRINODON VARIEGATUS*

11.6.15.1 Brood Stock

11.6.15.1.1 Adult sheepshead minnows for use as brood stock may be obtained by seine in Gulf of Mexico and Atlantic coast estuaries, from commercial sources, or from young fish raised to maturity in the laboratory. Feral brood stocks and first generation laboratory fish are preferred, to minimize inbreeding.

11.6.15.1.2 To detect disease and to allow time for acute mortality due to the stress of capture, field-caught adults are observed in the laboratory a minimum of two weeks before using as a source of gametes. Injured or diseased fish are discarded.

11.6.15.1.3 Sheepshead minnows can be continuously cultured in the laboratory from eggs to adults. The larvae, juvenile, and adult fish should be kept in appropriate size rearing tanks, maintained at ambient laboratory temperature. The larvae should be fed sufficient newly-hatched *Artemia* nauplii daily to assure that live nauplii are always present. Juveniles are fed frozen adult brine shrimp and a commercial flake food, such as TETRA SM-80® or MARDEL AQUARIAN® Tropical Fish Flakes or equivalent. Adult fish (age one month) are fed flake food three or four times daily, supplemented with frozen adult brine shrimp.

11.6.15.1.3.1 Sheepshead minnows reach sexual maturity in three-to-five months after hatch, and have an average standard length of approximately 27 mm for females and 34 mm for males. At this time, the males begin to exhibit sexual dimorphism and initiate territorial behavior. When the fish reach sexual maturity and are to be used for natural spawning, the temperature should be controlled at 18-20°C.

11.6.15.1.4 Adults can be maintained in natural or artificial seawater in a flow-through or recirculating, aerated system consisting of an all-glass aquarium , or equivalent.

11.6.15.1.5 The system is equipped with an undergravel or outside biological filter of shells (Spotte, 1973; Bower, 1983) for conditioning the biological filter), or a cartridge filter, such as a MAGNUM® Filter, or an EHEIM® Filter,

or equivalent, at a salinity of 20-30‰ and a photoperiod of 16 h light/8 h dark.

11.6.15.2 Obtaining Embryos for Toxicity Tests (See USEPA, 1978)

11.6.15.2.1 Embryos can be shipped to the laboratory from an outside source or obtained from adults held in the laboratory. Ripe eggs can be obtained either by natural spawning or by intraperitoneal injection of the females with human chorionic gonadotrophin (HCG) hormone. If the culturing system for adults is temperature controlled, natural spawning can be induced. Natural spawning is preferred because repeated spawnings can be obtained from the same brood stock, whereas with hormone injection, the brood stock is sacrificed in obtaining gametes.

11.6.15.2.2 It should be emphasized that the injection and hatching schedules given below are to be used only as guidelines. Response to the hormone varies from stock to stock and with temperature. Time to hatch and percent viable hatch also vary among stocks and among batches of embryos obtained from the same stock, and are dependent on temperature, DO, and salinity. The coordination of spawning and hatching is further complicated by the fact that, even under the most ideal conditions, embryos spawned over a 24-h period may hatch over a 72-h period. Therefore, it is advisable (especially if natural spawning is used) to obtain fertilized eggs over several days to ensure that a sufficient number of newly hatched larvae (less than 24 h old) will be available to initiate a test.

11.6.15.2.3 Forced Spawning

11.6.15.2.3.1 HCG is reconstituted with sterile saline or Ringer's solution immediately before use. The standard HCG vial contains l,000 IU to be reconstituted in 10 mL of saline. Freeze-dried HCG which comes with premeasured and sterilized saline is the easiest to use. Use of a 50 IU dose requires injection of 0.05 mL of reconstituted hormone solution. Reconstituted HCG may be used for several weeks if kept in the refrigerator.

11.6.15.2.3.2 Each female is injected intraperitoneally with 50 IU HCG on two consecutive days, starting at least 10 days prior to the beginning of a test. Two days following the second injection, eggs are stripped from the females and mixed with sperm derived from excised macerated testes. At least ten females and five males are used per test to ensure that there is a sufficient number (400) of viable embryos.

11.6.15.2.3.3 HCG is injected into the peritoneal cavity, just below the skin, using as small a needle as possible. A 50 IU dose is recommended for females approximately 27 mm in standard length. A larger or smaller dose may be used for fish which are significantly larger or smaller than 27 mm. With injections made on days one and two, females which are held at 25°C should be ready for stripping on days 4, 5, and 6. Ripe females should show pronounced abdominal swelling, and release at least a few eggs in response to a gentle squeeze. Injected females should be isolated from males. It may be helpful if fish that are to be injected are maintained at 20°C before injection, and the temperature raised to 25°C on the day of the first injection.

11.6.15.2.3.4 Prepare the testes immediately before stripping the eggs from the females. Remove the testes from three-to-five males. The testes are paired, dark grey organs along the dorsal midline of the abdominal cavity. If the head of the male is cut off and pulled away from the rest of the fish, most of the internal organs can be pulled out of the body cavity, leaving the testes behind. The testes are placed in a few mL of seawater until the eggs are ready.

11.6.15.2.3.5 Strip the eggs from the females, into a dish containing 50-100 mL of seawater, by firmly squeezing the abdomen. Sacrifice the females and remove the ovaries if all the ripe eggs do not flow out freely. Break up any clumps of ripe eggs and remove clumps of ovarian tissue and underripe eggs. Ripe eggs are spherical, approximately 1 mm in diameter, and almost clear.

11.6.15.2.3.6 While being held over the dish containing the eggs, the testes are macerated in a fold of NITEX® screen (250-500 µm mesh) dampened with seawater. The testes are then rinsed with seawater to remove the sperm from tissue, and the remaining sperm and testes are washed into the dish. Let the eggs and milt stand together for 10-15 min, swirling occasionally.

11.6.15.2.3.7 Pour the contents of the dish into a beaker, and insert an airstone. Aerate gently, such that the water moves slowly over the eggs, and incubate at 25°C for 60-90 min. After incubation, wash the eggs on a NITEX® screen and resuspend them in clean seawater. Examine the eggs periodically under a dissecting microscope until they are in the 2-8 cell stage. (The stage at which it is easiest to tell the developing embryos from the abnormal embryos and unfertilized eggs; see Figure 1). The eggs can then be gently rolled on a NITEX® screen and culled (see Section 6, Test Organisms).

11.6.15.2.4 Natural Spawning

11.6.15.2.4.1 Cultures of adult fish to be used for spawning are maintained at 18-20°C until embryos are required. When embryos are required, raise the temperature to 25°C in the morning, seven or eight days before the beginning of a test. That afternoon, transfer the adult fish (generally, at least five females and three males) to a spawning chamber (approximately, 20 × 35 × 22 cm high; USEPA, 1978), which is a basket constructed of 3-5 mm NITEX® mesh, made to fit a 57-L (15 gal) aquarium. Spawning generally will begin within 24 h or less. Embryos will fall through the bottom of the basket and onto a collecting screen (250-500 µm mesh) or tray below the basket. Allow the embryos to collect for 24 h. Embryos are washed from the screen, checked for viability, and placed in incubation dishes. Replace the screens until a sufficient number of embryos have been collected. One-to-three spawning aquaria can be used to collect the required number of embryos to run a toxicity test. To help keep the embryos clean, the adults are fed while the screens are removed.

11.6.15.2.5 Incubation

11.6.15.2.5.1 Four hours post-fertilization, the embryos obtained by natural or forced spawning are rolled gently with a finger on a 250-500 µm Nitex® screen to remove excess fibers and tissue. The embryos have adhesive threads and tend to adhere to each other. Gentle rolling on the screen facilitates the culling process described below. To reduce fungal contamination of the newly spawned embryos after they have been manipulated, they should be placed in a 250 µm sieve and briskly sprayed with seawater from a squeeze bottle.

11.6.15.2.5.2 Under a dissecting microscope, separate and discard abnormal embryos and unfertilized eggs. While they are checked, the embryos are maintained in seawater at 25°C. The embryos should be in Stages C-G, Figure 1.

11.6.15.2.5.3 If the test is prepared with four replicates of 15 larvae at each of six treatments (five effluent concentrations and a control), and the combined mortality of eggs and larvae prior to the start of the test is less than 20%, approximately 400 viable embryos are required at this stage.

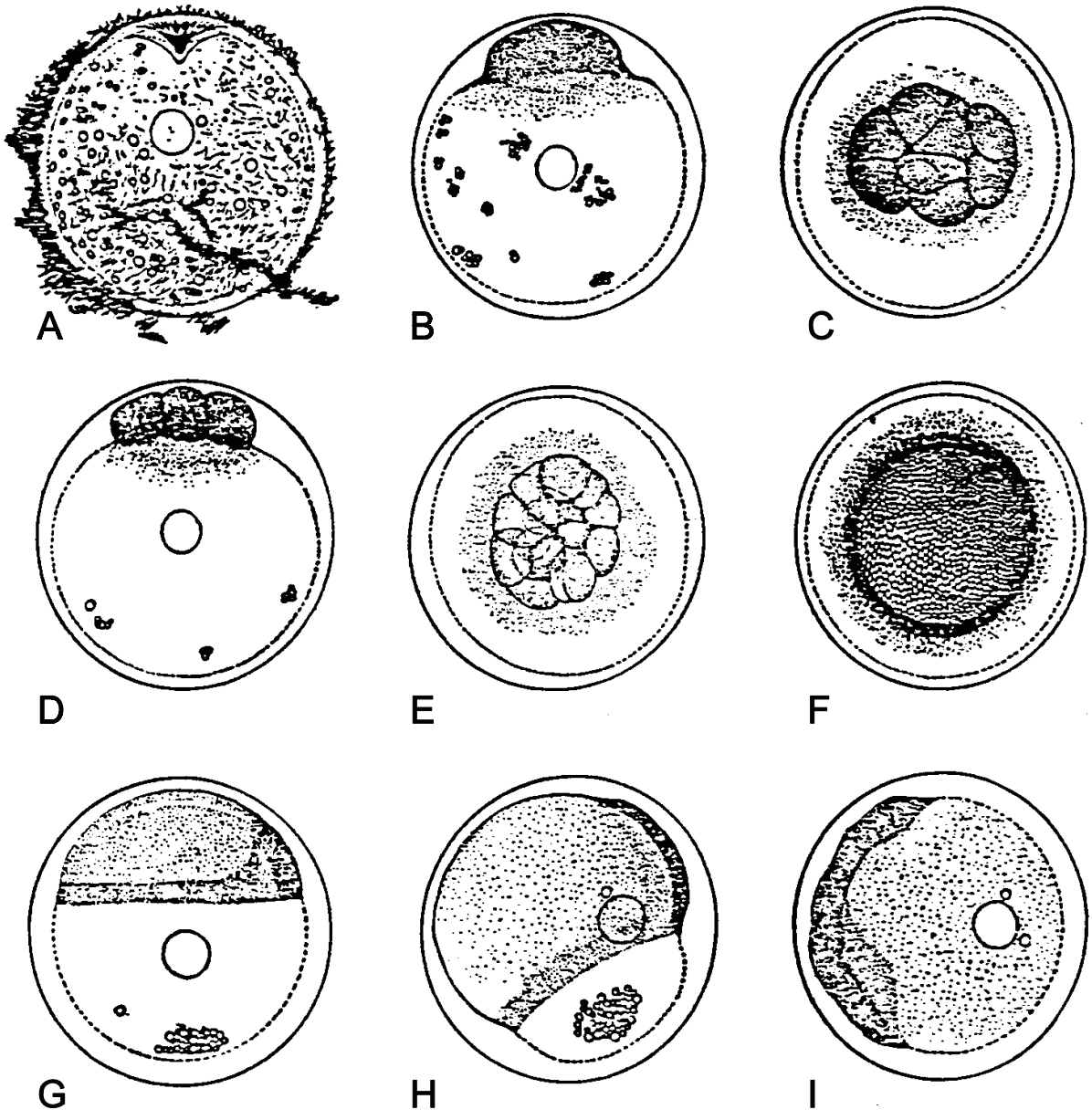


Figure 1. Embryonic development of sheepshead minnow, *Cyprinodon variegatus*: A. Mature unfertilized egg, showing attachment filaments and micropyle, X33; B. Blastodisc fully developed; C,D. Blastodisc, 8 cells; E. Blastoderm, 16 cells; F. Blastoderm, late cleavage stage; G. Blastoderm with germ ring formed, embryonic shield developing; H. Blastoderm covers over 3/4 of yolk, yolk noticeably constricted; I. Early embryo. From Kuntz (1916).

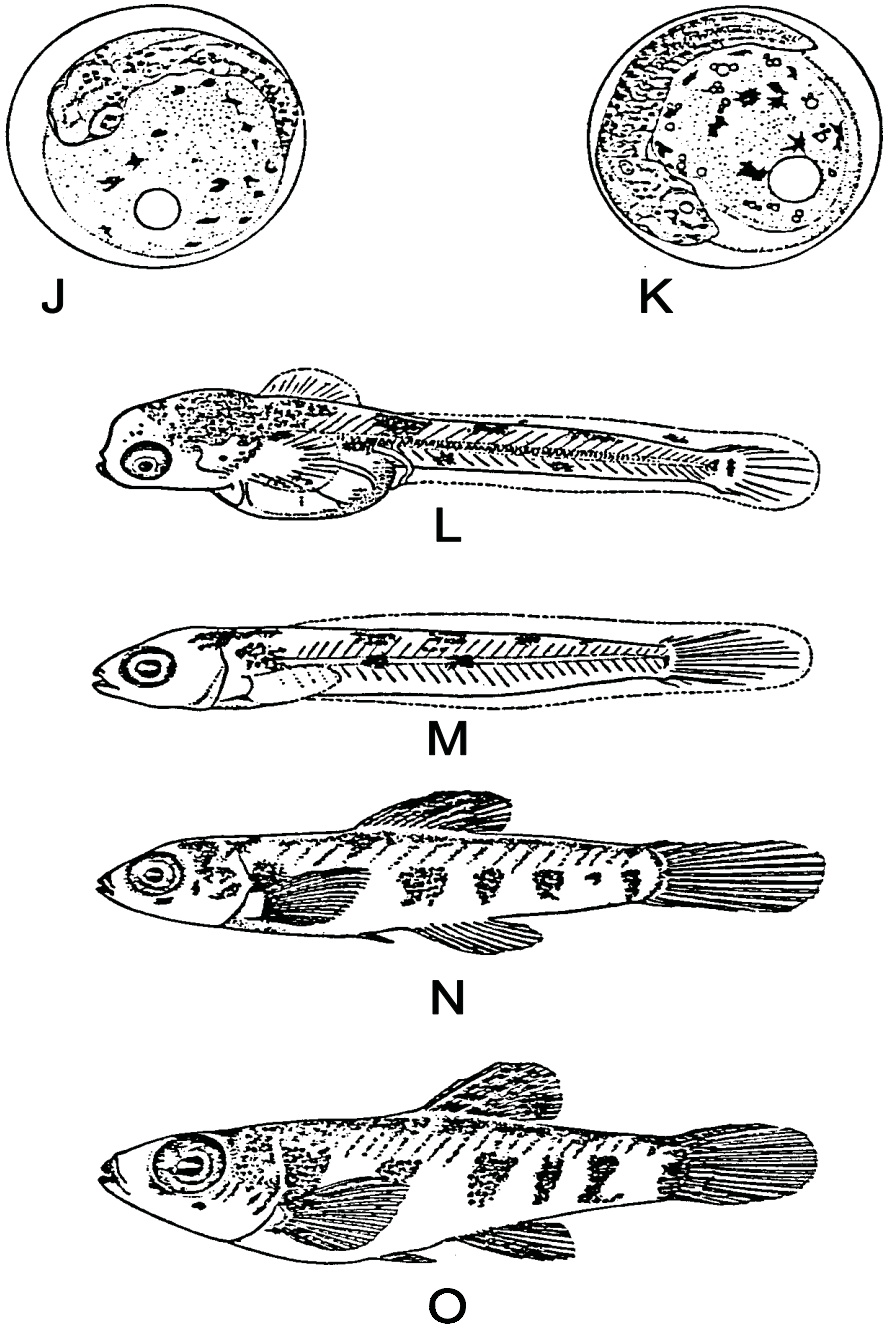


Figure 1. Embryonic development of sheepshead minnow, *Cyprinodon variegatus*: J. Embryo 48 h after fertilization, now segmented throughout, pigment on yolk sac and body, otoliths formed; K. Posterior portion of embryo free from yolk and moves freely within egg membrane, 72 h after fertilization; L. Newly hatched fish, actual length 4 mm; M. Larval fish 5 days after hatching, actual length 5 mm; N. Young fish 9 mm in length; O. Young fish 12 mm in length (CONTINUED). From Kuntz (1916).

11.6.15.2.5.4 Embryos are demersal. They should be aerated and incubated at 25°C, at a salinity of 20-30‰ and a 16-h photoperiod. The embryos can be cultured in either a flow-through or static system, using aquaria or crystallization dishes. However, if the embryos are cultured in dishes, it is essential that aeration and daily water changes be provided, and the dishes be covered to reduce evaporation that may cause increased salinity. One-half to three-quarters of the seawater from the culture vessels can be poured off and the incubating embryos retained. Embryos cultured in this manner should hatch in six or seven days.

11.6.15.2.5.5 At 48 h post-fertilization, embryos are examined under a microscope to determine development and survival. Embryos should be in Stages I and J, Figure 1. Discard dead embryos. Approximately 360 viable embryos are required at this stage.

11.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

11.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

11.8 CALIBRATION AND STANDARDIZATION

11.8.1 See Section 4, Quality Assurance.

11.9 QUALITY CONTROL

11.9.1 See Section 4, Quality Assurance.

11.10 TEST PROCEDURES

11.10.1 TEST SOLUTIONS

11.10.1.1 Receiving Waters

11.10.1.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 500-750 mL, and 400 mL for chemical analysis, would require approximately 2.4-3.4 L or more of sample per test per day.

11.10.1.2 Effluents

11.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and allows for testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25.0%, 50.0%, and 100%). Test precision shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor**. If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ salinity and 70% at 30‰ salinity.

11.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1-to-2 h of the test, additional dilutions at the lower range of effluent concentrations should be added.

11.10.1.2.3 The volume of effluent required to initiate the test and for daily renewal of four replicates per concentration for five concentrations of effluent and a control, each containing 750 mL of test solution, is approximately 5 L. Prepare enough test solution (approximately 3400 mL) at each effluent concentration to provide

400 mL additional volume for chemical analyses (Table 2).

11.10.1.2.4 The salinity of effluent and receiving water tests for sheepshead minnows should be between 20‰ and 30‰. If concurrent effluent and receiving water testing occurs, the effluent test salinity should closely approximate that of the receiving water test. If an effluent is tested alone, select a salinity between 20‰ and 30‰, whichever comes closest to the salinity of the receiving waters. Table 2 illustrates the quantities of effluent, artificial sea salts, hypersaline brine, or seawater needed to prepare 3 L of test solution at each treatment level for tests performed at 20‰ salinity.

11.10.1.2.5 Just prior to test initiation (approximately 1 h), the temperature of sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (25 ± 1°C) and maintained at that temperature during the addition of dilution water.

11.10.1.2.6 Higher effluent concentrations (i.e., 25%, 50%, and 100%) may require aeration to maintain adequate dissolved oxygen concentrations. However, if one solution is aerated, all concentrations must be aerated. Aerate effluent as it warms and continue to gently aerate test solutions in the test chambers for the duration of the test.

11.10.1.2.7 Effluent dilutions should be prepared for all replicates in each treatment in one beaker to minimize variability among the replicates. The test chambers are labelled with the test concentration and replicate number. Dispense into the appropriate effluent dilution chamber.

11.10.1.3 Dilution Water

11.10.1.3.1 Dilution water may be uncontaminated natural seawater (receiving water), HSB prepared from natural seawater, or artificial seawater prepared from FORTY FATHOMS® or GP2 sea salts (see Table 1 and Section 7, Dilution Water). Other artificial sea salts may be used for culturing sheepshead minnows and for the larval survival and growth test if the control criteria for acceptability of test data are satisfied.

11.10.2 START OF THE TEST

11.10.2.1 Tests should begin as soon as possible, preferably within 24 h after sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

TABLE 2. PREPARATION OF TEST SOLUTIONS AT A SALINITY OF 20‰ , USING 20‰ SALINITY DILUTION WATER PREPARED FROM NATURAL SEAWATER, HYPERSALINE BRINE, OR ARTIFICIAL SEA SALTS

Effluent Solution	Effluent Conc. (%)	Solutions To Be Combined	
		Volume of Effluent Solution	Volume of Diluent Seawater (20‰)
1	100[1,2]	6800 mL	----
2	50	3400 mL Solution 1	+ 3400 mL
3	25	3400 mL Solution 2	+ 3400 mL
4	12.5	3400 mL Solution 3	+ 3400 mL
5	6.25	3400 mL Solution 4	+ 3400 mL
Control	0.0		3400 mL
Total			17000 mL

[1] This illustration assumes: (1) the use of 750 mL of test solution in each of four replicates and 400 mL for chemical analysis (total of 3,400 mL) for the control and each of five concentrations of effluent (2) an effluent dilution factor of 0.5, and (3) the effluent lacks appreciable salinity. A sufficient initial volume (6,800 mL) of effluent is prepared by adjusting the salinity to the desired level. In this example, the salinity is adjusted by adding artificial sea salts to the 100% effluent, and preparing a serial dilution using 20‰ seawater (natural seawater, hypersaline brine, or artificial seawater). Following addition of salts, the effluent is stirred for 1 h to ensure that the salts have dissolved. The salinity of the initial 6,800 mL of 100% effluent is adjusted to 20‰ by adding 136 g of dry artificial sea salts (FORTY FATHOMS®). Test concentrations are then made by mixing appropriate volumes of salinity-adjusted effluent and 20‰ salinity dilution water to provide 6,800 mL of solution for each concentration. If hypersaline brine alone (100‰) is used to adjust the salinity of the effluent, the highest concentration of effluent that could be achieved would be 80% at 20‰ salinity. When dry sea salts are used to adjust the salinity of the effluent, it may be desirable to use a salinity control prepared under the same conditions and used to determine survival and growth.

[2] The same procedures would be followed in preparing test concentrations at other salinities between 20‰ and 30‰: (1) the salinity of the bulk (initial) effluent sample would be adjusted to the appropriate salinity using artificial sea salts or hypersaline brine, and (2) the remaining effluent concentrations would be prepared by serial dilution, using a large batch (17,000 mL) of seawater for dilution water, which had been prepared at the same salinity as the effluent, using natural seawater, or hypersaline or artificial sea salts and deionized water.

11.10.2.2 If the embryos have been incubating at 25°C, 30‰ salinity, and a 16-h photoperiod, for 5 to 6 days with aeration and daily water renewals, approximately 24 h prior to hatching, the salinity of the seawater in the incubation chamber may be reduced from 30‰ to the test salinity, if lower than 30‰. In addition to maintaining good water quality, reducing the salinity and/or changing the water may also help to initiate hatching over the next 24 h. A few larvae may hatch 24 h ahead of the majority. Remove these larvae and reserve them in a separate dish, maintaining the same culture conditions. It is preferable to use only the larvae that hatch in the 24 h prior to starting the test. However, if sufficient numbers of larvae do not hatch within the 24-h period, the larvae that hatch prior to 24 h are added to the test organisms. The test organisms are then randomly selected for the test. When eggs or larvae must be shipped to the test site from a remote location, it may be necessary to use larvae older than 24-h because of the difficulty in coordinating test organism shipments with field operations. However, in the latter case, the larvae should not be more than 48-h old at the start of the test and should all be within 24-h of the same age.

11.10.2.3 Label the test chambers with a marking pen. Use of color coded tape to identify each treatment and replicate. A minimum of five effluent concentrations and a control are used for each test. Each treatment (including

controls) must have a minimum of four replicates. For exposure chambers, use 1000 mL beakers, non-toxic disposable plasticware, or glass chambers with a sump area as illustrated in the inland silverside test method (see Section 13).

11.10.2.4 Prepare the test solutions and add to the test chambers.

11.10.2.5 The test is started by randomly placing larvae from the common pool into each test chamber until each chamber contains a minimum of 10 larvae, for a total of a minimum of 40 for each concentration (see Appendix A). The amount of water added to the chambers when transferring the larvae should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

11.10.2.6 The chambers may be placed on a light table to facilitate counting the larvae.

11.10.2.7 Randomize the position of the test chambers at the beginning of the test (see Appendix A). Maintain the chambers in this configuration throughout the test. Preparation of a position chart may be helpful.

11.10.3 LIGHT, PHOTOPERIOD, SALINITY, AND TEMPERATURE

11.10.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h light and 8 h darkness. The water temperature in the test chambers should be maintained at 25 ± 1°C. The test salinity should be in the range of 20 to 30‰ to accommodate receiving waters that may fall within this range. Conduct of this test at salinities less than 20‰ may cause an unacceptably low growth response and thereby invalidate the test. The salinity should vary by no more than ± 2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

11.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

11.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain a satisfactory DO. The DO should be measured on new solutions at the start of the test (Day 0) and before daily renewal of test solutions on subsequent days. The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed 100 bubbles/min, using a pipet with a 1-2 mm orifice, such as a 1-mL KIMAX® serological pipet, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue stress on the fish.

11.10.5 FEEDING

11.10.5.1 *Artemia* nauplii are prepared as described above.

11.10.5.2 Sheepshead minnow larvae are fed newly-hatched (less than 24-h old) *Artemia* nauplii once a day from hatch day 0 through day 6; larvae are not fed on day 7. Feed 0.10 g nauplii per test chamber on days 0-2, and 0.15 g nauplii per test chamber on days 3-6. Equal amounts of *Artemia* nauplii must be added to each replicate test chamber to minimize the variability of larval weight. Sufficient numbers of nauplii should be fed to ensure that some remain alive overnight in the test chambers. An adequate but not excessive amount should be provided to each replicate on a daily basis. Feeding excessive amounts of nauplii will result in a depletion in DO to a lower than acceptable level (below 4.0 mg/L). Siphon as much of the uneaten *Artemia nauplii* as possible from each chamber daily to ensure that the larvae principally eat newly hatched nauplii.

11.10.5.3 On days 0-2, weigh 4 g wet weight or pipette 4 mL of concentrated, rinsed *Artemia* nauplii for a test with five treatments and a control. Resuspend the 4 g *Artemia* in 80 mL of natural or artificial seawater in a 100 mL beaker. Aerate or swirl *Artemia* to maintain a thoroughly mixed suspension of nauplii. Dispense 2 mL *Artemia* suspension by pipette or adjustable syringe to each test chamber. Collect only enough *Artemia* in the pipette or syringe for one test

chamber or settling of *Artemia* may occur, resulting in unequal amounts of *Artemia* being distributed to the replicate test chambers.

11.10.5.4 On days 3-6, weigh 6 g wet weight or pipette 6 mL *Artemia* suspension for a test with five treatments and a control. Resuspend the 6 g *Artemia* in 80 mL of natural or artificial seawater in a 100 mL beaker. Aerate or swirl as 2 mL is dispensed to each test chamber.

11.10.5.5 If the survival rate in any test replicate on any day falls below 50%, reduce the volume of *Artemia* added to that test chamber by one-half (i.e., from 2 mL to 1 mL) and continue feeding one-half the volume through day 6. Record the time of feeding on data sheets (Figure 2).

11.10.6 DAILY CLEANING OF TEST CHAMBERS

11.10.6.1 Before the daily renewal of test solutions, uneaten and dead *Artemia*, dead fish larvae, and other debris are removed from the bottom of the test chambers with a siphon hose. As much of the uneaten *Artemia* as possible should be siphoned from each chamber to ensure that the larvae principally eat newly hatched nauplii. Alternately, a large pipet (50 mL), fitted with a safety pipet filler or rubber bulb, can be used. Because of their small size during the first few days of the tests, larvae are easily drawn into the siphon tube when cleaning the test chambers. By placing the test chambers on a light box, inadvertent removal of live larvae can be greatly reduced because they can be more easily seen. If the water siphoned from the test chambers is collected in a white plastic tray, the live larvae caught in the siphon can be retrieved and returned to the appropriate test chamber. Any incidence of removal of live larvae from the test chambers by the siphon during cleaning, and subsequent return to the chambers, should be noted in the test records.

11.10.7 OBSERVATIONS DURING THE TEST

11.10.7.1 Routine Chemical and Physical Determinations

1.10.7.1.1 DO is measured at the beginning and end of each 24-h exposure period in one test chamber at each test concentration and in the control.

11.10.7.1.2 Temperature, pH, and salinity are measured at the end of each 24-h exposure period in one test chamber at each test concentration and in the control. Temperature should also be monitored continuously, observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test vessels at least at the end of the test to determine the temperature variation in the environmental chamber.

11.10.7.1.3 The pH is measured in the effluent sample each day.

11.10.7.1.4 Record all the measurements on the data sheet (Figure 2).

Test Dates:_____ Species:_____

Type Effluent:_____ Field _____ Lab_____ Test _____

Effluent Tested:_____

CONCENTRATION:																															
REPLICATE:									REPLICATE:								REPLICATE:								REPLICATE:							
DAYS	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			
CONCENTRATION:																																
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			
CONCENTRATION:																																
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			

TIME FED							

COMMENTS:

Figure 2. Data form for the sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test. Daily record of larval survival and test conditions. (From USEPA, 1987b).

Test Dates:_____ Species:_____

Type Effluent:_____ Field _____ Lab_____ Test _____

Effluent Tested:_____

CONCENTRATION:																															
REPLICATE:									REPLICATE:								REPLICATE:								REPLICATE:							
DAYS	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			
CONCENTRATION:																																
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			
CONCENTRATION:																																
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			

TIME FED							

COMMENTS:

Figure 2. Data form for the sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test. Daily record of larval survival and test conditions. (CONTINUED) (From USEPA, 1987b).

11.10.7.2 Routine Biological Observations

11.10.7.2.1 The number of live larvae in each test chamber are recorded daily (Figure 2), and the dead larvae are discarded.

11.10.7.2.2 Protect the larvae from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of dead larvae, carefully. Make sure the larvae remain immersed during the performance of the above operations.

11.10.8 TEST SOLUTION RENEWAL

11.10.8.1 The test solutions are renewed daily using freshly prepared solution, immediately after cleaning the test chambers. For on-site toxicity studies, fresh effluent and receiving water samples used in toxicity tests should be collected daily, and no more than 24 h should elapse between collection of the sample and use in the test (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples must be collected, preferably on days one, three, and five. Maintain the samples at 0-6°C until used.

11.10.8.2 For test solution renewal, the water level in each chamber is lowered to a depth of 7 to 10 mm, which leaves 15 to 20% of the test solution. New test solution (750 mL) should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence and possible injury to the larvae.

11.10.9 TERMINATION OF THE TEST

11.10.9.1 The test is terminated after 7-d of exposure. At test termination, dead larvae are removed and discarded. The surviving larvae in each test chamber (replicate) are counted and immediately prepared as a group for dry weight determination, or are preserved as a group in 4% formalin or 70% ethanol. Preserved organisms are dried and weighed within 7 days. For safety, formalin should be used under a hood.

11.10.9.2 For immediate drying and weighing, siphon or pour live larvae onto a 500 µm mesh screen in a large beaker to retain the larvae and allow *Artemia* and debris to be rinsed away. Rinse the larvae with deionized water to wash away salts that might contribute to the dry weight. Sacrifice the larvae in an ice bath of deionized water.

11.10.9.3 Small aluminum weighing pans can be used to dry and weigh the larvae. Mark for identification an appropriate number of small aluminum weighing pans (one per replicate). Weigh to the nearest 0.01 mg, and record the weights (Figure 3).

Test Dates: _____ Species: _____

Pan No.	Conc. & Rep.	Initial Wt. (mg)	Final Wt. (mg)	Diff. (mg)	No. Larvae	Av. Wt./ Larvae (mg)

Figure 3. Data form for the sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test. Dry weights of larvae (from USEPA 1987b).

11.10.9.4 Immediately prior to drying, rinse the preserved larvae in distilled (or deionized) water. The rinsed larvae from each test chamber are transferred to a tared weighing pan and dried at 60°C for 24 h or at 105°C for a minimum of 6 h. Immediately upon removal from the drying oven, the weighing pans are placed in a desiccator until weighed, to prevent the absorption of moisture from the air. Weigh to the nearest 0.01 mg all weighing pans containing dried larvae and subtract the tare weight to determine the dry weight of larvae in each replicate. Record the weights (Figure 3). For each test chamber, divide the final dry weight by the number of original larvae in the test chamber to determine the average individual dry weight, and record (Figure 3). For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptable criteria (see Subsection 11.12). Complete the summary data sheet (Figure 4) after calculating the average measurements and statistically analyzing the dry weights and percent survival. Average dry weights should be expressed to the nearest 0.001 mg.

11.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

11.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

11.12 ACCEPTABILITY OF TEST RESULTS

11.12.1 The tests are acceptable if (l) the average survival of control larvae equals or exceeds 80%, and (2) the average dry weight per surviving unpreserved control larvae is equal to or greater than 0.60 mg, or (3) the average dry weight per surviving preserved control larvae is equal to or greater than 0.50 mg. The above minimum weights presume that the age of the larvae at the start of the test is less than or equal to 24 h.

11.13 DATA ANALYSIS

11.13.1 GENERAL

11.13.1.1 Tabulate and summarize the data. A sample set of survival and growth response data is listed in Table 4.

11.13.1.2 The endpoints of toxicity tests using the sheepshead minnow larvae are based on the adverse effects on survival and growth. The LC50, the IC25, and the IC50 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values, for survival and growth, are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50, IC25 and IC50. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for survival and growth, but included in the estimation of the LC50, IC25 and IC50. See the Appendices for examples of the manual computations, program listings, and examples of data input and program output.

11.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

Test Dates: _____ Species: _____

Effluent Tested: _____

TREATMENT						
NO. LIVE LARVAE						
SURVIVAL (%)						
MEAN DRY WT/ LARVAE (MG) ± SD						
SIGNIF. DIFF. FROM CONTROL (o)						
MEAN TEMPERATURE (°C) ± SD						
MEAN SALINITY ‰ ± SD						
AVE DISSOLVED OXYGEN (MG/L) ± SD						

COMMENTS:

Figure 4. Data form for the sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test. Summary of test results (from USEPA, 1987b).

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1004.0)[1]

1.	Test type:	Static renewal (required)
2.	Salinity:	20‰ to 32‰ (± 2‰ of the selected test salinity) (recommended)
3.	Temperature:	25 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum termperature) by more than 3°C during the test (required)
4.	Light quality:	Ambient laboratory illumination (recommended)
5.	Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (ambient laboratory levels) (recommended)
6.	Photoperiod:	16 h light, 8 h darkness (recommended)
7.	Test chamber size:	600 mL - 1 L beakers or equivalent (recommended)
8.	Test solution volume:	500-750 mL/replicate (loading and DO restrictions must be met) (recommended)
9.	Renewal of test solutions:	Daily (required)
10.	Age of test organisms	Newly hatched larvae (less than 24 h old; less than or equal to 24-h range in age) (required)
11.	No. larvae per test chamber:	10 (required minimum)
12.	No. replicate chambers per concentration	4 (required minimum)
13.	No. larvae per concentration:	40 (required minimum)
14.	Source of food:	Newly hatched *Artemia* nauplii, (less than 24-h old) (required)
15.	Feeding regime:	Feed once a day 0.10 g wet weight *Artemia* nauplii per replicate on Days 0-2; Feed 0.15 g wet weight *Artemia* nauplii per replicate on Days 3-6 (recommended)
16.	Cleaning:	Siphon daily, immediately before test solution renewal and feeding (required)
17.	Aeration:	None, unless DO falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/minimum (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1004.0) (CONTINUED)

18.	Dilution water:	Uncontaminated source of natural seawater; deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, GP2 or equivalent) (available options)
19.	Test concentrations:	Effluent: 5 and a control (required) Receiving Waters: 100% receiving water (or minimum of 5) and a control (recommended)
20.	Dilution factor:	Effluents: ≥ 0.5 (recommended) Receiving waters: None, or ≥ 0.5 (recommended)
21.	Test duration:	7 days (required)
22.	Endpoints:	Survival and growth (weight) (required)
23.	Test acceptability criteria:	80% or greater survival in controls; average dry weight per surviving organism in control chambers must be 0.60 mg or greater, if unpreserved, or 0.50 mg or greater after no more than 7 days in 4% formalin or 70% ethanol (required)
24.	Sampling requirements:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
25.	Sample volume required:	6 L per day (recommended)

TABLE 4. SUMMARY OF SURVIVAL AND GROWTH DATA FOR SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAE EXPOSED TO AN EFFLUENT FOR SEVEN DAYS[1]

Effl. Conc. (%)	Proportion of Survival in Replicate Chambers				Mean Prop. Surv	Avg Dry Wgt (mg) in Replicate Chambers				Mean Dry Wgt (mg)
	A	B	C	D		A	B	C	D	
0.0	1.0	1.0	1.0	1.0	1.00	1.29	1.32	1.59	1.27	1.368
6.25	1.0	1.0	0.9	1.0	0.98	1.27	1.00	0.97	0.97	1.053
12.5	1.0	1.0	1.0	1.0	1.00	1.32	1.37	1.35	1.34	1.345
25.0	1.0	1.0	1.0	0.8	0.95	1.29	1.33	1.20	0.94	1.190
50.0	0.8	0.8	0.7	0.6	0.73	1.62	0.56	0.46	0.46	0.525
100.0	0.0	0.0	0.0	0.0	0.00	---	---	---	---	---

[1] Four replicates of 10 larvae each.

11.13.2 EXAMPLE OF ANALYSIS OF SHEEPHEAD MINNOW, *CYPRINODON VARIEGATUS* SURVIVAL DATA

11.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 5 and 6. The response used in the analysis is the proportion of animals surviving in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the LC50 endpoint. Concentrations at which there is no survival in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC, EC, and LC endpoint.

11.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

11.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t-test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

11.13.2.4 Probit Analysis (Finney, 1971; see Appendix H) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, or the Graphical Method may be used (see Appendices H-K).

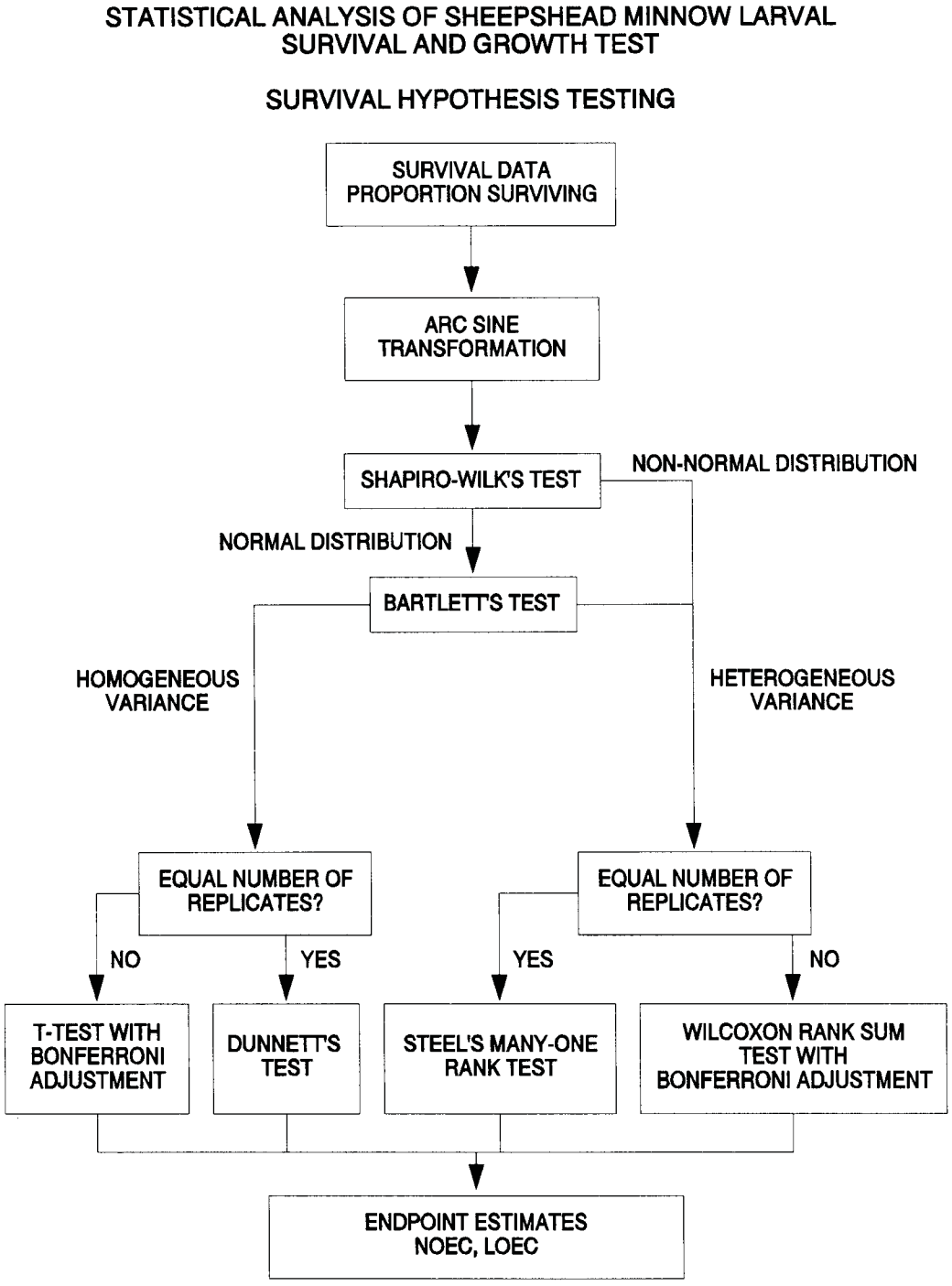


Figure 5. Flowchart for statistical analysis of the sheepshead minnow, *Cyprinodon variegatus*, larval survival data by hypothesis testing.

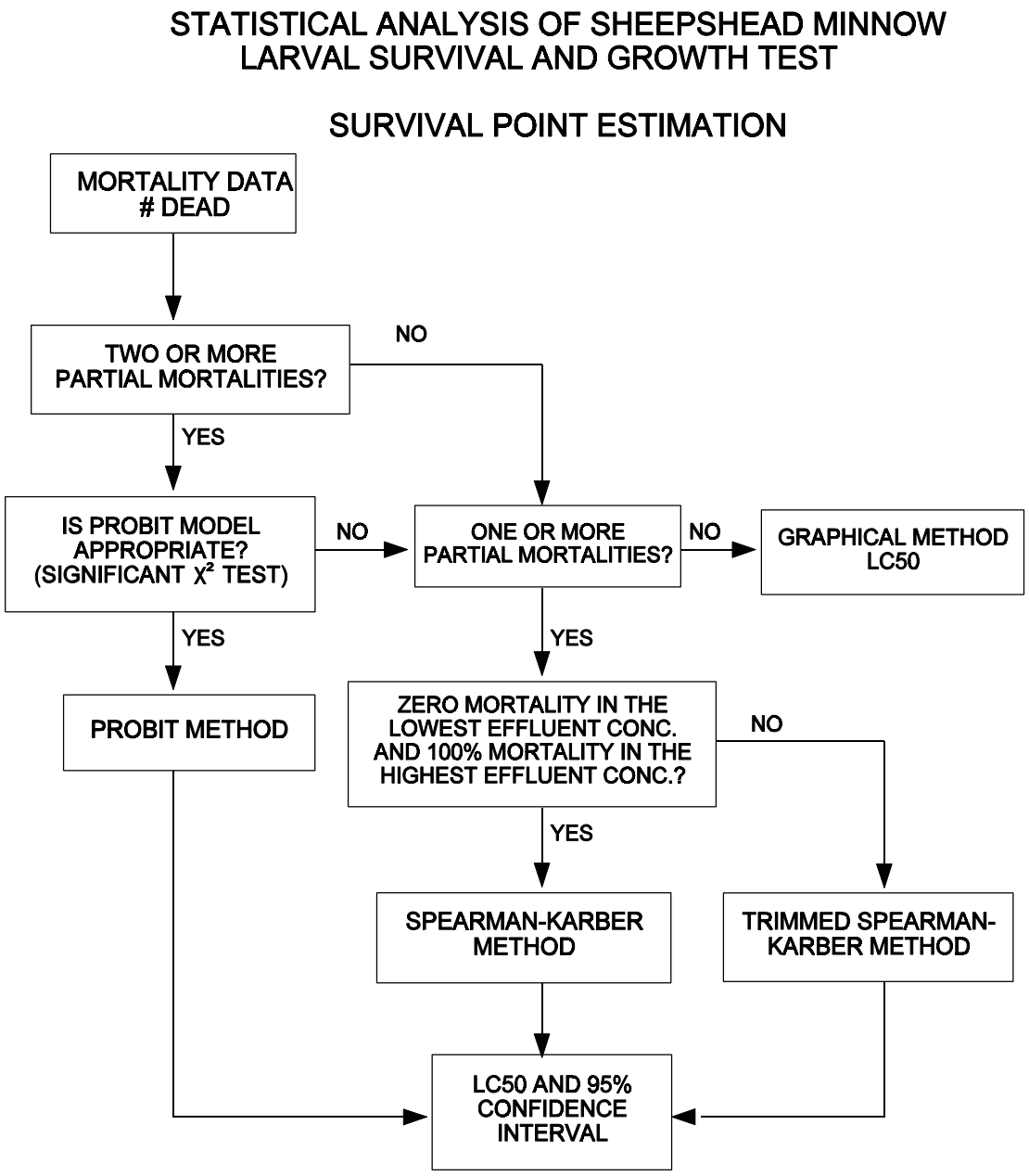


Figure 6. Flowchart for statistical analysis of the sheepshead minnow, *Cyprinodon variegatus*, larval survival data by point estimation.

11.13.2.5 Example of Analysis of Survival Data

11.13.2.5.1 This example uses the survival data from the Sheepshead Minnow Larval Survival and Growth Test. The proportion surviving in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and control are listed in Table 5. A plot of the survival proportions is provided in Figure 7. Since there was 100% mortality in all four replicates for the 100% concentration, it was not included in the statistical analysis and was considered a qualitative mortality effect.

11.13.2.6 Test for Normality

11.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

11.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

Where: X_i = the ith centered observation

$\overline{X}$ = the overall mean of the centered observations

n = the total number of centered observations

TABLE 5. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, SURVIVAL DATA

	Replicate	Control	Effluent Concentration (%)			
			6.25	12.5	25.0	50.0
RAW	A	1.0	1.0	1.0	1.0	0.8
	B	1.0	1.0	1.0	1.0	0.8
	C	1.0	0.9	1.0	1.0	0.7
	D	1.0	1.0	1.0	0.8	0.6
ARC SINE TRANSFORMED	A	1.412	1.412	1.412	1.412	1.107
	B	1.412	1.412	1.412	1.412	1.107
	C	1.412	1.249	1.412	1.412	0.991
	D	1.412	1.412	1.412	1.107	0.886
Mean ($\bar{Y}_i$)		1.412	1.371	1.412	1.336	1.023
S_i^2		0.0	0.007	0.0	0.023	0.011
i		1	2	3	4	5

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	Effluent Concentration (%)			
		6.25	12.5	25.0	50.0
A	0.0	0.041	0.0	0.076	0.084
B	0.0	0.041	0.0	0.076	0.084
C	0.0	-0.122	0.0	0.076	-0.032
D	0.0	0.041	0.0	-0.229	-0.137

11.13.2.6.3 For this set of data,

$$n = 20$$

$$\bar{X} = \frac{1}{20}(-0.001) = 0.000$$

$$D = 0.1236$$

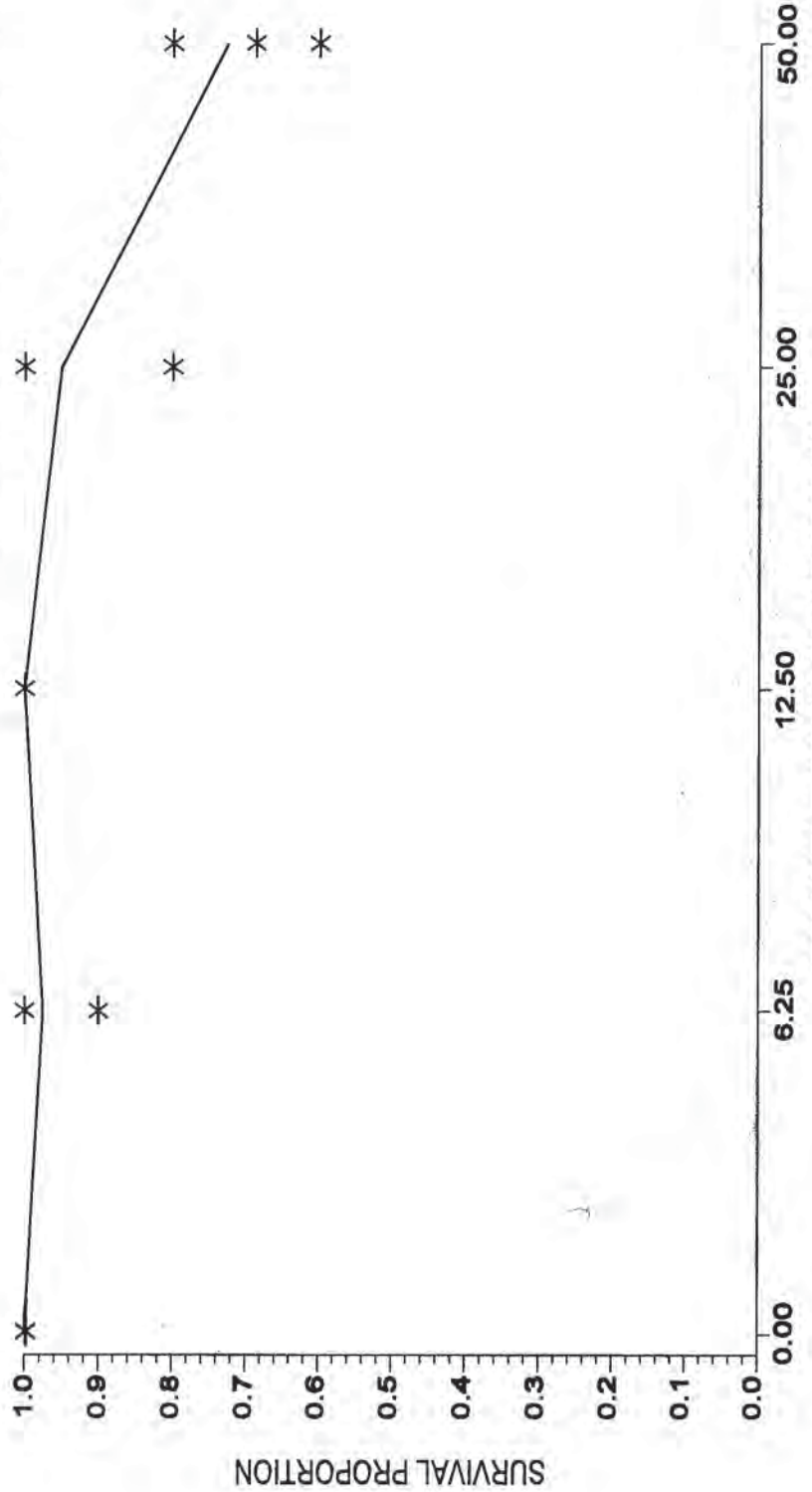


Figure 7. Plot of mean survival proportion data in Table 5.

11.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \le X^{(2)} \le \ldots \le X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 7.

11.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table 8.

11.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 8. For the data in this example,

$$W = \frac{1}{0.1236} (0.3178)^2 = 0.8171$$

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR THE SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.229	11	0.0
2	-0.137	12	0.0
3	-0.122	13	0.041
4	-0.032	14	0.041
5	0.0	15	0.041
6	0.0	16	0.076
7	0.0	17	0.076
8	0.0	18	0.076
9	0.0	19	0.084
10	0.0	20	0.084

11.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 11.13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 20 observations is 0.868. Since W = 0.817 is less than the critical value, conclude that the data are not normally distributed.

11.13.2.6.8 Since the data do not meet the assumption of normality, Steel's Many-one Rank Test will be used to analyze the survival data.

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.313	$X^{(20)} - X^{(1)}$
2	0.3211	0.221	$X^{(19)} - X^{(2)}$
3	0.2565	0.198	$X^{(18)} - X^{(3)}$
4	0.2085	0.108	$X^{(17)} - X^{(4)}$
5	0.1686	0.076	$X^{(16)} - X^{(5)}$
6	0.1334	0.041	$X^{(15)} - X^{(6)}$
7	0.1013	0.041	$X^{(14)} - X^{(7)}$
8	0.0711	0.041	$X^{(13)} - X^{(8)}$
9	0.0422	0.0	$X^{(12)} - X^{(9)}$
10	0.0140	0.0	$X^{(11)} - X^{(10)}$

11.13.2.7 Steel's Many-one Rank Test

11.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ..., 8) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

11.13.2.7.2 An example of assigning ranks to the combined data for the control and 6.25% effluent concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are next summed for each effluent concentration, as shown in Table 11.

11.13.2.7.3 For this example, determine if the survival in any of the effluent concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for the survival at each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control) and four replicates is 10 (see Table 5, Appendix E).

11.13.2.7.4 Since the rank sum for the 50% effluent concentration is equal to the critical value, the proportion surviving in the 50% concentration is considered significantly less than that in the control. Since no other rank sums are less than or equal to the critical value, no other concentrations have a significantly lower proportion surviving than the control. Hence, the NOEC and the LOEC are the 25% and 50% concentrations, respectively.

11.13.2.8 Calculation of the LC50

11.13.2.8.1 The data used for the calculation of the LC50 is summarized in Table 12. For estimating the LC50, the data for the 100% effluent concentration with 100% mortality is included.

11.13.2.8.2 Because there are at least two partial mortalities in this set of test data, calculation of the LC50 using Probit Analysis is recommended. For this set of data, however, the test for heterogeneity of variance was significant. Probit Analysis is not appropriate in this case. Inspection of the data reveals that, once the data is smoothed and adjusted, the proportion mortality in the lowest effluent concentration will not be zero although the proportion mortality in the highest effluent concentration will be one. Therefore, the Spearman-Karber Method is appropriate for this data.

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 6.25% EFFLUENT CONCENTRATION FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Surviving	Effluent Concentration (%)
1	1.249	6.25
5	1.412	6.25
5	1.412	6.25
5	1.412	6.25
5	1.412	Control
5	1.412	Control
5	1.412	Control
5	1.412	Control

TABLE 10. TABLE OF RANKS

		Effluent Concentration (%)			
Replicate	Control	6.25	12.5	25.0	50.0
A	1.412 (5,4.5,5,6.5)	1.412 (5)	1.412 (4.5)	1.412 (5)	1.107 (3.5)
B	1.412 (5,4.5,5,6.5)	1.412 (5)	1.412 (4.5)	1.412 (5)	1.107 (3.5)
C	1.412 (5,4.5,5,6.5)	1.249 (1)	1.412 (4.5)	1.412 (5)	0.991 (2)
D	1.412 (5,4.5,5,6.5)	1.412 (5)	1.412 (4.5)	1.107 (1)	0.886 (1)

TABLE 11. RANK SUMS

Effluent Concentration (%)	Rank Sum
6.25	16
12.5	18
25.0	16
50.0	10

11.13.2.8.3 Before the LC50 can be calculated the data must be smoothed and adjusted. For the data in this example, because the observed proportion mortality for the 12.5% effluent concentration is less than the observed response proportion for the 6.25% effluent concentration, the observed responses for the control and these two groups must be averaged:

$$p_o^s = p_1^s = p_2^s = \frac{0.00+0.025+0.00}{3} = \frac{0.025}{3} = 0.0083$$

Where: p^s_i = the smoothed observed mortality proportion for effluent concentration i

11.13.2.8.3.1 Because the rest of the responses are monotonic, additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table 12.

11.13.2.8.4 Because the smoothed observed proportion mortality for the control is now greater than zero, the data in each effluent concentration must be adjusted using Abbott's formula (Finney, 1971). The adjustment takes the form:

Where: p^s_0 = the smoothed observed proportion mortality for the control

$p^a_i = (p^s_i - p^s_0) / (1 - p^s_0)$

p^s_i = the smoothed observed proportion mortality for effluent concentration i

11.13.2.8.4.1 For the data in this example, the data for each effluent concentration must be adjusted for control mortality using Abbott's formula, as follows:

$$p_0^a = p_1^a = p_2^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.0083 - 0.0083}{1 - 0.0083} = \frac{0.00}{0.9917} = 0.0$$

$$p_3^a = \frac{p_3^s - p_0^s}{1 - p_0^s} = \frac{0.05 - 0.0083}{1 - 0.0083} = \frac{0.0417}{0.9917} = 0.042$$

$$p_4^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{0.275 - 0.0083}{1 - 0.0083} = \frac{0.2667}{0.9917} = 0.269$$

$$p_5^a = \frac{p_5^s - p_0^s}{1 - p_0^s} = \frac{1.000 - 0.0083}{1 - 0.0083} = \frac{0.9917}{0.9917} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table 12.

TABLE 12. DATA FOR EXAMPLE OF SPEARMAN-KARBER ANALYSIS

Effluent Concentration %	Number of Deaths	Number of Organisms Exposed	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0	40	0.000	0.0083	0.000
6.25	1	40	0.025	0.0083	0.000
12.5	0	40	0.000	0.0083	0.000
25.0	2	40	0.050	0.0500	0.042
50.0	11	40	0.275	0.2750	0.269
100.0	40	40	1.000	1.0000	1.000

11.13.2.8.5 Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k-1} \frac{(p_i^a + 1)\ (X_i + X_{i+1})}{2}$$

Where: p^a_i = the smoothed adjusted proportion mortality at concentration i

X_i = the $\log_{10}$ of concentration i

k = the number of effluent concentrations tested, not including the control

11.13.2.8.5.1 For this example, the $\log_{10}$ of the estimated LC50, m, is calculated as follows:

m = [(0.000 - 0.000) (0.7959 + 1.0969)]/2 +
[(0.042 - 0.000) (1.0969 + 1.3979)]/2 +
[(0.269 - 0.042) (1.3979 + 1.6990)]/2 +
[(1.000 - 0.269) (1.6990 + 2.0000)]/2

= 1.755873

11.13.2.8.6 Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k-1} \frac{p_i^a(1 - p_i^a)(X_{i+1} + X_{i-1})^2}{4(n_i - 1)}$$

Where: X_i = the $\log_{10}$ of concentration i

n_i = the number of organisms tested at effluent concentration i

p^a_i = the smoothed adjusted observed proportion mortality at effluent concentration i

k = the number of effluent concentrations tested, not including the control

11.13.2.8.6.1 For this example, the estimated variance of m, V(m), is calculated as follows:

V(m) = (0.000)(1.000)(1.3979 - 0.7959)^2/4(39) +
(0.042)(0.958)(1.6990 - 1.0969)^2/4(39)+
(0.269)(0.731)(2.0000 - 1.3979)^2/4(39)

= 0.0005505

11.13.2.8.7 Calculate the 95% confidence interval for m: $m \pm 2.0 \sqrt{V(m)}$

11.13.2.8.7.1 For this example, the 95% confidence interval for m is calculated as follows:

$$1.755873 \pm 2 \sqrt{0.0005505} = (1.754772, 1.756974)$$

11.13.2.8.8 The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking $base_{10}$ antilogs of the above values.

11.13.2.8.8.1 For this example, the estimated LC50 is calculated as follows:

LC50 = antilog(m) = antilog(1.755873) = 57.0%.

11.13.2.8.8.2 The limits of the 95% confidence interval for the estimated LC50 are calculated by taking the antilogs of the upper and lower limits of the 95% confidence interval for m as follows:

lower limit: antilog(1.754772) = 56.9%

upper limit: antilog(1.756974) = 57.1%

11.13.3 EXAMPLE OF ANALYSIS OF SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, GROWTH DATA

11.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 8. The response used in the statistical analysis is mean weight per original organism for each replicate. Because this measurement is based on the number of original organisms exposed (rather than the number surviving), the measured response is a combined survival and growth endpoint that can be termed biomass. The IC25 and IC50 can be calculated for the growth data via a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC and LOEC for growth. Concentrations above the NOEC for survival are excluded from the hypothesis test for growth effects.

11.13.3.2 The statistical analysis using hypothesis testing consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steels' Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

11.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative. For detailed information on the Bonferroni adjustment, see Appendix D.

11.13.3.4 The data, mean and variance of the observations at each concentration including the control are listed in Table 13. A plot of the mean weights for each treatment is provided in Figure 9. Since there is no survival in the 100% concentration, it is not considered in the growth analysis. Additionally, since there is significant mortality in the 50% effluent concentration, its effect on growth is not considered.

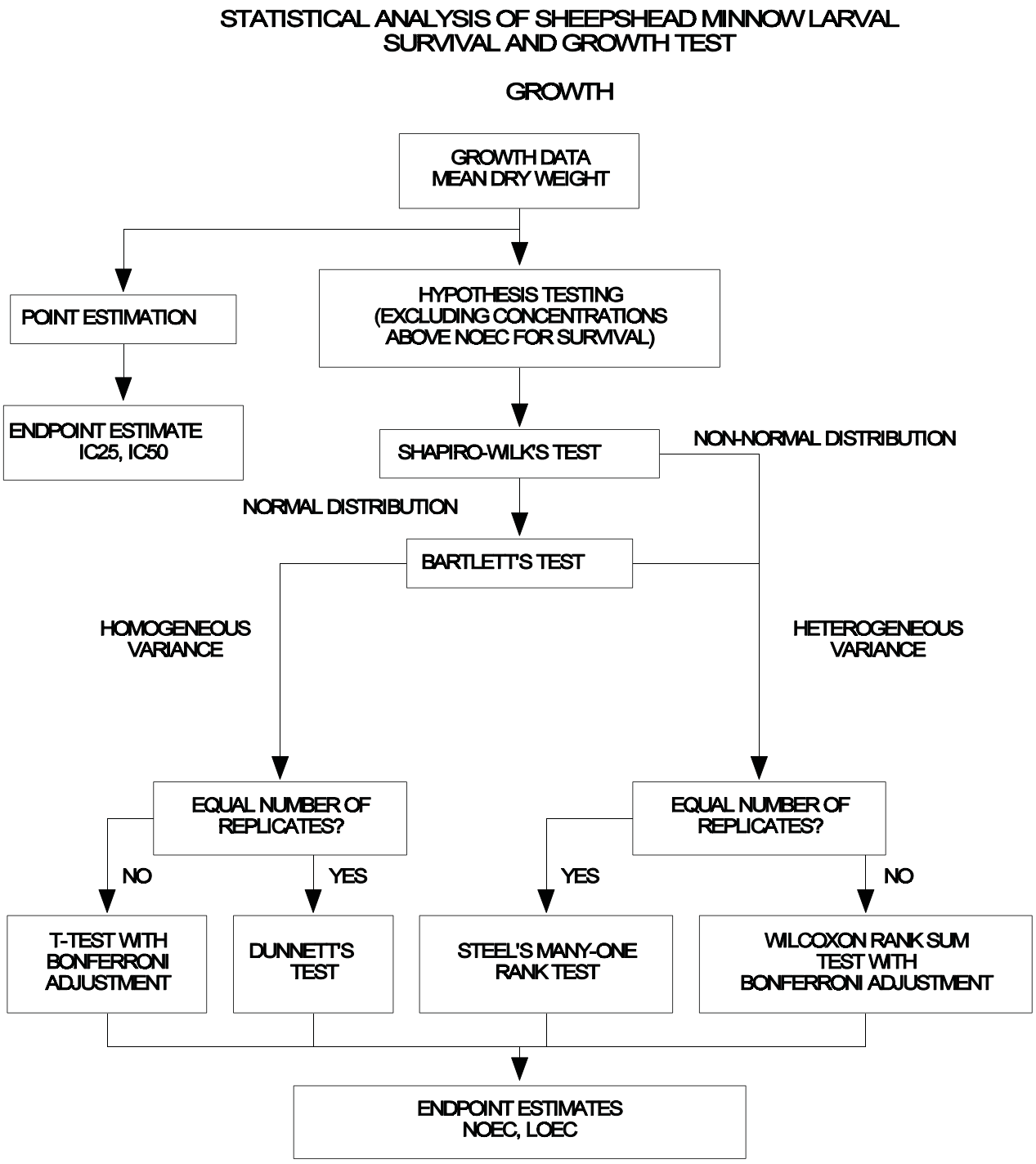


Figure 8. Flowchart for statistical analysis of the sheepshead minnow, *Cyprinodon variegatus*, larval growth data.

TABLE 13. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, GROWTH DATA

		Effluent Concentration (%)				
Replicate	Control	6.25	12.5	25.0	50.0	100.0
A	1.29	1.27	1.32	1.29	-	-
B	1.32	1.00	1.37	1.33	-	-
C	1.59	0.97	1.35	1.20	-	-
D	1.27	0.97	1.34	0.94	-	-
Mean ($\bar{Y}_i$)	1.368	1.053	1.345	1.190	-	-
S^2_i	0.0224	0.0212	0.0004	0.0307	-	-
i	1	2	3	4	5	6

11.13.3.5 Test for Normality

11.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 14.

TABLE 14. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Effluent Concentration (%)		
Replicate	Control	6.25	12.5	25.0
A	-0.078	0.217	-0.025	0.100
B	-0.048	-0.053	0.025	0.140
C	0.222	-0.083	0.005	0.010
D	-0.098	0.083	-0.005	-0.250

11.13.3.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations.

For this set of data, n = 16

$$\bar{X} = \frac{1}{16}(-0.004) = 0.00024 = 0.00$$

D = 0.2245

11.13.3.5.3 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 15.

TABLE 15. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.250	9	-0.005
2	-0.098	10	0.005
3	-0.083	11	0.010
4	-0.083	12	0.025
5	-0.078	13	0.100
6	-0.053	14	0.140
7	-0.048	15	0.217
8	-0.025	16	0.222

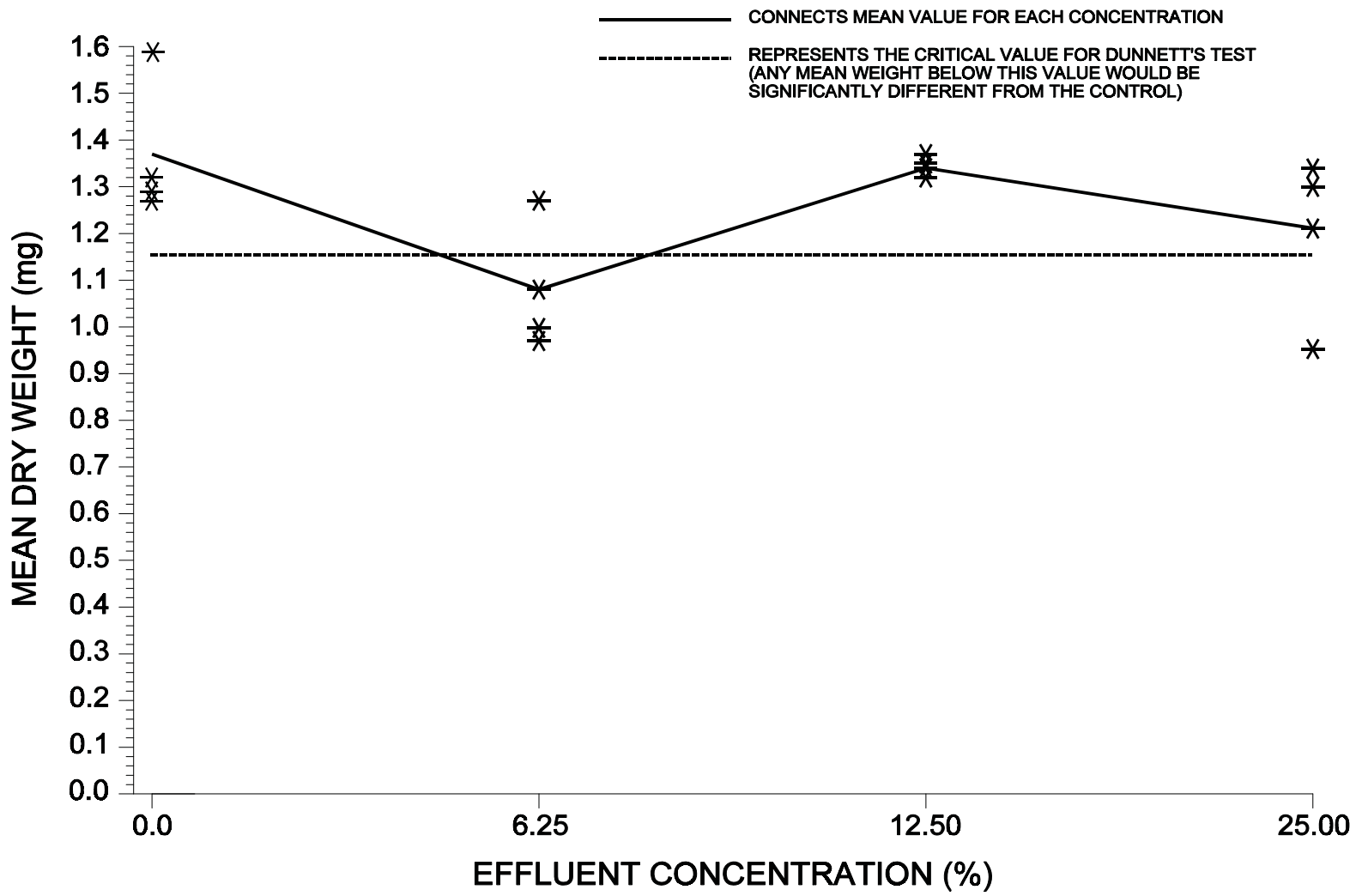


Figure 9. Plot of weight data from sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth test.

11.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ..., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 16 and k = 8. The a_i values are listed in Table 16.

TABLE 16. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.5056	0.472	$X^{(16)} - X^{(1)}$
2	0.3290	0.315	$X^{(15)} - X^{(2)}$
3	0.2521	0.223	$X^{(14)} - X^{(3)}$
4	0.1939	0.183	$X^{(13)} - X^{(4)}$
5	0.1447	0.103	$X^{(12)} - X^{(5)}$
6	0.1005	0.063	$X^{(11)} - X^{(6)}$
7	0.0593	0.053	$X^{(10)} - X^{(7)}$
8	0.0196	0.020	$X^{(9)} - X^{(8)}$

11.13.3.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 16.

For this set of data:

$$W = \frac{1}{0.2245}(0.4588)^2 = 0.938$$

11.13.3.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 16 observations (n) is 0.844. Since W = 0.938 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

11.13.3.6 Test for Homogeneity of Variance

11.13.3.6.1 The test used to examine whether the variation in mean dry weight is the same across all effluent concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i) \ln \overline{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, $V_i = (n_i - (1))$

n_i = the number of replicates for concentration i

p = number of levels of effluent concentration including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

11.13.3.6.2 For the data in this example (see Table 14), all effluent concentrations including the control have the same number of replicates ($n_i = 4$ for all i). Thus, $V_i = 3$ for all i.

11.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(12)\ln(0.0187) - 3\sum_{i=1}^{P} \ln(S_i^2)]/1.139$$

$$= [12(-3.979) - 3(-18.876)]/1.139$$

$$= 8.882/1.139$$

$$= 7.798$$

11.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with three degrees of freedom, is 11.345. Since B = 7.798 is less than the critical value of 11.345, conclude that the variances are not different.

11.13.3.7 Dunnett's Procedure

11.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 17.

TABLE 17. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	$S_B^2 = SSB/(p-1)$
Within	N - p	SSW	$S_W^2 = SSW/(N-p)$
Total	N - 1	SST	

Where: p = number of concentration levels including the control
N = total number of observations $n_1 + n_2 \ldots + n_p$
n_i = number of observations in concentration i

$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$ Between Sum of Squares

$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean dry weight of the mysids for concentration i in test chamber j)

11.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = 4$

$N = 16$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 5.47$

$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 4.21$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 5.38$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 4.76$

$G = T_1 + T_2 + T_3 + T_4 = 19.82$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$

$$= \frac{1}{4}(99.247) - \frac{(19.82)^2}{16} = 0.260$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 25.036 - \frac{(19.82)^2}{16} = 0.484$$

$$SSW = SST - SSB$$

$$= 0.484 - 0.260 = 0.224$$

$S_B^2 = SSB/(p-1) = 0.260/(4-1) = 0.087$

$S_W^2 = SSW/(N-p) = 0.224/(16-4) = 0.019$

11.13.3.7.3 Summarize these calculations in the ANOVA table (Table 18).

TABLE 18. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	3	0.260	0.087
Within	12	0.224	0.019
Total	15	0.484	

11.13.3.7.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(Y_1 - Y_i)}{S_w\sqrt{(1/n_1)+(1/n_i)}}$$

Where: $\overline{Y}_i$ = mean dry weight for effluent concentration i

$\overline{Y}_1$ = mean dry weight for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

11.13.3.7.5 Table 19 includes the calculated t values for each concentration and control combination. In this example, comparing the 6.25% concentration with the control, the calculation is as follows:

TABLE 19. CALCULATED T VALUES

Effluent Concentration (%)	i	t_i
6.25	2	3.228
12.5	3	0.236
25.0	4	1.824

11.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean weight, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 12 degrees of freedom for error and three concentrations (excluding the control) the critical value is 2.29. The mean weight for concentration i is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Since t_2 is greater than 2.29, the 6.25% concentration has significantly lower growth than the control. However, the 12.5% and 25% concentrations do not exhibit this effect. Hence the NOEC and the LOEC for growth cannot be calculated.

11.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\,S_w\sqrt{(1/n_1)+(1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

11.13.3.7.8 In this example:

$$MSD = 2.29(0.10)\sqrt{(1/4)+(1/4)}$$

$$= 2.29\ (0.138)(0.707)$$

$$= 0.223$$

11.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.223 mg.

11.13.3.7.10 This represents a 16% reduction in mean weight from the control.

11.13.3.8 Calculation of the ICp

11.13.3.8.1 The growth data from Table 4 are utilized in this example. As seen from Table 4 and Figure 7, the observed means are not monotonically non-increasing with respect to concentration (mean response for each higher concentration is not less than or equal to the mean response for the previous concentration and the responses between concentrations do not follow a linear trends). Therefore, the means are smoothed prior to calculating the IC. In the following discussion, the observed means are represented by $\overline{Y}_i$ and the smoothed

means by M_i.

11.13.3.8.2 Starting with the control mean, $\overline{Y}_1 = 1.368$ and $\overline{Y}_2 = 1.053$, we see that $\overline{Y}_1 > \overline{Y}_2$. Set $M_1 = \overline{Y}_1$. Comparing $\overline{Y}_2$ to $\overline{Y}_3$, $\overline{Y}_2 < \overline{Y}_3$.

11.13.3.8.3 Calculate the smoothed means:

$$M_2 = M_3 = (\overline{Y}_2 + \overline{Y}_3)/2 = 1.199$$

11.13.3.8.4 Since $\overline{Y}_6 = 0 < \overline{Y}_5 = 0.525 < \overline{Y}_4 = 1.190 < \overline{Y}_4 = 1.345$, set $M_3 = 1.199$, $M_4 = 1.190$, $M_5 = 0.525$, and set $M_6 = 0$.

11.13.3.8.5 Table 20 contains the response means and smoothed means and Figure 10 gives a plot of the smoothed response curve.

TABLE 20. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, MEAN GROWTH RESPONSE AFTER SMOOTHING

Effluent Conc. (%)	i	Response Means (mg) Y_i	Smoothed Means (mg) M_i
Control	1	1.368	1.368
6.25	2	1.053	1.199
12.50	3	1.345	1.199
25.00	4	1.189	1.189
50.00	5	0.525	0.525
100.00	6	0.0	0.0

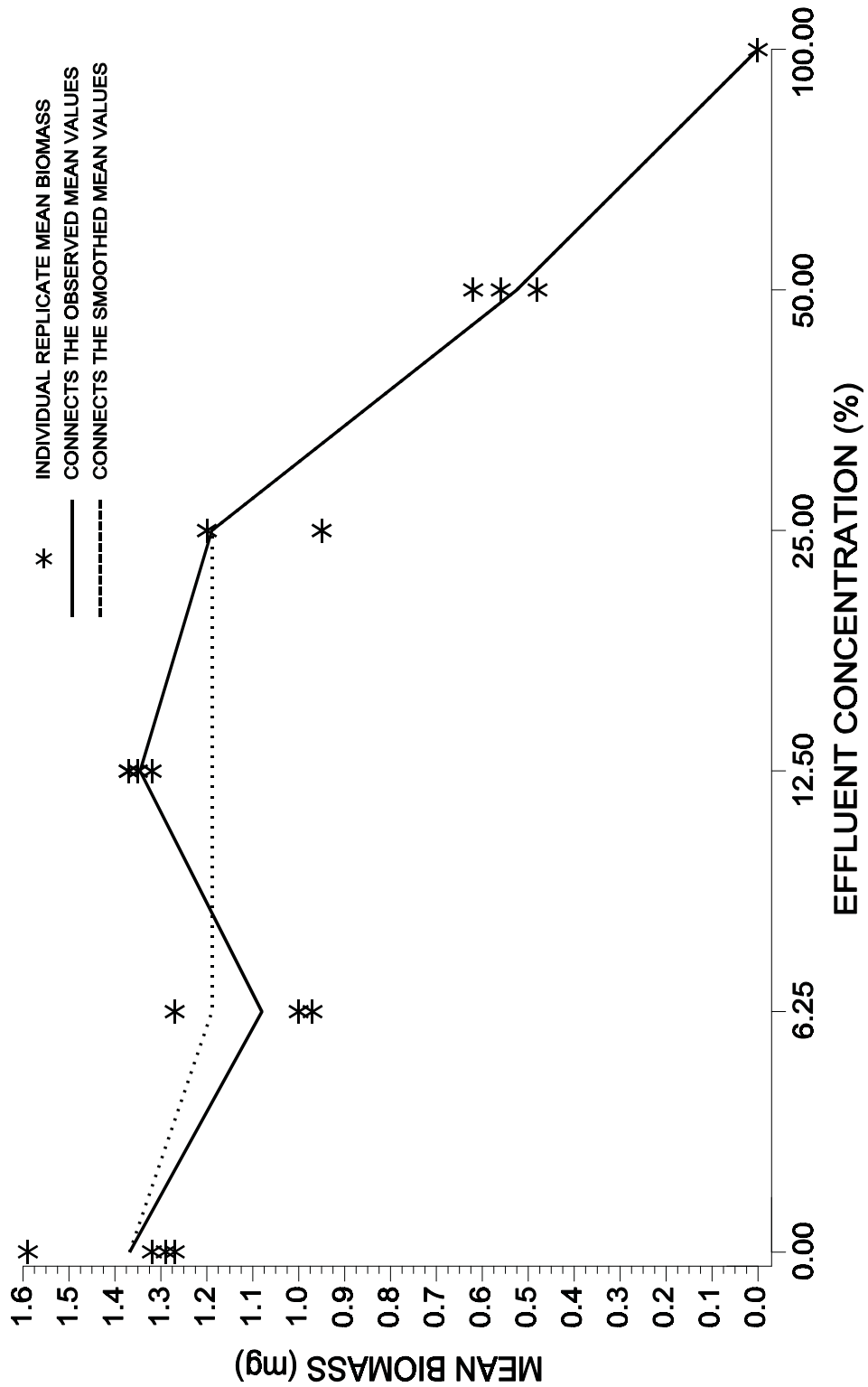


Figure 10. Plot of raw data, observed means, and smoothed means for the sheepshead minnow, *Cyprinodon variegatus*, growth data from Tables 4 and 20.

11.13.3.8.6 An IC25 and IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in weight, compared to the controls, would result in a mean dry weight of 1.026 mg, where $M_1(1\text{-}p/100) = 1.368(1\text{-}25/100)$. A 50% reduction in mean dry weight, compared to the controls, would result in a mean dry weight of 0.684 mg. Examining the smoothed means and their associated concentrations (Table 4), the response, 1.026 mg, is bracketed by C_4 = 25.0% effluent and C_5 = 50.0% effluent. The response (0.684 mg) is bracketed by C_4 = 25.0% effluent and C_5 = 50% effluent.

11.13.3.8.7 Using the equation from Section 4.2 of Appendix M, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 25.0 + [1.368(1 - 25/100) - 1.189]\ \frac{(50.00 - 25.00)}{(0.525 - 1.189)}$$

$$= 31.2\%$$

11.13.3.8.8 Using the equation from Section 4.2 of Appendix L, the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC50 = 50.0 + [1.368(1\text{-}50/100) - 0525]\ \frac{(100.00\text{-}50.00)}{(0.0 - 0.525)}$$

$$= 44.0\%$$

11.13.3.8.9 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 31.1512%. The empirical 95% confidence interval for the true mean was 22.0420% and 36.3613%. The computer program output for the IC25 for this data set is shown in Figure 11.

11.13.3.8.10 When the ICPIN program was used to analyze this set of data for the IC50, requesting 80 resamples, the estimate of the IC50 was 44.0230%. The empirical 95% confidence interval for the true mean was 39.1011% and 49.0679%. The computer program output is shown in Figure 12.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	6.25	12.5	25	50	100
Response 1	1.29	1.27	1.32	1.29	.62	0
Response 2	1.32	1	1.37	1.33	.560	0
Response 3	1.59	.972	1.35	1.2	.46	0
Response 4	1.27	.97	1.34	.936	.46	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: Cyprinodon variegatus
Test Duration: 7-d
DATA FILE: sheep.icp
OUTPUT FILE: sheep.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	1.368	0.150	1.368
2	4	6.250	1.053	0.145	1.199
3	4	12.500	1.345	0.021	1.199
4	4	25.000	1.189	0.177	1.189
5	4	50.000	0.525	0.079	0.525
6	4	100.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 31.1512 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 30.6175 Standard Deviation: 2.9490
Original Confidence Limits: Lower: 25.4579 Upper: 34.4075
Expanded Confidence Limits: Lower: 22.0420 Upper: 36.3613
Resampling time in Seconds: 1.70 Random Seed: -2137496326

Figure 11. ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	6.25	12.5	25	50	100
Response 1	1.29	1.27	1.32	1.29	.62	0
Response 2	1.32	1	1.37	1.33	.560	0
Response 3	1.59	.972	1.35	1.2	.46	0
Response 4	1.27	.97	1.34	.936	.46	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: Cyprinodon variegatus
Test Duration: 7-d
DATA FILE: sheep.icp
OUTPUT FILE: sheep.i50

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	1.368	0.150	1.368
2	4	6.250	1.053	0.145	1.199
3	4	12.500	1.345	0.021	1.199
4	4	25.000	1.189	0.177	1.189
5	4	50.000	0.525	0.079	0.525
6	4	100.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 44.0230 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 44.3444 Standard Deviation: 1.7372
Original Confidence Limits: Lower: 40.9468 Upper: 47.1760
Expanded Confidence Limits: Lower: 39.1011 Upper: 49.0679
Resampling time in Seconds: 1.70 Random Seed: -156164614

Figure 12. ICPIN program output for the IC50.

11.14 PRECISION AND ACCURACY

11.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 11.14.1.1 and 11.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

11.14.1.1 Single-Laboratory Precision

11.14.1.1.1 Data on the single-laboratory precision of the Sheepshead Minnow Larval Survival and Growth Test using FORTY FATHOMS® artificial seawater, natural seawater, and GP2 with copper sulfate, sodium dodecyl sulfate, and hexavalent chromium, as reference toxicants, are given in Tables 21-26. The IC25, IC50, or LC50 data (coefficient of variation), indicating acceptable precision for the reference toxicants (copper, sodium dodecyl sulfate, and hexavalent chromium), are also listed in these Tables.

11.14.1.1.2 EPA evaluated within-laboratory precision of the Sheepshead Minnow, *Cyprinodon variegatus*, Larval Survival and Growth Test using a database of routine reference toxicant test results from five laboratories (USEPA, 2000b). The database consisted of 57 reference toxicant tests conducted in 5 laboratories using reference toxicants including: cadmium and potassium chloride. Among the 5 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 13% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 9%; and in 75% of laboratories, the within-laboratory CV was less than 14%.

11.14.1.2 Multilaboratory Precision

11.14.1.2.1 Data from a study of multilaboratory test precision, involving a total of seven tests by four participating laboratories, are listed in Table 27. The laboratories reported very similar results, indicating good interlaboratory precision. The coefficient of variation (IC25) was 44.2% and (IC50) was 56.9%, indicating acceptable precision.

11.14.1.2.2 In 2000, EPA conducted an interlaboratory variability study of the Sheepshead Minnow, *Cyprinodon variegatus*, Larval Survival and Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 7 participant laboratories tested 4 blind test samples that included blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of bioassay-grade FORTY FATHOMS® synthetic seawater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a natural seawater spiked with KCl, and the reference toxicant sample consisted of bioassay-grade FORTY FATHOMS® synthetic seawater spiked with KCl. Of the 28 Sheepshead Minnow Larval Survival and Growth Tests conducted in this study, 100% were successfully completed and met the required test acceptability criteria. Of 7 tests that were conducted on blank samples, none showed false positive results for the survival endpoint or the growth endpoint. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 28 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 10.5% for IC25 results. Table 29 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned two concentrations for the reference toxicant sample type and one concentration for the effluent and

receiving water sample types. The percentage of values within one concentration of the median was 100% for each of the sample types. For the growth endpoint, NOEC values spanned one concentration for the reference toxicant sample type and two concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 100% for each of the sample types.

11.14.2 ACCURACY

11.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 21. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST PERFORMED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING COPPER (CU) SULFATE AS A REFERENCE TOXICANT[1,2,3,4,5]

Test Number	NOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)	Most Sensitive Endpoint[6]
1	50	113.3	152.3	S
2	<50[7]	54.3	97.5	G
3	<50[7]	41.8	71.4	G
4	50	63.2	90.8	S
5	<50[7]	57.7	99.8	S
6	50	48.3	132.5	G
7	50	79.6	159.7	G
8	50	123.5	236.4	G
n:	5	8	8	
Mean:	NA	72.7	130.0	
CV(%):	NA	41.82	40.87	

1 Data from USEPA (1988a) and USEPA (1991a).

2 Tests performed by Donald J. Klemm, Bioassessment and Ecotoxicology Branch, EMSL, Cincinnati, OH.

3 All tests were performed using FORTY FATHOMS® synthetic seawater. Three replicate exposure chambers, each with 15 larvae, were used for the control and each copper concentration. Copper concentrations used in Tests 1-6 were: 50, 100, 200, 400, and 800 mg/L. Copper concentrations in Tests 7-8 were: 25, 50, 100, 200 and 400 mg/L.

4 Adults collected in the field.

5 For a discussion of the precision of data from chronic toxicity test see Section 4, Quality Assurance.

6 Endpoints: G=growth; S=survival.

7 Lowest concentration tested was 50 µg/L (NOEC Range: < 50* - 50 µg/L).

TABLE 22. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST PERFORMED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT[1,2,3,4,5,6]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)	Most Sensitive Endpoint[7]
1	1.0	1.2799	1.5598	S
2	1.0	1.4087	1.8835	S
3	1.0	2.3051	2.8367	S
4	0.5	1.9855	2.6237	G
5	1.0	1.1901	1.4267	S
6	0.5	1.1041	1.4264	G
n:	6	6	6	
Mean:	NA	1.5456	1.9595	
CV(%):	NA	31.44	31.82	

1 Data from USEPA (1988a) and USEPA (1991a).

2 Tests performed by Donald J. Klemm, Bioassessment and Ecotoxicology Branch, EMSL, Cincinnati, OH.

3 All tests were performed using FORTY FATHOMS® synthetic seawater. Three replicate exposure chambers, each with 15 larvae, were used for the control and each SDS concentration. SDS concentrations in Tests 1-2 were: 1.0, 1.9, 3.9, 7.7, and 15.5 mg/L. SDS concentrations in Tests 3-6 were: 0.2, 0.5, 1.0, 1.9, and 3.9 mg/L.

4 Adults collected in the field.

5 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

6 NOEC Range: 0.5 -1.0 mg/L (this represents a difference of one exposure concentration).

7 Endpoints: G=growth; S=survival

TABLE 23. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST PERFORMED IN NATURAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN NATURAL SEAWATER, USING COPPER (CU) SULFATE AS A REFERENCE TOXICANT[1,2,3,4,5,6]

Test Number	NOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)	Most Sensitive Endpoint[7]
1	125	320.3	437.5	S
2	31	182.3	323.0	G
3	125	333.4	484.4	S
4	125	228.4	343.8	S
5	125	437.5	NC[8]	S
n:	5	5	4	
Mean:	NA	300.4	396.9	
CV(%):	NA	33.0	19.2	

1 Data from USEPA (1988a) and USEPA (1991a).

2 Tests performed by George Morrison and Elise Torello, ERL-N, USEPA, Narragansett, RI.

3 Three replicate exposure chambers, each with 10-15 larvae, were used for the control and each copper concentration. Copper concentrations were: 31, 63, 125, 250, and 500 µg/L.

4 NOEC Range: 31 - 125 µg/L (this represents a difference of two exposure concentrations).

5 Adults collected in the field.

6 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

7 Endpoints: G=growth; S=survival.

8 NC = No linear interpolation estimate could be calculated from the data, since none of the group response means were less than 50 percent of the control response mean.

TABLE 24. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST PERFORMED IN NATURAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN NATURAL SEAWATER, USING SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT[1,2,3,4,5,6]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)	Most Sensitive Endpoint[7]
1	2.5	2.9	3.6	S
2	1.3	NC1[8]	NC2[9]	G
3	1.3	1.9	2.4	S
4	1.3	2.4	NC2	G
5	1.3	1.5	1.8	S
n:	5	4	3	
Mean:	NA	2.2	2.6	
CV(%):	NA	27.6	35.3	

1 Data from USEPA (1988a) and USEPA (1991a).

2 Tests performed by George Morrison and Elise Torello, ERL-N, USEPA, Narragansett, RI.

3 Three replicate exposure chambers, each with 10-15 larvae, were used for the control and each SDS concentration. SDS concentrations were: 0.3, 0.6, 1.3, 2.5, and 5.0 mg/L.

4 NOEC Range: 1.3 - 2.5 mg/L (this represents a difference of one exposure concentration).

5 Adults collected in the field.

6 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

7 Endpoints: G=growth; S=survival.

8 NC1 = No linear interpolation estimate could be calculated from the data, since none of the group response means were less than 75 percent of the control response mean.

9 NC2 = No linear interpolation estimate could be calculated from the data, since none of the group response means were less than 50 percent of the control response mean.

TABLE 25. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST PERFORMED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, AND HEXAVALENT CHROMIUM AS THE REFERENCE TOXICANT[1,2,3,4,5]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)	Most Sensitive Endpoint[6]
1	2.0	5.8	11.4	G
2	1.0	2.9	9.9	G
3	4.0	6.9	11.5	G
4	2.0	2.4	9.2	G
5	1.0	3.1	10.8	G
n:	5	5	5	
Mean:	NA	4.2	10.6	
CV(%):	NA	47.6	9.7	

1 Tests performed by Donald Klemm, Bioassessment and Ecotoxicology Branch, EMSL, Cincinnati, OH.

2 All tests were performed using Forty Fathoms® synthetic seawater. Three replicate exposure chambers, each with 15 larvae, were used for the control and each hexavalent chromium concentration. Hexavalent chromium concentrations used in Tests 1-5 were: 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 mg/L.

3 NOEC Range: 1.0 - 4.0 mg/L (this represents a difference of four exposure concentrations)

4 Adults collected in the field.

5 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

6 Endpoints: G=growth; S=survival.

TABLE 26 COMPARISON OF LARVAL SURVIVAL (LC50) AND GROWTH (IC50) VALUES FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EXPOSED TO SODIUM DODECYL SULFATE (SDS) AND COPPER (CU) SULFATE IN GP2 ARTIFICIAL SEAWATER MEDIUM OR NATURAL SEAWATER[1,2,3,4]

	Survival		Growth	
SDS (mg/L)	GP2	NSW	GP2	NSW
	7.49	8.13	7.39	8.41
	8.70	8.87	8.63	8.51
	8.38	8.85	8.48	9.33
Mean	8.19	8.62	8.17	8.75
CV (%)	7.7	4.9	8.3	5.8
Copper(µg/L)	GP2	NSW	GP2	NSW
	455	412	341	333
	467	485	496	529
	390	528	467	776
Mean	437	475	435	546
CV (%)	9.4	12.3	18.9	40.7

1 Tests performed by George Morrison and Glen Modica, ERL-N, USEPA, Narragansett, RI.

2 Three replicate exposure chambers, each with 10-15 larvae, were used for the control and each SDS concentration. SDS concentrations were: 0.3, 0.6, 1.3, 2.5, and 5.0 mg/L.

3 Adults collected in the field.

4 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 27. DATA FROM INTERLABORATORY STUDY OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL SURVIVAL AND GROWTH TEST USING AN INDUSTRIAL EFFLUENT AS A REFERENCE TOXICANT[1,2,3]

		Most Sensitive Endpoint[4]		
	Test Number	NOEC (%)	IC25 (%)	IC50 (%)
Laboratory A	1	3.2 (S,G)	7.4 (S)	7.4 (G)
	2	3.2 (S,G)	7.6 (S)	14.3 (G)
Laboratory B	1	3.2 (S,G)	5.7 (G)	9.7 (G)
	2	3.2 (S,G)	5.7 (G)	8.8 (G)
Laboratory C	1	1.0 (S)	4.7 (S)	7.2 (S)
Laboratory D	1	3.2 (S,G)	7.4 (G)	24.7 (G)
	2	1.0 (G)	5.2 (S)	7.2 (S)
n:		7	7	7
Mean:		NA	5.5	11.3
CV(%):		NA	44.2	56.9

[1] Data from USEPA (1987b), USEPA (1988a), and USEPA (1991a).

[2] Effluent concentrations were: 0.32, 1.0, 3.2, 10.0, and 32.0%.

[3] NOEC Range: 1.0 - 3.2% (this represents a difference of one exposure concentration).

[4] Endpoints: G=growth; S=survival.

TABLE 28. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]
IC25	Reference toxicant	18.4
	Effluent	6.12
	Receiving water	7.15
	Average	10.5

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] CVs were calculated based on the total interlaboratory variability (including both within-laboratory and between-laboratory components of variability). Individual within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

TABLE 29. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	25%	57.1	42.9	0.00
	Effluent	25%	100	0.00	0.00
	Receiving water	25%	100	0.00	0.00
Growth NOEC	Reference toxicant	25%	100	0.00	0.00
	Effluent	12.5%	57.1	42.9	0.00
	Receiving water	12.5%	71.4	28.6	0.00

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.

[3] Percent of values two or more concentration intervals above or below the median.

SECTION 12

TEST METHOD

SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS* EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST METHOD 1005.0

12.1 SCOPE AND APPLICATION

12.1.1 This method, adapted in part from USEPA (1981) and USEPA (1987b), estimates the chronic toxicity of effluents and receiving waters to the sheepshead minnow, *Cyprinodon variegatus*, using embryos and larvae in a nine-day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms. The test is useful in screening for teratogens because organisms are exposed during embryonic development.

12.1.2 Daily observations on mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

12.1.3 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

12.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly volatile and highly degradable toxicants present in the source may not be detected in the test.

12.1.5 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

12.2 SUMMARY OF METHOD

12.2.1 Sheepshead minnow, *Cyprinodon variegatus*, embryos and larvae are exposed in a static renewal system to different concentrations of effluent or to receiving water starting shortly after fertilization of the eggs through four days posthatch. Test results are based on the total frequency of both mortality and gross morphological deformities (terata).

12.3 INTERFERENCES

12.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

12.3.2 Adverse effects of low dissolved oxygen concentrations (DO), high concentrations of suspended and/or dissolved solids, and extremes of pH may mask the effect of toxic substances.

12.3.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.3.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

12.3.5 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 12.3.5.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 12.3.5.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

12.3.5.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 12.3.5.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample after adjusting the sample salinity for use in marine testing.

12.3.5.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ± 0.3 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ± 0.3 pH units in pH-controlled tests (USEPA, 1996).

12.3.5.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

12.3.5.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 12.3.5.1.1).

12.3.5.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 12.3.5.2) is applied routinely to subsequent testing of the effluent.

12.3.5.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.9). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992);

or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the sample after adjusting the sample salinity for use in marine testing. USEPA (1996) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

12.4 SAFETY

12.4.1 See Section 3, Health and Safety.

12.5 APPARATUS AND EQUIPMENT

12.5.1 Facilities for holding and acclimating test organisms.

12.5.2 Sheepshead minnow culture unit -- see Subsection 12.6.12 below. To perform toxicity tests on-site or in the laboratory, sufficient numbers of newly fertilized eggs must be available, preferably from an in-house sheepshead minnow culture unit. If necessary, embryos can be obtained from outside sources if shipped in well oxygenated water in insulated containers.

12.5.2.1 A test using 15 embryos per test vessel and four replicates per concentration, will require 360 newly-fertilized embryos at the start of the test. A test with a minimum of 10 embryos per test vessel and three replicates per concentration, and with five effluent concentrations and a control, will require a minimum of 180 embryos at the start of the test.

12.5.3 Brine Shrimp, *Artemia*, Culture Unit -- for feeding sheepshead minnow larvae in the continuous culture unit (see Subsection 12.6.12 below).

12.5.4 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L, and maintain sample temperature at 4°C.

12.5.5 Environmental Chamber or Equivalent Facility with Temperature Control (25 ± 1°C).

12.5.6 Water Purification System -- Millipore Milli-Q®, deionized water (DI) or equivalent.

12.5.7 Balance -- analytical, capable of accurately weighing to 0.00001 g. Note: An analytical balance is not needed for this test but is needed for other specified toxicity test methods with growth endpoints.

12.5.8 Reference Weights, Class S -- for checking the performance of the balance. The reference weights should bracket the expected weights of reagents, and the expected weights of the weighing pans and the weights of the weighing pans plus larvae.

12.5.9 Air Pump -- for oil free air supply.

12.5.10 Air Lines, and Air Stones -- for aerating water containing embryos, larvae, or supplying air to test solution with low DO

12.5.11 Meters, pH and DO -- for routine physical and chemical measurements.

12.5.12 Standard or Micro-Winkler Apparatus -- for determining DO (optional).

12.5.13 Dissecting microscope -- for examining embryos and larvae.

12.5.14 Light box -- for counting and observing embryos and larvae.

12.5.15 Refractometer -- for determining salinity.

12.5.16 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

12.5.17 Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.

12.5.18 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, 1979b) -- to calibrate laboratory thermometers.

12.5.19 Test Chambers -- four (minimum of three), borosilicate glass or non-toxic plastic labware per test concentration. Care must be taken to avoid inadvertently removing embryos or larvae when test solutions are decanted from the chambers. To avoid potential contanimation from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic (6 mm thick). The covers are removed only for observation and removal of dead organisms.

12.5.20 Beakers -- six Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

12.5.21 Wash Bottles -- for deionized water, for washing embryos from substrates and containers, and for rinsing small glassware and instrument electrodes and probes.

12.5.22 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

12.5.23 Pipets, volumetric -- Class A, 1-100 mL.

12.5.24 Pipets, automatic -- adjustable, 1-100 mL.

12.5.25 Pipets, serological -- 1-10 mL, graduated.

12.5.26 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

12.5.27 Droppers and glass tubing with fire polished aperatures, 4 mm ID -- for transferring embryos and larvae.

12.5.28 Siphon with bulb and clamp -- for cleaning test chambers.

12.5.29 NITEX® or stainless steel mesh sieves, (≤150 µm, 500 µm, and 3-5 mm) -- for collecting *Artemia* nauplii and fish embryos, and for spawning baskets, respectively.

12.6 REAGENTS AND CONSUMABLE MATERIALS

12.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.6.2 Data sheets (One set per test) -- for data recording (see Figure 1).

12.6.3 Tape, colored -- for labelling test chambers.

12.6.4 Markers, waterproof -- for marking containers, etc.

12.6.5 Buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for Standards and Calibration Check (see USEPA Method 150.1, USEPA, 1979b).

12.6.6 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

12.6.7 Laboratory quality assurance samples and standards -- for calibration of the above methods.

12.6.8 Reference toxicant solutions -- see Section 4, Quality Assurance.

12.6.9 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

12.6.10 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

12.6.10.1 Saline test and dilution water -- The salinity of the test water must be in the range of 5 to 32‰. The salinity should vary no more than ±2‰ among chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of the water should be similar.

12.6.10.2 The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Exposure of sheepshead minnow embryos to these effluents will require adjustments in the salinity of the test solutions. It is important to maintain a constant salinity across all treatments. In addition, it may be desirable to match the test salinity with that of the receiving water. Two methods are available to adjust salinities -- a hypersaline brine derived from natural seawater or artificial sea salts.

Test Dates: _____ Species: _____

Type Effluent: _____ Field: _____ Lab: _____ Test: _____

Effluent Tested: _____

Original pH: _____ Salinity: _____ DO: _____

CONCENTRATION:
Replicate I:

DAY	0	1	2	3	4	5	6	7	8	9
#Live/Dead Embryo-Larvae										
Terata										
Temp. (°C)										
Salinity (ppt)										
DO (mg/L)										
pH										

CONCENTRATION:
Replicate II:

DAY	0	1	2	3	4	5	6	7	8	9
#Live/Dead Embryo-Larvae										
Terata										
Temp. (°C)										
Salinity (ppt)										
DO (mg/L)										
pH										

Comments: _____

Note: Final endpoint for this test is total mortality (combined total number of dead embryos, dead larvae, and deformed larvae) (see Subsection 12.10.9 and 12.13).

Figure 1. Data form for sheepshead minnow, *Cyprinodon variegatus*, embryo-larval survival/teratogenicity test. Daily record of embryo-larval survival/terata and test conditions.

CONCENTRATION:
Replicate III:

DAY	0	1	2	3	4	5	6	7	8	9
#Live/Dead Embryo-Larvae										
Terata										
Temp. (°C)										
Salinity (ppt)										
DO (mg/L)										
pH										

CONCENTRATION:
Replicate IV:

DAY	0	1	2	3	4	5	6	7	8	9
#Live/Dead Embryo-Larvae										
Terata										
Temp. (°C)										
Salinity (ppt)										
DO (mg/L)										
pH										

Comments:

Note: Final endpoint for this test is total mortality (combined total number of dead embryos, dead larvae, and deformed larvae) (see Subsection 12.10.9 and 12.13).

Figure 1. Data form for sheepshead minnow, *Cyprinodon variegatus*, embryo-larval survival/teratogenicity test. Daily record of embryo-larval survival/terata and test conditions (CONTINUED).

12.6.10.3 Hypersaline brine (HSB): HSB has several advantages that make it desirable for use in toxicity testing. It can be made from any high quality, filtered seawater by evaporation, and can be added to the effluent or to deionized water to increase the salinity. HSB derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and may be stored for prolonged periods without any apparent degradation. However if 100% HSB is used as a diluent, the maximum concentration of effluent that can be tested using HSB is limited to 80% at 20‰ salinity, and 70% at 30‰ salinity.

12.6.10.3.1 The ideal container for making HSB from natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is used, use only oil-free air compressors to prevent contamination.

12.6.10.3.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough deionized water rinses.

12.6.10.3.3 High quality (and preferably high salinity) seawater should be filtered to at least l0 µm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

12.6.10.3.4 The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on volume being generated) to ensure that salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

12.6.10.3.5 After the required salinity is attained, the HSB should be filtered a second time through a 1 µm filter and poured directly into portable containers (20 L) cubitainers or polycarbonate water cooler jugs are suitable. The containers should be capped and labelled with the date the brine was generated and its salinity. Containers of HSB should be stored in the dark and maintained at room temperature until used.

12.6.10.3.6 If a source of HSB is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and brine before mixing in the effluent.

12.6.10.3.7 Divide the salinity of the HSB by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the HSB is 100‰ and the test is to be conducted at 20‰, 100‰ divided by 20‰ = 5.0. The proportion of brine is one part in five (one part brine to four parts deionized water). To make 1 L of seawater at 20‰ salinity from a HSB of 100‰, divide 1 L (1000 mL) by 5.0. The result, 200 mL, is the quantity of HSB needed to make 1 L of sea water. The difference, 800 mL, is the quantity of deionized water required.

12.6.10.3.8 Table 1 illustrates the composition of test solutions at 20‰ if they are prepared by serial dilution of effluent with 20‰ salinity seawater.

12.6.10.4 Artificial sea salts: HW MARINEMIX® brand sea salts have been used successfully at the USEPA, Region 6, Houston laboratory to culture sheepshead minnows and perform the embryo-larval survival and teratogenicity test. EMSL-Cincinnati has found FORTY FATHOMS® artificial sea salts to be suitable for culturing sheepshead minnows and for performing the larval survival and growth test and embryo-larval test. Artificial sea salts may be used for culturing sheepshead minnows and for the embryo larval test if the criteria for acceptability of test data are satisfied (see Subsection 12.11).

12.6.10.4.1 Synthetic sea salts are packaged in plastic bags and mixed with deionized water or equivalent. The instructions on the package of sea salts should be followed carefully, and salts should be mixed in a separate container -- not the culture tank. The deionized water used in hydration should be in the temperature range of 21-26°C. Seawater made from artificial sea salts is conditioned (Spotte, 1973; Spotte et al., 1984; Bower, 1983) before it is used for culturing or testing. After adding the water, place an airstone in the container, cover, and aerate the solution mildly for at least 24 h before use.

12.6.11 BRINE SHRIMP, *ARTEMIA*, CULTURE -- for feeding cultures.

12.6.11.1 Newly-hatched *Artemia* nauplii are used as food in the sheepshead minnow culture, and a brine shrimp culture unit should be prepared (USEPA, 2002a). Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are currently preferred because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size.

12.6.11.2 Each new batch of *Artemia* cysts must be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (Leger, et al., 1985; Leger, et al., 1986) against known suitable reference cysts by performing a side by side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be a previously tested and acceptable batch of cysts. A sample of newly hatched *Artemia* nauplii from each new batch of cysts should be chemically analyzed. The *Artemia* cysts should not be used if the concentration of total organic chlorine pesticides exceeds 0.15 µg/g wet weight or the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight. (For analytical methods see USEPA, 1982).

TABLE 1. PREPARATION OF TEST SOLUTIONS AT A SALINITY OF 20‰, USING 20‰ NATURAL OR ARTIFICAL SEAWATER, HYPERSALINE BRINE, OR ARTIFICAL SEA SALTS

Effluent Solution	Effluent Conc. (%)	Solutions To Be Combined: Volume of Effluent Solution		Solutions To Be Combined: Volume of Diluent Seawater (20‰)
1	100[1,2]	4000 mL		- - -
2	50	2000 mL Solution 1	+	2000 mL
3	25	2000 mL Solution 2	+	2000 mL
4	12.5	2000 mL Solution 3	+	2000 mL
5	6.25	2000 mL Solution 4	+	2000 mL
Control	0.0			2000 mL
Total				10000 mL

[1] This illustration assumes: (1) the use of 400 mL of test solution in each of four replicates and 400 mL for chemical analysis (total of 2000 mL) for the control and five concentrations of effluent (2) an effluent dilution factor of 0.5, and (3) the effluent lacks appreciable salinity. A sufficient initial volume (4000 mL) of effluent is prepared by adjusting the salinity to the desired level. In this example, the salinity is adjusted by adding artificial sea salts to the 100% effluent, and preparing a serial dilution using 20‰ seawater (natural seawater, hypersaline brine, or artificial seawater). The salinity of the initial 4000 mL of 100% effluent is adjusted to 20‰ by adding 80 g of dry artificial sea salts (HW MARINEMIX or FORTY FATHOMS®), and mixing for 1 h. Test concentrations are then made by mixing appropriate volumes of salinity-adjusted effluent and 20‰ salinity dilution water to provide 4000 mL of solution for each concentration. If hypersaline brine alone (100‰) is used to adjust the salinity of the effluent, the highest concentration of effluent that could be achieved would be 80% at 20‰ salinity, and 70% at 30‰ salinity.

[2] The same procedures would be followed in preparing test concentrations at other salinities between 20‰ and 30‰: (1) The salinity of the bulk (initial) effluent sample would be adjusted to the appropriate salinity using artificial sea salts or hypersaline brine, and (2) the remaining effluent concentrations would be prepared by serial dilution, using a large batch (10 L) of seawater for dilution water, which had been prepared at the same salinity as the effluent, using natural seawater, hypersaline and deionized water.

12.6.11.3 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or a solution prepared by adding 35.0 g uniodized salt (NaCl) or artificial sea salts to 1 L of deionized water, to a 2 L separatory funnel, or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24 h at 27°C. (Hatching time varies with incubation temperature and the geographic strain of *Artemia* used (see USEPA, 1985d, USEPA, 2002a; and ASTM, 1993).
3. After 24 h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic, and will concentrate at the bottom of the funnel if it is covered for five to 10 minutes. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 minutes without aeration.

4. Drain the nauplii into a beaker or funnel fitted with a ≤150 µm NITEX® or stainless steel screen, and rinse with seawater or equivalent before use.

12.6.11.4 Testing *Artemia* nauplii as food for toxicity test organisms.

12.6.11.4.1 The primary criterion for acceptability of each new supply of brine shrimp cysts is the ability of the nauplii to support good survival and growth of the sheepshead minnow larvae. The larvae used to evaluate the suitability of the brine shrimp nauplii must be of the same geographical origin, species, and stage of development as those used routinely in the toxicity tests. Sufficient data to detect differences in survival and growth should be obtained by using three replicate test vessels, each containing a minimum of 15 larvae, for each type of food.

12.6.11.4.2 The feeding rate and frequency, test vessels, volume of control water, duration of the test, and age of the nauplii at the start of the test, should be the same as used for the routine toxicity tests.

12.6.11.4.3 Results of the brine shrimp nutrition assay, where there are only two treatments, can be evaluated statistically by use of a t test. The "new" food is acceptable if there are no statistically significant differences in the survival and growth of the larvae fed the two sources of nauplii.

12.6.11.4.4 The average seven-day survival of larvae should be 80% or greater, and (2) the average dry weight of larvae should be 0.60 mg or greater, if dried and weighed immediately after the test, or (3) the average dry weight of larvae should be 0.50 mg or greater, if the larvae are preserved in 4% formalin before drying and weighing. The above minimum weights presume that the age of the larvae at the start of the test is not greater than 24 h.

12.6.12 TEST ORGANISMS, SHEEPSHEAD MINNOWS, *CYPRINODON VARIEGATUS*

12.6.12.1 Brood stock

12.6.12.1.1 Adult sheepshead minnows for use as brood stock may be obtained by seine in Gulf of Mexico and Atlantic coast estuaries, from commercial sources, or from young fish raised to maturity in the laboratory. Feral brood stocks and first generation laboratory fish are preferred, to minimize inbreeding.

12.6.12.1.2 To detect disease and to allow time for acute mortality due to the stress of capture, field-caught adults are observed in the laboratory a minimum of two weeks before using as a source of gametes. Injured or diseased fish are discarded.

12.6.12.1.3 Sheepshead minnows can be continuously cultured in the laboratory from eggs to adults. The larvae, juvenile, and adult fish should be kept in appropriate size rearing tanks, maintained at ambient laboratory temperature. The larvae should be fed sufficient newly hatched *Artemia* nauplii daily to assure that live nauplii are always present. Juveniles are fed frozen adult brine shrimp and a commercial flake food, such as TETRA SM-80® or MARDEL AQUARIAN® Tropical Fish Flakes, or equivalent. Adult fish are fed flake food three or four times daily, supplemented with frozen adult brine shrimp.

12.6.12.1.3.1 Sheepshead minnows reach sexual maturity in three-to-five months after hatch, and have an average standard length of approximately 27 mm for females and 34 mm for males. At this time, the males begin to exhibit sexual dimorphism and initiate territorial behavior. When the fish reach sexual maturity and are to be used for natural spawning, the temperature should be controlled at 18-20°C.

12.6.12.1.4 Adults can be maintained in natural or artificial seawater in a flow-through or recirculating, aerated system consisting of an all-glass aquarium, or equivalent.

12.6.12.1.5 The system is equipped with an undergravel or outside biological filter of shells (see Spotte, 1973; Bower, 1983) for conditioning the biological filter, or a cartridge filter, such as a MAGNUM® Filter, or an EHEIM® Filter, or equivalent, at a salinity of 20-30‰ and a photoperiod of 16 h light/8 h dark.

12.6.12.2 Obtaining Embryos for Toxicity Tests

12.6.12.2.1 Embryos can be shipped to the laboratory from an outside source or obtained from adults held in the laboratory. Ripe eggs can be obtained either by natural spawning or by intraperitoneal injection of the females with human chorionic gonadotrophin (HCG) hormone. If the culturing system for adults is temperature controlled, natural spawning can be induced. Natural spawning is preferred because repeated spawnings can be obtained from the same brood stock, whereas with hormone injection, the brood stock is sacrificed in obtaining gametes.

12.6.12.2.2 It should be emphasized that the injection and hatching schedules given below are to be used only as guidelines. Response to the hormone varies from stock to stock and with temperature. Time to hatch and percent hatch also vary among stocks and among batches of embryos obtained from the same stock, and are dependent on temperature, DO, and salinity.

12.6.12.2.3 Forced Spawning

12.6.12.2.3.1 HCG is reconstituted with sterile saline or Ringer's solution immediately before use. The standard HCG vial contains 1,000 IU to be reconstituted in 10 mL of saline. Freeze-dried HCG which comes with premeasured and sterilized saline is the easiest to use. Use of a 50 IU dose requires injection of 0.05 mL of reconstituted hormone solution. Reconstituted HCG may be used for several weeks if kept in the refrigerator.

12.6.12.2.3.2 Each female is injected intraperitoneally with 50 IU HCG on two consecutive days, starting at least four days prior to the beginning of a test. Two days following the second injection, eggs are stripped from the females and mixed with sperm derived from excised macerated testes. At least 10 females and five males are used per test to ensure that there is a sufficient number of viable embryos.

12.6.12.2.3.3 HCG is injected into the peritoneal cavity, just below the skin, using as small a needle as possible. A 50 IU dose is recommended for females approximately 27 mm in standard length. A larger or smaller dose may be used for fish which are significantly larger or smaller than 27 mm. With injections made on days one and two, females which are held at 25°C should be ready for stripping on Day 4. Ripe females should show pronounced abdominal swelling, and release at least a few eggs in response to a gentle squeeze. Injected females should be isolated from males. It may be helpful if fish that are to be injected are maintained at 20°C before injection, and the temperature raised to 25°C on the day of the first injection.

12.6.12.2.3.4 Prepare the testes immediately before stripping the eggs from the females. Remove the testes from three to five males. The testes are paired, dark grey organs along the dorsal midline of the abdominal cavity. If the head of the male is cut off and pulled away from the rest of the fish, most of the internal organs can be pulled out of the body cavity, leaving the testes behind. The testes are placed in a few mL of seawater until the eggs are ready.

12.6.12.2.3.5 Strip the eggs from the females, into a dish containing 50-100 mL of seawater, by firmly squeezing the abdomen. Sacrifice the females and remove the ovaries if all the ripe eggs do not flow out freely. Break up any clumps of ripe eggs and remove clumps of ovarian tissue and underripe eggs. Ripe eggs are spherical, approximately 1 mm in diameter, and almost clear.

12.6.12.2.3.6 While being held over the dish containing the eggs, the testes are macerated in a fold of NITEX® screen (250-500 µm mesh) dampened with seawater. The testes are then rinsed with seawater to remove the sperm from tissue, and the remaining sperm and testes are washed into the dish. Let the eggs and milt stand together for 10-15 minutes, swirling occasionally.

12.6.12.2.3.7 Pour the contents of the dish into a beaker, and insert an airstone. Aerate gently, such that the water moves slowly over the eggs, and incubate at 25°C for 60-90 minutes. After incubation, wash the eggs on a NITEX® screen and resuspend them in clean seawater.

12.6.12.2.4 Natural Spawning

12.6.12.2.4.1 Short-term (Demand) Embryo Production

12.6.12.2.4.1.1 Adult fish should be maintained at 18-20°C in a temperature controlled system. To obtain embryos for a test, adult fish (generally, at least eight to 10 females and three males) are transferred to a spawning chamber, with a photoperiod of 16 h light/8 h dark and a temperature of 25°C, two days before the beginning of the test. The spawning chambers are approximately 20 x 35 x 22 cm high (USEPA, 1978), and consist of a basket of 3-5 mm NITEX® mesh, made to fit into a 57-L (15 gal) aquarium. Spawning generally will begin within 24 h or less. The embryos will fall through the bottom of the basket and onto a collecting screen (250-500 µm mesh) or tray below the basket. The collecting tray should be checked for embryos the next morning. The number of eggs produced is highly variable. The number of spawning units required to provide the embryos needed to perform a toxicity test is determined by experience. If the trays do not contain sufficient embryos after the first 24 h, discard the embryos, replace the trays, and collect the embryos for another 24 h or less. To help keep the embryos clean, the adults are fed while the screens are removed.

12.6.12.2.4.1.2 The embryos are collected in a tray placed on the bottom of the tank. The collecting tray consists of 250-500 µm NITEX® screen attached to a rigid plastic frame. The collecting trays with newly-spawned, embryos are removed from the spawning tank, and the embryos are collected from the screens by washing them with a wash bottle or removing them with a fine brush. The embryos from several spawning units may be pooled in a single container to provide a sufficient number to conduct the test(s). The embryos are transferred into a petri dish or equivalent, filled with fresh culture water, and are examined using a dissecting microscope or other suitable magnifying device. Damaged and infertile eggs are discarded (see Figure 2). It is strongly recommended that the embryos be obtained from fish cultured in-house, rather than from outside sources, to eliminate the uncertainty of damage caused by shipping and handling that may not be observable, but which might affect the results of the test.

12.6.12.2.4.1.3 After sufficient embryos are collected for the test, the adult fish are returned to the (18-20°C) culture tanks.

12.6.12.2.4.2 Sustained Natural Embryo Production

12.6.12.2.4.2.1 Sustained (long-term), daily, embryo production can be achieved by maintaining mature fish in tanks, such as a (285 L or 75 gal) LIVING STREAM® tank, at a temperature of 23-25°C. Embryos are produced daily, and when needed, embryo "collectors" are placed on the bottom of the tank on the afternoon preceding the start of the test. The next morning, the embryo collectors are removed and the embryos are washed into a shallow glass culture dish using artificial seawater.

12.6.12.2.4.2.2 Four embryo collectors, approximately 20 cm x 45 cm, will approximately cover the bottom of the 285 L tank. The collectors are fabricated from plastic fluorescent light fixture diffusors (grids), with cells approximately 14 mm deep X 14 mm square. A screen consisting of 500 µm mesh is attached to one side (bottom) of the grid with silicone adhesive. The depth and small size of the grid protects the embryos from predation by the adult fish.

12.6.12.2.4.2.3 The brood stock is replaced annually with feral stock.

12.6.12.2.5 Test Organisms

12.6.12.2.5.1 Embryos spawned over a less than 24-h period, are used for the test. These embryos may be used immediately to start a test or may be placed in a suitable container and transported for use at a remote location. When overnight transportation is required, embryos should be obtained when they are no more than 8-h old. This permits the tests at the remote site to be started with less than 24-h old embryos. Embryos should be transported or shipped in clean, insulated containers, in well aerated or oxygenated fresh seawater or aged artificial sea water of correct salinity, and should be protected from extremes of temperature and any other stressful conditions during

transport. Instantaneous changes of water temperature when embryos are transferred from culture unit water to test dilution water, or from transport container water to on-site test dilution, should be less than 2°C. Instantaneous changes of pH, dissolved ions, osmotic strength, and DO should also be kept to a minimum.

12.6.12.2.5.2 The number of embryos needed to start the test will depend on the number of tests to be conducted and the objectives. If the test is conducted with four replicate test chambers (minimum of three) at each toxicant concentration and in the control, with 15 embryos (minimum of 10) in each test chamber, and the combined mortality of embryos prior to the start of the test is less than 20%, 400 viable embryos are required for the test.

12.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

12.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests

12.8 CALIBRATION AND STANDARDIZATION

12.8.1 See Section 4, Quality Assurance

12.9 QUALITY CONTROL

12.9.1 See Section 4, Quality Assurance

12.10 TEST PROCEDURES

12.10.1 Test Solutions

12.10.1.1 Receiving Waters

12.10.1.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 400-500 mL, and 400 mL for chemical analysis, would require approximately 2.0-2.5 L or more of sample per test per day.

12.10.1.2 Effluents

12.10.1.2.1 The selection of the effluent test concentration should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ±100%, and allows for testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **Therefore, USEPA recommends the use of the ≥0.5 dilution factor.** If 100‰ salinity HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ and 70% at 30‰ salinity.

12.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1-2 h of the test, additional dilutions at the lower range of effluent concentrations should be added.

12.10.1.2.3 The volume of effluent required to initiate the test and for daily renewal of four replicates (minimum of three) per concentration for five concentrations of effluent and a control, each containing 400 mL of test solution, is approximately 4 L. Prepare enough test solution (approximately 3000 mL) at each effluent concentration to refill the test chambers and provide at least 400 mL additional volume for chemical analyses.

12.10.1.2.4 Maintain the effluent at 0-6°C. Plastic containers such as 8-20 L cubitainers have proven successful for effluent collection and storage.

12.10.1.2.5 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample(s) to make the test solutions should be adjusted to the test temperature (25 ± 1°C) and maintained at that temperature during the addition of dilution water.

12.10.1.2.6 Higher effluent concentrations (i.e., 25%, 50%, and 100%) may require aeration to maintain adequate dissolved oxygen concentrations. However, if one solution is aerated, all concentrations must be aerated. Aerate effluent as it warms and continue to gently aerate test solutions in the test chambers for the duration of the test.

12.10.1.2.7 Effluent dilutions should be prepared for all replicates in each treatment in one beaker to minimize variability among the replicates. The test chambers are labelled with the test concentration and replicate number. Dispense into the appropriate effluent dilution chamber.

12.10.1.3 Dilution Water

12.10.1.3.1 Dilution water may be uncontaminated natural seawater (receiving water), HSB prepared from natural seawater, or artifical seawater prepared from FORTY FATHOMS® or GP2 sea salts (see Table 3 in Section 7, Dilution Water). Other artificial sea salts may be used for culturing sheepshead minnows if the control criteria for acceptability of test data are satisfied.

12.10.2 START OF THE TEST

12.10.2.1 Tests should begin as soon as possible, preferably within 24 h after sample collection. For on-site toxicity studies, no more than 24 h should elapse between collection of the effluent and use in an embryo-larval study. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity studies unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.10.2.2 Label the test chambers with a marking pen. Use color-coded tape to identify each treatment and replicate. A minimum of five effluent concentrations and a control are used for each effluent test. Each concentration (including controls) is to have four replicates (minimum of three). Use 500 mL beakers, crystallization dishes, nontoxic disposable plastic labware, or equivalent for test chambers.

12.10.2.3 Prepare the test solutions (see Table 1) and add to the test chambers.

12.10.2.4 Gently agitate and mix the embryos to be used in the test in a large container so that eggs from different spawns are evenly dispersed.

12.10.2.5 The test is started by randomly placing embryos from the common pool, using a small bore (2 mm), fire polished, glass tube calibrated to contain approximately the desired number of embryos, into each of four replicate test chamber, until each chamber contains 15 embryos (minimum of 10), for a total of 60 embryos (minimum of 30) for each concentration (four replicates recommended, three minimum) (see Appendix A). The amount of water added to the chambers when transferring the embryos should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

12.10.2.6 After the embryos have been distributed to each test chamber, examine and count them. Remove and discard damaged or infertile eggs and replace with undamaged embryos. It may be more convenient and efficient to transfer embryos to intermediate containers of dilution water for examination and counting. After the embryos have been examined and counted in the intermediate container, assign them to the appropriate test chamber and transfer them with a minimum of dilution water.

12.10.2.7 Randomize the position of the test chambers at the beginning of the test (see Appendix A). Maintain the chambers in this configuration throughout the test. Preparation of a position chart may be helpful.

12.10.3 LIGHT, PHOTOPERIOD, SALINITY, AND TEMPERATURE

12.10.3.1 The light quality and intensity should be at ambient laboratory levels, approximately 10-20 μE/m^2/s, or 50-100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The test water temperature should be maintained at 25 ± 1 °C. The salinity should be 5 to 32‰ ± 2‰ to accommodate receiving waters that may fall within this range. The salinity should vary no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

12.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

12.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO. The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Holding, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed 100 bubbles/min, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® Serological Pipet, or equivalent. Care should be taken to ensure that turbulence resulting from the aeration does not cause undue physical stress to the fish.

12.10.5 FEEDING

12.10.5.1 Feeding is not required.

12.10.6 OBSERVATIONS DURING THE TEST

12.10.6.1 Routine Chemical and Physical Determinations

12.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period at each test concentration and in the control.

12.10.6.1.2 Temperature, pH, and salinity are measured at the end of each 24-h exposure period in one test chamber at each test concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at least at the end of the test to determine the temperature variation in the environmental chambers.

12.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

12.10.6.1.4 Record all measurements on the data sheet (Figure 1).

12.10.6.2 Routine Biological Observations

12.10.6.2.1 At the end of the first 24 h of exposure, before renewing the test solutions, examine and count the embryos. Remove the dead embryos (milky colored and opaque) and record the number. If the rate of mortality or fungal infection exceeds 20% in the control chambers, or if excessive nonconcentration related mortality occurs, terminate the test and start a new test with new embryos. If the above mortality conditions do not occur, continue the test for the full nine days.

12.10.6.2.2 At 25 °C, hatching begins on about the sixth day. After hatching begins, count the number of dead and live embryos and the number of hatched, dead, live, and deformed and/or debilitated larvae, daily (see Figure 2 for illustrations of morphological development of embryo and larva). Deformed larvae are those with gross morphological abnormalities such as curved spines, lack of appendages, lack of fusiform shape (non-distinct mass),

a colored beating heart in an opaque mass, lack of mobility, abnormal swimming, or other characteristics that preclude survival. Remove dead embryos and dead and deformed larvae as previously discussed and record the numbers for all test observations (see Figure 2).

12.10.6.2.3 Protect the embryos and larvae from unnecessary disturbance during the test by carefully carrying out the daily test observations, solution renewals, and removal of dead organisms. Make sure the test organisms remain immersed during the performance of the above operations.

12.10.7 DAILY CLEANING OF TEST CHAMBERS

12.10.7.1 Since feeding is not required, test chambers are not cleaned daily unless accumulation of particulate matter at the bottom of the tank causes a problem.

12.10.8 TEST SOLUTION RENEWAL

12.10.8.1 The test solutions are renewed daily using freshly prepared solution, immediately after cleaning the test chambers. For on-site toxicity studies, fresh effluent and receiving water samples used in toxicity tests should be collected daily, and no more than 24 h should elapse between collection of the sample and use in the test (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples must be collected, preferably on days 1, 3, and 5. Maintain the samples at 0-6°C until used.

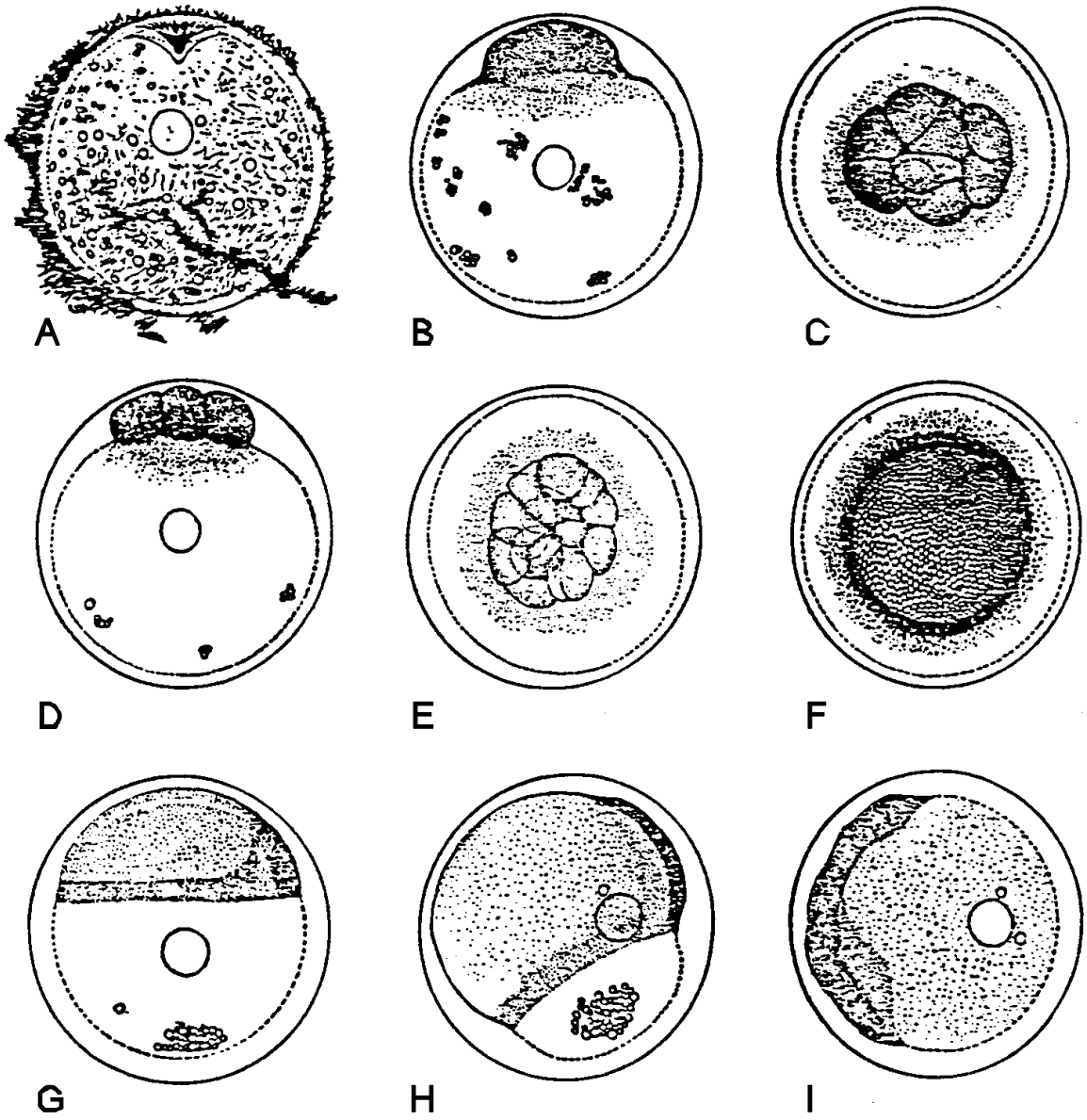


Figure 2 Embryonic development of sheepshead minnow, *Cyprinodon variegatus*: A. Mature unfertilized egg, showing attachment filaments and micropyle, X33; B. Blastodisc fully developed; C,D. Blastodisc, 8 cells; E. Blastoderm, 16 cells; F. Blastoderm, late cleavage stage; G. Blastoderm with germ ring formed, embryonic shield developing; H. Blastoderm covers over ¾ of yolk, yolk noticeably constricted; I. Early embryo. From Kuntz (1916).

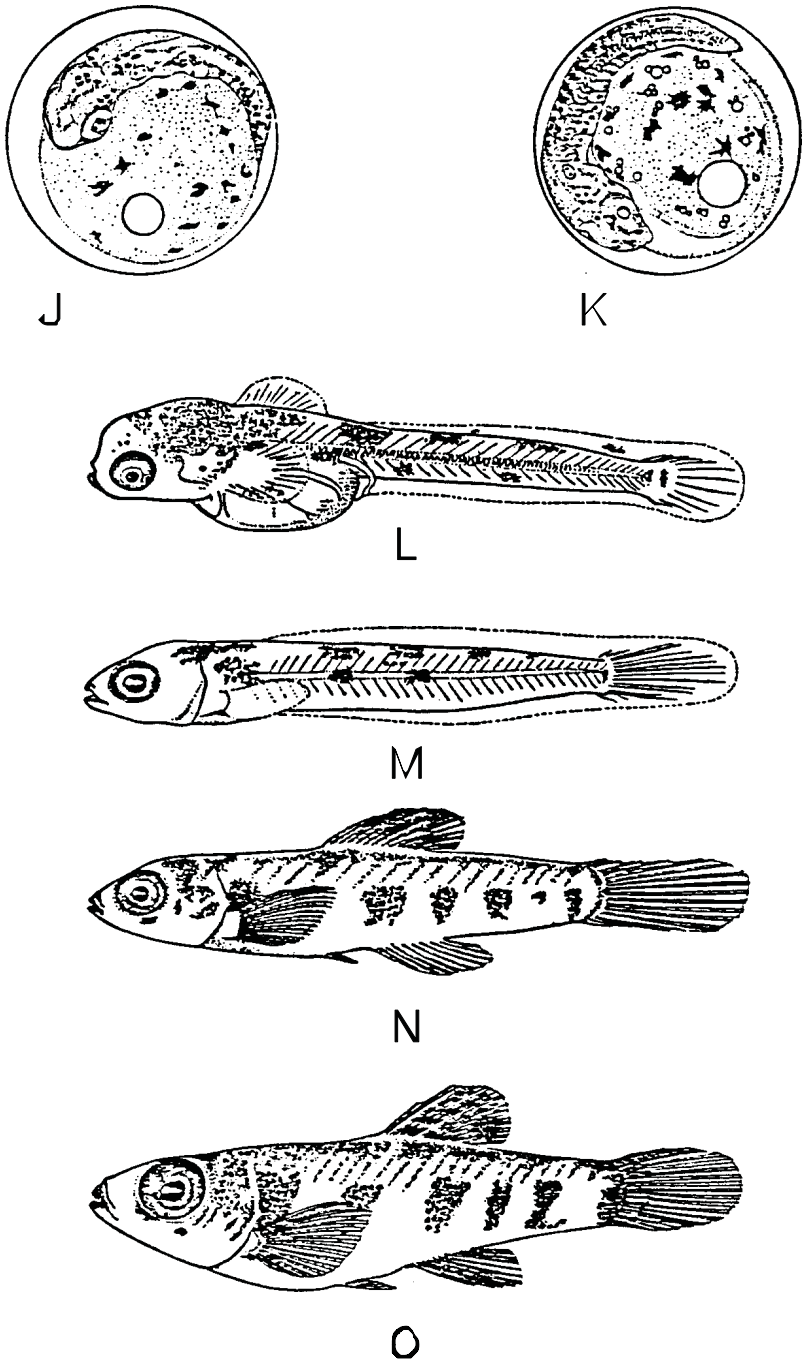


Figure 2. Embryonic development of sheepshead minnow, *Cyprinodon variegatus*: J. Embryo 48 h after fertilization, now segmented throughout, pigment on yolk sac and body, otoliths formed; K. Posterior portion of embryo free from yolk and moves freely within egg membrane, 72 h after fertilization; L. Newly hatched fish, actual length 4 mm; M. Larval fish five days after hatching, actual length 5 mm; N. Young fish 9 mm in length; O. Young fish 12 mm in length (CONTINUED). From Kuntz (1916).

12.10.8.2 The test solutions are adjusted to the correct salinity and renewed daily using freshly collected samples. During the daily renewal process, 7-10 mm of water is left in the chamber to ensure that the embryos and larvae remain submerged during the renewal process. New test solution (400 mL) should be added slowly by pouring down the side of the test chamber to avoid exposing the embryos and larvae to excessive turbulence.

12.10.8.3 Prepare test solutions daily, making a minimum of five concentrations and a control. If concurrent effluent and receiving water testing occurs, the effluent test salinity should closely approximate that of the receiving water test. If an effluent is tested alone, select a salinity which approximately matches the salinity of the receiving waters. Table 1 illustrates the quantities of effluent, seawater, deionized water, and artificial sea salts needed to prepare 3 L of test solution at each effluent concentration for tests conducted at 20‰ salinity.

12.10.9 TERMINATION OF THE TEST

12.10.9.1 The test is terminated after nine days of exposure, or four days post-hatch, whichever comes first. Count the number of surviving, dead, and deformed and/or debilitated larvae, and record the numbers of each. The deformed larvae are treated as dead. Keep a separate record of the total number of deformed larvae for use in reporting the teratogenicity of the test solution.

12.11 ACCEPTABILITY OF TEST RESULTS

12.11.1 For the test results to be acceptable, survival in the controls must be at least 80% or better.

12.12 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

12.12.1 A summary of test conditions and test acceptability criteria is listed in Table 2.

12.13 DATA ANALYSIS

12.13.1 General

12.13.1.1 Tabulate and summarize the data.

12.13.1.2 The endpoints of this toxicity test are based on total mortality, combined number of dead embryos, dead larvae, and deformed larvae. The EC endpoints are calculated using Probit Analysis (Finney, 1971). LOEC and NOEC values, for total mortality, are obtained using a hypothesis test approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981). See the Appendices for examples of the manual computations, program listings, and examples of data input and program output.

TABLE 2. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1005.0)[1]

1. Test type:	Static renewal (required)
2. Salinity:	5‰ to 32‰ (±2‰ of the selected test salinity) (recommended)
3. Temperature:	25 ± 1 °C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3 °C during the test (required)
4. Light quality:	Ambient laboratory light (recommended)
5. Light intensity:	10-20 μE/m^2/s, or 50-100 ft-c (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	400-500 mL (recommended)
8. Test solution volume:	250-400 mL per replicate (loading and DO restrictions must be met) (recommended)
9. Renewal of test solutions:	Daily (required)
10. Age of test organisms:	Less than 24 h old (required)
11. No. of embryos per chamber:	15 (recommended) 10 (required minimum)
12. No. replicate test chambers per concentration:	4 (recommended) 3 (required minimum)
13. No. embryos per concentration:	60 (recommended) 30 (required minimum)
14. Feeding regime:	Feeding not required
15. Aeration:	None unless DO falls below 4.0 mg/L (recommended)
16. Dilution water:	Uncontaminated source of natural seawater; deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, GP2, or equivalent) (available options)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 2. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1005.0)[1]

17. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving waters: 100% receiving water (or minimum of 5) and a control (recommended)
18. Dilution factor:	Effluent: ≥0.5 (recommended) Receiving waters: None, or ≥0.5 (recommended)
19. Test duration:	9 days (required)
20. Endpoints:	Percent hatch; percent larvae dead or with debilitating morphological and/or behavior abnormalities such as: gross deformities; curved spine; disoriented, abnormal swimming behavior; surviving normal larvae from original embryos (required)
21. Test acceptability criteria:	80% or greater survival in controls (required)
22. Sampling requirements:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
23. Sample volume required:	5 L per day (recommended)

12.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

12.13.2 EXAMPLE OF ANALYSIS OF SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS,* EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY DATA

12.13.2.1 Formal statistical analysis of the total mortality data is outlined in Figure 3. The response used in the analysis is the total mortality proportion in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the EC endpoint. Concentrations at which there is 100% mortality in all of the test chambers are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the EC endpoints.

12.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's

Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

12.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

12.13.2.4 Probit Analysis (Finney, 1971; see Appendix H) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method or the Graphical Method may be used (see Appendices H-K).

12.13.2.5 Example of Analysis of Survival Data

12.13.2.5.1 The data for this example are listed in Table 3. Total mortality, expressed as a proportion (combined total number of dead embryos, dead larvae and deformed larvae divided by the number of embryos at start of test), is the response of interest. The total mortality proportion in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variences of the transformed observations at each SDS concentration and control are listed in Table 3. A plot of the data is provided in Figure 4. Since there is 100% mortality in all replicates for the 8.0 mg/L concentration, it is not included in this statistical analysis and is considered a qualitative mortality effect.

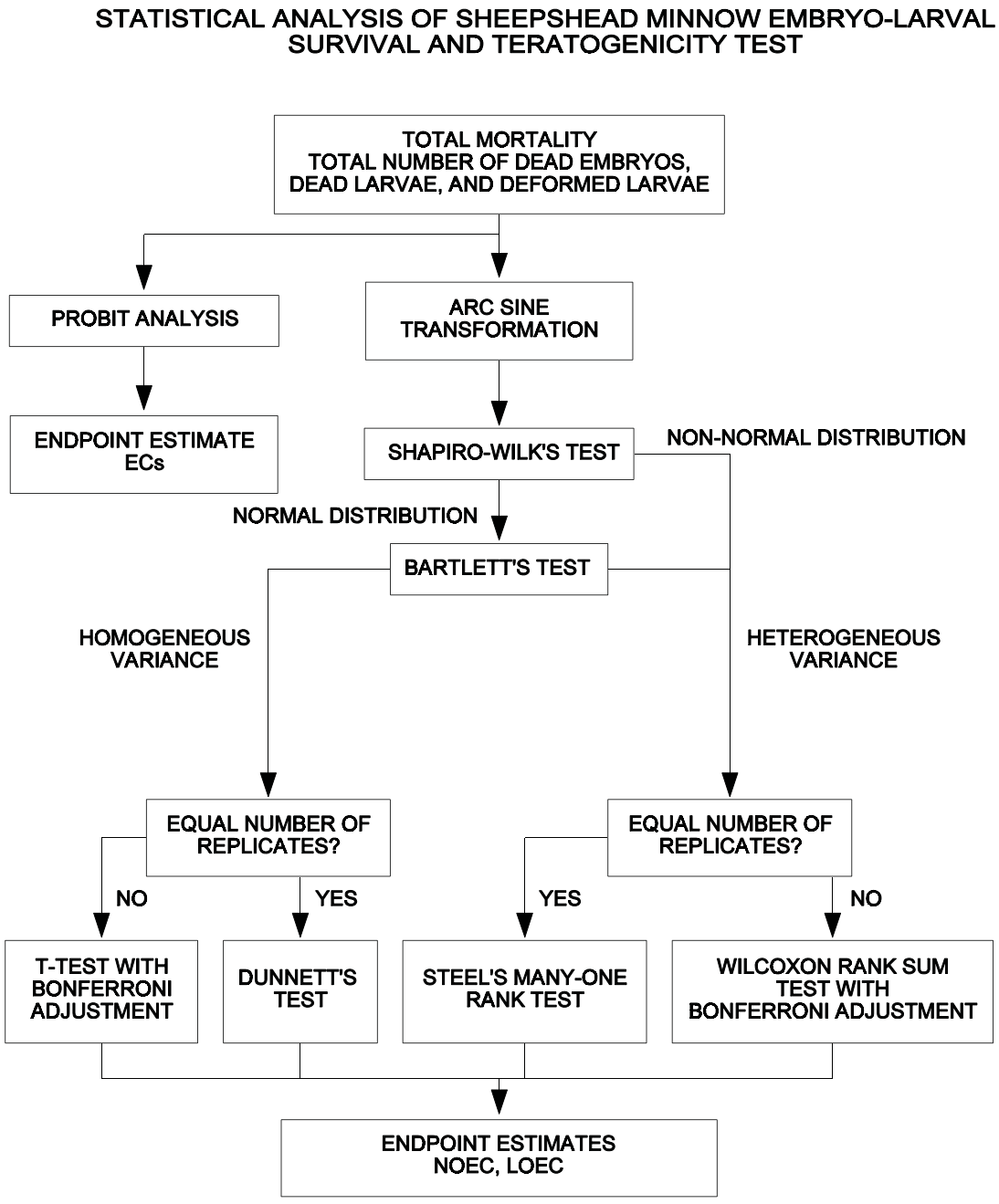


Figure 3. Flowchart for statistical analysis of sheepshead minnow, *Cyprinodon variegatus*, embryo-larval survival and teratogenicity test. Survival and terata data.

TABLE 3. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EMBRYO-LARVAL TOTAL MORTALITY DATA

			SDS Concentration (mg/L)				
	Replicate	Control	0.5	1.0	2.0	4.0	8.0
RAW	A	0.1	0.0	0.0	0.3	0.9	1.0
	B	0.0	0.2	0.1	0.1	0.7	1.0
	C	0.1	0.2	0.1	0.2	0.8	1.0
	D	0.0	0.1	0.2	0.4	0.8	1.0
ARC SINE	A	0.322	0.159	0.159	0.580	1.249	–
TRANS-	B	0.159	0.464	0.322	0.322	0.991	–
FORMED	C	0.322	0.464	0.322	0.464	1.107	–
	D	0.159	0.322	0.464	0.685	1.107	–
Mean ($\overline{Y}_i$)		0.241	0.352	0.317	0.513	1.114	
S^2_i		0.009	0.021	0.016	0.024	0.011	
i		1	2	3	4	5	

12.13.2.6 Test for Normality

12.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 4.

TABLE 4: CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		SDS Concentration (mg/L)				
Replicate	Control	0.5	1.0	2.0	4.0	8.0
A	0.081	-0.193	-0.158	0.067	0.135	–
B	-0.082	0.112	0.005	-0.191	-0.123	–
C	0.081	0.112	0.005	-0.049	-0.007	–
D	-0.082	-0.030	0.147	0.172	-0.007	–

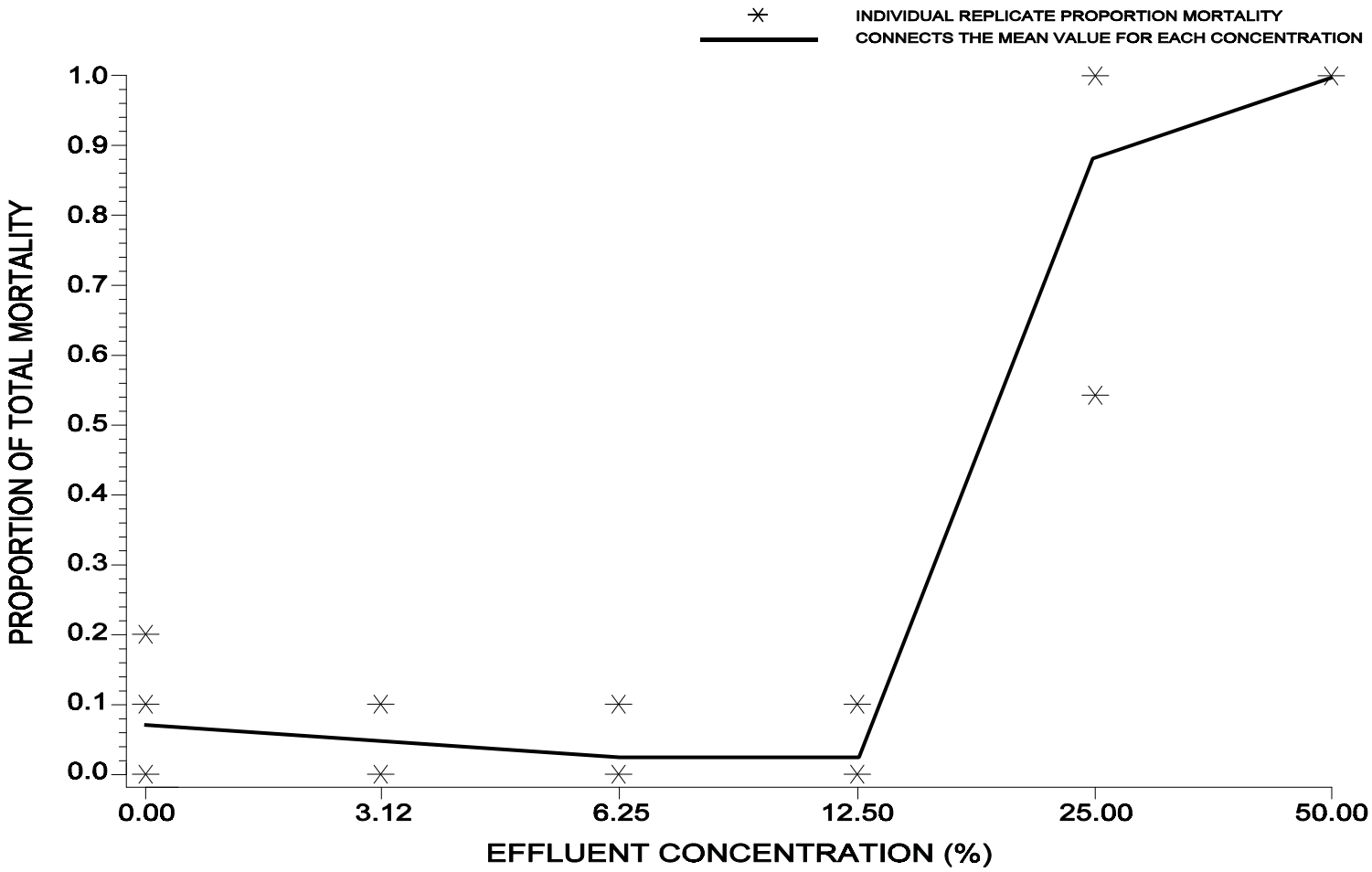


Figure 4. Plot of Sheepshead minnow, *Cyprinodon variegatus*, total mortality data from the embryo-larval test

12.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

12.13.2.6.3 For this set of data, n = 20

$$\bar{X} = \frac{1}{20} (-0.005) = 0.000$$

$$D = 0.2428$$

12.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where: $X^{(i)}$ = the ith ordered observation

The ordered observations for this example are listed in Table 5.

TABLE 5. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.193	11	0.005
2	-0.191	12	0.005
3	-0.158	13	0.067
4	-0.123	14	0.081
5	-0.082	15	0.081
6	-0.082	16	0.112
7	-0.049	17	0.112
8	-0.030	18	0.135
9	-0.007	19	0.147
10	-0.007	20	0.172

12.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ..., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table 6.

TABLE 6. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.365	$X^{(20)} - X^{(1)}$
2	0.3211	0.338	$X^{(19)} - X^{(2)}$
3	0.2565	0.293	$X^{(18)} - X^{(3)}$
4	0.2085	0.295	$X^{(17)} - X^{(4)}$
5	0.1686	0.194	$X^{(16)} - X^{(5)}$
6	0.1334	0.163	$X^{(15)} - X^{(6)}$
7	0.1013	0.130	$X^{(14)} - X^{(7)}$
8	0.0711	0.097	$X^{(13)} - X^{(8)}$
9	0.0422	0.012	$X^{(12)} - X^{(9)}$
10	0.0140	0.012	$X^{(11)} - X^{(10)}$

12.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D} \left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 6. For the data in this example,

$$W = \frac{1}{0.2428} (0.4807)^2 = 0.952$$

12.13.2.6.7 The decision rule for this test is to compare W as calculated in Section 12.13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 20 observations is 0.868. Since W = 0.952 is greater than the critical value, conclude that the data are normally distributed.

12.13.2.7 Test for Homogeneity of Variance

12.13.2.7.1 The test used to examine whether the variation in mean proportion mortality is the same across all toxicant concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{\left[\left(\sum_{i=1}^{P} V_i\right) \ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2\right]}{C}$$

Where: V_i = degrees of freedom for each copper concentration and control, $V_i = (n_i - 1)$

p = number of concentration levels including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i = the number of replicates for concentration i

$$\bar{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i)^2}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3\ (p-1)]^{-1}\ [\sum_{i=1}^{p} i/v_i\ -\ (\sum_{i=1}^{p} v_i)-1]$$

12.13.2.7.2 Since B is approximately distributed as chi-square with p-1 degrees of freedom when the variances are equal, the appropriate critical value is obtained from a table of the chi-square distribution for p-1 degrees of freedom and a significance level of 0.01. If B is less than the critical value then the variances are assumed to be equal.

12.13.2.7.3 For the data in this example, $V_i = 3$, p=5, $\bar{S}^2 = 0.0162$, and C = 1.133. The calculated B value is:

$$B = \frac{(15)\ [\ln\ (0.01262)]-3\sum_{i=1}^{p} \ln(S_i^2)}{1.33}$$

$$= \frac{15\ (-4.1227)\ -\ 3\ (-20.9485)}{1.33}$$

$$= 0.886$$

12.13.2.7.4 Since B is approximately distributed as chi-square with p-1 degrees of freedom when the variances are equal, the appropriate critical value for the test is 13.277 for a significance level of 0.01. Since B = 0.886 is less than the critical value of 13.277, conclude that the variances are not different.

12.13.2.8 Dunnett's Procedure

12.13.2.8.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 7.

TABLE 7. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean dry weight of the mysids for concentration i in test chamber j)

12.13.2.8.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 4$

$N = 20$

$$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 0.962$$
$$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 1.409$$
$$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 1.267$$
$$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.051$$
$$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 4.454$$

$$G = T_1 + T_2 + T_3 + T_4 = 10.143$$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= \frac{1}{4}(28.561) - \frac{(10.143)^2}{20} = 1.996$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 7.383 - \frac{(10.143)^2}{20} = 1.996$$

$$SSW = SST - SSB = 2.239 - 1.996 = 0.243$$

$$S_w^2 = SSB/(p-1) = 1.996/(5-1) = 0.499$$

$$S_w^2 = SSW/(N-p) = 0.243/(20-5) = 0.016$$

12.13.2.8.3 Summarize these calculations in the ANOVA table (Table 8).

TABLE 8. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	4	1.996	0.499
Within	15	0.243	0.016
Total	19	2.239	

12.13.2.8.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean proportion surviving for concentration i

$\bar{Y}_1$ = mean proportion surviving for the control

S_w = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

Since we are looking for an increased response in percent of total mortality over control, the control mean is subtracted from the mean at a concentration.

12.13.2.8.5 Table 9 includes the calculated t values for each concentration and control combination. In this example, comparing the 0.5 mg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{0.352 - 0.241}{[0.1265\sqrt{(1/4) + (1/4)}]} = 1.241$$

12.13.2.8.6 Since the purpose of this test is to detect a significant increase in total mortality, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 15 degrees of freedom for error and four concentrations (excluding the control) the critical value is 2.36. The mean proportion of total mortality for concentration "i" is considered significantly less than the mean proportion of total mortality for the control if t_i is greater than the critical value. Therefore, the 2.0 mg/L and the 4.0 mg/L concentrations have significantly higher mean proportions of total mortality than the control. Hence the NOEC is 1.0 mg/L and the LOEC is 2.0 mg/L.

TABLE 9. CALCULATED T VALUES

SDS Concentration (mg/L)	i	t_i
0.5	2	1.241
1.0	3	0.850
2.0	4	3.041
4.0	5	9.760

12.13.2.8.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be detected statistically may be calculated.

$$MSD = d\ S_w\sqrt{(1/n_1) + (1/n)}$$

Where: d = the critical value for Dunnett's procedure

S_W = the square root of the within mean square

n_1 = the number of replicates in the control

n = The common number of replicates at each concentration (this assumes equal replication at each concentration)

12.13.2.8.8 In this example:

$$MSD = 2.36\ (0.1265)\ \sqrt{(1/4) + (1/4)}$$

$$= 2.36\ (0.1265)\ (0.7071)$$

$$= 0.211$$

12.13.2.8.9 The MSD (0.450) is in transformed units. To determine the MSD in terms of percent survival, carry out the following conversion.

1. Add the MSD to the transformed control mean.

   0.241 + 0.211 = 0.452

2. Obtain the untransformed values for the control mean and the sum calculated in 1.

   $[\ \text{Sine}\ (0.241)\ ]^2 = 0.057$

   $[\ \text{Sine}\ (0.452)\ ]^2 = 0.191$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from Step 2.

   $MSD_u = 0.191 - 0.057 = 0.134$

12.13.2.8.10 Therefore, for this set of data, the minimum difference in mean proportion of total mortality between the control and any SDS concentration that can be detected as statistically significant is 0.134.

12.13.2.8.11 This represents a 268% increase in mortality from the control.

12.13.2.9 Calculation of the LC50

12.13.2.9.1 The data used for the Probit Analysis is summarized in Table 10. To perform the Probit Analysis, run the USEPA Probit Analysis Program. An example of the program input and output is supplied in Appendix H.

TABLE 10. DATA FOR PROBIT ANALYSIS

	SDS Concentration (mg/L)					
	Control	0.5	1.0	2.0	4.0	8.0
Number Dead	2	5	4	10	32	40
Number Exposed	40	40	40	40	40	40

12.13.2.9.2 For this example, the chi-square test for heterogeneity was not significant. Thus Probit Analysis appears appropriate for this set of data.

12.13.2.9.3 Figure 5 shows the output data for the Probit Analysis of the data from Table 10 using the USEPA Probit Program.

USEPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

Probit Analysis of Sheepshead Minnow Embryo-Larval Survival and Teratogenicity Data

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	40	2	0.5000	0.0000
0.5000	40	5	0.1250	0.0369
1.0000	40	4	0.1000	0.0094
2.0000	40	10	0.2500	0.1745
4.0000	40	32	0.8000	0.7799
8.0000	40	40	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 0.782
Chi - Square for Heterogeneity (tabular value) = 7.815

Probit Analysis of Sheephead Minnow Embryo-Larval Survival and Teratogenicity Data

Estimated LC/EC Values and Confidence Limits

Point		Exposure Conc.	Lower 95% Confidence	Upper Limits
LC/EC	1.00	1.187	0.643	1.601
LC/EC	50.00	2.912	2.432	3.361

Figure 5. Output for USEPA Probit Analysis Program, Version 1.5

12.14 PRECISION AND ACCURACY

12.14.1 PRECISION

12.14.1.1 Single-Laboratory Precision

12.14.1.1.1 Data on the single-laboratory precision of the Sheepshead Minnow Embryo-larval Survival and Teratogenicity test are available for eight tests with copper sulfate and five tests with sodium dodecyl sulfate (USEPA, 1989a). The data for the first five tests show that the same NOEC and LOEC, 240 µg Cu/L and 270 µg Cu/L, respectively, were obtained in all five tests, which is the maximum level of precision that can be attained. Three additional tests (6-8) were performed with narrower (20 µg) concentration intervals, to more precisely identify the threshold concentration. The NOEC and LOEC for these tests are 200 µg and 220 µg Cu/L, respectively. For sodium dodecyl sulfate, the NOEC's and LOEC's for all tests are 2.0 and 4.0 mg/L, respectively. The precision, expressed as the coefficient of variation (CV%), is indicated in Tables 11-12. For copper (Cu), the coefficient of variation, depending on the endpoint used, ranges from 2.5 to 6.1% which indicates excellent precision. For sodium dodecyl sulfate (SDS), the coefficient of variation, depending on the endpoint used, ranges from 11.7 to 51.2%, indicating acceptable precision.

12.14.1.2 Multilaboratory Precision

12.14.1.2.1 Data on the multilaboratory precision of this test are not yet available.

12.14.2 Accuracy

12.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 11. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST PERFORMED IN HW MARINEMIX® ARTIFICIAL SEAWATER, USING EMBRYOS FROM FISH MAINTAINED AND SPAWNED IN HW MARINEMIX® ARTIFICIAL SEAWATER USING COPPER (CU) SULFATE AS REFERENCE TOXICANT[1,2,3,4,5,6,7]

Test Number	EC1 (µg/L)	EC5 (µg/L)	EC10 (µg/L)	EC50 (µg/L)	NOEC (µg/L)
1	173	189	198	234	240
2	*	*	*	*	240
3	*	*	*	*	240
4	182	197	206	240	240
5	171	187	197	234	240
6	*	*	*	*	< 200
7	*	*	*	*	220
8	195	203	208	226	220
n:	4	4	4	4	7
Mean:	180	194	202	233	NA
CV (%):	6.1	3.8	2.8	2.5	NA

1 Data from USEPA (1988a) and USEPA (1991a).

2 Tests performed by Terry Hollister, Aquatic Biologist, Houston Facility, Environmental Services Division, Region 6, USEPA, Houston, Texas.

3 *Cyprinodon variegatus* embryos used in the tests were less than 20 h old when the tests began. Two replicate test chambers were used for the control and each toxicant concentration. Ten embryos were randomly added to each test chamber containing 250 mL of test or control water. Solutions were renewed daily. The temperature and salinity of the test solutions were 24 ± 1 °C and 20‰, respectively.

4 Copper test concentrations were prepared using copper sulfate. Copper concentrations for Tests 1-5 were: 180, 210, 240, 270, and 300 µg/L. Copper concentrations for Test 6 were: 220, 240, 260, 280, and 300 µg/L. Copper concentrations for Tests 7-8 were: 200, 220, 240, 260, and 280 µg/L. Tests were conducted over a two-week period.

5 Adults collected in the field.

6 NOEC Range: 200-240 µg/L (this represents a difference of two exposure concentrations).

7 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

* = Data did not fit the Probit model.

TABLE 12. SINGLE-LABORATORY PRECISION OF THE SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST PERFORMED IN HW MARINEMIX® ARTIFICIAL SEAWATER, USING EMBRYOS FROM FISH MAINTAINED AND SPAWNED IN HW MARINEMIX® ARTIFICIAL SEAWATER USING SODIUM DODECYL SULFATE (SDS) AS REFERENCE TOXICANT[1,2,3,4,5,6,7]

Test Number	EC1 (mg/L)	EC5 (mg/L)	EC10 (mg/L)	EC50 (mg/L)	NOEC (mg/L)
1	1.7	2.0	2.2	3.1	2.0
2	*	*	*	*	4.0
3	0.4	0.7	0.9	2.5	2.0
4	1.9	2.2	2.4	3.3	2.0
5	1.3	1.7	1.9	3.0	2.0
n:	4	4	4	4	5
Mean:	1.3	1.6	1.9	2.9	NA
CV (%):	51.2	41.6	35.0	11.7	NA

1 Data from USEPA (1988a) and USEPA (1991a).

2 Tests performed by Terry Hollister, Aquatic Biologist, Houston Facility, Environmental Services Division, Region 6, USEPA, Houston, Texas.

3 *Cyprinodon variegatus* embryos used in the tests were less than 20 h old when the tests began. Two replicate test chambers were used for the control and each toxicant concentration. Ten embryos were randomly added to each test chamber containing 250 mL of test or control water. Solutions were renewed daily. The temperature and salinity of the test solutions were 24 ± 1 °C and 20‰, respectively.

4 SDS concentrations for all tests were: 0.5, 1.0, 2.0, 4.0, and 8.0 mg/L. Tests were conducted over a three-week period.

5 Adults collected in the field.

6 NOEC Range: 2.0-4.0 mg/L (this represents a difference of two exposure concentrations).

7 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

* = Data did not fit the Probit model.

SECTION 13

TEST METHOD

INLAND SILVERSIDE, *MENIDIA BERYLLINA*, LARVAL SURVIVAL AND GROWTH METHOD 1006.0

13.1 SCOPE AND APPLICATION

13.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the inland silverside, *Menidia beryllina*, using seven to 11-day old larvae in a seven day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test species.

13.1.2 Daily observations on mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

13.1.3 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

13.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly volatile and highly degradable toxicants present in the source may not be detected in the test.

13.1.5 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

13.2 SUMMARY OF METHOD

13.2.1 Inland silverside, *Menidia beryllina*, seven to 11-day old larvae are exposed in a static renewal system for seven days to different concentrations of effluent or to receiving water. Test results are based on the survival and growth of the larvae.

13.3 INTERFERENCES

13.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

13.3.2 Adverse effects of low dissolved oxygen (DO) concentrations, high concentrations of suspended and/or dissolved solids, and extremes of pH, may mask or confound the effects of toxic substances.

13.3.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.3.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

13.3.5 Food added during the test may sequester metals and other toxic substances and confound test results.

13.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with

increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 13.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 13.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

13.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 13.3.6.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample after adjusting the sample salinity for use in marine testing.

13.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ± 0.3 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ± 0.3 pH units in pH-controlled tests (USEPA, 1996).

13.3.6.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

13.3.6.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 13.3.6.1.1).

13.3.6.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 13.3.6.2) is applied routinely to subsequent testing of the effluent.

13.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.9). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992); or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents.

If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the sample after adjusting the sample salinity for use in marine testing. USEPA (1996) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

13.4 SAFETY

13.4.1 See Section 3, Health and Safety.

13.5 APPARATUS AND EQUIPMENT

13.5.1 Facilities for holding and acclimating test organisms.

13.5.2 Brine shrimp, *Artemia*, Culture Unit -- see Subsection 13.6.16 below and Section 4, Quality Assurance.

13.5.3 *Menidia Beryllina* Culture Unit -- see Subsection 13.6.17 below, Middaugh and Hemmer (1984), Middaugh et al. (1986), USEPA (1987g) and USEPA (2002a) for detailed culture methods. This test requires from 180-360 7 to 11 day-old larvae. It is preferable to obtain the test organisms from an in-house culture unit. If it is not feasible to culture fish in-house, embryos or larvae can be obtained from other sources by shipping them in well oxygenated saline water in insulated containers.

13.5.4 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L.

13.5.5 Environmental chamber or equivalent facility with temperature control (25 ± 1°C).

13.5.6 Water purification system -- Millipore Milli-Q®, deionized water (DI) or equivalent.

13.5.7 Balance, analytical -- capable of accurately weighing to 0.00001 g.

13.5.8 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the weighing pans plus fish.

13.5.9 Drying oven -- 50-105°C range, for drying larvae.

13.5.10 Air pump -- for oil-free air supply.

13.5.11 Air lines, plastic or pasteur pipettes, or air stones -- for gently aerating water containing the fragile larvae or for supplying air to test solution with low DO

13.5.12 Meters, pH and DO -- for routine physical and chemical measurements.

13.5.13 Standard or micro-Winkler apparatus -- for calibrating DO (optional).

13.5.14 Desiccator -- for holding dried larvae.

13.5.15 Light box -- for counting and observing larvae.

13.5.16 Refractometer -- for determining salinity.

13.5.17 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

13.5.18 Thermometers, bulb-thermograph or electronic chart type -- for continuously recording temperature.

13.5.19 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, 1979b) -- to calibrate laboratory thermometers.

13.5.20 Test chambers -- four chambers per concentration. The chambers should be borosilicate glass or nontoxic disposable plastic labware. To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered during the test with safety glass plates or sheet plastic (6 mm thick).

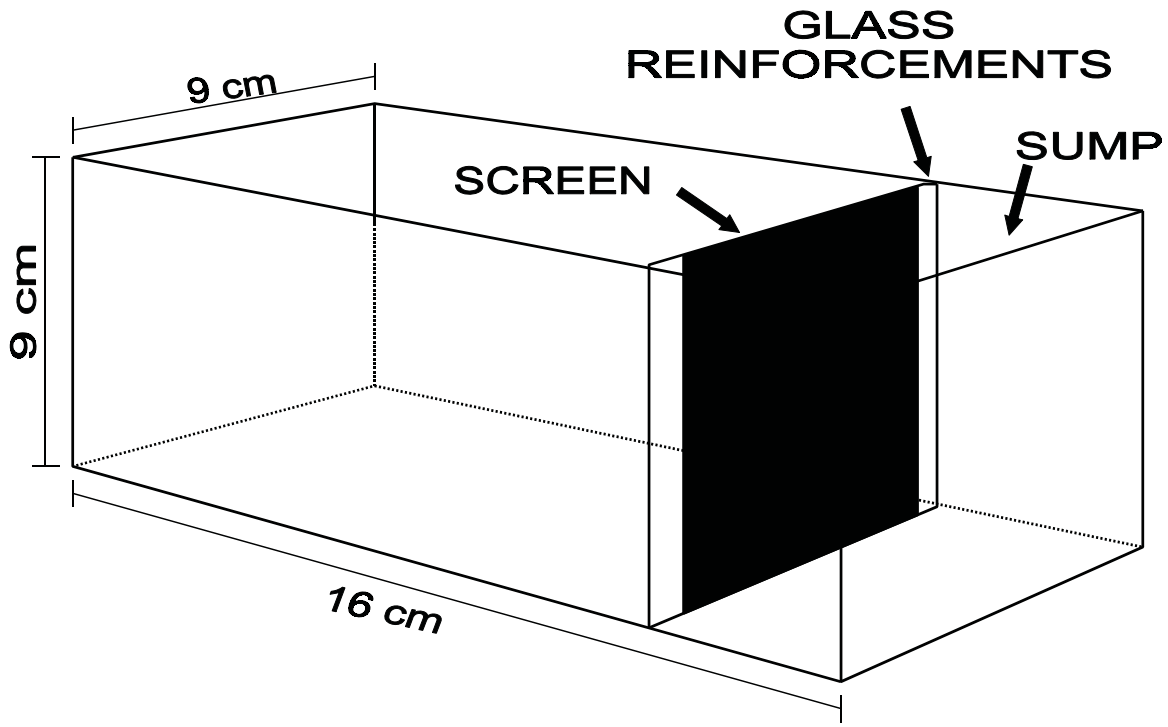


Figure 1. Glass chamber with sump area. Modified from Norberg and Mount (1985). From USEPA (1987c).

13.5.20.1 Each test chamber for the inland silverside should contain a minimum of 750 mL of test solution. A modified Norberg and Mount (1985) chamber (Figure 1), constructed of glass and silicone cement, has been used successfully for this test. This type of chamber holds an adequate column of test solution and incorporates a sump area from which test solutions can be siphoned and renewed without disturbing the fragile inland silverside larvae. Modifications for the chamber are as follows: 1) 200 µm mesh NITEX® screen instead of stainless steel screen; and 2) thin pieces of glass rods cemented with silicone to the NITEX® screen to reinforce the bottom and sides to

produce a sump area in one end of the chamber. Avoid excessive use of silicone, while still ensuring that the chambers do not leak and the larvae cannot get trapped or escape into the sump area. Once constructed, check the chambers for leaks and repair if necessary. Soak the chambers overnight in seawater (preferably in flowing water) to cure the silicone cement before use. Other types of glass test chambers, such as the 1000 mL beakers used in the short-term Sheepshead Minnow Larval Survival and Growth Test, may be used. It is recommended that each chamber contain a minimum of 50 mL per larvae and allow adequate depth of test solution (5.0 cm).

13.5.21 Beakers -- six Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

13.5.22 Mini-Winkler bottles -- for dissolved oxygen calibrations.

13.5.23 Wash bottles -- for deionized water, for washing embryos from substrates and containers, and for rinsing small glassware and instrument electrodes and probes.

13.5.24 Crystallization dishes, beakers, culture dishes, or equivalent -- for incubating embryos.

13.5.25 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

13.5.26 Separatory funnels, 2 L -- Two - four for culturing *Artemia*.

13.5.27 Pipets, volumetric -- Class A, 1-100 mL.

13.5.28 Pipets, automatic -- adjustable, 1-100 mL.

13.5.29 Pipets, serological -- 1-10 mL, graduated.

13.5.30 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

13.5.31 Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring larvae.

13.5.32 Siphon with bulb and clamp -- for cleaning test chambers.

13.5.33 Forceps -- for transferring dead larvae to weighing pans.

13.5.34 NITEX® Mesh Sieves (≤ 150 µm, 500 µm, 3-5 mm) -- for collecting *Artemia* nauplii and fish larvae.

13.6 REAGENTS AND CONSUMABLE MATERIALS

13.6.1 Sample Containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.6.2 Data sheets (one set per test) -- for data recording.

13.6.3 Tape, colored -- for labelling test chambers.

13.6.4 Markers, waterproof -- for marking containers, etc.

13.6.5 Vials, marked -- 24/test, containing 4% formalin or 70% ethanol, to preserve larvae (optional).

13.6.6 Weighing pans, aluminum -- 26/test (two extra).

13.6.7 Buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see USEPA Method 150.1, USEPA, 1979b).

13.6.8 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

13.6.9 Laboratory quality assurance samples and standards -- for the above methods.

13.6.10 Reference toxicant solutions -- see Section 4, Quality Assurance.

13.6.11 Ethanol (70%) or formalin (4%) -- for use as a preservative for the fish larvae.

13.6.12 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

13.6.13 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Surface Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

13.6.13.1 Saline test and dilution water -- the salinity of the test water must be in the range of 5 to 32‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

13.6.13.2 The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Exposure of *Menidia beryllina* larvae to these effluents will require adjustments in the salinity of the test solutions. It is important to maintain a constant salinity across all treatments. In addition, it may be desirable to match the test salinity with that of the receiving water. Artificial sea salts or hypersaline brine (100‰) derived from natural seawater may be used to adjust the salinities.

13.6.13.3 Hypersaline brine (HSB): HSB has several advantages that make it desirable for use in toxicity testing. It can be made from any high quality, filtered seawater by evaporation, and can be added to the effluent or to deionized water to increase the salinity. HSB derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and may be stored for prolonged periods without any apparent degradation. However, if 100% HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 70% at 30‰ salinity and 80% at 20‰ salinity.

13.6.13.3.1 The ideal container for making HSB from natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a noncorrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is used, use only oil free air compressors to prevent contamination.

13.6.13.3.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough deionized water rinses.

13.6.13.3.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

13.6.13.3.4 The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on

volume being generated) to ensure that salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

13.6.13.3.5 After the required salinity is attained, the HSB should be filtered a second time through a 1 µm filter and poured directly into portable containers (20 L cubitainers or polycarbonate water cooler jugs are suitable). The containers should be capped and labelled with the date the brine was generated and its salinity. Containers of HSB should be stored in the dark and maintained at room temperature until used.

13.6.13.3.6 If a source of HSB is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and brine before mixing in the effluent.

13.6.13.3.7 Divide the salinity of the HSB by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the HSB is 100‰ and the test is to be conducted at 20‰, 100‰ divided by 20‰ = 5.0. The proportion of brine is one part in five (one part brine to four parts deionized water). To make 1 L of seawater at 20‰ salinity from a HSB of 100‰, divide 1 L (1000 mL) by 5.0. The result, 200 mL, is the quantity of HSB needed to make 1 L of seawater. The difference, 800 mL, is the quantity of deionized water required.

13.6.13.3.8 Table 1 illustrates the composition of test solutions at 20‰ if they are made by combining effluent (0‰), deionized water and HSB at 100‰ salinity. The volume (mL) of brine required is determined by using the amount calculated above. In this case, 200 mL of brine is required for 1 L; therefore, 600 mL would be required for 3 L of solution. The volumes of HSB required are constant. The volumes of deionized water are determined by subtracting the volumes of effluent and brine from the total volume of solution: 3,000 mL - mL effluent - mL HSB = mL deionized water.

13.6.13.4 Artificial sea salts: A modified GP2 artificial seawater formulation (Table 2) has been successfully used to perform the inland silverside survival and growth test. The use of GP2 for holding and culturing of adults is not recommended at this time.

13.6.13.4.1 The GP2 artificial sea salts (Table 2) should be mixed with deionized (DI) water or its equivalent in a container other than the culture or testing tanks. The deionized water used for hydration should be between 21-26°C. The artificial seawater must be conditioned (aerated) for 24-h before use as the testing medium. If the solution is to be autoclaved, sodium bicarbonate is added after the solution has cooled. A stock solution of sodium bicarbonate is made up by dissolving 33.6 gm $NaHCO_3$ in 500 mL deionized water. Add 2.5 mL of this stock solution for each liter of the GP2 artificial seawater.

13.6.14 ROTIFER CULTURE --for feeding cultures and test organisms

13.6.14.1 At hatching *Menidia beryllina* larvae are too small to ingest *Artemia* nauplii and must be fed rotifers, *Brachionus plicatilis*. The rotifers can be maintained in continuous culture when fed algae (see Section 6 and USEPA, 1987g). Rotifers are cultured in 10-15 L Pyrex® carboys (with a drain spigot near the bottom) at 25-28°C and 25-35‰ salinity. Four 12 L culture carboys should be maintained simultaneously to optimize production. Clean carboys should be filled with autoclaved seawater. Alternatively, an immersion heater may be used to heat saline water in the carboy to 70-80°C for 1-h.

TABLE 1: PREPARATION OF 3 L SALINE WATER FROM DEIONIZED WATER AND A HYPERSALINE BRINE OF 100‰ NEEDED FOR TEST SOLUTIONS AT 20‰ SALINITY

Effluent Concentration	Volume of Effluent (0‰) (mL)	Volume of Deionized Water (mL)	Volume of Hypersaline Brine (mL)	Total Volume (mL)
80	2400	0	600	3000
40	1200	1200	600	3000
20	600	1800	600	3000
10	300	2100	600	3000
5	150	2250	600	3000
Control	0	2400	600	3000
Total	4,650	9,750	3,600	18,000

TABLE 2. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR THE INLAND SILVERSIDE, *MENIDIA BERYLLINA*, TOXICITY TEST[1,2,3]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
KCl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

[1] Modified GP2 from Spotte et al. (1984)
[2] The constituent salts and concentrations were taken from USEPA (l990b). The salinity is 30.89 g/L.
[3] GP2 can be diluted with deionized (DI) water to the desired test salinity.

13.6.14.2 When the water has cooled to 25-28°C, aerate and add a start-up sample of rotifers (50 rotifers/mL) and food (about 1 L of a dense algal culture). The carboys should be checked daily to ensure that adequate food is available and that the rotifer density is adequate. If the water appears clear, drain 1 L of culture water and replace it with algae. Excess water can be removed through the spigot drain and filtered through a ≤ 60 µm mesh screen. Rotifers collected on the screen should be returned to the culture. If a more precise measure of the rotifer population is needed, rotifers collected from a known volume of water can be resuspended in a smaller volume, killed with formalin and counted in a Sedgwick-Rafter cell. If the density exceeds 50 rotifers/mL, the amount of food per day should be increased to 2 L of algae suspension. The optimum density of approximately 300-400 rotifers/mL may be reached in seven to 10 days and is sustainable for two to three weeks. At these densities, the rotifers should be cropped daily. Keeping the carboys away from light will reduce the amount of algae attached to the carboy walls. When detritus accumulates, populations of ciliates, nematodes, or harpacticoid copepods that may have been inadvertently introduced can rapidly take over the culture. If this occurs, discard the cultures.

13.6.15 ALGAL CULTURES -- for feeding rotifer cultures

13.6.15.1 *Tetraselmus suecica* or *Chlorella* sp. (see USEPA, 1987a) can be cultured in 20 L polycarbonate carboys that are normally used for bottled drinking water. Filtered seawater is added to the carboys and then autoclaved (110°C for 30 minutes). After cooling to room temperature, the carboys are placed in a temperature chamber controlled at 18-20°C. One liter of *T. suecica* or *Chlorella* sp. starter culture and 100 mL of nutrients are added to each carboy.

13.6.15.2 Formula for algal culture nutrients.

13.6.15.2.1 Add 180 g $NaNO_3$, 12 g NaH_2PO_4, and 6.16 g EDTA to 12 L of deionized water. Mix with a magnetic stirrer until all salts are dissolved (at least 1-h).

13.6.15.2.2 Add 3.78 g $FeCl_3 \cdot 6\ H_2O$ and stir again. The solution should be bright yellow.

13.6.15.2.3 The algal culture is vigorously aerated via a pipette inserted through a foam stopper at the top of the carboy. A dense algal culture should develop in 7 to 10 days and should be used by Day 14. Thus, start-up of cultures should be made on a daily or every second day basis. Approximately 6 to 8 continuous cultures will meet the feeding requirements of four 12 L rotifer cultures. When emptied, carboys are washed with soap and water and rinsed thoroughly with deionized water before reuse.

13.6.16 BRINE SHRIMP, *ARTEMIA*, NAUPLII -- for feeding cultures and test organisms

13.6.16.1 Newly hatched *Artemia* nauplii are used as food for inland silverside larvae in toxicity tests. Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are being used because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size.

13.6.16.2 Each new batch of *Artemia* cysts must be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (see Leger et al., 1985; Leger et al., 1986) against known suitable reference cysts by performing a side by side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be a previously tested and acceptable batch of cysts. A sample of newly-hatched *Artemia* nauplii from each new batch of cysts should be chemically analyzed. The *Artemia* cysts should not be used if the concentration of total organochlorine pesticides exceeds 0.15 µg/g wet weight or that the total concentration of organochlorine pesticides plus PCBs does not exceed 0.30 µg/g wet weight. (For analytical methods, see USEPA 1982).

13.6.16.2.1 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or a solution prepared by adding 35.0 g uniodized salt (NaCl) or artificial sea salts to 1 L of deionized water, to a 2 L separatory funnel or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24 h at 27°C. (Hatching time varies with incubation temperature and the geographic strain of *Artemia* used (see USEPA, 1985d; USEPA, 2002a; and ASTM, 1993.)
3. After 24-h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic and will concentrate at the bottom of the funnel if it is covered for 10-15 minutes to prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 minutes without aeration.
4. Drain the nauplii into a beaker or funnel fitted with ≤ 150 µm NITEX® or stainless steel screen, and rinse with seawater or equivalent before use.

13.6.16.3 Testing *Artemia* nauplii as food for toxicity test organisms.

13.6.16.3.1 The primary criterion for acceptability of each new supply of brine shrimp cysts is the ability of the nauplii to support good survival and growth of the inland silverside larvae (see Subsection 13.11). The larvae used to evaluate the suitability of the brine shrimp nauplii must be of the same geographical origin, species, and stage of development as those used routinely in the toxicity tests. Sufficient data to detect differences in survival and growth should be obtained by using three replicate test chambers each containing a minimum of 15 larvae, for each type of food.

13.6.16.3.2 The feeding rate and frequency, test vessels and volume of control water, duration of the test, and age of the nauplii at the start of the test, should be the same as used for the routine toxicity tests.

13.6.16.3.3 Results of the brine shrimp nutrition assay, where there are only two treatments, can be evaluated statistically by use of a t test. The "new" food is acceptable if there are no statistically significant differences in the survival and growth of the larvae fed the two sources of nauplii.

13.6.16.4 Use of *Artemia* nauplii as food for inland silverside, *Menidia beryllina*, larvae.

13.6.16.4.1 *Menidia beryllina* larvae begin feeding on newly hatched *Artemia* nauplii about five days after hatching, and are fed *Artemia* nauplii daily throughout the 7-day larval survival and growth test. Survival of *Menidia beryllina* larvae seven to nine days old is improved by feeding newly hatched (< 24-h old) *Artemia* nauplii. Equal amounts of *Artemia* nauplii must be fed to each replicate test chamber to minimize the variability of larval weight. Sufficient numbers of nauplii should be fed to ensure that some remain alive overnight in the test chambers. An adequate but not excessive amount should be provided to each replicate on a daily basis. Feeding excessive amounts of nauplii will result in a depletion in DO to below an acceptable level (below 4.0 mg/L). As much of the uneaten *Artemia* nauplii as possible should be siphoned from each chamber prior to test solution renewal to ensure that the larvae principally eat newly hatched nauplii.

13.6.17 TEST ORGANISMS, INLAND SILVERSIDE, *MENIDIA BERYLLINA*

13.6.17.1 The inland silverside, *Menidia beryllina*, is one of three species in the atherinid family that are amenable to laboratory culture; and one of four atherinid species used for chronic toxicity testing. Several atherinid species have been utilized successfully for early life stage toxicity tests using field collected (Goodman et al., 1985) and laboratory reared adults (Middaugh and Takita, 1983; Middaugh and Hemmer, 1984; and USEPA, 1987g). The inland silverside, *Menidia beryllina*, populates a variety of habitats from Cape Cod, Massachusetts, to Florida and west to Vera Cruz, Mexico (Johnson, 1975). It can tolerate a wide range of temperature, 2.9-32.5°C (Tagatz and Dudley, 1961; Smith, 1971) and salinity, of 0-58‰ (Simmons, 1957; Renfro, 1960), having been reported from the freshwaters of the Mississippi River drainage basin (Chernoff et al., 1981) to hypersaline lagoons (Simmons, 1957). Ecologically, *Menidia* spp. are important as major prey for many prominent commercial species (e.g., bluefish (*Pomatomus saltatrix*), mackerel (*Scomber scombrus*), and striped bass (*Morone saxatilis*) (Bigelow and Schroeder, 1953). The inland silverside, *Menidia beryllina*, is a serial spawner, and will spawn under controlled laboratory conditions. Spawning can be induced by diurnal interruption in the circulation of water in the culture tanks (Middaugh et al., 1986; USEPA, 1987a). The eggs are demersal, approximately 0.75 mm in diameter (Hildebrand and Schroeder, 1928), and adhere to vegetation in the wild, or to filter floss in laboratory culture tanks. The larvae hatch in six to seven days when incubated at 25°C and maintained in seawater ranging from 5-30‰ (USEPA, 1987a). Newly hatched larvae are 3.5-4.0 mm in total length (Hildebrand, 1922).

13.6.17.2 Inland silverside, *Menidia beryllina*, adults (see USEPA, 1987g and USEPA, 2002a for detailed culture methods) may be cultured in the laboratory or obtained from the Gulf of Mexico or Atlantic coast estuaries throughout the year (Figure 2). Gravid females can be collected from low salinity waters along the Atlantic coast during April to July, depending on the latitude. The most productive and protracted spawning stock can be obtained from adults brought into the laboratory. Broodstocks, collected from local estuaries twice each year (in April and October), will become sexually active after one to two months and will generally spawn for 4-6 months.

13.6.17.3 The fish can be collected easily with a beach seine (3-6 mm mesh), but the seine should not be completely landed onto the beach. Silversides are very sensitive to handling and should never be removed from the water by net -- only by beaker or bucket.

13.6.17.4 Samples may contain a mixture of inland silverside, *Menidia beryllina*, and Atlantic silverside, *Menidia menidia*, on the Atlantic coast or inland silverside and tidewater silverside, *Menidia peninsulae*, on the Gulf Coast (see USEPA, 1987g for additional information on morphological differences for identification). Johnson (1975) and Chernoff et al. (1981) have attempted to differentiate these species. In the northeastern United States, *M. beryllina* juveniles and adults are usually considerably smaller than *M. menidia* juveniles and adults (Bengtson, 1984), and can be separated easily in the field on that basis.

13.6.17.5 Record the water temperature and salinity at each collection site. Aerate (portable air pump, battery operated) the fish and transport to the laboratory as quickly as possible after collection. Upon arrival at the laboratory, the fish and the water in which they were collected are transferred to a tank at least 0.9 m in diameter. A filter system should be employed to maintain water quality (see USEPA, 1987g). Laboratory water is added to the tank slowly, and the fish are acclimated at the rate of 2°C per day, to a final temperature of 25°C, and about 5‰ salinity per day, to a final salinity in the range of 20-32‰. The seawater in each tank should be brought to a minimum volume of 150 L. A density of about 50 fish/tank is appropriate. Maintain a photoperiod of 16 h light/8 h dark. Feed the adult fish flake food or frozen brine shrimp twice daily and *Artemia* nauplii once daily. Siphon the detritus from the bottom of the tanks weekly.

13.6.17.6 Larvae for a toxicity test can be obtained from the broodstock by spawning onto polyester aquarium filter-fiber substrates, 15 cm long x 10 cm wide x 10 cm thick, which are suspended with a string 8-10 cm below the surface of the water and in contact with the side of the holding tanks for 24-48 h, 14 days prior to the beginning of a test. The floss should be gently aerated by placing it above an airstone, and weighted down with a heavy non-toxic object. The embryos, which are light yellow in color, can be seen on the floss, and are round and hard to the touch compared to the soft floss.

13.6.17.7 Remove as much floss as possible from the embryos. The floss should be stretched and teased to prevent the embryos from clumping. The embryos should be incubated at the test salinity and lightly aerated. At 25°C, the embryos will hatch in about six to eight days. Larvae are fed about 500 rotifer larvae/day from hatch through four days post-hatch. On Days 5 and 6, newly hatched (less than 12 h old) *Artemia* nauplii are mixed with the rotifers, to provide a transition period. After Day 7, only nauplii are fed, and the age range for the nauplii can be increased from 12 h old to 24 h old.

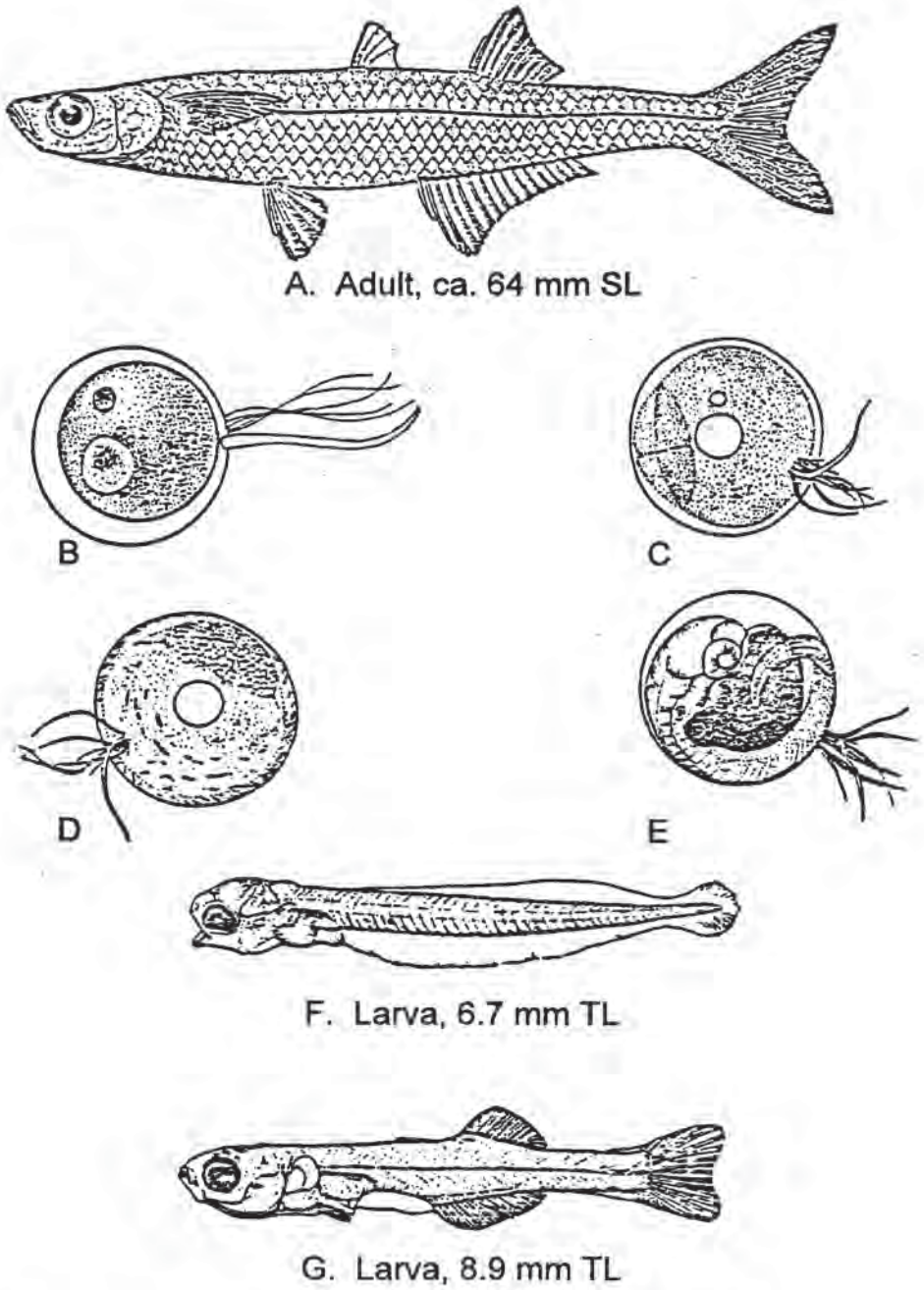


Figure 2. Inland silverside, *Menidia beryllina*: A. Adult, ca. 64 mm SL; B. Egg (diagrammatic), only bases of filaments shown; C. Egg, 2-cell stage; D. Egg, morula stage; E. Advanced embryo, two and one half days after fertilization. From Martin and Drewry (1978).

13.6.17.8 Silverside larvae are very sensitive to handling and shipping during the first week after hatching. For this reason, if organisms must be shipped to the test laboratory, it may be impractical to use larvae less than 11 days old because the sensitivity of younger organisms may result in excessive mortality during shipment. If organisms are to be shipped to a test site, they should be shipped only as (1) early embryos, so that they hatch after arrival, or (2) after they are known to be feeding well on *Artemia* nauplii (8-10 days of age). Larvae shipped at 8 - 10 days of age would be 9 to 11 days old when the test is started. Larvae that are hatched and reared in the test laboratory can be used at seven days of age.

13.6.17.9 If four replicates of 15 larvae are used at each effluent concentration and in the control, 360 larvae will be needed for each test.

13.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

13.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

13.8 CALIBRATION AND STANDARDIZATION

13.8.1 See Section 4, Quality Assurance.

13.9 QUALITY CONTROL

13.9.1 See Section 4, Quality Assurance.

13.10 TEST PROCEDURES

13.10.1 TEST SOLUTIONS

13.10.1.1 Receiving Waters

13.10.1.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 500-750 mL, and 400 mL for chemical analysis, would require approximately 2.4-3.4 L or more of sample per day.

13.10.1.2 Effluents

13.10.1.2. The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ±100%, and allows for testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **Therefore, USEPA recommends the use of the ≥0.5 dilution factor.** If 100% salinity HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ salinity, and 70% at 30‰ salinity.

13.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1-2 h of the test, additional dilutions at the lower range of effluent concentrations should be added.

13.10.1.2.3 The volume of effluent required to initiate the test and for daily renewal of four replicates per treatment for five concentrations of effluent and a control, each containing 750 mL of test solution, is approximately

5 L. Prepare enough test solution at each effluent concentration to provide 400 mL additional volume for chemical analyses.

13.10.1.2.4 Tests should begin as soon as possible after sample collection, preferably within 24 h. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity studies unless permission is granted by the permitting authority. In no case should the test be started more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4).

13.10.1.2.5 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solution should be adjusted to the test temperature (25 ± 1°C) and maintained at that temperature during the addition of dilution waters.

13.10.1.2.6 Effluent dilutions should be prepared for all replicates in each treatment in one beaker to minimize variability among the replicates. The test chambers are labeled with the test concentration and replicate number. Dispense into the appropriate effluent dilution chamber.

13.10.1.3 Dilution Water

13.10.1.3.1 Dilution water may be uncontaminated natural seawater (receiving water), HSB prepared from natural seawater, or artificial seawater prepared from FORTY FATHOMS® or GP2 sea salts (see Table 3 in Section 7, Dilution Water). Other artificial sea salts may be used for culturing inland silverside minnows and for the larval survival and growth test if the control criteria for acceptability of test data are satisfied.

13.10.2 START OF THE TEST

13.10.2.1 Inland silverside larvae 7 to 11 days old can be used to start the survival and growth test. At this age, the inland silverside feed on newly-hatched *Artemia* nauplii. At 25°C, tests with inland silverside larvae can be performed at salinities ranging from 5 to 32‰. If the test salinity ranges from 16 to 32‰, the salinity for spawning, incubation, and culture of the embryos and larvae should be maintained within this salinity range. If the test salinity is in the range of 5 to 15‰, the embryos may be spawned at 30‰, but egg incubation and larval rearing should be at the test salinity. If the specific salinity required for the test differs from the rearing salinity, adjustments of 5‰ daily should be made over the three days prior to start of test.

13.10.2.2 One day Prior to Beginning of Test

13.10.2.2.1 Set up the *Artemia* culture so that newly hatched nauplii will be available on the day the test begins. (see Section 7).

13.10.2.2.2 Increase the temperature of water bath, room, or incubator to the required test temperature (25 ± 1°C).

13.10.2.2.3 Label the test chambers with a marking pen. Use of color coded tape to identify each concentration and replicate is helpful. A minimum of five effluent concentrations and a control should be selected for each test. Glass test chambers, such as crystallization dishes, beakers, or chambers with a sump area (Figure 1), with a capacity for 500-750 mL of test solution, should be used.

13.10.2.2.4 Randomize the position of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a table of random numbers or similar process (see Appendix A for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart.

13.10.2.2.5 Because inland silverside larvae are very sensitive to handling, it is advisable to distribute them to their respective test chambers which contain control water on the day before the test is to begin. Each test chamber

should contain a minimum of 10 larvae and it is required that there be four replicates minimum for each concentration and control.

13.10.2.2.6 Seven to 11 day old larvae are active and difficult to capture and are subject to handling mortality. Carefully remove larvae (two to three at a time) by concentrating them in a corner of the aquarium or culture vessel, and capture them with a wide-bore pipette, small petri dish, crystallization dish, 3-4 cm in diameter, or small pipette. They are active and will readily escape from a pipette. Randomly transfer the larvae (two to three at a time) into each test chamber until the desired number (15) is attained. See Appendix A for an example of randomization. After the larvae are dispensed, use a light table to verify the number in each chamber.

13.10.2.3 Before beginning the test remove and replace any dead larvae from each test chamber. The test is started by removing approximately 90% of the clean seawater from each test chamber and replacing with the appropriate test solution.

13.10.3 LIGHT, PHOTOPERIOD, SALINITY, AND TEMPERATURE

13.10.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 $\mu E/m^2/s$, or 50-100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The water temperature in the test chambers should be maintained at 25 ± 1°C. The test salinity should be in the range of 5-32‰, and the salinity should not vary by more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

13.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

13.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO The DO should be measured on new solutions at the start of the test (Day 0) and before daily renewal of test solutions on subsequent days. The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all concentrations and the control should be aerated. The aeration rate should not exceed 100 bubbles/min., using a pipet with a 1-2 mm orifice such as a 1 mL KIMAX® serological pipet, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue stress to the fish.

13.10.5 FEEDING

13.10.5.1 *Artemia* nauplii are prepared as described above.

13.10.5.2 The test larvae are fed newly-hatched (less than 24 h old) *Artemia* nauplii once a day from Day 0 through Day 6; larvae are not fed on Day 7. Equal amounts of *Artemia* nauplii must be fed to each replicate test chamber to minimize the variability of larval weight. Sufficient numbers of nauplii should be fed to ensure that some remain alive overnight in the test chambers. An adequate, but not excessive amount of *Artemia* nauplii, should be provided to each replicate on a daily basis. Feeding excessive amounts of *Artemia* nauplii will result in a depletion in DO to below an acceptable level. Siphon as much of the uneaten *Artemia* nauplii as possible from each chamber daily to ensure that the larvae principally eat newly hatched nauplii.

13.10.5.3 On Days 0-2, transfer 4 g wet weight or pipette 4 mL of concentrated, rinsed *Artemia* nauplii to seawater in a 100 mL beaker, and bring to a volume of 80 mL. Aerate or swirl the suspension to equally distribute the nauplii while withdrawing individual 2 mL portions of the *Artemia* nauplii suspension by pipette or adjustable syringe to transfer to each replicate test chamber. Because the nauplii will settle and concentrate at the tip of the pipette during the transfer, limit the volume of concentrate withdrawn each time to a 2 mL portion for one test chamber helps ensure an equal distribution to the replicate chambers. Equal distribution of food to the replicates is critical for successful tests.

13.10.5.4 On Days 3-6, transfer 6 g wet weight or 6 mL of the *Artemia* nauplii concentrate to seawater in a 100 mL beaker. Bring to a volume of 80 mL and dispense as described above.

13.10.5.5 If the larvae survival rate in any replicate on any day falls below 50%, reduce the volume of *Artemia* nauplii suspension added to that test chamber by one-half (i.e., reduce from 2 mL to 1 mL) and continue feeding one-half the volume through Day 6. Record the time of feeding on the data sheets.

13.10.6 DAILY CLEANING OF TEST CHAMBERS

13.10.6.1 Before the daily renewal of test solutions, uneaten and dead *Artemia*, and other debris are removed from the bottom of the test chambers with a siphon hose. Alternately, a large pipet (50 mL), fitted with a safety pipet filler or rubber bulb, can be used. If the test chambers illustrated in Figure 1 are used, remove only as much of the test solution from the chamber as is necessary to clean, and siphon the remainder of the test solution from the sump area. Because of their small size during the first few days of the test, larvae are easily drawn into a siphon tube when cleaning the test chambers. By placing the test chambers on a light box, inadvertent removal of larvae can be greatly reduced because they can be more easily seen. If the water siphoned from the test chambers is collected in a white plastic tray, the live larvae caught up in the siphon can be retrieved, and returned by pipette to the appropriate test chamber and noted on data sheet. Any incidence of removal of live larvae from the test chambers by the siphon during cleaning, and subsequent return to the chambers should be noted in the test records.

13.10.7 OBSERVATIONS DURING THE TEST

13.10.7.1 Routine Chemical and Physical Determinations

13.10.7.l.l DO is measured at the beginning and end of each 24 h exposure period in one test chamber at all test concentrations and in the control.

13.10.7.1.2 Temperature, pH, and salinity are measured at the end of each 24 h exposure period in one test chamber at all test concentrations and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at least the end of the test to determine the temperature variation in the environmental chamber.

13.10.7.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

13.10.7.1.4 Record all measurements on the data sheet (Figure 3)

Test Dates:_____ Species:_____

Type Effluent:_____ Field _____ Lab _____ Test _____

Effluent Tested:_____

CONCENTRATION:																															
REPLICATE:									REPLICATE:								REPLICATE:								REPLICATE:							
DAYS	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			
CONCENTRATION:																																
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				**MEAN WEIGHT/ LARVAE (mg) ± SD**					**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**				**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**				**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**			
CONCENTRATION:																																
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				**MEAN WEIGHT/ LARVAE (mg) ± SD**					**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**				**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**				**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**			

TIME FED							

COMMENTS:

Figure 3. Data form for the inland silverside, *Menida beryllina,* larval survival and growth test. Daily record of larval survival and test conditions. (From USEPA, 1987c).

Test Dates:_____ Species:_____

Type Effluent:_____ Field _____ Lab _____ Test _____

Effluent Tested:_____

CONCENTRATION:																															
REPLICATE:									REPLICATE:								REPLICATE:								REPLICATE:							
DAYS	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD					# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD				# LARVAE/ DRY WT				MEAN WEIGHT/ LARVAE (mg) ± SD			
CONCENTRATION:																																
# LIVE LARVAE																																
TEMP (°C)																																
SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				**MEAN WEIGHT/ LARVAE (mg) ± SD**					**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**				**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**				**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**			
CONCENTRATION:																																
# LIVE LARVAE																																
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SALINITY (‰)																																
DO (mg/L)																																
# LARVAE/ DRY WT				**MEAN WEIGHT/ LARVAE (mg) ± SD**					**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**				**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**				**# LARVAE/ DRY WT**				**MEAN WEIGHT/ LARVAE (mg) ± SD**			

TIME FED							

COMMENTS:

Figure 3. Data form for inland silverside, *Menida beryllina*, larval survival and growth test. Daily record of larval survival and test conditions. (CONTINUED)(From USEPA, 1987c).

13.10.7.2 Routine Biological Observation

13.10.7.2.1 The number of live larvae in each test chamber are recorded daily (Figure 3), and the dead larvae are discarded.

13.10.7.2.2 Protect the larvae from unnecessary disturbances during the test by carrying out the daily test observations, solution renewals, and removal of dead larvae. Make sure the larvae remain immersed at all times during the performance of the above operations.

13.10.8 TEST SOLUTION RENEWAL

13.10.8.1 The test solutions are renewed daily using freshly prepared solutions, immediately after cleaning the test chambers. The water level in each chamber is lowered to a depth of 7-10 mm, leaving 10-15% of the test solution. New test solution is added slowly by refilling each chamber with the appropriate amount of test solution without excessively disturbing the larvae. If the modified chamber is used (Figure 1), renewals should be poured into the sump area using a narrow bore (approximately 9 mm ID) funnel.

13.10.8.2 The effluent or receiving water used in the test is stored in an incubator or refrigerator at 0-6°C. Plastic containers such as 8-20 L cubitainers have proven suitable for effluent collection and storage. For on-site toxicity studies no more than 24 h should elapse between collection of the effluent and use in a toxicity test (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.10.8.3 Approximately 1 h before test initiation, a sufficient quantity of effluent or receiving water sample is warmed to 25 ± 1°C to prepare the test solutions. A sufficient quantity of effluent should be warmed to make the daily test solutions.

13.10.8.3.1 An illustration of the quantities of effluent and seawater needed to prepare test solution at the appropriate salinity is provided in Table 2.

13.10.9 TERMINATION OF THE TEST

13.10.9.1 The test is terminated after seven days of exposure. At test termination dead larvae are removed and discarded. The surviving larvae in each test chamber (replicate) are counted, and immediately prepared as a group for dry weight determination, or are preserved in 4% formalin or 70% ethanol. Preserved organisms are dried and weighed within seven days. For safety, formalin should be used under a hood.

13.10.9.2 For immediate drying and weighing, siphon or pour live larvae onto a 500 µm mesh screen in a large beaker to retain the larvae and allow *Artemia* to be rinsed away. Rinse the larvae with deionized water to remove salts that might contribute to the dry weight. Sacrifice the larvae in an ice bath of deionized water.

13.10.9. Small aluminum weighing pans can be used to dry and weigh larvae. An appropriate number of aluminum weigh pans (one per replicate) are marked for identification and weighed to 0.01 mg, and the weights are recorded (Figure 4) on the data sheets.

13.10.9.4 Immediately prior to drying, rinse the preserved larvae in distilled (or deionized) water. The rinsed larvae from each test chamber are transferred, using forceps, to a tared weighing pan and dried at 60°C for 24 h, or at 105°C for a minimum of 6 h. Immediately upon removal from the drying oven, the weighing pans are placed in a desiccator to cool and to prevent the adsorption of moisture from the air until weighed. Weigh all weighing pans containing the dried larvae to 0.01 mg, subtract the tare weight to determine dry weight of larvae in each replicate. Record (Figure 4) the weights. Divide the dry weight by the number of original larvae per replicate to determine the average dry weight, and record (Figures 4 and 5) on the data sheets. For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptability criteria (see Subsection 13.11). Complete the summary data sheet (Figure 5) after calculating the average measurements and

statistically analyzing the dry weights and percent survival for the entire test. Average weights should be expressed to the nearest 0.001 mg.

13.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

13.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

13.12 ACCEPTABILITY OF TEST RESULTS

13.12.1 Test results are acceptable if (1) the average survival of control larvae is equal to or greater than 80%, and (2) where the test starts with seven-day old larvae, the average dry weight per surviving control larvae, when dried immediately after test termination, is equal to or greater than 0.50 mg, or the average dry weight of the control larvae preserved not more than seven days in 4% formalin or 70% ethanol equals or exceeds 0.43 mg.

13.13 DATA ANALYSIS

13.13.1 GENERAL

13.13.1.1 Tabulate and summarize the data.

13.13.1.2 The endpoints of toxicity tests using the inland silverside are based on the adverse effects on survival and growth. The LC50, the IC25, and the IC50 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values, for survival and growth, are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50, IC25, and IC50. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for survival and growth but included in the estimation of the LC50, IC25, and IC50. See the Appendices for examples of the manual computations and examples of data input and program output.

13.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

Test Dates:_____ Species:_____

Pan No.	Conc. & Rep.	Initial Wt. (mg)	Final Wt. (mg)	Diff. (mg)	No. Larvae	Av. Wt./ Larvae (mg)

Figure 4. Data form for the inland silverside, *Menidia beryllina*, larval survival and growth test. Dry weights of larvae (from USEPA, 1987b).

Test Dates: _____ Species:_____

Effluent Tested: _____

TREATMENT						
NO. LIVE LARVAE						
SURVIVAL (%)						
MEAN DRY WT/ LARVAE (MG) ± SD						
SIGNIF. DIFF. FROM CONTROL (o)						
MEAN TEMPERATURE (°C) ± SD						
MEAN SALINITY ‰ ± SD						
AVE. DISSOLVED OXYGEN (MG/L) ± SD						

COMMENTS:

Figure 5. Data form for the inland silverside, *Menidia beryllina*, larval survival and growth test. Summary of test results (from USEPA, 1987c).

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE INLAND SILVERSIDE, *MENIDIA BERYLLINA*, LARVAL SURVIVAL AND GROWTH TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1006.0)[1]

1.	Test type:	Static renewal (required)
2.	Salinity:	5‰ to 32‰ (± 2‰ of the selected test salinity) (recommended)
3.	Temperature:	25 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4.	Light quality:	Ambient laboratory illumination (recommended)
5.	Light intensity:	l0-20 µE/m^2/s (50-100 ft-c) (Ambient laboratory levels) (recommended)
6.	Photoperiod:	16 h light, 8 h darkness (recommended)
7.	Test chamber size:	600 mL-1 L containers (recommended)
8.	Test solution volume:	500-750 mL/replicate (loading and DO restrictions must be met) (recommended)
9.	Renewal of test solutions:	Daily (required)
10.	Age of test organisms:	7-11 days post hatch; less than or equal to 24-h range in age (required)
11.	No. larvae per test chamber:	10 (required minimum)
12.	No. replicate chambers per concentration:	4 (required minimum)
13.	No. larvae per concentration:	40 (required minimum)
14.	Source of food:	Newly hatched *Artemia* nauplii (survival of 7-9 days old *Menidia beryllina* larvae improved by feeding 24 h old *Artemia*) (required)
15.	Feeding regime:	Feed 0.l0 g wet weight *Artemia* nauplii per replicate on days 0-2; Feed 0.15 g wet weight *Artemia* nauplii per replicate on days 3-6 (recommended)
16.	Cleaning:	Siphon daily, immediately before test solution renewal and feeding (required)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE INLAND SILVERSIDE, *MENIDIA BERYLLINA*, LARVAL SURVIVAL AND GROWTH TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1006.0) (CONTINUED)

17.	Aeration:	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/minimum (recommended)
18.	Dilution water:	Uncontaminated source of natural sea water, artificial seawater; deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, GP2 or equivalent) (available options)
19.	Test concentrations:	Effluent: 5 and a control (required) Receiving Waters: 100% receiving water (or minimum of 5) and a control (recommended)
20.	Dilution factor:	Effluents: ≥ 0.5 (recommended) Receiving waters: None, or ≥ 0.5 (recommended)
21.	Test duration:	7 days (required)
22.	Endpoints:	Survival and growth (weight) (required)
23.	Test acceptability criteria:	80% or greater survival in controls, 0.50 mg average dry weight of control larvae where test starts with 7-days old larvae and dried immediately after test termination, or 0.43 mg or greater average dry weight per surviving control larvae, preserved not more than 7 days in 4% formalin or 70% ethanol (required)
24.	Sampling requirement:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
25.	Sample volume required:	6 L per day (recommended)

13.13.2 EXAMPLE OF ANALYSIS OF INLAND SILVERSIDE, *MENIDIA BERYLLINA*, SURVIVAL DATA

13.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 6 and 7. The response used in the analysis is the proportion of animals surviving in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the LC50 endpoint. Concentrations at which there is no survival in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC, EC, and LC endpoint.

13.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's

Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for the homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

13.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

13.13.2.4 Probit Analysis (Finney, 1971; see Appendix H) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit model, the Spearman-Karber method, the Trimmed Spearman-Karber method, or the Graphical method may be used (see Appendices H-K).

13.13.2.5 Example of Analysis of Survival Data

13.13.2.5.1 This example uses the survival data from the inland silverside larval survival and growth test. The proportion surviving in each replicate in this example must first be transformed by the arc sine transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and control are listed in Table 4. A plot of the data is provided in Figure 8. Since there is 100% mortality in all three replicates for the 50% and 100% concentrations, they are not included in this statistical analysis and are considered a qualitative mortality effect.

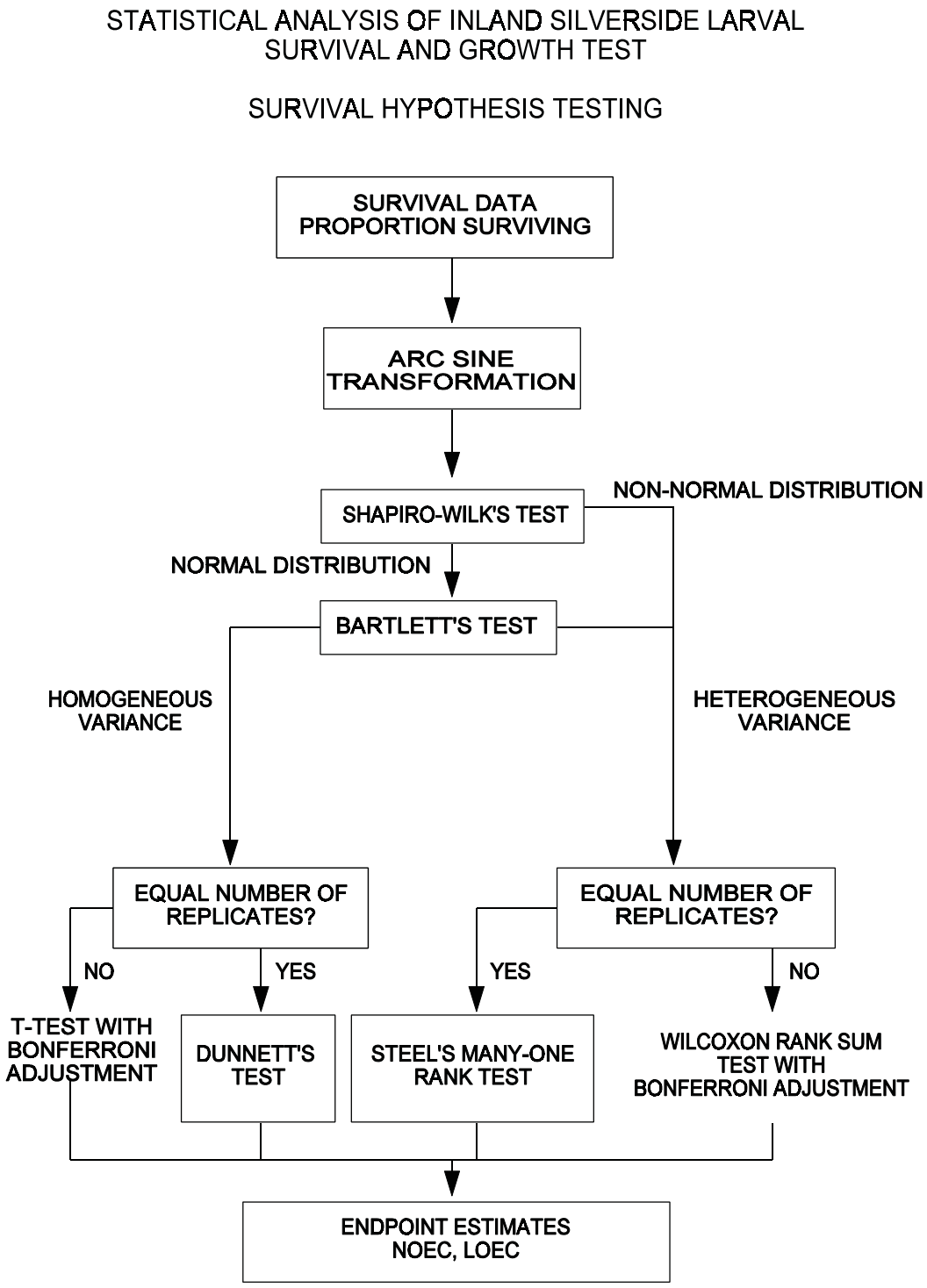

Figure 6. Flowchart for statistical analysis of the inland silverside, *Menidia beryllina*, survival data by hypothesis testing.

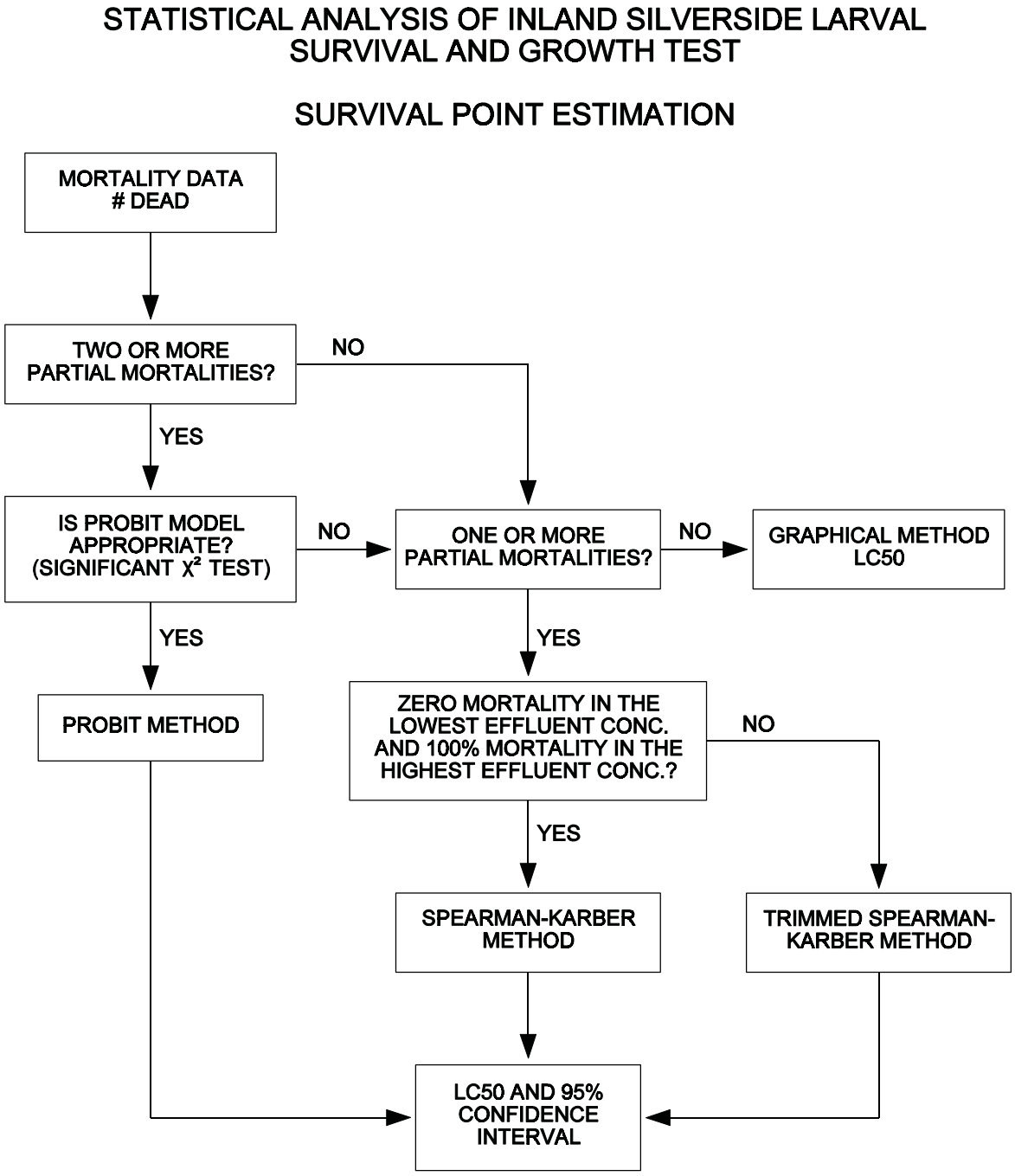

Figure 7. Flowchart for statistical analysis of the inland silverside, *Menidia beryllina*, survival data by point estimation.

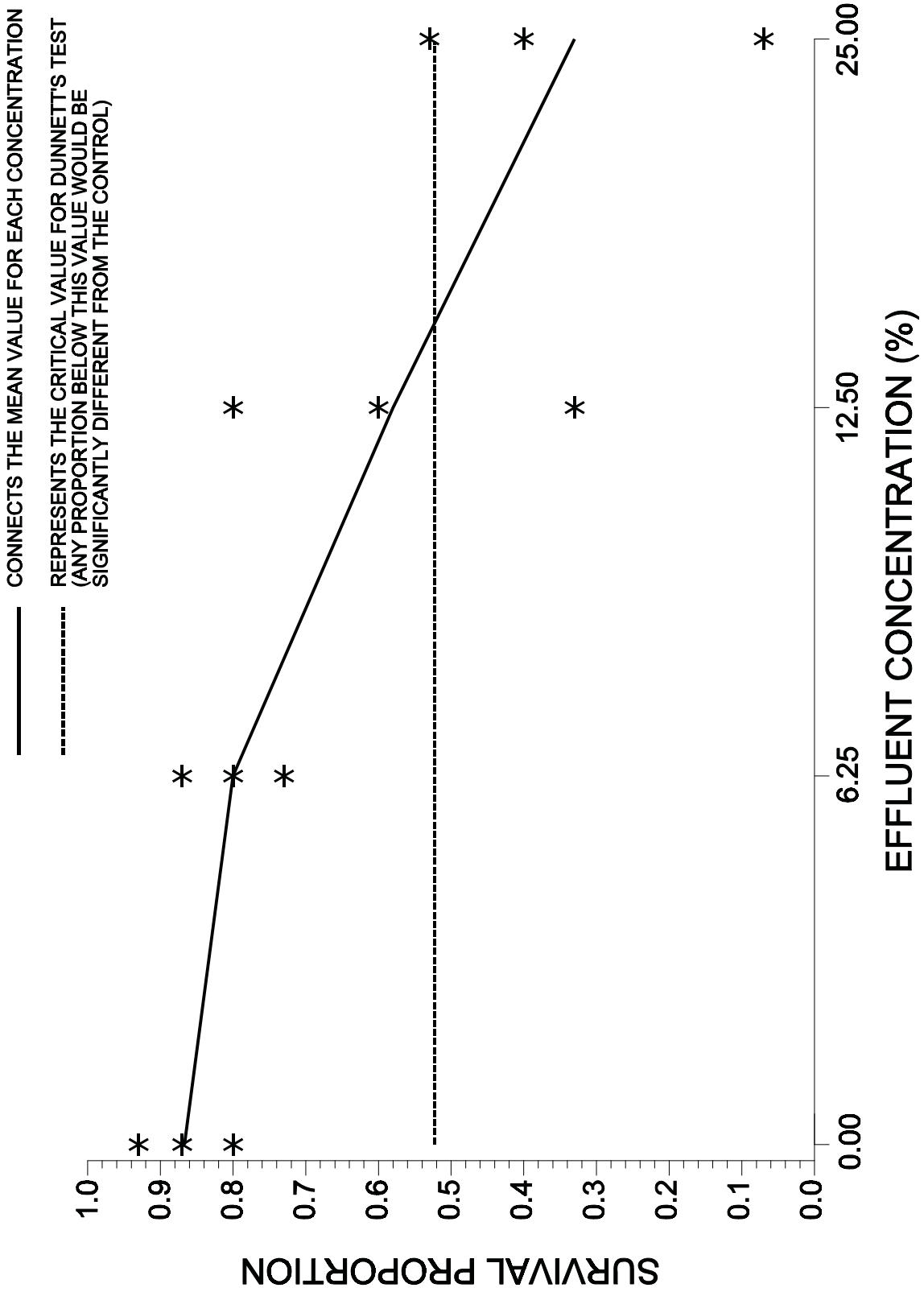

Figure 8. Plot of mean survival proportion of the inland silverside, *Menidia beryllina*, larvae.

TABLE 4. INLAND SILVERSIDE, *MENIDIA BERYLLINA*, LARVAL SURVIVAL DATA

			Concentration				
	Replicate	Control	6.25	12.5	25.0	50.0	100.0
RAW	A	0.80	0.73	0.80	0.40	0.0	0.0
	B	0.87	0.80	0.33	0.53	0.0	0.0
	C	0.93	0.87	0.60	0.07	0.0	0.0
ARC SINE TRANS-FORMED	A	1.107	1.024	1.107	0.685	-	-
	B	1.202	1.107	0.612	0.815	-	-
	C	1.303	1.202	0.886	0.268	-	-
Mean ($\bar{Y}_i$)		1.204	1.111	0.868	0.589		
S_i^2		0.010	0.008	0.061	0.082		
i		1	2	3	4		

13.13.2.6 Test for Normality

13.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 5.

TABLE 5. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Effluent Concentration (%)		
Replicate	Control	6.25	12.5	25.0
A	-0.097	-0.087	0.239	0.096
B	-0.002	-0.004	-0.256	0.226
C	0.099	0.091	0.018	-0.321

13.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

13.13.2.6.3 For this set of data, n = 12

$$\bar{X} = \frac{1}{12}(0.002) = 0.0$$

$$D = 0.3214$$

13.13.2.6.4 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 6.

TABLE 6. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.321	7	0.018
2	-0.256	8	0.091
3	-0.097	9	0.096
4	-0.087	10	0.099
5	-0.004	11	0.226
6	-0.002	12	0.239

13.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 12 and k = 6. The a_i values are listed in Table 7.

13.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-1+1)} - X^{(i)})\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 7. For the data in this example,

$$W = \frac{1}{0.3214}(0.5513)^2 = 0.945$$

TABLE 7. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.5475	0.560	$X^{(12)} - X^{(1)}$
2	0.3325	0.482	$X^{(11)} - X^{(2)}$
3	0.2347	0.196	$X^{(10)} - X^{(3)}$
4	0.1586	0.183	$X^{(9)} - X^{(4)}$
5	0.0922	0.095	$X^{(8)} - X^{(5)}$
6	0.0303	0.020	$X^{(7)} - X^{(6)}$

13.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 13.13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 12 observations is 0.805. Since W = 0.945 is greater than the critical value, conclude that the data are normally distributed.

13.13.2.7 Test for Homogeneity of Variance

13.13.2.7.1 The test used to examine whether the variation in survival is the same across all effluent concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i) \ln \overline{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, $V_i = (n_i - 1)$

p = number of levels of effluent concentration including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i = the number of replicates for concentration i.

$$\overline{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i^2)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{p} 1/V_i - (\sum_{i=1}^{p} V_i)^{-1}]$$

13.13.2.7.2 For the data in this example (See Table 4), all effluent concentrations including the control have the same number of replicates ($n_i = 3$ for all i). Thus, $V_i = 2$ for all i.

13.13.2.7.3 Bartlett's statistic is therefore:

$$B = [(8)\ln(0.0402) - 2\sum_{i=1}^{p} \ln(S_i^2)]/1.2083$$

$$= [8(-3.21391) - 2(-14.731)]/1.2083$$

$$= 3.7508/1.2083$$

$$= 3.104$$

13.13.2.7.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with three degrees of freedom, is 11.345. Since B = 3.104 is less than the critical value of 11.345, conclude that the variances are not different.

13.13.2.8 Dunnett's Procedure

13.13.2.8.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 8.

TABLE 8. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2= SSW/(N-p)
Total	N - 1	SST	

Where: p = number of SDS concentration levels including the control

N = total number of observations $n_1 + n_2 ... + n_p$

n_i = number of observations in concentration i

$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$ Between Sum of Squares

$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_j} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{p} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the proportion surviving for toxicant concentration i in test chamber j)

13.13.2.8.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = 3$

N = 12

$T_1 = Y_{11} + Y_{12} + Y_{13} = 3.612$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 3.333$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 2.605$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 1.768$

$G = T_1 + T_2 + T_3 + T_4 = 11.318$

$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$

$= \frac{1}{3}(34.067) - \frac{(11.318)^2}{12} = 0.681$

$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_j} Y_{ij}^2 - G^2/N$

$= 11.677 - \frac{(11.318)^2}{12} = 1.002$

$SSW = SST - SSB = 1.002 - 0.681 = 0.321$

S_B^2 = SSB/(p-1) = 0.681/(4-1) = 0.227

S_W^2 = SSW/(N-p) = 0.321/(12-4) = 0.040

13.13.2.8.3 Summarize these calculations in the ANOVA table (Table 9).

TABLE 9. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	3	0.681	0.227
Within	8	0.321	0.040
Total	11	1.002	

13.13.2.8.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_i - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean proportion surviving for effluent concentration i

$\bar{Y}_1$ = mean proportion surviving for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

13.13.2.8.5 Table 10 includes the calculated t values for each concentration and control combination. In this example, comparing the 1.0% concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.204 - 1.111)}{[0.020\sqrt{(1/3) + (1/3)}]} = 0.570$$

TABLE 10. CALCULATED T VALUES

Effluent Concentration (%)	i	t_i
6.25	2	0.570
12.5	3	2.058
25.0	4	3.766

13.13.2.8.6 Since the purpose of this test is to detect a significant reduction in survival, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, eight degrees of freedom for error and three concentrations (excluding the control) the critical value is 2.42. The mean proportion surviving for concentration i is considered significantly less than the mean proportion surviving for the control if t_i is greater than the critical value. Therefore, only the 25.0% concentration has a significantly lower mean proportion surviving than the control. Hence the NOEC is 12.5% and the LOEC is 25.0%.

13.13.2.8.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be detected statistically may be calculated.

$$MSD = d\ S_w\sqrt{(1/n_1) + (1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

13.13.2.8.8 In this example:

$$MSD = 2.42\,(0.20)\sqrt{(1/3) + (1/3)}$$

$$= 2.42\ (0.20)\ (0.817)$$

$$= 0.395$$

13.13.2.8.9 The MSD (0.395) is in transformed units. To determine the MSD in terms of percent survival, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

   1.204 - 0.395 = 0.809

2. Obtain the untransformed values for the control mean and the difference calculated in step 1.

   [Sine (1.204)]^2 = 0.871

   [Sine (0.809)]^2 = 0.524

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from step 2.

   MSD_u = 0.871 - 0.524 = 0.347

13.13.2.8.10 Therefore, for this set of data, the minimum difference in mean proportion surviving between the control and any effluent concentration that can be detected as statistically significant is 0.347.

13.13.2.8.11 This represents a 40% decrease in survival from the control.

13.13.2.9 Calculation of the LC50

13.13.2.9.1 The data used for the Probit Analysis is summarized in Table 11. To perform the Probit Analysis, run the USEPA Probit Analysis Program. An example of the program input and output is supplied in Appendix H.

TABLE 11. DATA FOR PROBIT ANALYSIS

		Effluent Concentration (%)				
	Control	6.25	12.5	25.0	50.0	100.0
Number Dead	6	9	19	45	45	45
Number Exposed	45	45	45	45	45	45

13.13.2.9.2 For this example, the chi-square test for heterogeneity was not significant. Thus Probit Analysis appears to be appropriate for this set of data.

13.13.2.9.3 Figure 9 shows the output data for the Probit Analysis of the data from Table 11 using the USEPA Probit Program.

13.13.3 ANALYSIS OF INLAND SILVERSIDE, *MENIDIA BERYLLINA,* GROWTH DATA

13.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 10. The response used in the statistical analysis is mean weight per original organism for each replicate. Because this measurement is based on the number of original organisms exposed (rather than the number surviving), the measured response is a combined survival and growth endpoint that can be termed biomass. The IC25 and IC50 can be calculated for the growth data via a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC and LOEC for growth. Concentrations above the NOEC for survival are excluded from the hypothesis test for growth effects.

13.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's

Test is used to test for homogeneity of variance. If either of these test fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

13.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative. For detailed information on the Bonferroni adjustment, see Appendix D.

Probit Analysis of Inland Silverside Larval Survival Data

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	45	6	0.1333	0.0000
6.2500	45	9	0.2000	0.0488
12.5000	45	19	0.4222	0.3130
25.0000	45	30	0.6667	0.6037
50.0000	45	45	1.0000	1.0000
100.0000	45	45	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 4.149
Chi - Square for Heterogeneity (tabular value) = 7.815

Probit Analysis of Inland Silverside Larval Survival Data

Estimated LC/EC Values and Confidence Limits

	Point	Exposure Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
LC/EC	1.00	4.980	2.023	7.789
LC/EC	50.00	18.302	13.886	22.175

Figure 9. Output for USEPA Probit Analysis Program, Version 1.5.

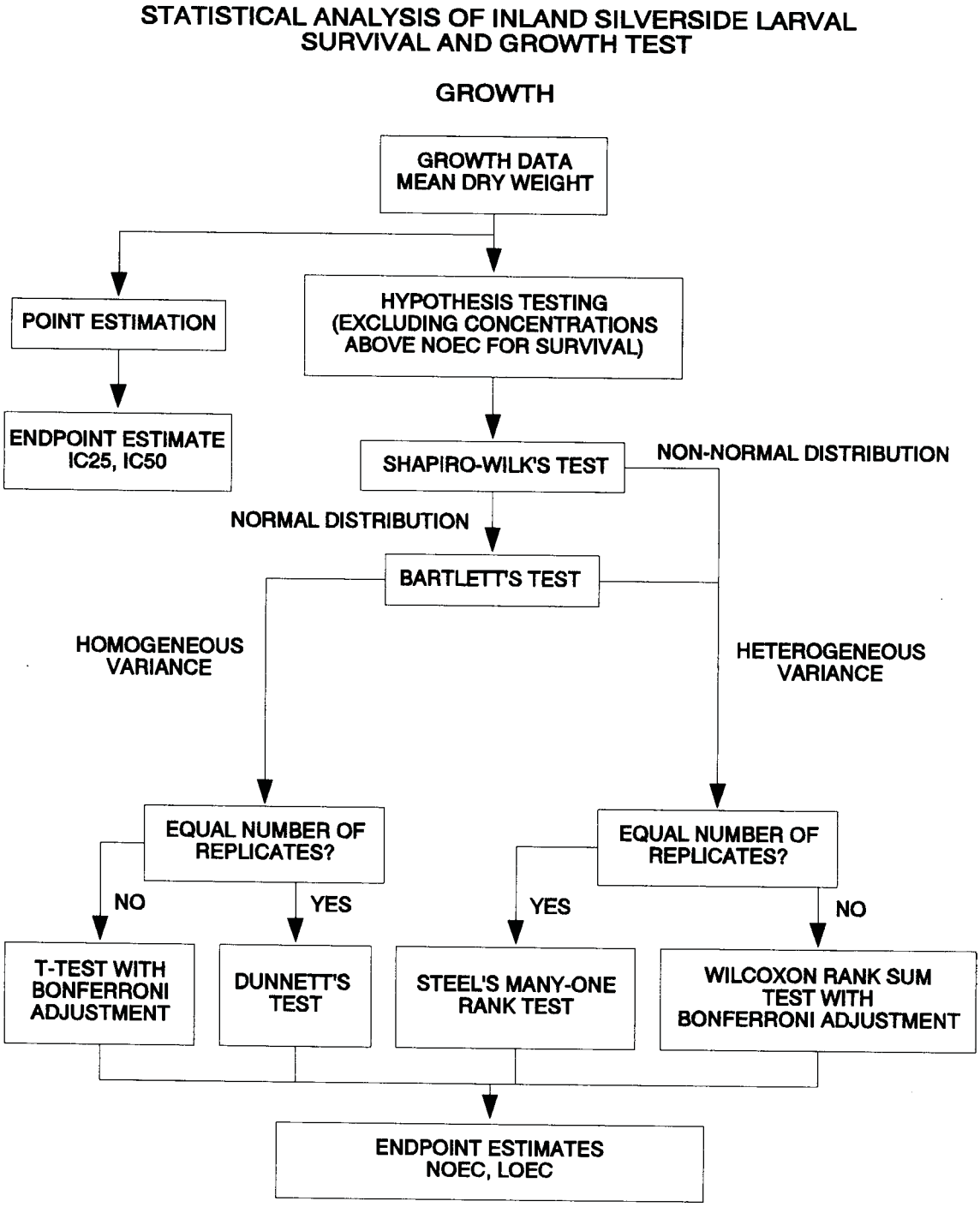

Figure 10. Flowchart for statistical analysis of the inland silverside, *Menida beryllina*, growth data.

13.13.3.4 The data, mean and variance of the growth observations at each concentration including the control are listed in Table 12. A plot of the data is provided in Figure 11. Since there was no survival in the 50% and 100% concentrations, these are not considered in the growth analysis. Additionally, since there is significant mortality in the 25% effluent concentration, its effect on growth is not considered.

TABLE 12. INLAND SILVERSIDE, *MENIDIA BERYLLINA*, GROWTH DATA

		Effluent Concentration %				
Replicate	Control	6.25	12.5	25.0	50.0	100.0
A	0.751	0.737	0.722	0.196	-	-
B	0.849	0.922	0.285	0.312	-	-
C	0.907	0.927	0.718	0.079	-	-
Mean($\bar{Y}_i$)	0.836	0.862	0.575	0.196	-	-
S_i^2	0.0062	0.0117	0.0631	0.0136	-	-
i	1	2	3	4	5	6

13.13.3.5 Test for Normality

13.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 13.

TABLE 13. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Effluent Concentration (%)		
Replicate	Control	6.25	12.5
A	-0.085	-0.125	0.147
B	0.013	0.060	-0.290
C	0.071	0.065	0.143

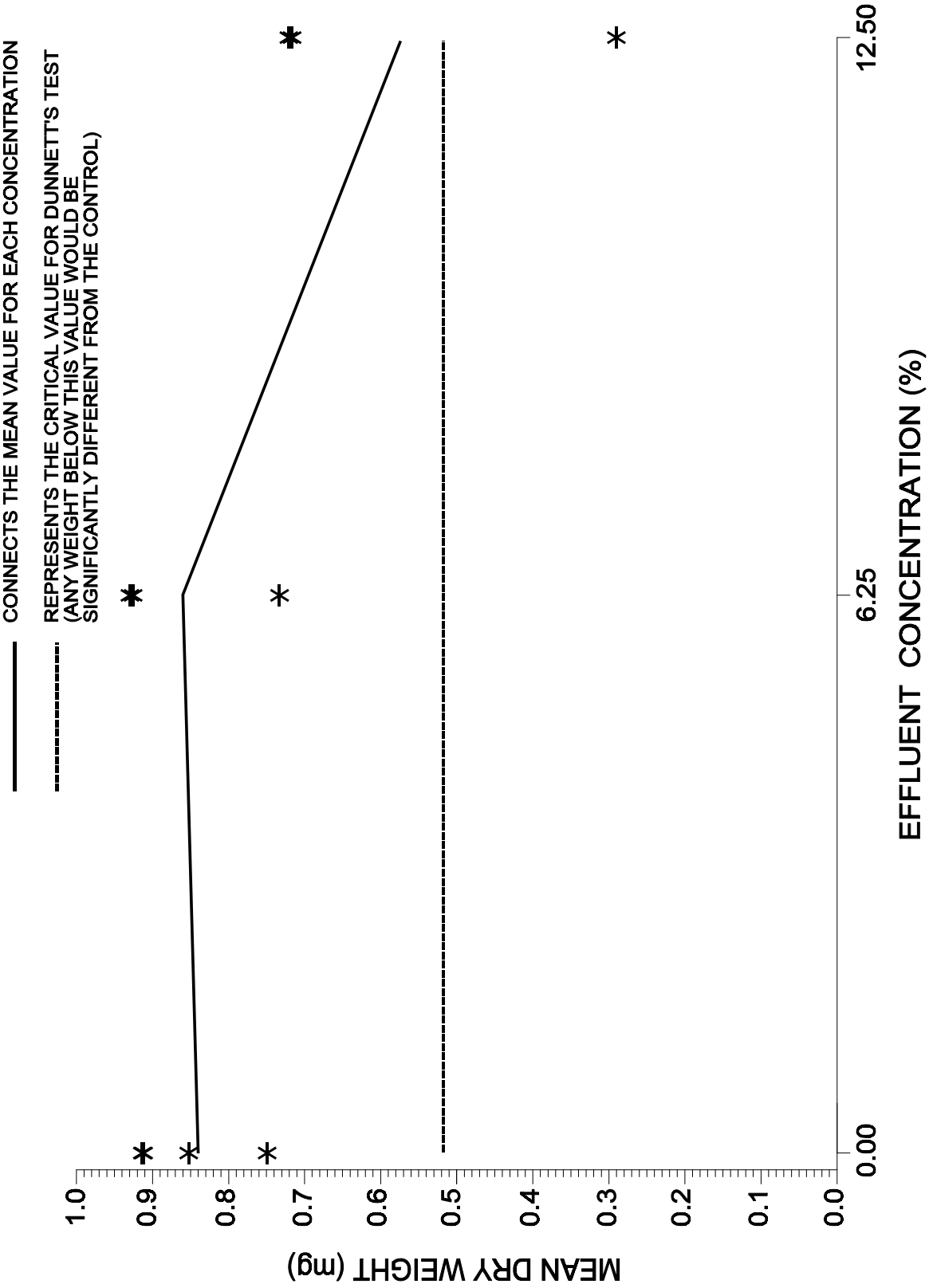

Figure 11. Plot of mean weights of inland silverside, *Menidia beryllina*, larval survival and growth test.

13.13.3.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations.

For this set of data, n = 9

$\bar{X} = \frac{1}{9}(-0.002) = 0.000$

D = 0.162

13.13.3.5.3 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 14.

TABLE 14. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.290	6	0.065
2	-0.125	7	0.071
3	-0.085	8	0.143
4	0.013	9	0.147
5	0.060		

13.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 9 and k = 4. The a_i values are listed in Table 15.

13.13.3.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 15. For this set of data:

$$W = \frac{1}{0.162}(0.3800)^2 = 0.89$$

TABLE 15. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.5888	0.437	$X^{(9)} - X^{(1)}$
2	0.3244	0.268	$X^{(8)} - X^{(2)}$
3	0.1976	0.156	$X^{(7)} - X^{(3)}$
4	0.0947	0.052	$X^{(6)} - X^{(4)}$

13.13.3.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and nine observations (n) is 0.764. Since W = 0.964 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

13.13.3.6 Test for Homogeneity of Variance

13.13.3.6.1 The test used to examine whether the variation in mean dry weight is the same across all effluent concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i)\ \ln \overline{S}^2 - \sum_{i=1}^{p} V_i\ \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, $V_i = (n_i - 1)$

p = number of levels of effluent concentration including the control

i = 1,2, ..., p where p is the number of concentrations including the control

ln = $\log_e$

n_i = number of replicates for concentration i

$$\overline{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i^2)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{p} 1/V_i - (\sum_{i=1}^{p} V_i)^{-1}]$$

13.13.3.6.2 For the data in this example, (See Table 13) all effluent concentrations including the control have the same number of replicates ($n_i = 3$ for all i). Thus, $V_i = 2$ for all i.

13.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(6)\ln(0.027) - 2\sum_{i=1}^{p} \ln(S_i^2)/1.222$$

$$= [6(-3.612)-2(-12.290)]/1.222$$

$$= 2.909/1.222$$

$$= 2.38$$

13.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with 2 degrees of freedom, is 9.210. Since B = 2.38 is less than the critical value of 9.210, conclude that the variances are not different.

13.13.3.7 Dunnett's Procedure

13.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 16.

TABLE 16. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	= SSB/(p-1)
Within	N - p	SSW	= SSW/(N-p)
Total	N - 1	SST	

Where: p = number of effluent concentrations including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N \qquad \text{Between Sum of Squares}$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_j} Y_{ij}^2 - G^2/N \qquad \text{Total Sum of Squares}$$

$$SSW = SST - SSB \qquad \text{Within Sum of Squares}$$

G = the grand total of all sample observations,

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean dry weight of the fish for toxicant concentration i in test chamber j)

13.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = 3$

N = 9

$T_1 = Y_{11} + Y_{12} + Y_{13} = 0.751 + 0.849 + 0.907 = 2.507$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 0.727 + 0.922 + 0.927 = 2.576$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 0.722 + 0.285 + 0.718 = 1.725$

$G = T_1 + T_2 + T_3 = 6.808$

$$= \frac{1}{3}(15.896) - \frac{(6.808)^2}{9} = 0.1488$$

$$= 5.463 - \frac{(6.808)^2}{9} = 0.3131$$

$$= 0.3131 - 0.1488 = 0.1643$$

$$= SSB/(p-1) = 0.1488/(3-1) = 0.0744$$

$$= SSW/(N-p) = 0.1643/(9-3) = 0.0274$$

13.13.3.7.3 Summarize these calculations in the ANOVA table (Table 17).

TABLE 17. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	2	0.1488	0.0744
Within	6	0.1643	0.0274
Total	8	0.3131	

13.13.3.7.4 To perform the individual comparisons, calculate the t statistic for each concentration and control combination as follows:

$$t_i = \frac{(\overline{Y_1} - \overline{Y_i})}{S_w\sqrt{(1/n_1 + 1/n_i)}}$$

Where: Y_i = mean dry weight for effluent concentration i

Y_1 = mean dry weight for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

13.13.3.7.5 Table 18 includes the calculated t values for each concentration and control combination. In this example, comparing the 6.25% concentration with the control the calculation is as follows:

$$t_2 = \frac{(0.836 - 0.859)}{[0.1655\sqrt{(1/3) + (1/3)}]} = -0.120$$

TABLE 18. CALCULATED T VALUES

Effluent Concentration (ppb)	i	t_i
6.25	2	-0.120
12.5	3	1.931

13.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean weight, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, six degrees of freedom for error and two concentrations (excluding the control) the critical value is 2.34. The mean weight for concentration i is considered significantly less than mean weight for the control if t_i is greater than the critical value. Therefore, all effluent concentrations in this example do not have significantly lower mean weights than the control. Hence the NOEC and the LOEC for growth cannot be calculated.

13.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be detected statistically may be calculated.

$$MSD = dS_w\sqrt{(1/n_1) + (1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

13.13.3.7.8 In this example:

$$MSD = 2.34(0.1655)\sqrt{(1/3) + (1/3)}$$

$$= 2.34\ (0.1655)(0.8165)$$

$$= 0.316$$

13.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.316 mg.

13.13.3.7.10 This represents a 37.8% reduction in mean weight from the control.

13.13.3.8 Calculation of the ICp

13.13.3.8.1 The growth data from Tables 4 and 12 are utilized in this example. As seen in Table 19 and Figure 11, the observed means are not monotonically non-increasing with respect to concentration (the mean response for each higher concentration is not less than or equal to the mean response for the previous concentration, and the reponses between concentrations do not follow a linear trend). Therefore, the means are smoothed prior to calculating the IC. In the following discussion, the observed means are represented by $\bar{Y}_i$ and the smoothed means by M_i.

13.13.3.8.2 Starting with the control mean, $\bar{Y}_1 = 0.836$ and $\bar{Y}_2 = 0.859$, we see that $\bar{Y}_1 < \bar{Y}_2$. Set $M_i = Y_i$.

13.13.3.8.3 Calculate the smoothed means:

$$M_1 = M_2 = (\bar{Y}_1 + \bar{Y}_2)/2 = 0.847$$

13.13.3.8.4 Since $\bar{Y}_5 = 0 < \bar{Y}_4 = 0.196 < \bar{Y}_3 = 0.575 < M_2$, set $M_3 = 0.575$, $M_4 = 0.196$, and $M_5 = 0$.

13.13.3.8.5 Table 19 contains the response means and the smoothed means and Figure 12 gives a plot of the smoothed response curve.

TABLE 19. INLAND SILVERSIDE MEAN GROWTH RESPONSE AFTER SMOOTHING

Effluent Conc. (%)	i	Response Means, Y_i (mg)	Smoothed Means, M_i (mg)
Control	1	0.836	0.847
6.25	2	0.859	0.847
12.50	3	0.575	0.575
25.00	4	0.196	0.196
50.00	5	0.00	0.0

13.13.3.8.6 An IC25 and IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in weight, compared to the controls, would result in a mean dry weight of 0.627 mg, where $M_1(1\text{-}p/100) = 1.847(1\text{-}25/100)$. A 50% reduction in mean dry weight, compared to the controls, would result in a mean weight of 0.418 mg. Examining the smoothed means and their associated concentrations (Table 20), the response, 0.627 mg, is bracketed by C_2 = 6.25% effluent and C_3 = 25.0% effluent. The response (0.418) is bracketed by C_3 = 12.5% and by C_4 = 25% effluent.

13.13.3.8.7 Using the equation from Section 4.2 of Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [m_1(1 - p/100) - M_j]\frac{(C_{j+1} - C_j)}{(m_{j+1} - M_j)}$$

$$IC25 = 6.25 + [0.847(1 - 25/100) - 0.847]\frac{(12.50 - 6.25)}{(0.575 - 0.847)}$$

$$= 11.1\%.$$

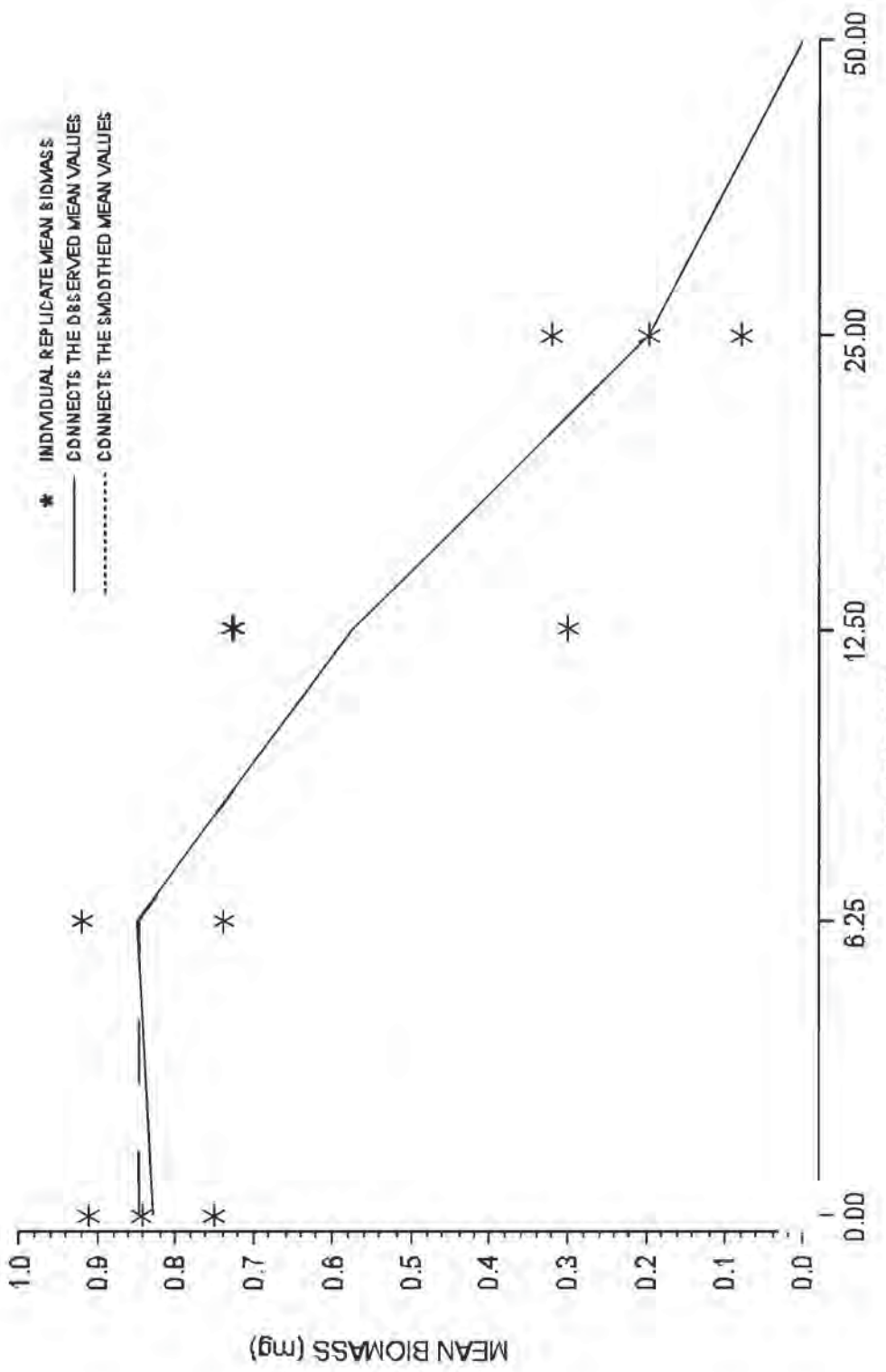

Figure 12. Plot of the raw data, observed means, and smoothed means from Tables 12 and 19.

13.13.3.8.8 Using the equation from Section 4.2 of Appendix L, the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [m_1(1 - p/100) - M_j]\frac{(C_{j+1} - C_j)}{(M_{j+1} - M_j)}$$

$$IC50 = 6.25 + [0.847(1 - 50/100) - 0.847] \frac{(12.50 - 6.25)}{(0.575 - 0.847)}$$

$$= 17.5\%.$$

13.13.3.8.9 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 11.1136%. The empirical 95% confidence interval for the true mean was 5.7119% to 19.2112%. The computer program output for the IC25 for this data set is shown in Figure 13.

13.13.3.8.10 When the ICPIN program was used to analyze this set of data for the IC50, requesting 80 resamples, the estimate of the IC50 was 17.4896%. The empirical 95% confidence interval for the true mean was 6.4891% to 22.4754% effluent. The computer program output is shown in Figure 14.

13.14 PRECISION AND ACCURACY

13.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 13.14.1.1 and 13.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

13.14.1.1 Single-Laboratory Precision

13.14.1.1.1 Data on the single-laboratory precision of the inland silverside larval survival and growth test using copper (CU) sulfate and sodium dodecyl sulfate (SDS) as reference toxicants, in natural seawater and GP2 are provided in Tables 20-22. In Tables 20-21, the coefficient of variation for copper based on the IC25 is 43.2% and for SDS is 43.2% indicating acceptable precision. In the five tests with each reference toxicant, the NOEC's varied by only one concentration interval, indicating good precision. The coefficient of variation for all reference toxicants based on the IC50 in two types of seawater (GP2 and natural) ranges from l.8% to 50.7% indicating acceptable precision. Data in Table 22 show no detectable differences between tests conducted in natural and artificial seawaters.

13.14.1.1.2 EPA evaluated within-laboratory precision of the Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test using a database of routine reference toxicant test results from 16 laboratories (USEPA, 2000b). The database consisted of 193 reference toxicant tests conducted in 16 laboratories using a variety of reference toxicants including: chromium, copper, potassium chloride, and sodium dodecyl sulfate. Among the 16 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 27% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 18%; and in 75% of laboratories, the within-laboratory CV was less than 43%.

13.14.1.2 Multilaboratory Precision

13.14.1.2.1 In 2000, EPA conducted an interlaboratory variability study of the Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 10 participant laboratories tested 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of bioassay-grade FORTY FATHOMS® synthetic seawater, the effluent sample was an industrial wastewater spiked with $CuSO_4$, the receiving water sample was a natural seawater spiked with $CuSO_4$, and the reference toxicant sample consisted of bioassay-grade FORTY FATHOMS® synthetic seawater spiked with $CuSO_4$. Of the 40 *Menidia beryllina* Larval Survival and Growth tests conducted in this study, 100% were successfully completed and met the required test acceptability criteria. Of seven tests that were conducted on blank samples, none showed false positive results for survival endpoints or for the growth endpoint. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 23 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 43.8% for IC25 results. Table 24 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned five concentrations for the effluent, four concentrations for the reference toxicant sample type, and three concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 90.9%, 84.6%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively. For the growth endpoint, NOEC values spanned four concentrations for the reference toxicant and effluent sample types and three concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 90.9%, 91.7%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively.

13.14.2 ACCURACY

13.14.2.1 The accuracy of toxicity tests cannot be determined.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	6.25	12.5	25	50	100
Response 1	.751	.727	.722	.196	0	0
Response 2	.849	.922	.285	.312	0	0
Response 3	.907	.927	.718	.079	0	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: Menidia beryllina
Test Duration: 7-d
DATA FILE: silver.icp
OUTPUT FILE: silver.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	0.836	0.079	0.847
2	3	6.250	0.859	0.114	0.847
3	3	12.500	0.575	0.251	0.575
4	3	25.000	0.196	0.117	0.196
5	3	50.000	0.000	0.000	0.000
6	3	100.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 11.1136 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 11.5341 Standard Deviation: 2.1155
Original Confidence Limits: Lower: 8.5413 Upper: 14.9696
Expanded Confidence Limits: Lower: 5.7119 Upper: 19.2112
Resampling time in Seconds: 1.43 Random Seed: -1912403737

Figure 13. ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	6.25	12.5	25	50	100
Response 1	.751	.727	.722	.196	0	0
Response 2	.849	.922	.285	.312	0	0
Response 3	.907	.927	.718	.079	0	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: Menidia beryllina
Test Duration: 7-d
DATA FILE: silver.icp
OUTPUT FILE: silver.i50

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	0.836	0.079	0.847
2	3	6.250	0.859	0.114	0.847
3	3	12.500	0.575	0.251	0.575
4	3	25.000	0.196	0.117	0.196
5	3	50.000	0.000	0.000	0.000
6	3	100.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 17.4896 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 16.9032 Standard Deviation: 2.49.73
Original Confidence Limits: Lower: 12.2513 Upper: 19.8638
Expanded Confidence Limits: Lower: 6.4891 Upper: 22.4754
Resampling time in Seconds: 1.43 Random Seed: -1440337465

Figure 14. ICPIN program output for the IC50.

TABLE 20. SINGLE-LABORATORY PRECISION OF THE INLAND SILVERSIDE, *MENIDIA BERYLLINA*, SURVIVAL AND GROWTH TEST PERFORMED IN NATURAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN NATURAL SEAWATER, AND COPPER (CU) AS A REFERENCE TOXICANT[1,2,3,4,5,6,7]

Test Number	NOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)	Most Sensitive Endpoint[6]
1	63	96.2	148.6	S
2	125	207.2	NC[8]	S
3	63	218.9	493.4	G
4	125	177.5	241.4	S
5	31	350.1	479.8	G
n:	5	5	4	
Mean:	NA	209.9	340.8	
CV(%):	NA	43.7	50.7	

1 Data from USEPA (1988a) and USEPA (1991a)
2 Tests performed by George Morrison and Elise Torello, ERL-N, USEPA, Narragansett, RI.
3 Three replicate exposure chambers with 10-15 larvae were used for the control and each copper concentration. Copper concentrations were: 31, 63, 125, 250, and 500 µg/L.
4 Adults collected in the field.
5 S = Survival effects. G = Growth data at these toxicant concentrations were disregarded because there was a significant reduction in survival.
6 NOEC Range: 31 - 125 µg/L (this represents a difference of two exposure concentrations).
7 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.
8 NC = No linear interpolation estimate could be calculated from the ata, since none of the group response means were less than 50 percent of the control response mean.

TABLE 21. SINGLE-LABORATORY PRECISION OF THE INLAND SILVERSIDE, *MENIDIA BERYLLINA*, SURVIVAL AND GROWTH TEST PERFORMED IN NATURAL SEAWATER, USING LARVAE FROM FISH MAINTAINED AND SPAWNED IN NATURAL SEAWATER, AND SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT[1,2,3,4,5,6,7]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)	Most Sensitive Endpoint
1	1.3	0.3	1.7	S
2	1.3	1.6	1.9	S
3	1.3	1.5	1.9	S
4	1.3	1.5	1.9	S
5	1.3	1.6	2.2	S
n:	5	5	5	
Mean:	NA	1.3	1.9	
CV(%):	NA	43.2	9.4	

1 Data from USEPA (1988a) and USEPA (1991a)
2 Tests performed by George Morrison and Elise Torello, ERL-N, USEPA, Narragansett, RI.
3 Three replicate exposure chambers with 10-15 larvae were used for the control and each SDS concentration. SDS concentrations were: 0.3, 0.6, 1.3, 2.5, and 5.0 mg/L.
4 Adults collected in the field.
5 S = Survival Effects. Growth data at these toxicant concentrations were disregarded because there was a significant reduction in survival.
6 NOEC Range: 1.3 mg/L.
7 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 22. COMPARISON OF THE SINGLE-LABORATORY PRECISION OF THE INLAND SILVERSIDE, *MENIDIA BERYLLINA*, LARVAL SURVIVAL (LC50) AND GROWTH (IC50) VALUES EXPOSED TO SODIUM DODECYL SULFATE (SDS) OR COPPER (CU) SULFATE IN GP2 ARTIFICIAL SEAWATER MEDIUM OR NATURAL SEAWATER (NSW)[1,2,3,4]

	Survival		Growth	
SDS (mg/L)	GP2	NSW	GP2	NSW
	3.59	3.69	3.60	3.55
	4.87	4.29	5.54	5.27
	5.95	8.05	6.70	8.53
Mean	4.81	5.34	5.28	5.79
CV (%)	24.6	44.2	29.6	43.8
Copper (µg/L)	GP2	NSW	GP2	NSW
	247	256	260	277
	215	211	236	223
	268	240	NC[5]	238
Mean	243	236	248	246
CV (%)	10.9	9.8	6.9	11.2

1 Tests performed by George Morrison and Glen Modica, ERL-N, USEPA, Narragansett, RI.
2 Three replicate exposure chambers with 10-15 larvae per treatment.
3 Adults collected in the field.
4 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.
5 NC= No linear interpolation estimate could be calculated from the data, since none of the group response means were less than 50 percent of the control response mean.

TABLE 23. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	22.0	29.1	36.4
	Effluent	7.24	55.5	56.0
	Receiving water	-	-	39.1
	Average	14.6	42.3	43.8

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

3 The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

4 The between-laboratory component of variability for duplicate samples tested at different laboratories.

5 The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 24. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	12.5%	72.7	18.2	9.09
	Effluent	25%	38.5	46.1	15.4
	Receiving water	25%	57.1	28.6	14.3
Growth NOEC	Reference toxicant	12.5%	72.7	18.2	9.09
	Effluent	25%	41.7	50.0	8.33
	Receiving water	25%	57.1	28.6	14.3

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.

SECTION 14
TEST METHOD

MYSID, *MYSIDOPSIS BAHIA*, SURVIVAL, GROWTH, AND FECUNDITY TEST METHOD 1007.0

14.1 SCOPE AND APPLICATION

14.1.1 This method, adapted in part from USEPA (1987d), estimates the chronic toxicity of effluents and receiving waters to the mysid, *Mysidopsis bahia*, during a seven-day, static renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and additive components which adversely affect the physiological and biochemical functions of the test organisms.

14.1.2 Daily observations on mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

14.1.3 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

14.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants present in the source may not be detected in the test.

14.1.5 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

14.2 SUMMARY OF METHOD

14.2.1 *Mysidopsis bahia* 7-day old juveniles are exposed to different concentrations of effluent, or to receiving water in a static system, during the period of egg development. The test endpoints are survival, growth (measured as dry weight), and fecundity (measured as the percentage of females with eggs in the oviduct and/or brood pouch).

14.3 INTERFERENCES

14.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

14.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.3.3 The test results can be confounded by (1) the presence of pathogenic and/or predatory organisms in the dilution water, effluent, and receiving water, (2) the condition of the brood stock from which the test animals were taken, (3) the amount and type of natural food in the effluent, receiving water, or dilution water, (4) nutritional value of the brine shrimp, *Artemia* nauplii, fed during the test, and (5) the quantity of brine shrimp, *Artemia* nauplii, or other food added during the test, which may sequester metals and other toxic substances, and lower the DO.

14.3.4 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed

to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 14.3.4.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 14.3.4.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

14.3.4.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 14.3.4.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample after adjusting the sample salinity for use in marine testing.

14.3.4.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ± 0.3 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ± 0.3 pH units in pH-controlled tests (USEPA, 1996).

14.3.4.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

14.3.4.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 14.3.4.1.1).

14.3.4.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 14.3.4.2) is applied routinely to subsequent testing of the effluent.

14.3.4.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.9). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992); or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the

receiving water, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the sample after adjusting the sample salinity for use in marine testing. USEPA (1996) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

14.4 SAFETY

14.4.1 See Section 3, Health and Safety.

14.5 APPARATUS AND EQUIPMENT

14.5.1 Facilities for holding and acclimating test organisms.

14.5.2 Brine shrimp, *Artemia*, culture unit -- see Subsection 14.6.12 below and Section 4, Quality Assurance.

14.5.3 Mysid, *Mysidopsis bahia*, culture unit -- see Subsection 14.13 below. This test requires a minimum of 240 7-day old (juvenile) mysids. It is preferable to obtain the test organisms from an in-house culture unit. If it is not feasible to culture mysids in-house, juveniles can be obtained from other sources, if shipped in well oxygenated saline water in insulated containers.

14.5.4 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L.

14.5.5 Environmental chamber or equivalent facility with temperature control (26 ± 1°C).

14.5.6 Water purification system -- Millipore Milli-Q®, deionized water or equivalent.

14.5.7 Balance -- Analytical, capable of accurately weighing to 0.00001 g.

14.5.8 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and weighing pans plus organisms.

14.5.9 Drying oven -- 50-105°C range, for drying organisms.

14.5.10 Desiccator -- for holding dried organisms.

14.5.11 Air pump -- for oil-free air supply.

14.5.12 Air lines, and air stones -- for aerating cultures, brood chambers, and holding tanks, and supplying air to test solutions with low DO.

14.5.13 Meters, pH and DO -- for routine physical and chemical measurements.

14.5.14 Tray -- for test vessels; approximately 90 X 48 cm to hold 56 vessels.

14.5.15 Standard or micro-Winkler apparatus -- for determining DO and checking DO meters.

14.5.16 Dissecting microscope (350-400X magnification) -- for examining organisms in the test vessels to determine their sex and to check for the presence of eggs in the oviducts of the females.

14.5.17 Light box -- for illuminating organisms during examination.

14.5.18 Refractometer or other method -- for determining salinity.

14.5.19 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

14.5.20 Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.

14.5.21 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, 1979b) -- to calibrate laboratory thermometers.

14.5.22 Test chambers -- 200 mL borosilicate glass beakers or non-toxic 8 oz disposable plastic cups or other similar containers. Forty-eight (48) test vessels are required for each test (eight replicates at each of five effluent concentrations and a control). To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic (6 mm thick).

14.5.23 Beakers or flasks -- six, borosilicate glass or non-toxic plasticware, 2000 mL for making test solutions.

14.5.24 Wash bottles -- for deionized water, for washing organisms from containers and for rinsing small glassware and instrument electrodes and probes.

14.5.25 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-2000 mL for making test solutions.

14.5.26 Separatory funnels, 2-L -- Two-four for culturing *Artemia*.

14.5.27 Pipets, volumetric -- Class A, 1-100 mL.

14.5.28 Pipets, automatic -- adjustable, 1-100 mL.

14.5.29 Pipets, serological -- 1-10 mL, graduated.

14.5.30 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

14.5.31 Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring organisms.

14.5.32 Forceps -- for transferring organisms to weighing pans.

14.5.33 NITEX® or stainless steel mesh sieves (≤150 µm, 500-1000 µm, 3-5 mm) -- for concentrating organisms.

14.5.34 Depression glass slides or depression spot plates -- two, for observing organisms.

14.6 REAGENTS AND CONSUMABLE MATERIALS

14.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.6.2 Data sheets (one set per test) -- for data recording (Figures 2, 7, and 8).

14.6.3 Tape, colored -- for labeling test chambers.

14.6.4 Markers, waterproof -- for marking containers, etc.

14.6.5 Weighing pans, aluminum -- to determine the dry weight of organisms.

14.6.6 Buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979b).

14.6.7 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979b), or reagents for modified Winkler analysis.

14.6.8 Laboratory quality assurance samples and standards -- for the above methods.

14.6.9 Reference toxicant solutions -- see Section 4, Quality Assurance.

14.6.10 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

14.6.11 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests. Dilution water containing organisms that might prey upon or otherwise interfere with the test organisms should be filtered through a fine mesh net (with 150 µm or smaller openings).

14.6.11.1 Saline test and dilution water -- The salinity of the test water must be in the range of 20‰ to 30‰. The salinity should vary by no more than ± 2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

14.6.11.2 The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Exposure of mysids to these effluents will require adjustments in the salinity of the test solutions. It is important to maintain a constant salinity across all treatments. In addition, it may be desirable to match the test salinity with that of the receiving water. Two methods are available to adjust salinities – a hypersaline brine (HSB) derived from natural seawater or artificial sea salts.

14.6.11.3 HSB has several advantages that make it desirable for use in toxicity testing. It can be made from any high quality, filtered seawater by evaporation, and can be added to the effluent or to deionized water to increase the salinity. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and may be stored for prolonged periods without any apparent degradation. However, if 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested is 80% effluent at 30‰ salinity and 70% effluent at 30‰ salinity.

14.6.11.3.1 The ideal container for making brine from natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is used, only oil-free air compressors should be used to prevent contamination.

14.6.11.3.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough deionized water rinses.

14.6.11.3.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

14.6.11.3.4 The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being enerated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

14.6.11.3.5 After the required salinity is attained, the HSB should be filtered a second time through a 1 mm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The containers should be capped and labeled with the date the brine was generated and its salinity. Containers of HSB should be stored in the dark and maintained under room temperature until used.

14.6.11.3.6 If a source of HSB is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and HSB before mixing in the effluent.

14.6.11.3.7 Divide the salinity of the HSB by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 20‰, 100‰ divided by 20‰ = 5.0. The proportion of brine is 1 part in 5 (one part brine to four parts deionized water). To make 1 L of seawater at 20‰ salinity from a HSB of 100‰, 200 mL of brine and 800 mL of deionized water are required.

14.6.11.3.8 Table 2 illustrates the composition of 1800 mL test solutions at 20‰ if they are made by combining effluent (0‰), deionized water and HSB of 100‰ (only). The volume (mL) of brine required is determined by using the amount calculated above. In this case, 200 mL of brine is required for 1 L; therefore, 360 mL would be required for 1.8 L of solution. The volumes of HSB required are constant. The volumes of deionized water are determined by subtracting the volumes of effluent and brine from the total volume of solution: 1800 mL - mL effluent - mL brine = mL deionized water.

14.6.11.4 Artificial sea salts: FORTY FATHOMS® brand sea salts have been used successfully to culture and perform life cycle tests with mysids (Horne, et al., 1983; ASTM, 1993) (see Section 7, Dilution Water). HW MARINEMIX® sea salts have been used successfully to culture mysids and perform the mysid toxicity test (USEPA Region 6 Houston Laboratory; EMSL-Cincinnati). In addition, a slightly modified version of the GP2 medium (Spotte et al., 1984) has been successfully used to perform the mysid survival, growth, and fecundity test (Table 1).

14.6.11.4.1 Synthetic sea salts are packaged in plastic bags and mixed with deionized water or equivalent. The instructions on the package of sea salts should be followed carefully, and the salts should be mixed in a separate container -- not in the culture tank. The deionized water used in hydration should be in the temperature range of 21-26°C. Seawater made from artificial sea salts is conditioned (Spotte, 1973; Spotte, et al., 1984; Bower, 1983) before it is used for culturing or testing. After adding the water, place an airstone in the container, cover, and aerate the solution mildly for 24 h before use.

14.6.11.4.2 The GP2 reagent grade chemicals (Table 1) should be mixed with deionized (DI) water or its equivalent in a container other than the culture or testing tanks. The deionized water used for hydration should be between 21-26°C. The artificial seawater must be conditioned (aerated) for 24 h before use as the testing medium. If the solution is to be autoclaved, sodium bicarbonate is added after the solution has cooled. A stock solution of sodium bicarbonate is made up by dissolving 33.6 g $NaHCO_3$ in 500 mL of deionized water. Add 2.5 mL of this stock solution for each liter of the GP2 artificial seawater.

TABLE 1. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR THE MYSID, *MYSIDOPSIS BAHIA*, TOXICITY TEST[1,2,3]

Compound	Concentration (g/L)	Amount (g) Required for 20L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
Kcl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

[1] Modified GP2 from Spotte et al. (1984).
[2] The constituent salts and concentrations were taken from USEPA (1990b). The salinity is 30.89 g/L.
[3] GP2 can be diluted with deionized (DI) water to the desired test salinity.

14.6.12 BRINE SHRIMP, *ARTEMIA*, NAUPLII -- for feeding cultures and test organisms.

14.6.12.1 Newly hatched *Artemia* nauplii are used for food for the stock cultures and test organisms. Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are preferred because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size.

TABLE 2. QUANTITIES OF EFFLUENT, DEIONIZED WATER, AND HYPERSALINE BRINE (100‰) NEEDED TO PREPARE 1800 ML VOLUMES OF TEST SOLUTION WITH A SALINITY OF 20‰

Effluent Concentration (%)	Volume of Effluent (0‰) (mL)	Volume of Deionized Water (mL)	Volume of Hypersaline Brine (mL)	Total Volume (mL)
80	1440	0	360	1800
40	720	720	360	1800
20	360	1080	360	1800
10	180	1260	360	1800
5	90	1350	360	1800
Control	0	1440	360	1800
Total	2790	5850	2160	10800

14.6.12.2 Each new batch of *Artemia* cysts must be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (Leger, et al., 1985, Leger, et al., 1986) against known suitable reference cysts by performing a side-by-side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be a previously tested and acceptable batch of cysts. A sample of newly-hatched *Artemia* nauplii from each new batch of cysts should be chemically analyzed. The *Artemia* cysts should not be used if the concentration of total organic chlorine exceeds 0.15 µg/g wet weight or the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight (For analytical methods see USEPA, 1982).

14.6.12.2.1 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or an aqueous uniodized salt (NaCl) solution prepared with 35 g salt or artificial sea salts to 1 L of deionized water, to a 2-L separatory funnel, or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24 h at 27°C. Hatching time varies with incubation temperature and the geographic strain of *Artemia* used (see USEPA, 1985a; USEPA, 2002a; ASTM, 1993).
3. After 24 h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic, and will concentrate at the bottom of the funnel if it is covered for 5-10 minutes. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 min without aeration.
4. Drain the nauplii into a beaker or funnel fitted with a 150 µm NITEX® or stainless steel screen, and rinse with seawater or equivalent before use.

14.6.12.3 Testing *Artemia* nauplii as food for toxicity test organisms.

14.6.12.3.1 The primary criteria for acceptability of each new supply of brine shrimp, cysts is adequate survival, growth, and reproduction of the mysids. The mysids used to evaluate the acceptability of the brine shrimp nauplii must be of the same geographical origin and stage of development (7 days old) as those used routinely in the toxicity tests. Sufficient data to detect differences in survival and growth should be obtained by using eight replicate test chambers, each containing 5 mysids, for each type of food.

14.6.12.3.2 The feeding rate and frequency, test vessels, volume of control water, duration of the test, and age of the *Artemia* nauplii at the start of the test, should be the same as used for the routine toxicity tests.

14.6.12.3.3 Results of the brine shrimp, *Artemia*, nauplii nutrition assay, where there are only two treatments, can be evaluated statistically by use of a t test. The "new" food is acceptable if there are no statistically significant differences in the survival, growth, and reproduction of the mysids fed the two sources of nauplii.

14.6.13 TEST ORGANISMS, *Mysidopsis bahia* (see Rodgers et al., 1986 and USEPA, 2002a for information on mysid ecology). The genus name of this organism was formally changed to *Americamysis* (Price *et al.*, 1994); however, the method manual will continue to refer to *Mysidopsis bahia* to maintain consistency with previous versions of the method.

14.6.13.1 Brood Stock

14.6.13.1.1 To provide an adequate supply of juveniles for a test, mysid, *Mysidopsis bahia*, cultures should be started at least four weeks before the test animals are needed. At least 200 mysids, *Mysidopsis bahia*, should be placed in each culture tank to ensure that 1500 to 2000 animals will be available by the time preparations for a test are initiated.

14.6.13.1.2 Mysids, *Mysidopsis bahia*, may be shipped or otherwise transported in polyethylene bottles or CUBITAINERS®. Place 50 animals in 700 mL of seawater in a 1-L shipping container. To control bacterial growth and prevent DO depletion during shipment, do not add food. Before closing the shipping container, oxygenate the water for 10 min. The mysids, *Mysidopsis bahia*, will starve if not fed within 36 h, therefore, they should be shipped so that they are not in transit more than 24 h.

14.6.13.1.3 The identification of the *Mysidopsis bahia* stock culture should be verified using the key from Heard (1982), Price (1978), Price, (1982), Stuck et al. (1979a), and Stuck et al. (1979b). Records of the verification should be retained along with a few of the preserved specimens.

14.6.13.1.4 Glass aquaria (120- to 200-L) are recommended for cultures. Other types of culture chambers may also be convenient. Three or more separate cultures should be maintained to protect against loss of the entire culture stock in case of accident, low DO, or high nitrite levels, and to provide sufficient numbers of juvenile mysids, *Mysidopsis bahia*, for toxicity tests. Fill the aquaria about three-fourths full of seawater. A flow-through system is recommended if sufficient natural seawater is available, but a closed, recirculating or static renewal system may be used if proper water conditioning is provided and care is exercised to keep the pH above 7.8 and nitrite levels below 0.05 mg/L.

14.6.13.1.5 Standard aquarium undergravel filters should be used with either the flow-through or recirculating system to provide aeration and a current conducive to feeding (Gentile et al., 1983). The undergravel filter is covered with a prewashed, coarse (2-5 mm) dolomite substrate, 2.5 cm deep for flow-through cultures or 10 cm deep for recirculating cultures.

14.6.13.1.6 The recirculating culture system is conditioned as follows:

1. After the dolomite has been added, the filter is attached to the air supply and operated for 24 h.
2. Approximately 4 L of seed water obtained from a successfully operating culture is added to the culture chamber.
3. The nitrite level is checked daily with an aquarium test kit or with EPA Method 354.1 (USEPA, 1979b).
4. Add about 30 mL of concentrated *Artemia* nauplii every other day until the nitrite level reaches at least 2.0 mg/L. The nitrite will continue to rise for several days without adding more *Artemia* nauplii and will then slowly decrease to less than 0.05 mg/L.

5. After the nitrite level falls below 0.05 mg/L, add another 30 mL of *Artemia* nauplii concentrate and check the nitrite concentration every day.
6. Continue this cycle until the addition of *Artemia* nauplii does not cause a rise in the nitrite concentration. The culture chamber is then conditioned and is ready to receive mysids.
7. Add only a few (5-20) mysids at first, to determine if conditions are favorable. If these mysids are still doing well after a week, several hundred more can be added.

14.6.13.1.7 It is important to add enough food to keep the adult animals from cannibalizing the young, but not so much that the DO is depleted or that there is a buildup of toxic concentrations of ammonia and nitrite. Just enough newly-hatched *Artemia* nauplii are fed twice a day so that each feeding is consumed before the next feeding.

14.6.13.1.8 Natural seawater is recommended as the culture medium, but HSB may be used to make up the culture water if natural seawater is not available. EMSL-Cincinnati has successfully used FORTY FATHOMS® artificial sea salts for culturing and toxicity tests of mysids, and USEPA, Region 6 has used HW MARINEMIX® artificial sea salts.

14.6.13.1.9 Mysids, *Mysidopsis bahia*, should be cultured at a temperature of 26 ± 1°C. No water temperature control equipment is needed if the ambient laboratory temperature remains in the recommended range, and if there are no frequent, rapid, large temperature excursions in the culture room.

14.6.13.1.10 The salinity should be maintained at 30 ± 2‰, or at a lower salinity (but not less than 20‰) if most of the tests will be conducted at a lower salinity.

14.6.13.1.11 Day/night cycles prevailing in most laboratories will provide adequate illumination for normal growth and reproduction. A 16-h/8-h day/night cycle in which the light is gradually increased and decreased to simulate dawn and dusk conditions, is recommended.

14.6.13.1.12 Mysid, *Mysidopsis bahia*, culture may suffer if DOs fall below 5 mg/L for extended periods. The undergravel filter will usually provide sufficient DO. If the DO drops below 5 mg/L at 25°C and 30‰, additional aeration should be provided. Measure the DO in the cultures daily the first week and then at least weekly thereafter.

14.6.13.1.13 Suspend a clear glass or plastic panel over the cultures, or use some other means of excluding dust and dirt, but leave enough space between the covers and culture tanks to allow circulation of air over the cultures.

14.6.13.1.14 If hydroids or worms appear in the cultures, remove the mysids and clean the chambers thoroughly, using soap and hot water. Rinse once with acid (10% HCl) and three times with distilled or deionized water. Mysids with attached hydroids should be discarded. Those without hydroids should be transferred by hand pipetting into three changes of clean seawater before returning them to the cleaned culture chamber. To guard against predators, natural seawater should be filtered through a net with 30 μm mesh openings before entering the culture vessels.

14.6.13.1.15 Mysids, *Mysidopsis bahia*, are very sensitive to low pH and sudden changes in temperature. Care should be taken to maintain the pH at 8.0 ± 0.3, and to limit rapid changes in water temperature to less than 3°C.

14.6.13.1.16 Mysids, *Mysidopsis bahia*, should be handled carefully and as little as possible so that they are not unnecessarily stressed or injured. They should be transferred between culture chambers with long handled cups with netted bottoms. Animals should be transferred to the test vessels with a large bore pipette (4-mm), taking care to release the animals under the surface of the water. Discard any mysids that are injured during handling.

14.6.13.1.17 Culture Maintenance (Also See USEPA, 2002a)

14.6.13.1.17.1 Cultures in closed, recirculating systems are fed twice a day. If no nauplii are present in the culture chamber after four hours, the amount of food should be increased slightly. In flow-through systems, excess food can be a problem by promoting bacterial growth and low dissolved oxygen.

14.6.13.1.17.2 Careful culture maintenance is essential. The organisms should not be allowed to become too crowded. The cultures should be cropped as often as necessary to maintain a density of about 20 mysids per liter. At this density, at least 70% of the females should have eggs in their brood pouch. If they do not, the cultures are probably under stress, and the cause should be found and corrected. If the cause cannot be found, it may be necessary to restart the cultures with a clean culture chamber, a new batch of culture water, and clean gravel.

14.6.13.1.17.3 In closed, recirculating systems, about one third of the culture water should be replaced with newly prepared seawater every week. Before siphoning the old media from the culture, it is recommended that the sides of the vessel be scraped and the gravel carefully turned over to prevent excessive buildup of algal growth. Twice a year the mysids should be removed from the recirculating cultures, the gravel rinsed in clean seawater, the sides of the chamber washed with clean seawater, and the gravel and animals returned to the culture vessel with newly conditioned seawater. No detergent should be used, and care should be taken not to rinse all the bacteria from the gravel.

14.6.13.2 Test Organisms

14.6.13.2.1 The test is begun with 7-day-old juveniles. To have the test animals available and acclimated to test conditions at the start of the test, gravid females must be obtained from the stock culture eight days in advance of the test. Whenever possible, brood stock should be obtained from cultures having similar salinity, temperature, light regime, etc., as are to be used in the toxicity test.

14.6.13.2.2 Eight days before the test is to start, sufficient gravid females are placed in brood chambers. Assuming that 240 juveniles are needed for each test, approximately half this number (120) of gravid females should be transferred to brood chambers. The mysids are removed from the culture tank with a net or netted cup and placed in 20-cm diameter finger bowls. The gravid females are transferred from the finger bowls to the brood chambers with a large-bore pipette or, alternatively, are transferred by pouring the contents of the finger bowls into the water in the brood chambers.

14.6.13.2.3 The mysid juveniles may be collected for the toxicity tests by transferring gravid females from the stock cultures to netted (1000 µm) flow-through containers (Figure 1) held within 4-L glass, wide-mouth separatory funnels. Newly released juveniles can pass through the netting, whereas the females are retained. The gravid females are fed newly hatched *Artemia* nauplii, and are held overnight to permit the release of young. The juvenile mysids are collected by opening the stopcock on the funnel and collecting them in another container from which they are transferred to holding tanks using a wide bore (4 mm ID) pipette. The brood chambers usually require aeration to maintain sufficient DO and to keep the food in suspension.

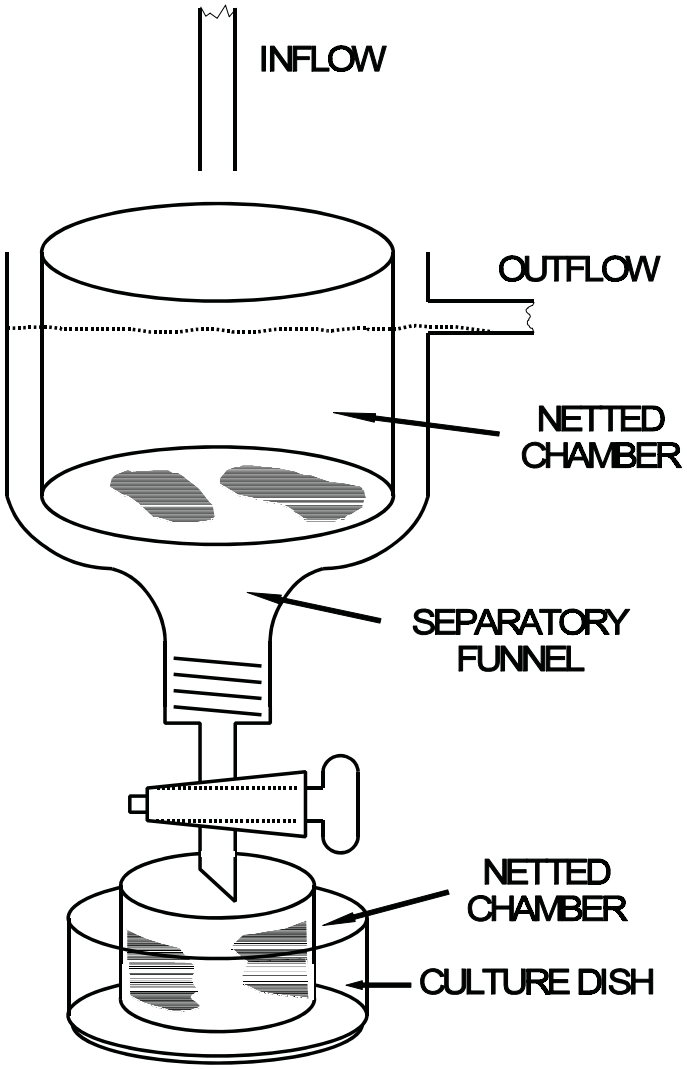

Figure 1. Apparatus (brood chamber) for collection of juvenile mysids, *Mysidopsis bahia*. From USEPA (1987d).

14.6.13.2.4 The temperature in the brood chamber should be maintained at the upper acceptable culture limit (26 - 27°C), or 1°C higher than the cultures, to encourage faster brood release. At this temperature, sufficient juveniles should be produced for the test.

14.6.13.2.5 The newly released juveniles (age = 0 days) are transferred to 20-L glass aquaria (holding vessels) which are gently aerated. Smaller holding vessels may be used, but the density of organisms should not exceed 10 mysids per liter. The test animals are held in the holding vessel for six days prior to initiation of the test. The holding medium is renewed every other day.

14.6.13.2.6 During the holding period, the mysids are acclimated to the salinity at which the test will be conducted, unless already at that salinity. The salinity should be changed no more than 2‰ per 24 h to minimize stress on the juveniles.

14.6.13.2.7 The temperature during the holding period is critical to mysid development, and must be maintained at 26 ± 1°C. If the temperature cannot be maintained in this range, it is advisable to hold the juveniles an additional day before beginning the test.

14.6.13.2.8 During the holding period, just enough newly-hatched *Artemia* nauplii are fed twice a day (a total of at least 150 nauplii per mysid per day) so that some food is constantly present.

14.6.13.2.9 If the test is to be performed in the field, the juvenile mysids, *Mysidopsis bahia*, should be gently siphoned into 1-L polyethylene wide-mouth jars with screw-cap lids filled two-thirds full with clean seawater from the holding tank. The water in these jars is aerated for 10 min, and the jars are capped and packed in insulated boxes for shipment to the test site. Food should not be added to the jars to prevent the development of excessive bacterial growth and a reduction in DO.

14.6.13.2.10 Upon arrival at the test site (in less than 24 h) the mysids, *Mysidopsis bahia*, are gently poured from the jars into 20-cm diameter glass culture dishes. The jars are rinsed with salt water to dislodge any mysids that may adhere to the sides. If the water appears milky, siphon off half of it with a netted funnel (to avoid siphoning the mysids) and replace with clean salt water of the same salinity and temperature. If no *Artemia* nauplii are present in the dishes, feed about 150 *Artemia* nauplii per mysid.

14.6.13.2.11 The pre-test holding conditions of test organisms (as well as the test conditions) have been shown to significantly influence the success of achieving the test acceptability criteria for the fecundity endpoint (egg production by 50% or more of control females). Temperature, feeding, and organism density are important factors in the rate of mysid development. Laboratories should optimize these factors (within the limits of the test procedure) during both the pre-test holding period and the testing period to encourage achieving the test acceptability criteria for the fecundity endpoint. If test organisms are purchased, the testing laboratory should also confer with the supplier to ensure that pre-test holding conditions are optimized to successfully achieve the fecundity endpoint. Lussier *et al.* (1999) found that by increasing holding temperature and test temperature from 26°C ± 1°C to 26°C - 27°C and maintaining holding densities to ≤10 organisms / L, the percentage of tests meeting the test acceptability criteria for fecundity increased from 60% to 97%. While the fecundity endpoint is an optional endpoint, it is often the most sensitive measure of toxicity, and the 7-d mysid test estimates the chronic toxicity of effluents most effectively when all three endpoints (survival, growth, and fecundity) are measured (Lussier *et al.* 1999).

14.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

14.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

14.8 CALIBRATION AND STANDARDIZATION

14.8.1 See Section 4, Quality Assurance.

14.9 QUALITY CONTROL

14.9.1 See Section 4, Quality Assurance.

14.9.2 The reference toxicant recommended for use with the mysid 7-day test is copper sulfate or sodium dodecyl sulfate.

14.10 TEST PROCEDURES

14.10.1 TEST DESIGN

14.10.1.1 The test consists of at least five effluent concentrations plus a site water control and a reference water treatment (natural seawater or seawater made up from hypersaline brine, or equivalent).

14.10.1.2 Effluent concentrations are expressed as percent effluent.

14.10.1.3 Eight replicate test vessels, each containing 5 to 7 day old animals, are used per effluent concentration and control.

14.10.2 TEST SOLUTIONS

14.10.2.1 Receiving waters

14.10.2.1.1 The sampling point(s) is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using eight replicate chambers per test, each containing 150 mL, and 400 mL for chemical analysis, would require approximately 1.6 L or more of sample per test per day.

14.10.2.2 Effluents

14.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.** If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ and 70% at 30‰ salinity.

14.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If high mortality is observed during the first 1-to-2 h of the test, additional dilutions at the lower range of effluent concentrations should be added.

14.10.2.2.3 The volume of effluent required for daily renewal of eight replicates per concentration for five concentrations of effluent and a control, each containing 150 mL of test solution, is approximately 1200 mL. Prepare enough test solution (approximately 1600 mL) at each effluent concentration to provide 400 mL additional volume for chemical analyses.

14.10.2.2.4 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (26 ± 1°C) and maintained at that temperature during the addition of dilution water.

14.10.2.2.5 Higher effluent concentrations (i.e., 25%, 50%, and 100%) may require aeration to maintain adequate dissolved oxygen concentrations. However, if one solution is aerated, all concentrations must be aerated. Aerate effluent as it warms and continue to gently aerate test solutions in the test chambers for the duration of the test.

14.10.2.2.6 Effluent dilutions should be prepared for all replicates in each treatment in one flask to minimize variability among the replicates. The test chambers (cups) are labeled with the test concentration and replicate number. Dispense 150 mL of the appropriate effluent dilution to each test chamber.

14.10.2.3 Dilution Water

14.10.2.3.1 Dilution water may be uncontaminated natural seawater (receiving water), HSB prepared from natural seawater, or artificial seawater prepared from FORTY FATHOMS® or GP2 sea salts (see Table 1 and Section 7, Dilution Water). Other artificial sea salts may be used for culturing mysid and for the survival, growth, and fecundity test if the control criteria for acceptability of test data are satisfied.

14.10.3 START OF THE TEST

14.10.3.1 The test should begin as soon as possible, preferably within 24 h after sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the test be started more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.10.3.2 Begin the test by randomly placing five animals (one at a time) in each test cup of each treatment using a large bore (4 mm ID) pipette (see Appendix A for an example of randomization). It is easier to capture the animals if the volume of water in the dish is reduced and the dish is placed on a light table. It is recommended that the transfer pipette be rinsed frequently because mysids tend to adhere to the inside surface.

14.10.4 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

14.10.4.1 The light quality and intensity under ambient laboratory conditions are generally adequate. Light intensity of 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h light and 8 h darkness. It is critical that the test water temperature be maintained at 26 ± 1°C. It is recommended that the test water temperature be continuously recorded. The salinity should vary no more than ± 2‰ among chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

14.10.4.1.1 If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be at least 2.5 cm deep.

14.10.4.1.2 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test cups with clear polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

14.10.5 DISSOLVED OXYGEN (DO) CONCENTRATION

14.10.5.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain a satisfactory DO. The DO should be measured on new solutions at the start of the test (Day 0) and before daily renewal of test solutions on subsequent days. The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1-mL KIMAX® serological pipet, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue stress on the mysid.

14.10.6 FEEDING

14.10.6.1 *Artemia* nauplii are prepared as described above.

14.10.6.2 During the test, the mysids in each test chamber should be fed *Artemia* nauplii, (less than 24-h old), at the rate of 150 nauplii per mysid per day. Adding the entire daily ration at a single feeding immediately after test solution renewal may result in a significant DO depression. Therefore, it is preferable to feed half of the daily

ration immediately after test solution renewal, and the second half 8 - 12 h later. Increase the feeding if the nauplii are consumed in less than 4 h. It is important that the nauplii be washed before introduction to the test chamber.

14.10.7 DAILY CLEANING OF TEST CHAMBERS

14.10.7.1 Before the daily renewal of test solutions, uneaten and dead *Artemia*, dead mysids and other debris are removed from the bottom of the test chambers with a pipette. As much of the uneaten *Artemia* as possible should be removed from each chamber to ensure that the mysids principally eat new hatched nauplii. By placing the test chambers on a light box, inadvertent removal of live mysids can be greatly reduced because they can be more easily seen. Any incidence of removal of live mysids from the test chambers during cleaning, and subsequent return to the chambers should be noted in the test records.

14.10.8 OBSERVATIONS DURING THE TEST

14.10.8.1 Routine Chemical and Physical Determinations

14.10.8.1.1 DO is measured at the beginning and end of each 24-h exposure period in one test chamber at each test concentration and in the control.

14.10.8.1.2 Temperature, pH, and salinity are measured at the end of each 24-h exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at least at the end of the test to determine temperature variation in environmental chamber.

14.10.8.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

14.10.8.2 Routine Biological Observations

14.10.8.2.1 The number of live mysids are counted and recorded each day when the test solutions are renewed (Figure 7). Dead animals and excess food should be removed with a pipette before test solutions are renewed.

14.10.8.2.2 Protect the mysids from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of the dead mysids, carefully. Make sure the mysids remain immersed during the performance of the above operations.

14.10.9 TEST SOLUTION RENEWAL

14.10.9.1 Before the daily renewal of test solutions, slowly pour off all but 10 mL of the old test medium into a 20 cm diameter culture dish on a light table. Be sure to check for animals that may have adhered to the sides of the test chamber. Rinse them back into the test cups. Add 150 mL of new test solution slowly to each cup. Check the culture dish for animals that may have been poured out with the old media, and return them to the test chamber.

14.10.10 TERMINATION OF THE TEST

14.10.10.1 After measuring the DO, pH, temperature, and salinity and recording survival, terminate the test by pouring off the test solution in all the cups to a one cm depth and refilling the cups with clean seawater. This will keep the animals alive, but not exposed to the toxicant, while waiting to be examined for sex and the presence of eggs.

14.10.10.2 The live animals must be examined for eggs and the sexes determined within 12 h of the termination of the test. If the test was conducted in the field, and the animals cannot be examined on site, the live animals should be shipped back to the laboratory for processing. Pour each replicate into a labeled 100 mL plastic screw capped

jar, and send to the laboratory immediately.

14.10.10.3 If the test was conducted in the laboratory, or when the test animals arrive in the laboratory from the field test site, the test organisms must be processed immediately while still alive as follows:

14.10.10.3.1 Examine each replicate under a stereomicroscope (240X) to determine the number of immature animals, the sex of the mature animals, and the presence or absence of eggs in the oviducts or brood sacs of the females (see Figures 3-6). This must be done while the mysids are alive because they turn opaque upon dying. This step should not be attempted by a person who has not had specialized training in the determination of sex and presence of eggs in the oviduct. NOTE: Adult females without eggs in the oviduct or brood sac look like immature mysids (see Figure 6).

TEST: _____

START DATE:_____

SALINITY:_____

	TRTMT	TEMP	SALINITY	D.O.	pH	TRTMT	TEMP	SALINITY	D.O.	pH	
DAY 1	REP										
	REP										
DAY 2	REP										
	REP										
DAY 3	REP										
	REP										
DAY 4	REP										
	REP										
DAY 5	REP										
	REP										
DAY 6	REP										
	REP										
DAY 7	REP										
	REP										
	TRTMT	TEMP	SALINITY	D.O.	pH	TRTMT	TEMP	SALINITY	D.O	pH	
DAY 1	REP										
	REP										
DAY 2	REP										
	REP										
DAY 3	REP										
	REP										
DAY 4	REP										
	REP										
DAY 5	REP										
	REP										
DAY 6	REP										
	REP										
DAY 7	REP										
	REP										

Figure 2. Data form for the mysid, *Mysidopsis bahia*, water quality measurements. From USEPA (1987d).

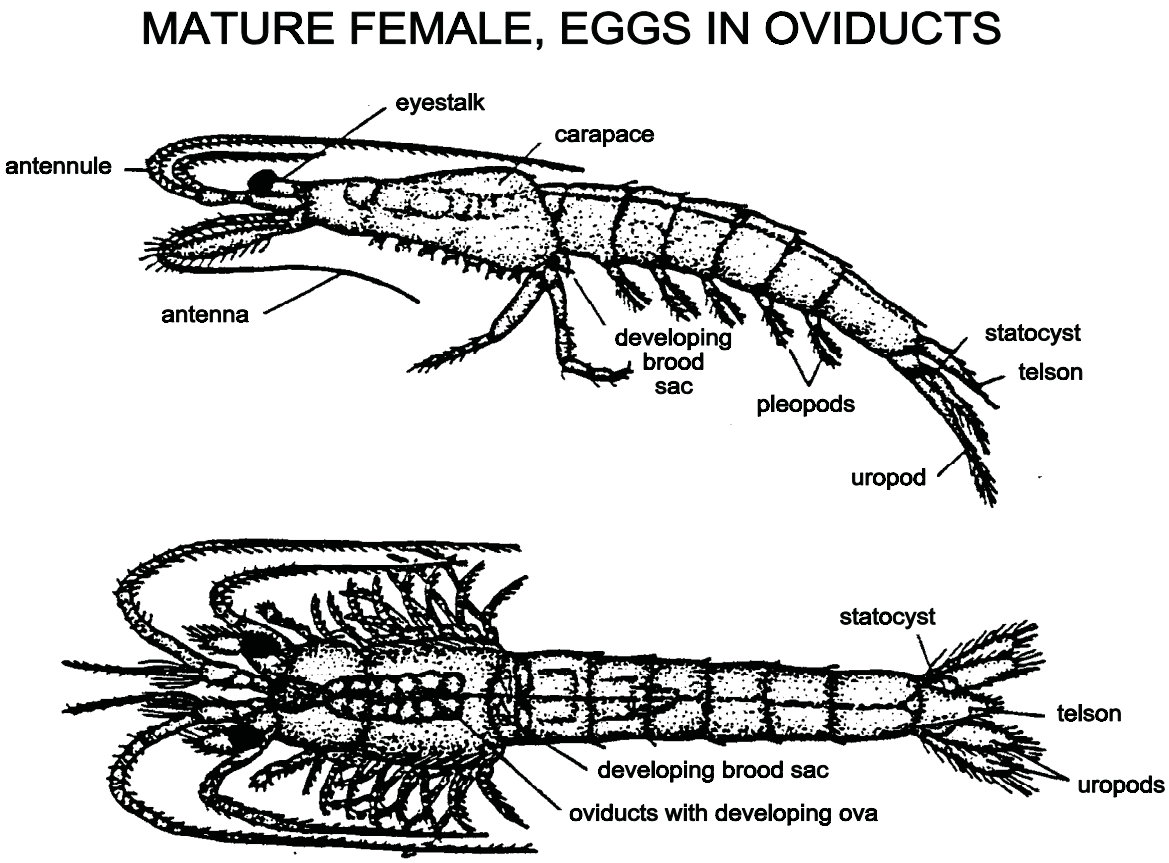

Figure 3. Mature female mysid, *Mysidopsis bahia*, with eggs in oviducts. From USEPA (1987d).

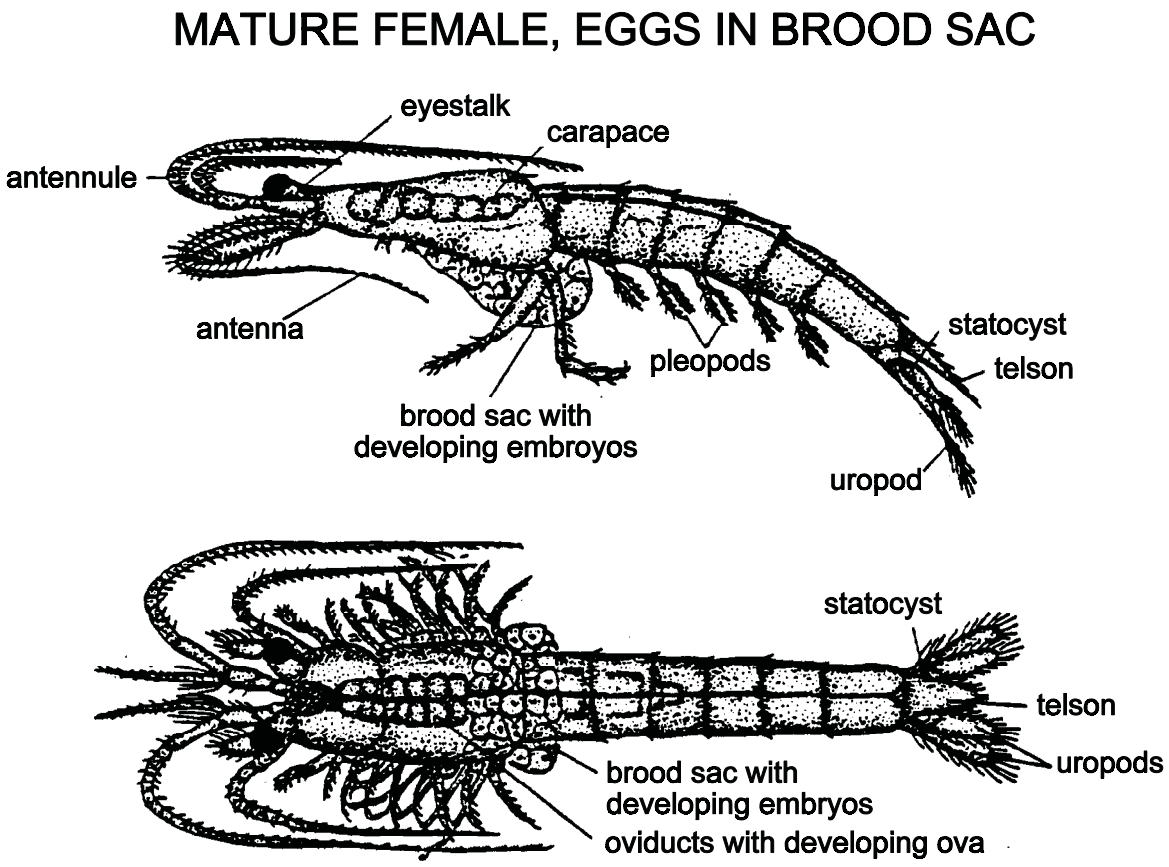

Figure 4. Mature female mysid, *Mysidopsis bahia*, with eggs in oviducts and developing embryos in the brood sac. Above: lateral view. Below: dorsal view. From USEPA (1987d).

14.10.10.3.2 Record the number of immatures, males, females with eggs and females without eggs on data sheets (Figure 7).

14.10.10.3.3 Rinse the mysids by pipetting them into a small netted cup and dipping the cup into a dish containing deionized water. Using forceps, place the mysids from each replicate cup on tared weighing boats and dry at 60°C for 24 h or at 105°C for at least 6 h.

14.10.10.3.4 Immediately upon removal from the drying oven, the weighing pans were placed in a dessicator until weighed, to prevent absorption of moisture from the air. Weigh to the nearest mg. Record weighing pans and subtract the tare weight to determine the dry weight of the mysid in each replicate. Record the weights (Figure 8). For each test chamber, divide the first dry weight by the number of original mysids per replicate to determine the average individual dry weight and record data. For the controls also calculate the mean weight per surviving mysid in the test chamber to evaluate if weights met test acceptability criteria (see Subsection 14.12).

14.10.9.3.5 Pieces of aluminum foil (1-cm square) or small aluminum weighing pans can be used for dry weight analyses. The weighing pans should not exceed 10 mg in weight.

14.10.9.3.6 Number each pan with a waterproof pen with the treatment concentration and replicate number. Forty-eight (48) weigh pans are required per test if all the organisms survive.

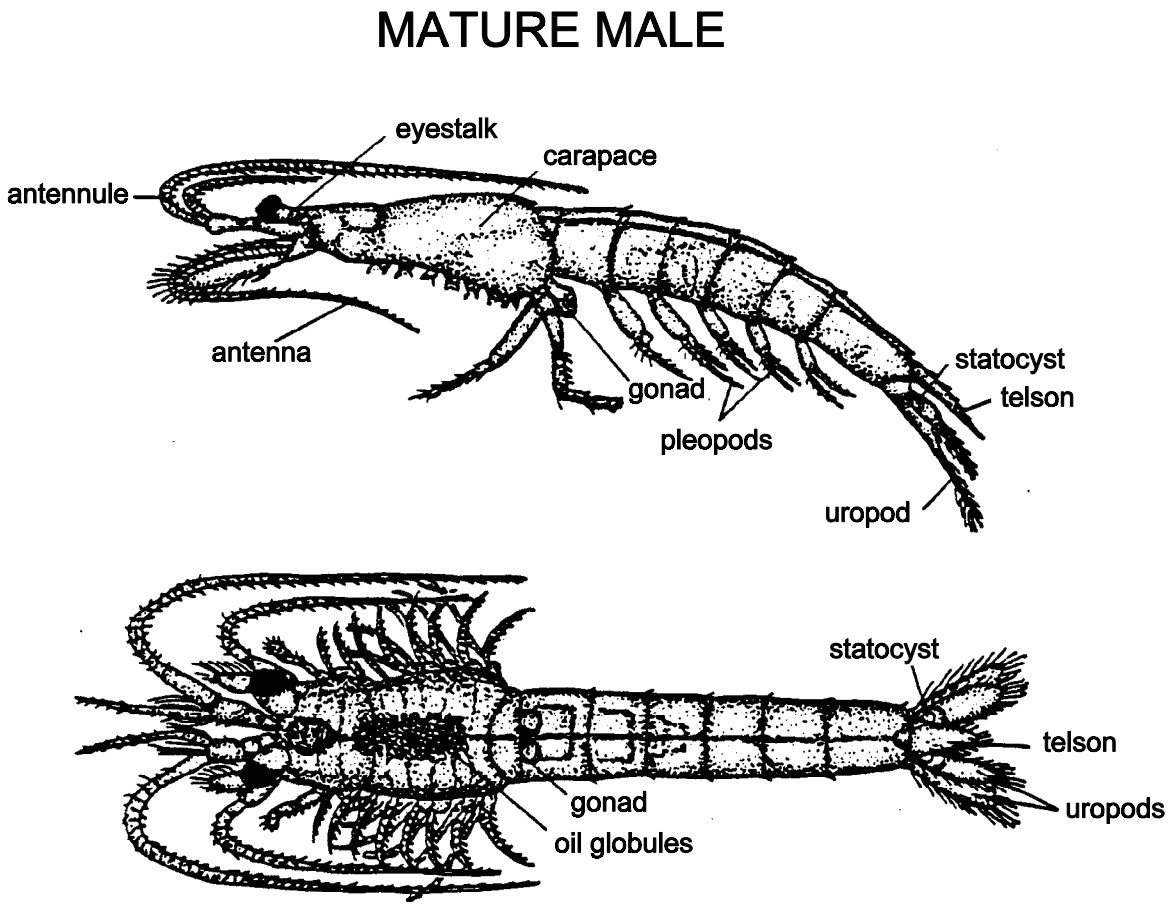

Figure 5. Mature male mysid, *Mysidopsis bahia*. From USEPA (1987d).

14.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

14.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

14.12 ACCEPTABILITY OF TEST RESULTS

14.12.1 The minimum requirements for an acceptable test are 80% survival and an average weight of at least 0.20 mg/surviving mysid in the controls. If fecundity in the controls is adequate (egg production by 50% of females), fecundity should be used as a criterion of effect in addition to survival and growth.

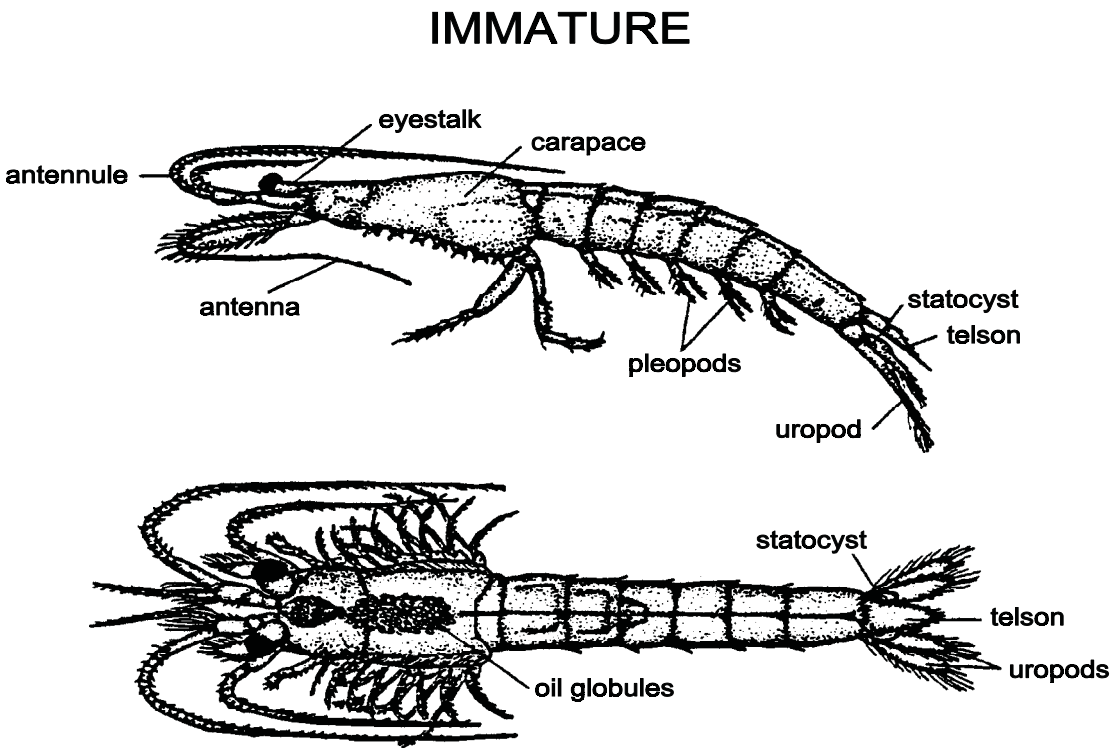

Figure 6. Immature mysid, *Mysidopsis bahia*, (A) lateral view, (B) dorsal view. From USEPA (1987d).

14.13 DATA ANALYSIS

14.13.1 GENERAL

14.13.1.1 Tabulate and summarize the data. Table 4 presents a sample set of survival, growth, and fecundity data.

14.13.1.2 The endpoints of the mysid 7-day chronic test are based on the adverse effects on survival, growth, and egg development. The LC50, the IC25, and the IC50 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for survival, growth, and fecundity are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50, IC25, and IC50. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for survival, growth, and fecundity, but included in the estimation of the LC50, IC25, and IC50. See the Appendices for examples of the manual computations, and examples of data input and program output.

14.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

TEST: _____

START DATE: _____

SALINITY: _____

TREATMENT/ REPLICATE	DAY 1 # ALIVE	DAY 2 # ALIVE	DAY 3 # ALIVE	DAY 4 # ALIVE	DAY 5 # ALIVE	DAY 6 # ALIVE	DAY 7 # ALIVE	FEMALES W/EGGS	FEMALES NO EGGS	MALES	IMMATURES
C											
1											
2											

Figure 7. Data form for the mysid, *Mysidopsis bahia*, survival and fecundity data. From USEPA (1987d).

TEST: _____

START DATE: _____

SALINITY: _____

TREATMENT/ REPLICATE		DAY 1 # ALIVE	DAY 2 # ALIVE	DAY 3 # ALIVE	DAY 4 # ALIVE	DAY 5 # ALIVE	DAY 6 # ALIVE	DAY 7 # ALIVE	FEMALES W/EGGS	FEMALES NO EGGS	MALES	IMMATURES
	1											
	2											
	3											
3	4											
	5											
	6											
	7											
	8											
	1											
	2											
	3											
4	4											
	5											
	6											
	7											
	8											
	1											
	2											
	3											
5	4											
	5											
	6											
	7											
	8											

Figure 7. Data form for the mysid, *Mysidopsis bahia*, survival and fecundity data (CONTINUED). From USEPA (1987d).

TEST: _____

START DATE: _____

SALINITY: _____

TREATMENT/REPLICATE		PAN #	TARE WT.	TOTAL WT.	ANIMAL WT.	# OF ANIMALS	WT./ANIMAL
C	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
1	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
2	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						

Figure 8. Data form for the mysid, *Mysidopsis bahia*, dry weight measurements. From USEPA (1987d).

TEST: _____

START DATE: _____

SALINITY: _____

TREATMENT/REPLICATE		PAN #	TARE WT.	TOTAL WT.	ANIMAL WT.	# OF ANIMALS	WT./ANIMAL
	1						
	2						
	3						
3	4						
	5						
	6						
	7						
	8						
	1						
	2						
	3						
4	4						
	5						
	6						
	7						
	8						
	1						
	2						
	3						
5	4						
	5						
	6						
	7						
	8						

Figure 8. Data form for the mysid, *Mysidopsis bahia*, dry weight measurements (CONTINUED). From USEPA (1987d).

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE MYSID, *MYSIDOPSIS BAHIA*, SEVEN DAY SURVIVAL, GROWTH, AND FECUNDITY TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1007.0)[1]

1.	Test type:	Static renewal (required)
2.	Salinity:	20‰ to 30‰ (± 2‰ of the selected test salinity) (recommended)
3.	Temperature:	26 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4.	Light quality:	Ambient laboratory illumination (recommended)
5.	Light intensity:	10-20 μE/m^2/s (50-100 ft-c.) (ambient laboratory levels) (recommended)
6.	Photoperiod:	16 h light, 8 h darkness, with phase in/out period (recommended)
7.	Test chamber:	8 oz plastic disposable cups, or 400 mL glass beakers (recommended)
8.	Test solution volume:	150 mL per replicate (recommended minimum)
9.	Renewal of test solutions:	Daily (required)
10.	Age of test organisms:	7 days (required)
11.	No. organisms per test chamber:	5 (required minimum)
12.	No. replicate chambers per concentration:	8 (required minimum)
13.	No. larvae per concentration:	40 (required minimum)
14.	Source of food:	Newly hatched *Artemia* nauplii (less than 24 h old)(required)
15.	Feeding regime:	Feed 150 24 h old nauplii per mysid daily, half after test solution renewal and half after 8-12 h (recommended)
16.	Cleaning:	Pipette excess food from cups daily immediately before test solution renewal and feeding (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE MYSID, *MYSIDOPSIS BAHIA*, SEVEN DAY SURVIVAL, GROWTH, AND FECUNDITY TEST WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1007.0) (CONTINUED)

17. Aeration:	None unless DO falls below 4.0 mg/L, then gently aerate in all cups (recommended)
18. Dilution water:	Uncontaminated source of natural seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, GP2 or equivalent) (available options)
19. Test concentrations:	Effluents: 5 and a control (required) Receiving waters: 100% receiving water (or minimum of 5) and a control (recommended)
20. Dilution factor:	Effluents: ≥ 0.5 series (required) Receiving waters: None, or ≥ 0.5 (recommended)
21. Test duration:	7 days (required)
22. Endpoints:	Survival and growth (required); and egg development (recommended)
23. Test acceptability criteria:	80% or greater survival, average dry weight 0.20 mg or greater in controls (required); fecundity may be used if 50% or more of females in controls produce eggs (required if fecundity endpoint used)
24. Sampling requirements:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
25. Sample volume required:	3 L per day (recommended)

TABLE 4. DATA FOR *MYSIDOPSIS BAHIA* 7-DAY SURVIVAL, GROWTH, AND FECUNDITY TEST[1]

Treatment	Replicate Chamber	Total Mysids	No. Alive	Total Females	Females w/Eggs	Mean Weight
Control	1	5	4	1	1	0.146
	2	5	4	2	2	0.118
	3	5	5	3	2	0.216
	4	5	5	1	1	0.199
	5	5	5	2	2	0.176
	6	5	5	5	4	0.243
	7	5	5	2	2	0.213
	8	5	4	3	3	0.144
50 ppb	1	5	4	2	1	0.154
	2	5	5	3	1	0.193
	3	5	4	3	2	0.190
	4	5	4	0	0	0.190
	5	5	5	5	2	0.256
	6	5	5	2	1	0.191
	7	5	4	4	1	0.122
	8	5	5	3	1	0.177
100 ppb	1	5	3	3	1	0.114
	2	5	5	2	1	0.172
	3	5	5	1	0	0.160
	4	5	5	2	1	0.199
	5	5	5	3	2	0.165
	6	5	3	1	0	0.145
	7	5	4	4	1	0.207
	8	5	4	0	0	0.186
210 ppb	1	5	5	1	0	0.153
	2	5	4	2	0	0.094
	3	5	1	1	0	0.017
	4	5	4	3	0	0.122
	5	5	3	1	0	0.052
	6	5	4	2	0	0.154
	7	5	4	1	0	0.110
	8	5	4	3	0	0.103
450 ppb	1	5	0	0	0	- -
	2	5	1	0	0	0.012
	3	5	0	0	0	- -
	4	5	1	0	0	0.002
	5	5	0	0	0	- -
	6	5	0	0	0	- -
	7	5	0	0	0	- -
	8	5	2	1	0	0.081

[1] Data provided by Lussier, Kuhn and Sewall, Environmental Research Laboratory, U.S. Environmental Protection Agency, Narragansett, RI.

14.13.2 EXAMPLE OF ANALYSIS OF MYSID, *MYSIDOPSIS BAHIA*, SURVIVAL DATA

14.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 9 and 10. The response used in the analysis is the proportion of animals surviving in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the LC50 endpoint. Concentrations at which there is no survival in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the LC, EC, and IC endpoints.

14.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

14.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t-test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

14.13.2.4 Probit Analysis (Finney, 1971; see Appendix H) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit model, the Spearman-Karber method, the Trimmed Spearman-Karber method, or the Graphical method may be used (see Appendices I-K).

14.13.2.5 The proportion of survival in each replicate must first be transformed by the arc sine transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each concentration including the control are listed in Table 5. A plot of the survival data is provided in Figure 11.

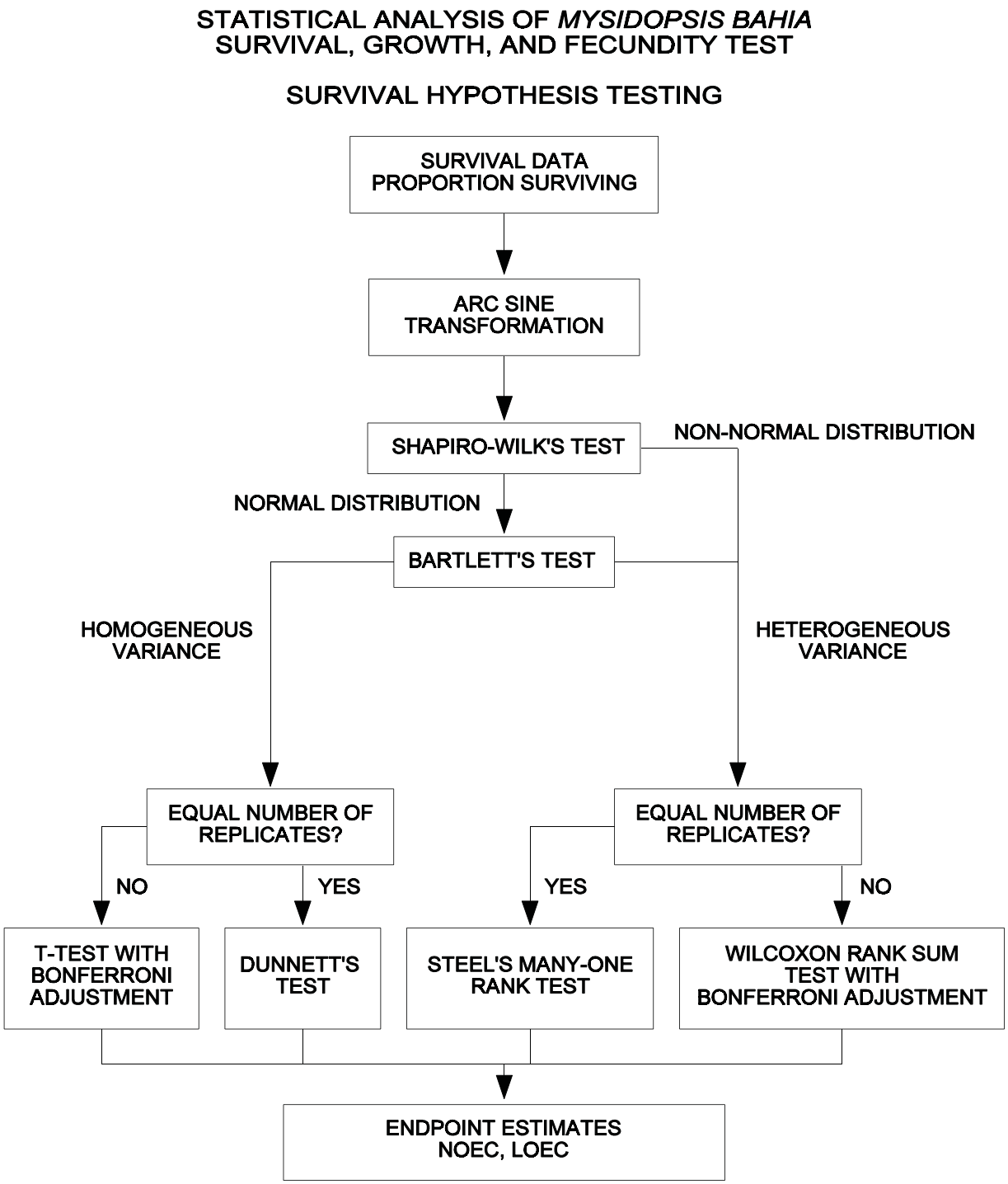

Figure 9. Flowchart for statistical analysis of mysid, *Mysidopsis bahia*, survival data by hypothesis testing.

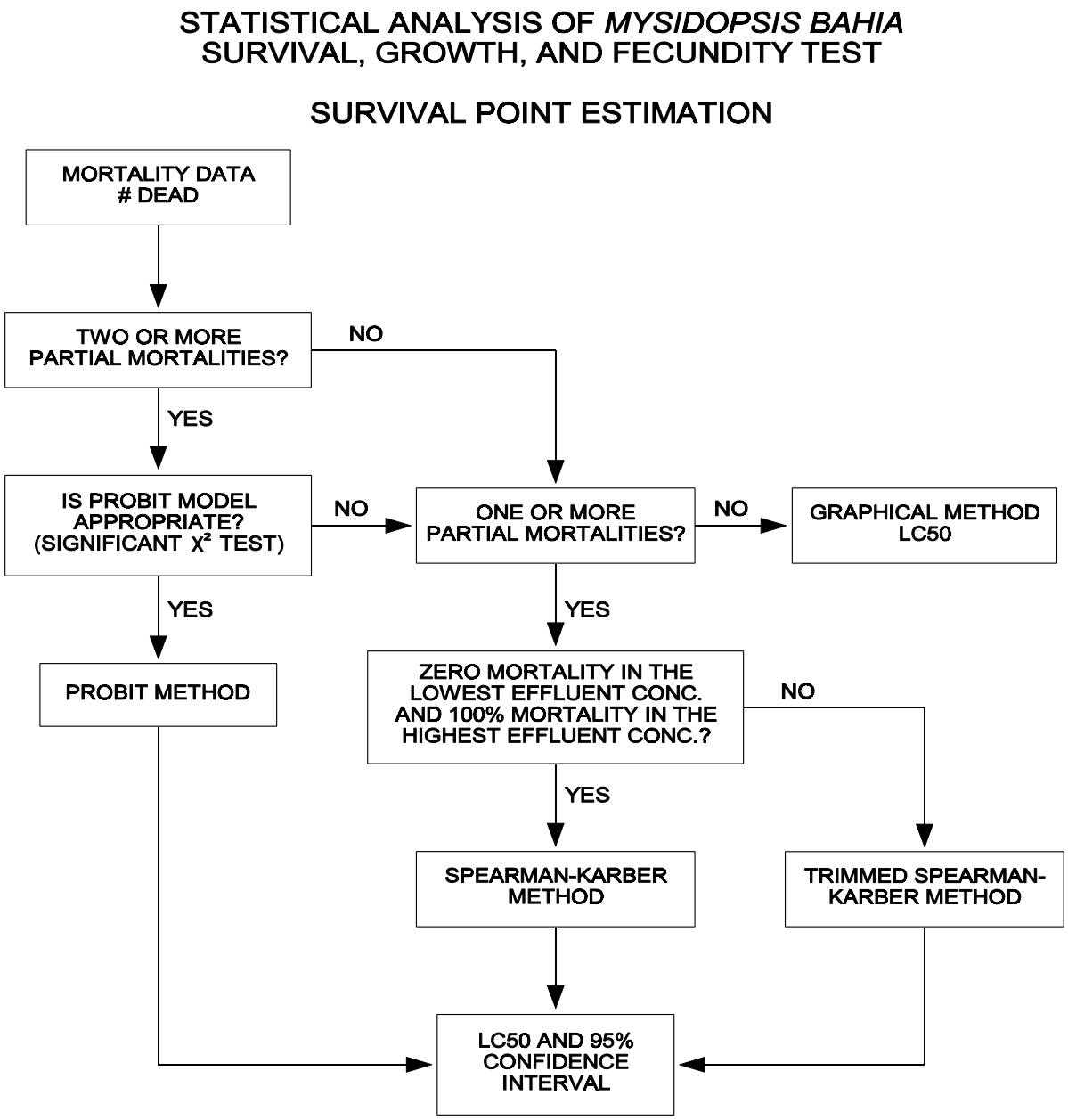

Figure 10. Flowchart for statistical analysis of mysid, *Mysidopsis bahia*, survival data by point estimation.

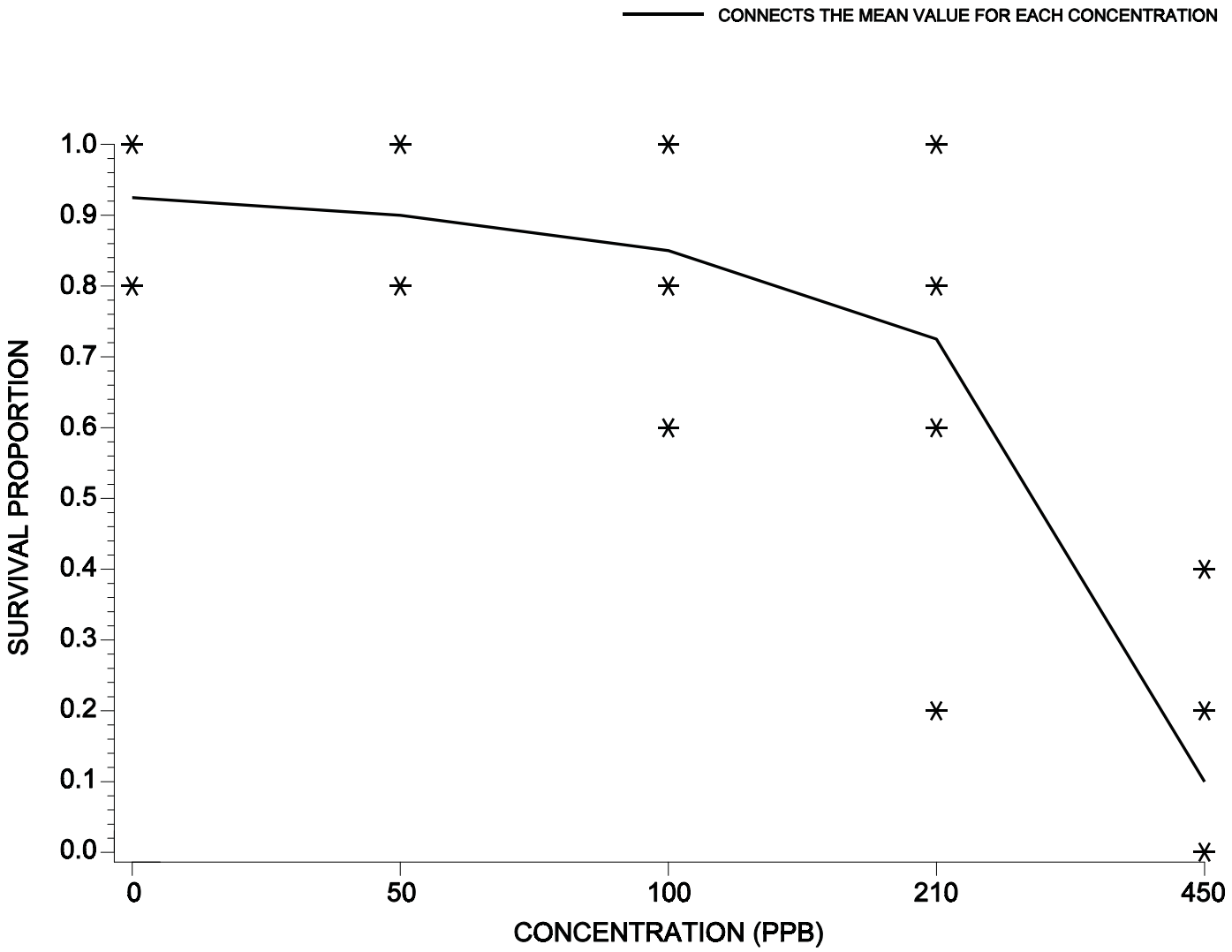

Figure 11. Plot of survival proportions of mysids, *Mysidopsis bahia*, at each treatment level.

TABLE 5. MYSID, *MYSIDOPSIS BAHIA*, SURVIVAL DATA

			Concentration (ppb)			
	Replicate	Control	50.0	100.0	210.0	450.0
RAW	1	0.80	0.80	0.60	1.00	0.00
	2	0.80	1.00	1.00	0.80	0.20
	3	1.00	0.80	1.00	0.20	0.00
	4	1.00	0.80	1.00	0.80	0.20
	5	1.00	1.00	1.00	0.60	0.00
	6	1.00	1.00	0.60	0.80	0.00
	7	1.00	0.80	0.80	0.80	0.00
	8	0.80	1.00	0.80	0.80	0.40
ARC SINE TRANS-FORMED	1	1.107	1.107	0.886	1.345	0.225
	2	1.107	1.345	1.345	1.107	0.464
	3	1.345	1.107	1.345	0.464	0.225
	4	1.345	1.107	1.345	1.107	0.464
	5	1.345	1.345	1.345	0.886	0.225
	6	1.345	1.345	0.886	1.107	0.225
	7	1.345	1.107	1.107	1.107	0.225
	8	1.107	1.345	1.107	1.107	0.685
Mean (Y_i)		1.256	1.226	1.171	1.029	0.342
S_i^2		0.015	0.016	0.042	0.067	0.031
i		1	2	3	4	5

14.13.2.6 Test for Normality

14.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 6.

14.13.2.6.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations.

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control (Site Water)	Concentration (ppb) 50.0	100.0	210.0	450.0
1	-0.149	-0.119	-0.285	0.316	-0.117
2	-0.149	0.119	0.174	0.078	0.121
3	0.089	-0.119	0.174	-0.565	-0.117
4	0.089	-0.119	0.174	0.078	0.121
5	0.089	0.119	0.174	-0.142	-0.117
6	0.089	0.119	-0.285	0.078	-0.117
7	0.089	-0.119	-0.064	0.078	-0.117
8	-0.149	0.119	-0.064	0.078	0.342

14.13.2.6.3 For this set of data, $n = 40$

$$\overline{X} = \frac{1}{40}(-0.006) = 0.0$$

$$D = 1.197$$

14.13.2.6.4 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 7.

14.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 40 and k = 20. The a_i values are listed in Table 8.

14.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 8. For this data in this example:

$$W = \frac{1}{1.197}(1.0475)^2 = 0.9167$$

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.565	21	0.078
2	-0.285	22	0.078
3	-0.285	23	0.078
4	-0.149	24	0.089
5	-0.149	25	0.089
6	-0.149	26	0.089
7	-0.143	27	0.089
8	-0.119	28	0.089
9	-0.119	29	0.119
10	-0.119	30	0.119
11	-0.119	31	0.119
12	-0.117	32	0.119
13	-0.117	33	0.121
14	-0.117	34	0.121
15	-0.117	35	0.174
16	-0.117	36	0.174
17	-0.064	37	0.174
18	-0.064	38	0.174
19	0.078	39	0.316
20	0.078	40	0.342

14.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 14.13.2.6.6 with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 40 observations is 0.919. Since W = 0.9167 is less than the critical value, conclude that the data are not normally distributed.

14.13.2.6.8 Since the data do not meet the assumption of normality, Steel's Many-one Rank Test will be used to analyze the survival data.

14.13.2.7 Steel's Many-one Rank Test

14.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ... , 16) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

14.13.2.7.2 An example of assigning ranks to the combined data for the control and 50.0 ppb concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are then summed for each concentration level, as shown in Table 11.

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.3964	0.907	$X^{(40)} - X^{(1)}$
2	0.2737	0.601	$X^{(39)} - X^{(2)}$
3	0.2368	0.459	$X^{(38)} - X^{(3)}$
4	0.2098	0.323	$X^{(37)} - X^{(4)}$
5	0.1878	0.323	$X^{(36)} - X^{(5)}$
6	0.1691	0.323	$X^{(35)} - X^{(6)}$
7	0.1526	0.264	$X^{(34)} - X^{(7)}$
8	0.1376	0.240	$X^{(33)} - X^{(8)}$
9	0.1237	0.238	$X^{(32)} - X^{(9)}$
10	0.1108	0.238	$X^{(31)} - X^{(10)}$
11	0.0986	0.238	$X^{(30)} - X^{(11)}$
12	0.0870	0.236	$X^{(29)} - X^{(12)}$
13	0.0759	0.206	$X^{(28)} - X^{(13)}$
14	0.0651	0.206	$X^{(27)} - X^{(14)}$
15	0.0546	0.206	$X^{(26)} - X^{(15)}$
16	0.0444	0.206	$X^{(25)} - X^{(16)}$
17	0.0343	0.153	$X^{(24)} - X^{(17)}$
18	0.0244	0.142	$X^{(23)} - X^{(18)}$
19	0.0146	0.0	$X^{(22)} - X^{(19)}$
20	0.0049	0.0	$X^{(21)} - X^{(20)}$

14.13.2.7.3 For this example, determine if the survival in any of the concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus compare the rank sums for the survival at each of the various concentration levels with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control) and eight replicates is 47 (See Table 5, Appendix E).

14.13.2.7.4 Since the rank sum for the 450 ppb concentration level is less than the critical value, the proportion surviving in that concentration is considered significantly less than that in the control. Since no other rank sums are less than or equal to the critical value, no other concentrations have a significantly lower proportion surviving than the control. Hence, the NOEC and the LOEC are assumed to be 210.0 ppb and 450.0 ppb, respectively.

14.13.2.8 Calculation of the LC50

14.13.2.8.1 The data used for the Probit Analysis is summarized in Table 12. For the Probit Analysis, run the USEPA Probit Analysis Program. An example of the program output is provided in Figure 12.

14.13.2.8.2 For this example, the chi-square test for heterogeneity was not significant. Thus Probit Analysis appears to be appropriate for this set of data.

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 50 PPB CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion of Total Mortality	Concentration
4	1.107	Control
4	1.107	Control
4	1.107	Control
4	1.107	50 ppb
4	1.107	50 ppb
4	1.107	50 ppb
4	1.107	50 ppb
12	1.571	Control
12	1.571	Control
12	1.571	Control
12	1.571	Control
12	1.571	Control
12	1.571	50 ppb
12	1.571	50 ppb
12	1.571	50 ppb
12	1.571	50 ppb

14.13.3 EXAMPLE OF ANALYSIS OF MYSID, *MYSIDOPSIS BAHIA,* GROWTH DATA

14.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 13. The response used in the statistical analysis is mean weight per original of males and females combined per replicate. Because this measurement is based on the number of original organisms exposed (rather than the number surviving), the measured response is a combined survival and growth endpoint that can be termed biomass. The IC25 and IC50 can be calculated for the growth data via a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC and LOEC for growth. Concentrations above the NOEC for survival are excluded from the hypothesis test for growth effects.

TABLE 10. TABLE OF RANKS[1]

		Concentration (ppb)			
Replicate	Control	50	100	210	450
1	1.107(4,5,6.5,10)	1.107(4)	0.886(1.5)	1.345(13.5)	0.225(3)
2	1.107(4,5,6.5,10)	1.345(12)	1.345(12)	1.107(6.5)	0.464(6.5)
3	1.345(12,12,13.5,14)	1.107(4)	1.345(12)	0.464(1)	0.225(3)
4	1.345(12,12,13.5,14)	1.107(4)	1.345(12)	1.107(6.5)	0.464(6.5)
5	1.345(12,12,13.5,14)	1.345(12)	1.345(12)	0.886(2)	0.225(3)
6	1.345(12,12,13.5,14)	1.345(12)	0.886(1.5)	1.107(6.5)	0.225(3)
7	1.345(12,12,13.5,14)	1.107(4)	1.107(5)	1.107(6.5)	0.225(3)
8	1.107(4,5,6.5,10)	1.345(12)	1.107(5)	1.107(6.5)	0.685(8)

[1]Control ranks are given in the order of the concentration with which they were ranked.

TABLE 11. RANK SUMS

Concentration	Rank Sum
50	64
100	61
210	49
450	36

14.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

14.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative. For detailed information on the Bonferroni adjustment, see Appendix D.

Probit Analysis of *Mysidopsis bahia* Survival Data

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	40	3	0.0750	0.0000
50.0000	40	4	0.1000	-0.0080
100.0000	40	6	0.1500	0.0480
210.0000	40	11	0.2750	0.1880
450.0000	40	36	0.9000	0.8880

Chi - Square for Heterogeneity (calculated) = 0.725
Chi - Square for Heterogeneity (tabular value) = 5.991

Probit Analysis of *Mysidopsis bahia* Survival Data

Estimated LC/EC Values and Confidence Limits

Point		Exposure Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
LC/EC	1.00	123.112	65.283	165.552
LC/EC	50.00	288.873	239.559	335.983

Figure 12. Output for USEPA Probit Analysis Program, Version 1.5.

TABLE 12. DATA FOR PROBIT ANALYSIS

		Concentration (ppb)			
	Control	50.0	100.0	210.0	450.0
No Dead	3	4	6	11	36
No Exposed	40	40	40	40	40

14.13.3.4 The data, mean and variance of the observations at each concentration including the control for this example are listed in Table 13. A plot of the data is provided in Figure 14. Since there is significant mortality in the 450 ppb concentration, its effect on growth is not considered.

TABLE 13. MYSID, *MYSIDOPSIS BAHIA*, GROWTH DATA

		Concentration (ppb)			
Replicate	Control	50.0	100.0	210.0	450.0
1	0.146	0.157	0.114	0.153	-
2	0.118	0.193	0.172	0.071	0.012
3	0.216	0.190	0.160	0.017	-
4	0.199	0.190	0.199	0.112	0.002
5	0.176	0.256	0.165	0.052	-
6	0.243	0.191	0.145	0.154	-
7	0.213	0.122	0.207	0.110	-
8	0.144	0.177	0.186	0.103	0.081
Mean (Y_i)	0.182	0.184	0.168	0.101	-
S_i^2	0.00186	0.00145	0.00091	0.00222	-
i	1	2	3	4	5

14.13.3.5 Test for Normality

14.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 14.

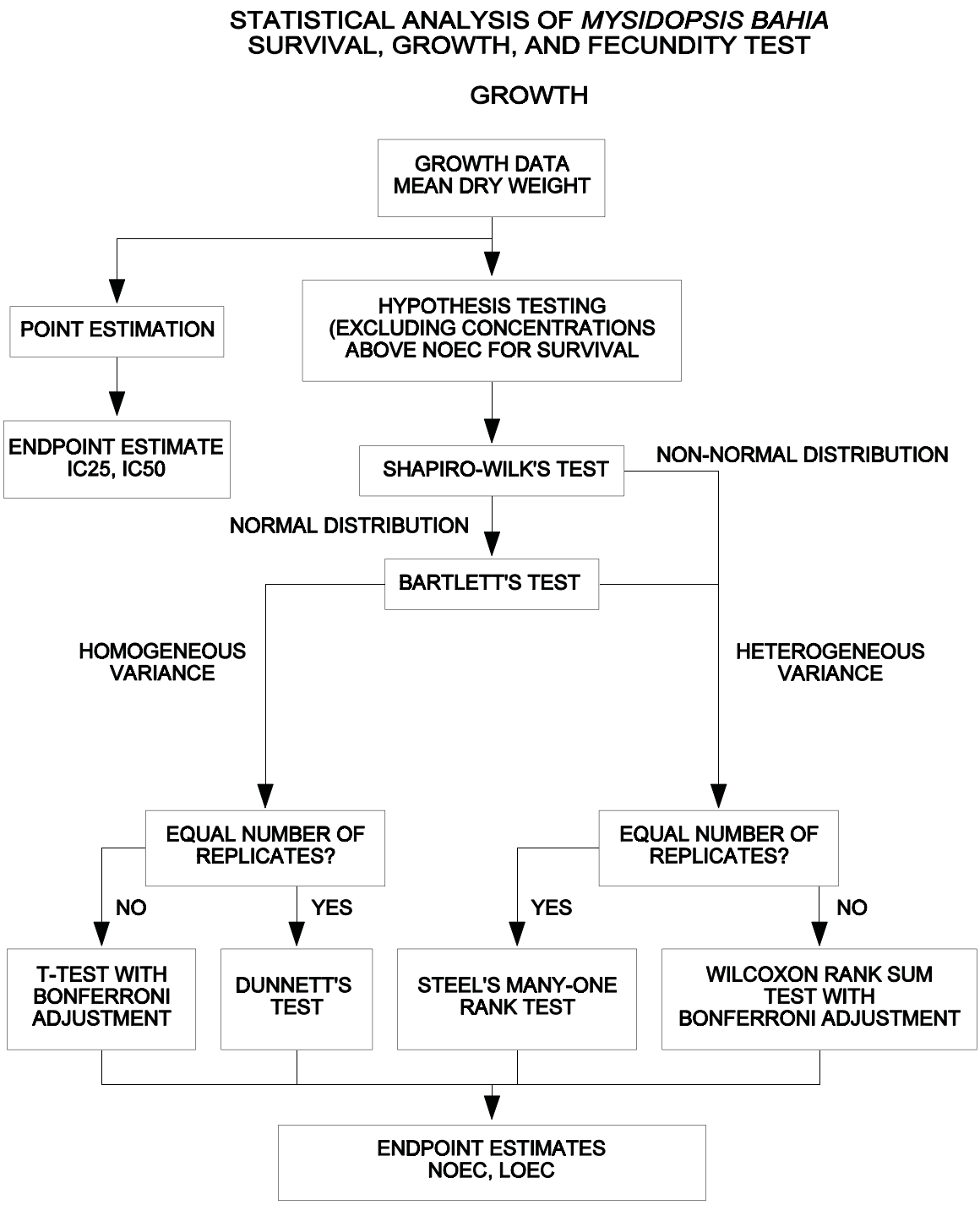

Figure 13. Flowchart for statistical analysis of mysid, *Mysidopsis bahia*, growth data.

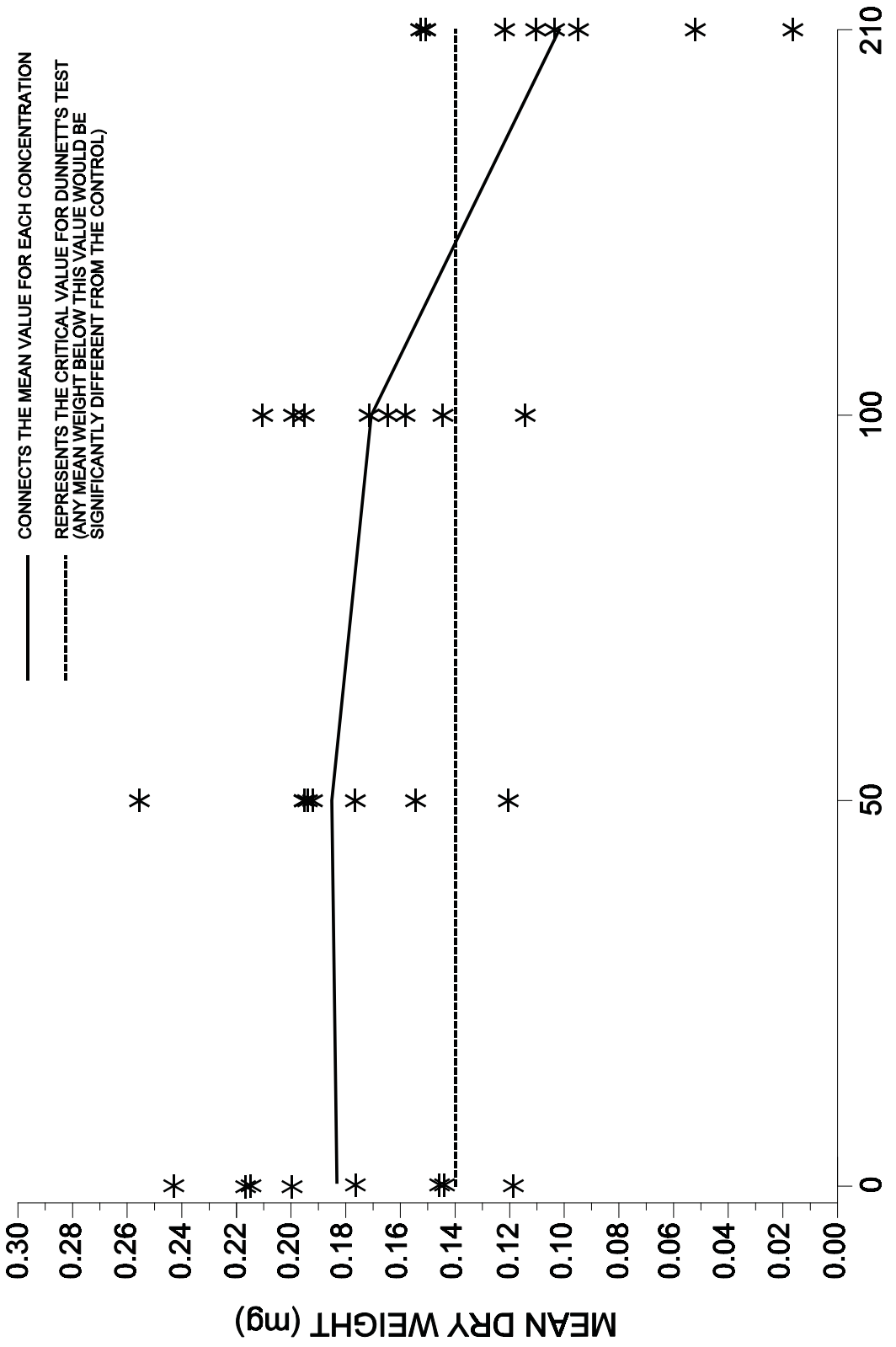

Figure 14. Plot of mean growth data for mysid, *Mysidopsis bahia*, test.

TABLE 14. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Concentration (ppb)		
Replicate	Control	50.0	100.0	210.0
1	-0.036	-0.030	-0.054	0.052
2	-0.064	0.009	0.004	-0.007
3	0.034	0.006	-0.008	-0.084
4	0.017	0.006	0.031	0.021
5	-0.006	0.072	-0.003	-0.049
6	0.061	0.007	-0.023	0.053
7	0.031	-0.062	0.039	0.009
8	-0.038	-0.007	0.018	0.002

14.13.3.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

14.13.3.5.3 For this set of data, n = 32

$$\bar{X} = \frac{1}{32}(0.007) = 0.000$$

D = 0.0451

14.13.3.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 15.

TABLE 15. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.084	17	0.006
2	-0.064	18	0.006
3	-0.062	19	0.007
4	-0.054	20	0.009
5	-0.049	21	0.009
6	-0.038	22	0.017
7	-0.036	23	0.018
8	-0.030	24	0.021
9	-0.023	25	0.031
10	-0.008	26	0.031
11	-0.007	27	0.034
12	-0.007	28	0.039
13	-0.006	29	0.052
14	-0.003	30	0.053
15	0.002	31	0.061
16	0.004	32	0.072

14.13.3.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 32 and k = 16. The a_i values are listed in Table 16.

14.13.3.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(x^{(n-i+1)} - x^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 16. For this set of data:

$$W = \frac{1}{0.045}(0.2097)^2 = 0.9752$$

14.13.3.5.7 The decision rule for this test is to compare W as calculated in Subsection 14.13.3.5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 32 observations is 0.904. Since W = 0.9752 is greater than the critical value, conclude that the data are normally distributed.

TABLE 16. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	$a_{(i)}$	$X^{(n-i+1)}$	
1	0.4188	0.156	$X^{(32)} - X^{(1)}$
2	0.2898	0.125	$X^{(31)} - X^{(2)}$
3	0.2462	0.115	$X^{(30)} - X^{(3)}$
4	0.2141	0.106	$X^{(29)} - X^{(4)}$
5	0.1878	0.088	$X^{(28)} - X^{(5)}$
6	0.1651	0.072	$X^{(27)} - X^{(6)}$
7	0.1449	0.067	$X^{(26)} - X^{(7)}$
8	0.1265	0.061	$X^{(25)} - X^{(8)}$
9	0.1093	0.044	$X^{(24)} - X^{(9)}$
10	0.0931	0.026	$X^{(23)} - X^{(10)}$
11	0.0777	0.024	$X^{(22)} - X^{(11)}$
12	0.0629	0.016	$X^{(21)} - X^{(12)}$
13	0.0485	0.015	$X^{(20)} - X^{(13)}$
14	0.0344	0.010	$X^{(19)} - X^{(14)}$
15	0.0206	0.004	$X^{(18)} - X^{(15)}$
16	0.0068	0.002	$X^{(17)} - X^{(16)}$

14.13.3.6 Test for Homogeneity of Variance

14.13.3.6.1 The test used to examine whether the variation in mean weight of the mysids is the same across all concentration levels including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i) \ln \bar{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each copper concentration and control, $V_i = (n_i - 1)$

p = number of concentration levels including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i = the number of replicates for concentration i.

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{p} 1/V_i - (\sum_{i=1}^{p} V_i)^{-1}$$

14.13.3.6.2 For the data in this example (see Table 13), all concentrations including the control have the same number of replicates ($n_i = 8$ for all i). Thus, $V_i = 7$ for all i.

14.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(28)\ln(0.00162) - 7\sum_{i=1}^{p} \ln(S_1^2)]/1.06$$

$$= [28(-6.427) - 7(-25.9329)]/1.06$$

$$= [-179.973 - (-181.530)]/1.06$$

$$= 1.469$$

14.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with three degrees of freedom, is 11.34. Since B = 1.469 is less than the critical value of 11.34, conclude that the variances are not different.

14.13.3.7 Dunnett's Procedure

14.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 17.

TABLE 17. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n} -j\ Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{p} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean dry weight of the mysids for concentration i in test chamber j)

14.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = 8$

$N = 32$

$T_1 = Y_{11} + Y_{12} + \ldots + Y_{18} = 1.455$

$T_2 = Y_{21} + Y_{22} + \ldots + Y_{28} = 1.473$

$T_3 = Y_{31} + Y_{32} + \ldots + Y_{38} = 1.348$

$T_4 = Y_{41} + Y_{42} + \ldots + Y_{48} = 0.805$

$$G = T_1 + T_2 + T_3 + T_4 = 5.081$$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= \frac{1}{8}(6.752) - \frac{(5.081)^2}{32} = 0.0372$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_j} Y_{ij}^2 - G^2/N$$

$$= 0.889 - \frac{(5.081)^2}{32} = 0.0822$$

$$SSW = SST - SSB = 0.0822 - 0.0372 = 0.0450$$

$$S_B^2 = \text{SSB} / (p - 1) = 0.0372 / (4 - 1) = 0.0124$$

$$S_W^2 = \text{SSW} / (N - p) = 0.0450 / (32 - 4) = 0.0016$$

14.13.3.7.3 Summarize these calculations in the ANOVA table (Table 18).

TABLE 18. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	3	0.0372	0.0127
Within	28	0.0450	0.0016
Total	31	0.0822	

14.13.3.7.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean dry weight for concentration i

$\bar{Y}_1$ = mean dry weight for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

14.13.3.7.5 Table 19 includes the calculated t values for each concentration and control combination. In this example, comparing the 50.0 ppb concentration with the control the calculation is as follows:

$$t_2 = \frac{(0.182 - 0.184)}{[0.040\sqrt{(1/8) + (1/8)}]}$$

$$= -0.100$$

TABLE 19. CALCULATED T VALUES

Concentration (ppb)	i	t_i
50.0	2	-0.150
100.0	3	0.700
210.0	4	4.050

14.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean weight, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 28 degrees of freedom for error and three concentrations (excluding the control) the approximate critical value is 2.15. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Therefore, the 210.0 ppb concentration has significantly lower mean weight than the control. Hence the NOEC and the LOEC for growth are 100.0 ppb and 210.0 ppb, respectively.

14.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be detected statistically may be calculated.

$$MSD = d\ S_w\sqrt{(1/n_1) + (1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration
(this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

14.13.3.7.8 In this example:

$$MSD = 2.15(0.04)\sqrt{(1/8) + (1/8)}$$

$$= 2.15\ (0.04)(0.5)$$

$$= 0.043$$

14.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.043 mg.

14.13.3.7.10 This represents a 23.6% reduction in mean weight from the control.

14.13.3.8 Calculation of the ICp

14.13.3.8.1 The growth data from Table 13 are utilized in this example. As seen in, the observed means are not monotonically non-increasing with respect to concentration. Therefore, it is necessary to smooth the means prior to calculating the ICp. In the following discussion, the observed means are represented by $\bar{Y}_i$ and the smoothed means by M_i.

14.13.3.8.2 Starting with the control mean, $\bar{Y}_1 = 0.182$ and $\bar{Y}_2 = 0.184$, we see that $\bar{Y}_1 < \bar{Y}_2$. Calculate the smoothed means:

$$M_1 = M_2 = (\bar{Y}_1 + \bar{Y}_2)/2 = 0.183$$

14.13.3.8.3 Since $\bar{Y}_5 = 0.025 < \bar{Y}_4 = 0.101 < \bar{Y}_3 = 0.168 < M_2$, set $M_3 = 0.168$ and $M_4 = 0.101$, and $M_5 = 0.025$. Table 20 contains the smoothed means and Figure 15 gives a plot of the smoothed response curve.

TABLE 20. MYSID, *MYSIDOPSIS BAHIA*, MEAN GROWTH RESPONSE AFTER SMOOTHING

Toxicant Conc. (ppb)	i	Response Means Y_i (mg)	Smoothed Mean M_i (mg)
Control	1	0.182	0.183
50.0	2	0.184	0.183
100.0	3	0.168	0.168
210.0	4	0.101	0.101
450.0	5	0.012	0.012

14.13.3.8.4 An IC25 and IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in weight, compared to the controls, would result in a mean weight of 0.136 mg, where $M_1(1\text{-}p/100) = 0.183(1\text{-}25/100)$. A 50% reduction in mean dry weight, compared to the controls, would result in a mean weight of 0.091 mg. Examining the smoothed means and their associated concentrations (Table 20), the response, 0.136 mg, is bracketed by $C_3 = 100$ ppb and $C_4 = 210$ ppb. The response, 0.091 mg, is bracketed by $C_4 = 210$ ppb and $C_5 = 450$ ppb.

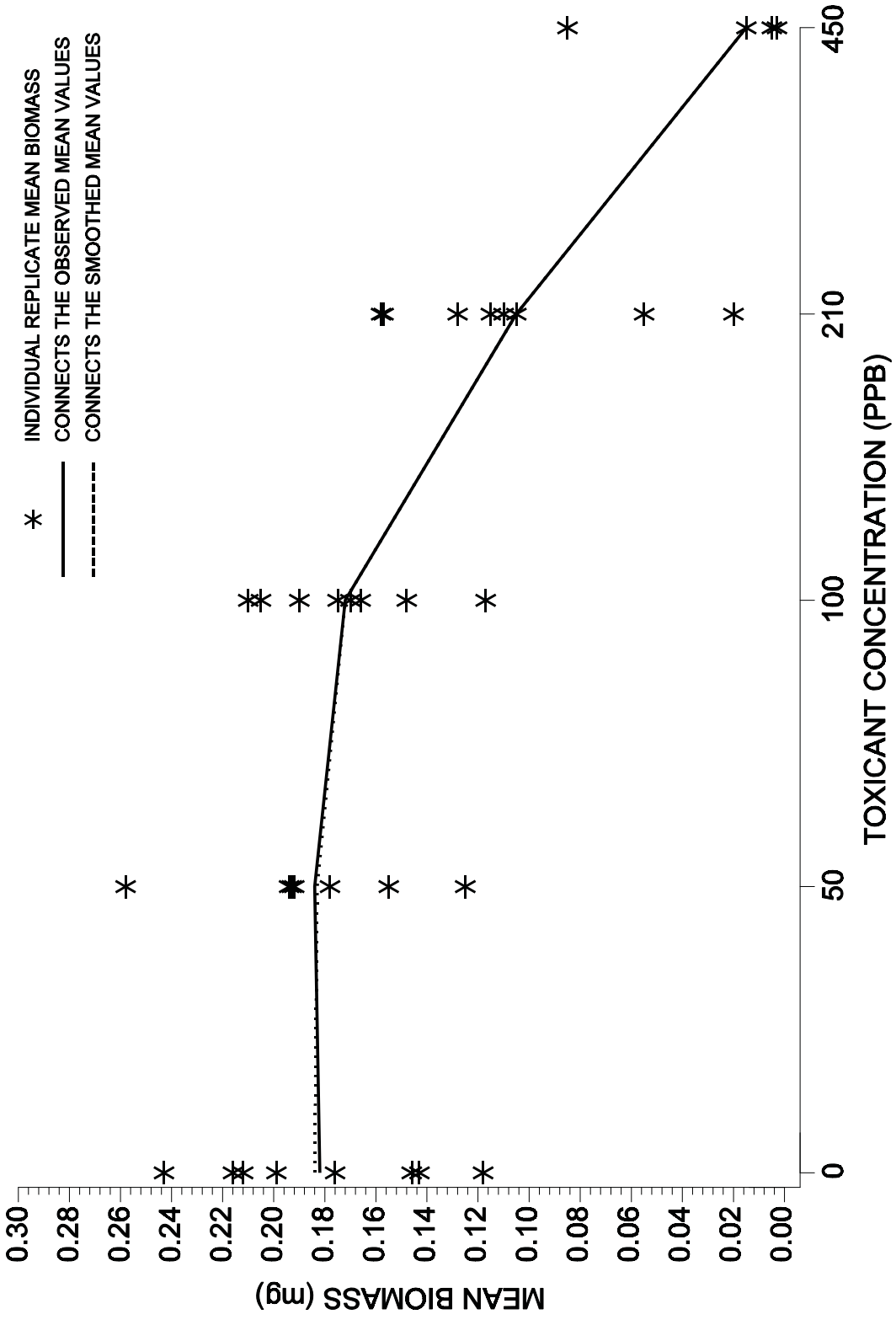

Figure 15. Plot of raw data, observed means, and smoothed means for the mysid, *Mysidopsis bahia*, growth data from Tables 13 and 20.

14.13.3.8.5 Using the equation in Section 4.2 from Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{M_{(j+1)} - M_j}$$

$$IC25 = 100 + [0.183(1 - 25/100) - 0.168] \quad \frac{(210 - 100)}{(0.101 - 0.168)}$$

$$= 151 \text{ ppb.}$$

14.13.3.8.6 Using Equation 1 from Appendix L, the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{M_{(j=1)} - M_j}$$

$$IC50 = 210 + [0.183(1 - 50/100) - 0.101] \quad \frac{(450 - 210)}{(0.012 - 0.101)}$$

$$= 236 \text{ ppb.}$$

14.13.3.8.7 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 150.6446 ppb. The empirical 95.0% confidence interval for the true mean was 97.0905 ppb and 186.6383 ppb. The computer program output for the IC25 for this data set is shown in Figure 16.

14.13.3.8.8 When the ICPIN program was used to analyze this set of data for the IC50, requesting 80 resamples, the estimate of the IC50 was 234.6761 ppb. The empirical 95.0% confidence interval for the true mean was (183.8187 ppb to 277.9211 ppb). The computer program output for the IC50 for this data set is shown in Figure 17.

14.13.4 EXAMPLE OF ANALYSIS OF MYSID, *MYSIDOPSIS BAHIA*, FECUNDITY DATA

14.13.4.1 Formal statistical analysis of the fecundity data is outlined in Figure 18. The response used in the statistical analysis is the proportion of females with eggs in each test or control chamber. If no females were present in a replicate, a response of zero should not be used. Instead there are no data available for that replicate and the number of replicates for that level of concentration or the control should be reduced by one. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints, and for the estimation of the EC, LC, and IC endpoints. The data for a concentration are excluded from the statistical analysis of the NOEC and LOEC endpoints if no eggs were produced in all of the replicates in which females existed. However, all data are included in the estimation of the IC25 and IC50.

Conc. ID	1	2	3	4.	5
Conc. Tested	0	50	100	210	450
Response 1	.146	.154	.114	.153	0
Response 2	.118	.19	.172	.094	.012
Response 3	.216	.193	.160	.017	0
Response 4	.199	.190	.199	.122	.002
Response 5	.176	.190	.165	.052	0
Response 6	.243	.191	.145	.154	0
Response 7	.213	.122	.207	.110	0
Response 8	.144	.177	.186	.103	.081

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: MYSID SHRIMP, Mysidopsis bahia
Test Duration: growth test
DATA FILE: mysidwt.icp
OUTPUT FILE: mysid.i25

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Standard. Dev.	Pooled Response Means
1	8	0.000	0.182	0.043	0.183
2	8	50.000	0.184	0.038	0.183
3	8	100.000	0.168	0.030	0.168
4	8	210.000	0.101	0.047	0.101
5	8	450.000	0.102	0.028	0.012

The Linear Interpolation Estimate: 150.6446 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 147.1702 Standard Deviation: 23.7984
Original Confidence Limits: Lower: 97.0905 Upper: 186.6383
Resampling time in Seconds: 0.11 Random Seed: -1623038650

Figure 16. ICPIN program output for the IC25.

Conc. ID	1	2	3	4.	5
Conc. Tested	0	50	100	210	450
Response 1	.146	.154	.114	.153	0
Response 2	.118	.193	.172	.094	.012
Response 3	.216	.190	.160	.017	0
Response 4	.199	.190	.199	.122	.002
Response 5	.176	.256	.165	.052	0
Response 6	.243	.191	.145	.154	0
Response 7	.213	.122	.207	.110	0
Response 8	.144	.177	.186	.103	.081

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent:
Test Start Date: Test Ending Date:
Test Species: MYSID SHRIMP, Mysidopsis bahia
Test Duration: growth test
DATA FILE: mysidwt.icp
OUTPUT FILE: mysidwt.i50

Conc. ID	Number Replicates	Concentration µg/L	Response Means	Standard. Dev. Response Means	Pooled
1	8	0.000	0.182	0.043	0.183
2	8	50.000	0.184	0.038	0.183
3	8	100.000	0.168	0.030	0.168
4	8	210.000	0.101	0.047	0.101
5	8	450.000	0.012	0.028	0.01

The Linear Interpolation Estimate: 234.6761 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 230.7551 Standard Deviation: 30.6781
Original Confidence Limits: Lower: 183.8197 Upper: 277.9211
Resampling time in Seconds: 0.16 Random Seed: -628896314

Figure 17. ICPIN program output for the IC50.

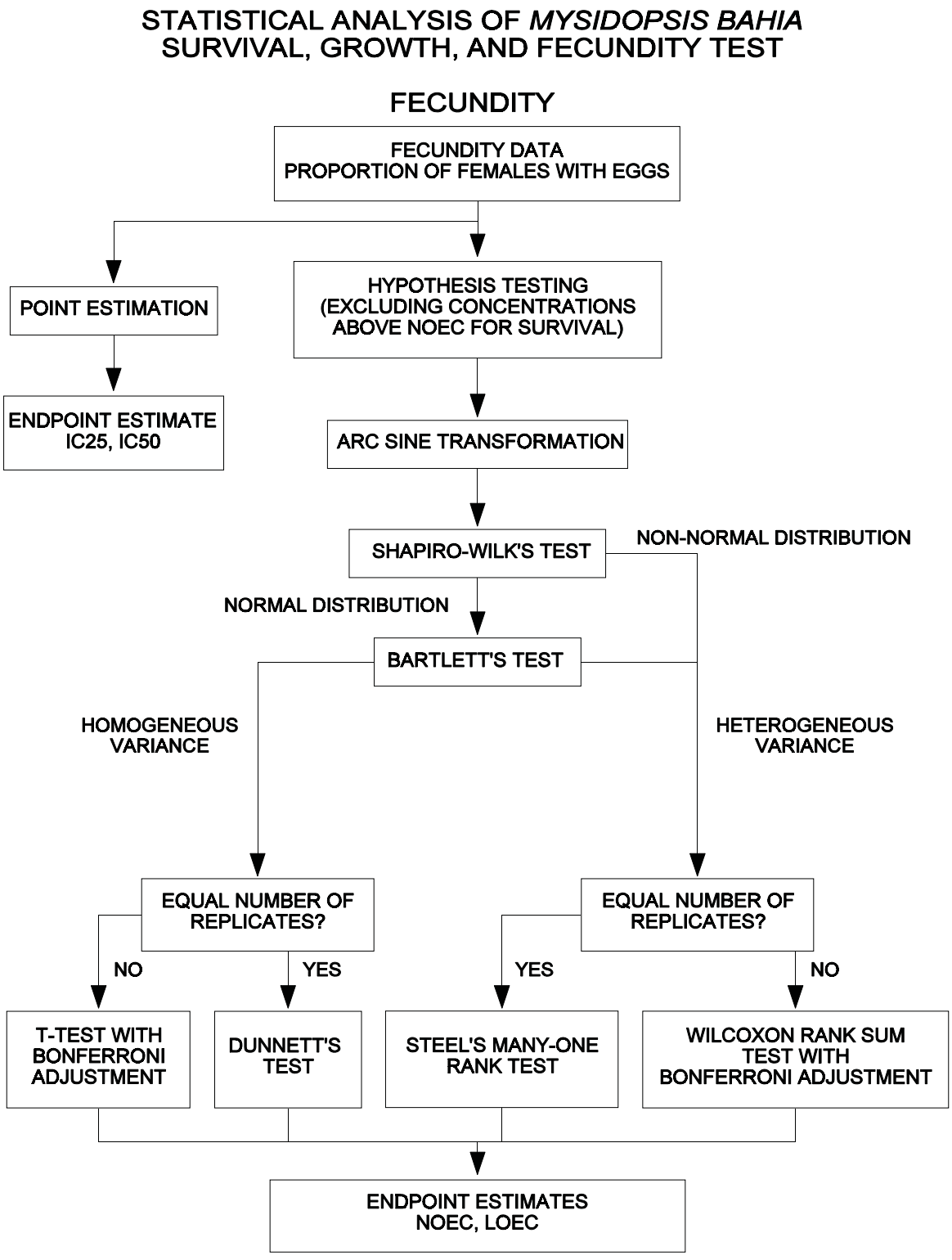

Figure 18. Flowchart for statistical analysis of mysid, *Mysidopsis bahia*, fecundity data.

14.13.4.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

14.13.4.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

14.13.4.4 The proportion of female mysids, *Mysidopsis bahia*, with eggs in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. Since the denominator of the proportion of females with eggs varies with the number of females occurring in that replicate, the adjustment of the arc sine square root transformation for 0% and 100% is not used for this data. The raw and transformed data, means and variances of the transformed observations at each test concentration including the control are listed in Table 21. Since there is significant mortality in the 450 ppb concentration, its effect on reproduction is not considered. Additionally, since no eggs were produced by females in any of the replicates for the 210 ppb concentration, it is not included in this statistical analysis and is considered a qualitative reproductive effect. A plot of the mean proportion of female mysids with eggs is illustrated in Figure 19.

14.13.4.5 Test for Normality

14.13.4.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 22.

14.13.4.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

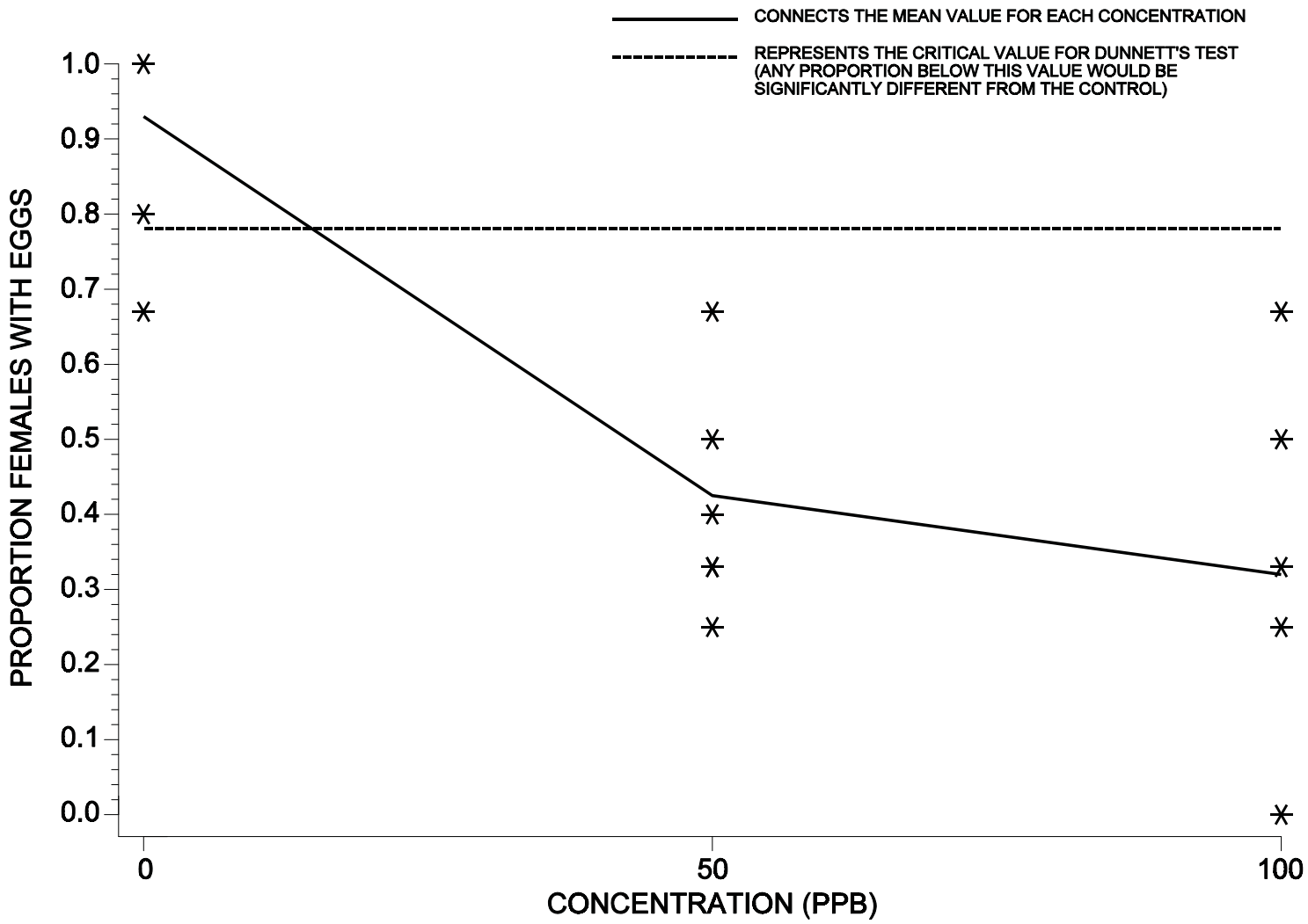

Figure 19. Proportion of female mysids, *Mysidopsis bahia*, with eggs.

TABLE 21. MYSID, *MYSIDOPSIS BAHIA*, FECUNDITY DATA: PERCENT FEMALES WITH EGGS

		Test Concentration (ppb)			
	Replicate	Control	50.0	100.0	210.0
RAW	1	1.00	0.50	0.33	0.0
	2	1.00	0.33	0.50	0.0
	3	0.67	0.67	0.00	0.0
	4	1.00	-	0.50	0.0
	5	1.00	0.40	0.67	0.0
	6	0.80	0.50	0.00	0.0
	7	1.00	0.25	0.25	0.0
	8	1.00	0.33	-	0.0
ARC SINE TRANS-FORMED[1]	1	1.57	0.78	0.61	-
	2	1.57	0.61	0.78	-
	3	0.96	0.96	0.00	-
	4	1.57	-	0.78	-
	5	1.57	0.68	0.96	-
	6	1.12	0.78	0.00	-
	7	1.57	0.52	0.52	-
	8	1.57	0.61	-	-
Mean(Y_i)		1.44	0.71	0.52	-
S^2_i		0.064	0.021	0.147	-
i		1	2	3	4

[1] Since the denominator of the proportion of females with eggs varies with the number of females occurring in that replicate, the adjustment of the arc sine square root transformation for 0% and 100% is not used for this data.

TABLE 22. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Test Concentration (ppb)	
Replicate	Control	50.0	100.0
1	0.13	0.07	0.09
2	0.13	-0.10	0.26
3	-0.48	0.25	-0.52
4	0.13	-	0.26
5	0.13	-0.03	0.44
6	-0.32	0.07	-0.52
7	0.13	-0.19	0.00
8	0.13	-0.10	-

14.13.4.5.3 For this set of data, n = 22

$$X = \frac{1}{22}(0.000) = 0.000$$

$$D = 1.4412$$

14.13.4.5.4 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 23.

14.13.4.5.5. From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 22 and k = 11. The a_i values are listed in Table 24.

14.13.4.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences $X^{(n-i+1)} - X^{(I)}$ are listed in Table 24. For the data in this example:

$$W = \frac{1}{1.4412}(1.1389)^2 = 0.900$$

TABLE 23. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.52	12	0.09
2	-0.52	13	0.13
3	-0.48	14	0.13
4	-0.32	15	0.13
5	-0.19	16	0.13
6	-0.10	17	0.13
7	-0.10	18	0.13
8	0.03	19	0.25
9	0.00	20	0.26
10	0.07	21	0.26
11	0.07	22	0.44

TABLE 24. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

	i	a_i	$X^{(n-i+1)} - X^{(i)}$
1	0.4590	0.96	$X^{(22)} - X^{(1)}$
2	0.3156	0.78	$X^{(21)} - X^{(2)}$
3	0.2571	0.74	$X^{(20)} - X^{(3)}$
4	0.2131	0.57	$X^{(19)} - X^{(4)}$
5	0.1764	0.32	$X^{(18)} - X^{(5)}$
6	0.1443	0.23	$X^{(17)} - X^{(6)}$
7	0.1150	0.23	$X^{(16)} - X^{(7)}$
8	0.0878	0.16	$X^{(15)} - X^{(8)}$
9	0.0618	0.13	$X^{(14)} - X^{(9)}$
10	0.0368	0.06	$X^{(13)} - X^{(10)}$
11	0.0122	0.02	$X^{(12)} - X^{(11)}$

14.13.4.5.7 The decision rule for this test is to compare W as calculated in Subsection 14.13.4.5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 22 observations is 0.878. Since W = 0.900 is greater than the critical value, conclude that the data are normally distributed.

14.13.4.6 Test for Homogeneity of Variance

14.13.4.6.1 The test used to examine whether the variation in proportion of female mysids with eggs is the same across all concentration levels including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i) \ln \overline{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each copper concentration and control, $V_i = (n_i - 1)$

p = number of concentration levels including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i^2)}{\sum_{i=1}^{p} V_i}$$

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i = the number of replicates for concentration i.

$$C = 1+[3(p-1)^{-1}\ [\sum_{i=1}^{p} 1/V_i - (\sum_{i=1}^{p} V_i)^{-1}]$$

14.13.4.6.2 For the data in this example (see Table 21), $n_1 = 8$, $n_2 = 7$ and $n_3 = 7$. Thus, the respective degrees of freedom are 7, 6 and 6.

14.13.4.6.3 Bartlett's statistic is therefore:

$$B = [(19)\ln(0.077) - (7\ln(0.064) + 6\ln(0.021) + 6\ln(0.147))]/1.07$$

$$= [19(-2.564) - (-53.925)]/1.07$$

$$= [-48.716 - (-53.925)]/1.07$$

$$= 4.868$$

14.13.4.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with two degrees of freedom, is 9.210. Since B = 4.868 is less than the critical value of 9.210, conclude that the variances are not different.

14.13.4.7 T test with the Bonferroni Adjustment

14.13.4.7.1 A t test with the Bonferroni adjustment is used as an alternative to Dunnett's Procedure when, as in this set of data, the number of replicates is not the same for all concentrations. Like Dunnett's Procedure, it uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance. To obtain an estimate of the pooled variance, construct an ANOVA table as described in Table 25.

TABLE 25. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{p} \sum_{j-1}^{n_j} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{p} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean dry weight of the mysids for concentration i in test chamber j)

14.13.4.7.2 For the data in this example:

$n_1 = 8$ $n_2 = 7$ $n_3 = 7$

$N = 22$

$T_1 = Y_{11} + Y_{12} + ... + Y_{18} = 11.5$

$T_2 = Y_{21} + Y_{22} + ... + Y_{27} = 4.94$

$T_3 = Y_{31} + Y_{32} + ... + Y_{37} = 3.65$

$G = T_1 + T_2 + T_3 = 20.09$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= \frac{132.25}{8} + \frac{24.40}{7} + \frac{13.32}{7} - \frac{403.61}{22} = 3.57$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_j} Y_{ij}^2 - G^2/N$$

$$= 23.396 - \frac{403.61}{22} = 5.05$$

SSW = SST - SSB = 5.05 - 3.57 = 1.48

S_B^2 = SSB/(p-1) = 3.57/(3-1) = 1.785

S_W^2 = SSW/(N-p) = 1.48/(22-3) = 0.078

14.13.4.7.3 Summarize these calculations in the ANOVA table (Table 26).

TABLE 26. ANOVA TABLE FOR THE T TEST WITH BONFERRONI'S ADJUSTMENT EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	2	3.57	1.785
Within	19	1.48	0.078
Total	21	5.05	

14.13.4.7.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean proportion of females with eggs for concentration i

$\bar{Y}_1$ = mean proportion of females with eggs for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

14.13.4.7.5 Table 27 includes the calculated t values for each concentration and control combination. In this example, comparing the 50.0 ppb concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.44 - 0.52)}{[0.279\sqrt{(1/8) - (1/7)}]}$$

$$= 5.05$$

TABLE 27. CALCULATED T VALUES

Test Concentration (ppb)	i	t_i
50.0	2	5.05
100.0	3	6.37

14.13.4.7.6 Since the purpose of this test is to detect a significant reduction in mean proportion of females with eggs, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix D, Critical Values for the t test with Bonferroni's adjustment. For an overall alpha level of 0.05, 19 degrees of freedom for error and two concentrations (excluding the control) the approximate critical value is 2.094. The mean proportion for concentration "i" is considered significantly less than the mean proportion for the control if t_i is greater than the critical value. Therefore, the 50.0 ppb and the 100.0 ppb concentrations have significantly lower mean proportion of females with eggs than the control. Hence the LOEC for fecundity is 50.0 ppb.

14.13.4.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be detected statistically may be calculated.

$$MSD = t\ S_w\sqrt{(1/n_1) + (1/n)}$$

Where: t = the critical value for the t test with Bonferroni's adjustment

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control

14.13.4.7.8 In this example:

$$MSD = 2.094\,(0.279)\sqrt{(1/8) + (1/7)}$$

$$= 2.094\ (0.279)(0.518)$$

$$= 0.303$$

14.13.4.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.30.

14.13.4.7.10 The MSD (0.30) is in transformed units. To determine the MSD in terms of percent of females with eggs, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

   1.44 - 0.30 = 1.14

2. Obtain the untransformed values for the control mean and the difference calculated in 4.10.1.

   [Sine (1.44)]^2 = 0.983

   [Sine (1.14)]^2 = 0.823

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from 14.13.4.7.10.2.

   MSD_u = 0.983 - 0.823 = 0.16

14.13.4.7.11 Therefore, for this set of data, the minimum difference in mean proportion of females with eggs between the control and any copper concentration that can be detected as statistically significant is 0.16.

14.13.4.7.12 This represents a 17% decrease in proportion of females with eggs from the control.

14.13.4.8 Calculation of the ICp

14.13.4.8.1 The fecundity data in Table 4 are utilized in this example. Table 28 contains the mean proportion of females with eggs for each toxicant concentration. As can be seen, the observed means are monotonically nonincreasing with respect to concentration. Therefore, it is not necessary to smooth the means prior to calculating the IC. Figure 20 gives a plot of the response curve.

TABLE 28. MYSID, *MYSIDOPSIS BAHIA*, MEAN MEAN PROPORTION OF FEMALES WITH EGGS

Toxicant Conc. (ppb)	i	Response Means Y^i (mg)	Smoothed Mean M_i (mg)
Control	1	0.934	0.934
50.0	2	0.426	0.426
100.0	3	0.317	0.317
210.0	4	0.000	0.000
450.0	5	0.010	0.000

14.13.4.8.2 An IC25 and IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of females with eggs, compared to the controls, would result in a mean proportion of 0.701, where $M_1(1-p/100)$ = 0.934(1-25/100). A 50% reduction in mean proportion of females with eggs, compared to the could would result in a mean proportion of 0.467. Examining the means and their associated concentrations (Table 28), the response, 0.701, is bracketed by C_1 = 0 ppb and C_2 = 50 ppb. The response, 0.467, is bracketed by C_1 = 0 ppb and C_2 = 50 ppb.

14.13.4.8.3 Using the equation in Section 4.2 from Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j] \frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 0 + [0.934(1 - 25/100) - 0.934] \quad \frac{(50 - 0)}{(0.426 - 0.934)}$$

$$= 23 \text{ ppb.}$$

14.13.4.8.4 Using the equation in Section 4.2 from Appendix L, the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [M_1 \ (1 - p/100) - M_j] \frac{C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC50 = 0 + [0.934(1 - 50/100) - 0.934] \quad \frac{(50 - 0)}{(0.426 - 0.934)}$$

$$= 46 \text{ ppb.}$$

14.13.4.8.5 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 29.9745 ppb. The empirical 95.0% confidence interval for the true mean was 20.0499 ppb to 30.5675 ppb. The computer program output for the IC25 for this data set is shown in Figure 21. This value is extrapolated below the lowest test concentration and data should be used cautiously.

14.13.4.8.6 When the ICPIN program was used to analyze this set of data for the IC50, requesting 80 resamples, the estimate of the IC50 was 45.9490 ppb. The empirical 95.0% confidence interval for the true mean was 40.1467 ppb to 63.0931 ppb. The computer program output for the IC50 for this data set is shown in Figure 22.

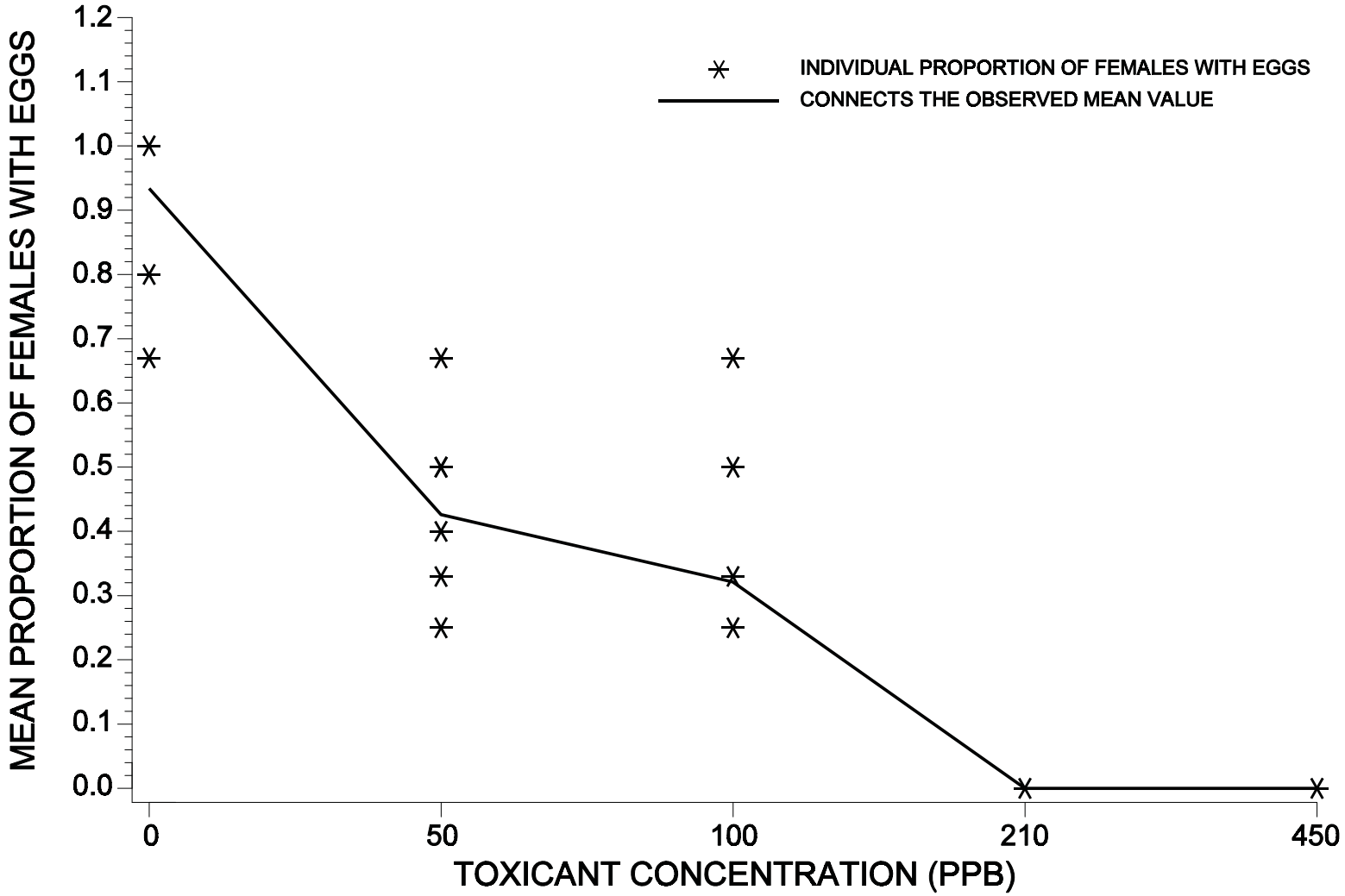

Figure 20. Plot of the mean proportion of female mysids, *Mysidopsis bahia*, with eggs

Conc. ID	1	2	3	4
Conc. Tested	0	50	100	210
Response 1	1	.5	.3	0
Response 2	1	.33	.5	0
Response 3	.67	.67	0	0
Response 4	1	.4	.5	0
Response 5	1	.5	.67	0
Response 6	.8	.25	0	0
Response 7	1	.33	.25	0
Response 8	1			0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: MYSID SHRIMP, Mysidopsis bahia
Test Duration: fecundity
DATA FILE: mysidfe.icp
OUTPUT FILE: mysidfe.i25

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Standard. Dev.	Pooled Response Means
1 8	0.000	0.934	0.127	0.934	
2 7	50.000	0.426	0.142	0.426	
3 7	100.000	0.317	0.257	0.317	
4 8	210.000	0.000	0.000	0.000	

The Linear Interpolation Estimate: 29.9745 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 23.8871 Standard Deviation: 3.0663
Original Confidence Limits: Lower: 20.0499 Upper: 30.5765
Resampling time in Seconds: 1.37 Random Seed: 1918482350

Figure 21. ICPIN program output for the IC25.

Conc. ID	1	2	3	4
Conc. Tested	0	50	100	210
Response 1	1	.5	.3	0
Response 2	1	.33	.5	0
Response 3	.67	.67	0	0
Response 4	1	.4	.5	0
Response 5	1	.5	.67	0
Response 6	.8	.25	0	0
Response 7	1	.33	.25	0
Response 8	1			0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: MYSID SHRIMP
Test Duration: fecundity
DATA FILE: mysidfe.icp
OUTPUT FILE: mysidfe.i50

-Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1 8	0.000	0.934	0.127	0.934	
2 7	50.000	0.426	0.142	0.426	
3 7	100.000	0.317	0.257	0.317	
4 8	210.000	0.000	0.000	0.000	

The Linear Interpolation Estimate: 45.9490 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 47.8720 Standard Deviation: 8.2908
Original Confidence Limits: Lower: 40.1467 Upper: 63.0931
Resampling time in Seconds: 1.32 Random Seed: -391064242

Figure 22. ICPIN program output for the IC50.

14.14 PRECISION AND ACCURACY

14.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 14.14.1.1 and 14.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

14.14.1.1 Single-Laboratory Precision

14.14.1.1.1 Data on the single-laboratory precision of the mysid survival, growth, and fecundity using copper (Cu) sulfate and sodium dodecyl sulfate (SDS) in natural seawater and in artificial seawater (GP2) are shown in Tables 29-33. In Tables 29-30 the coefficient of variation for the IC25, ranges from 18.0 to 35.0 and the IC50, ranges from 5.8 to 47.8, indicating acceptable test precision. Data in Tables 31-33 show no detectable differences between tests conducted in natural or artificial seawaters.

14.14.1.1.2 EPA evaluated within-laboratory precision of the Mysid, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test using a database of routine reference toxicant test results from 10 laboratories (USEPA, 2000b). The database consisted of 130 reference toxicant tests conducted in 10 laboratories using a variety of reference toxicants including: chromium, copper, and potassium chloride. Among the 10 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 28% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 24%; and in 75% of laboratories, the within-laboratory CV was less than 32%.

14.14.1.2 Multilaboratory Precision

14.14.1.2.1 In 2000, EPA conducted an interlaboratory variability study of the Mysid, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 11 participant laboratories tested 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of bioassay-grade FORTY FATHOMS® synthetic seawater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a natural seawater spiked with KCl, and the reference toxicant sample consisted of bioassay-grade FORTY FATHOMS® synthetic seawater spiked with KCl. Of the 44 *Mysidopsis bahia* Survival, Growth, and Fecundity tests conducted in this study, 97.7% were successfully completed and met the required test acceptability criteria. Of seven tests that were conducted on blank samples, none showed false positive results for survival, growth, or fecundity endpoints. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 34 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 41.3% for growth IC25 results. Table 35 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned three concentrations for the reference toxicant, effluent, and receiving water sample types. The percentage of values within one concentration of the median was 100% for each of the sample types. For the growth endpoint, NOEC values spanned four concentrations for the reference toxicant sample type and three concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 92.3%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively. For the fecundity endpoint, NOEC values spanned three concentrations for the reference toxicant, the effluent, and the receiving water sample types. The percentage of values within one concentration of the median was 75.0%, 87.5%, and 66.7% for the reference toxicant, effluent, and receiving water sample types, respectively.

14.14.2 ACCURACY

14.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 29. SINGLE-LABORATORY PRECISION OF THE MYSID, *MYSIDOPSIS BAHIA*, SURVIVAL, GROWTH, AND FECUNDITY TEST PERFORMED IN NATURAL SEAWATER, USING JUVENILES FROM MYSIDS CULTURED AND SPAWNED IN NATURAL SEAWATER, AND COPPER (Cu) SULFATE AS A REFERENCE TOXICANT[1,2,3,4,5,6]

Test Number	NOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)	Most Sensitive Endpoint[7]
1	63	96.1	NC[8]	S
2	125	138.3	175.5	S
3	125	156.3	187.5	S
4	125	143.0	179.9	S
5	125	157.7	200.3	S
n:	5	5	4	
Mean:	NA	138.3	185.8	
CV(%):	NA	18.0	5.8	

1 Data from USEPA (1988a) and USEPA (1991a).
2 Tests performed by Randy Cameleo, ERL-N, USEPA, Narragansett, RI.
3 Eight replicate exposure chambers, each with five juveniles, were used for the control and each toxicant concentration. The temperature of the test solutions was maintained at 26 ± 1°C.
4 Copper concentrations in Tests 1-2 were: 8, 16, 31, 63, and 125 mg/L. Copper concentrations in Tests 3-6 were, 16, 31, 63, 125, and 250 µg/L.
5 NOEC Range: 63 - 125 µg/L (this represents a difference of two exposure concentrations).
6 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.
7 Endpoints: G=Growth; S=Survival.
8 NC = No linear interpolation estimate could be calculated from the data, since none of the group response means were less than 50 percent of the control concentrations.

TABLE 30. SINGLE-LABORATORY PRECISION OF THE MYSID, *MYSIDOPSIS BAHIA*, SURVIVAL, GROWTH, AND FECUNDITY TEST PERFORMED IN NATURAL SEAWATER, USING JUVENILES FROM MYSIDS CULTURED AND SPAWNED IN NATURAL SEAWATER, AND SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT[1,2,3,4,5,6]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)	Most Sensitive Endpoint[7]
1	2.5	4.5	NC[9]	S
2	< 0.3	NC[8]	NC[9]	S
3	< 0.6	NC[8]	NC[9]	S
4	5.0	7.8	NC[9]	S
5	2.5	3.6	4.6	S
6	5.0	7.0	9.3	S
n:	4	4	2	
Mean:	NA	5.7	6.9	
CV(%):	NA	35.0	47.8	

1 Data from USEPA (1988a) and USEPA (1991a).
2 Tests performed by Randy Cameleo, ERL-N, USEPA, Narragansett, RI.
3 Eight replicate exposure chambers, each with five juveniles, were used for the control and each toxicant concentration. The temperature of the test solutions was maintained at 26 ± 1°C.
4 SDS concentrations in Tests 1-2 were: 0.3, 0.6, 1.3, 2.5, and 5.0 mg/L. SDS concentrations in Tests 3-4 were: 0.6, 1.3, 2.5, 5.0 and 10.0 mg/L. SDS concentrations in Tests 5-6 were: 1.3, 2.5, 5.0, 10.0, and 20.0 mg/L.
5 NOEC Range: < 0.3 - 5.0 mg/L (this represents a difference of four exposure concentrations).
6 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.
7 Endpoints: G=Growth; S=Survival.
8 NC = No linear interpolation estimate could be calculated from the data, since none of the group response means were less than 75 percent of the control response mean.
9 NC = No linear interpolation estimate could be calculated from the data, since none of the group response means were less than 50 percent of the control response mean.

TABLE 31. COMPARISON OF SURVIVAL (LC50)[1], GROWTH AND FECUNDITY (IC50)[1] RESULTS FROM 7-DAY TESTS WITH THE MYSID, *MYSIDOPSIS BAHIA*, USING NATURAL SEAWATER (NSW) AND ARTIFICIAL SEAWATER (GP2) AS DILUTION WATER AND SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT

	Survival LC50		Growth IC50		Fecundity IC50	
Test	NSW	GP2	NSW	GP2	NSW	GP2
1	16.2	16.3	16.8	16.3	12.0	10.9
2	20.5	19.2	24.2	23.3	20.1	18.5
3	--[2]	21.9	--[2]	24.4	--[2]	21.7

[1] All LC50/IC50 values in mg/L.
[2] No test performed.

TABLE 32. COMPARISON OF SURVIVAL (LC50)[1], GROWTH AND FECUNDITY (IC50)[1] RESULTS FROM 7-DAY TESTS WITH THE MYSID, *MYSIDOPSIS BAHIA*, USING NATURAL SEAWATER (NSW) AND ARTIFICIAL SEAWATER (GP2) AS DILUTION WATER AND COPPER (Cu) SULFATE AS A REFERENCE TOXICANT

	Survival LC50		Growth IC50		Fecundity IC50	
Test	NSW	GP2	NSW	GP2	NSW	GP2
1	177	182	208	186	177	125
2	--[2]	173	--[2]	210	--[2]	142
3	190	174	195	179	168	186

[1] All LC50/IC50 values in µg/L.
[2] No test performed.

TABLE 33. CONTROL RESULTS FROM 7-DAY SURVIVAL, GROWTH, AND FECUNDITY TESTS WITH THE MYSID, *MYSIDOPSIS BAHIA*, USING NATURAL SEAWATER AND ARTIFICIAL SEAWATER (GP2) AS A DILUTION WATER

	Control [1]					
	Survival (%)		Growth (mg)		Fecundity (%)	
Test	NSW	GP2	NSW	GP2	NSW	GP2
1	98	93	0.32	0.32	73	77
2	80	90	0.40	0.43	100	95
3	--[2]	95	--[2]	0.40	--[2]	100
4	94	84	0.34	0.37	89	83
5	--[2]	94	--[2]	0.36	--[2]	83
6	80	75	0.40	0.41	79	93

[1] Survival as percent of mysids alive after 7 days; growth as mean individual dry weight; fecundity as percent females with eggs.
[2] No test performed.

TABLE 34. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25 for Growth	Reference toxicant	8.69	40.0	40.9
	Effluent	5.26	36.6	37.0
	Receiving water	-	-	45.9
	Average	6.98	38.3	41.3

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

[3] The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

[4] The between-laboratory component of variability for duplicate samples tested at different laboratories.

[5] The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 35. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	25%	53.8	46.2	0.00
	Effluent	12.5%	46.7	53.3	0.00
	Receiving water	12.5%	37.5	62.5	0.00
Growth NOEC	Reference toxicant	25%	53.8	38.5	7.69
	Effluent	12.5%	46.7	53.3	0.00
	Receiving water	12.5%	50.0	50.0	0.00
Fecundity NOEC	Reference toxicant	18.8%	-[4]	75.0	25.0
	Effluent	25%	62.5	25.0	12.5
	Receiving water	9.38%	-[4]	66.7	33.3

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.
[4] The median NOEC fell between test concentrations, so no test results fell precisely on the median.

SECTION 15

TEST METHOD

SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST METHOD 1008.0

15.1 SCOPE AND APPLICATION

15.1.1 This method, adapted in part from USEPA (1987e), measures the toxicity of effluents and receiving water to the gametes of the sea urchin, *Arbacia punctulata*, during a 1 h and 20 min exposure. The purpose of the sperm cell toxicity test is to determine the concentration of a test substance that reduces fertilization of exposed gametes relative to that of the control.

15.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

15.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

15.1.4 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

15.2 SUMMARY OF METHOD

15.2.1 The method consists of exposing dilute sperm suspensions to effluents or receiving waters for 1 h. Eggs are then added to the sperm suspensions. Twenty minutes after the eggs are added, the test is terminated by the addition of preservative. The percent fertilization is determined by microscopic examination of an aliquot from each treatment. The test results are reported as the concentration of the test substance which causes a statistically significant reduction in fertilization.

15.3 INTERFERENCES

15.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

15.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

15.4 SAFETY

15.4.1 See Section 3, Health and Safety.

15.5. APPARATUS AND EQUIPMENT

15.5.1 Facilities for holding and acclimating test organisms.

15.5.2 Laboratory sea urchins, *Arbacia punctulata*, culture unit -- See Subsection 15.6.19, culturing methods below and Section 4, Quality Assurance. To test effluent or receiving water toxicity, sufficient eggs and sperm must be available.

15.5.3 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L.

15.5.4 Environmental chamber or equivalent facility with temperature control (20 ± 1°C).

15.5.5 Water purification system -- Millipore Milli-Q®, deionized water (DI) or equivalent.

15.5.6 Balance -- Analytical, capable of accurately weighing to 0.00001 g.

15.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of materials to be weighed.

15.5.8 Air pump -- for oil-free air supply.

15.5.9 Air lines, and air stones -- for aerating water containing adults, or for supplying air to test solutions with low DO.

15.5.10 Vacuum suction device -- for washing eggs.

15.5.11 Meters, pH and DO -- for routine physical and chemical measurements.

15.5.12 Standard or micro-Winkler apparatus -- for determining DO (optional).

15.5.13 Transformer, 10-12 Volt, with steel electrodes -- for stimulating release of eggs and sperm.

15.5.14 Centrifuge, bench-top, slant-head, variable speed -- for washing eggs.

15.5.15 Fume hood -- to protect the analyst from formaldehyde fumes.

15.5.16 Dissecting microscope -- for counting diluted egg stock.

15.5.17 Compound microscope -- for examining and counting sperm cells and fertilized eggs.

15.5.18 Sedgwick-Rafter counting chamber -- for counting egg stock and examining fertilized eggs.

15.5.19 Hemacytometer, Neubauer -- for counting sperm.

15.5.20 Count register, 2-place -- for recording sperm and egg counts.

15.5.21 Refractometer -- for determining salinity.

15.5.22 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

15.5.23 Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.

15.5.24 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, 1979b) -- to calibrate laboratory thermometers.

15.5.25 Ice bucket, covered -- for maintaining live sperm.

15.5.26 Centrifuge tubes, conical -- for washing eggs.

15.5.27 Cylindrical glass vessel, 8-cm diameter -- for maintaining dispersed egg suspension.

15.5.28 Beakers -- six Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

15.5.29 Glass dishes, flat bottomed, 20-cm diameter -- for holding urchins during gamete collection.

15.5.3 Wash bottles -- for deionized water, for rinsing small glassware and instrument electrodes and probes.

15.5.31 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

15.5.32 Syringes, 1-mL, and 10-mL, with 18 gauge, blunt-tipped needles (tips cut off) -- for collecting sperm and eggs.

15.5.33 Pipets, volumetric -- Class A, 1-100 mL.

15.5.34 Pipets, automatic -- adjustable 1-100 mL.

15.5.35 Pipets, serological -- 1-10 mL, graduated.

15.6.36 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

15.6 REAGENTS AND CONSUMABLE MATERIALS

15.6.1 Sea Urchins, *Arbacia punctulata* minimum 12 of each sex.

15.6.2 Food -- kelp, *Laminaria* sp., or romaine lettuce for the sea urchin, *Arbacia punctulata*.

15.6.3 Standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 15°C) -- with appropriate filtration and aeration system.

15.6.4 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

15.6.5 Scintillation vials, 20 mL, disposable -- to prepare test concentrations.

15.6.6 Tape, colored -- for labeling tubes.

15.6.7 Markers, waterproof -- for marking containers, etc.

15.6.8 Parafilm -- to cover tubes and vessels containing test materials.

15.6.9 Gloves, disposable; labcoat and protective eyewear – for personal protection from contamination.

15.6.10 Data sheets (one set per test) -- for data recording (see Figures 1, 2, and 3).

15.6.11 Acetic acid, 10%, reagent grade, in seawater -- for preparing killed sperm dilutions.

15.6.12 Formalin, 1%, in 2 mL of seawater -- for preserving eggs (see Subsection 15.10.9 Termination of the Test).

15.6.13 Buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979b).

15.6.14 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

15.6.15 Laboratory quality assurance samples and standards -- for the above methods.

15.6.16 Reference toxicant solutions -- see Section 4, Quality Assurance.

15.6.17 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.

15.6.18 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

TEST DATE: _____

SAMPLE: _____

COMPLEX EFFLUENT SAMPLE: _____

COLLECTION DATE: _____

SALINITY/ADJUSTMENT: _____

PH/ADJUSTMENT REQUIRED: _____

PHYSICAL CHARACTERISTICS: _____

STORAGE: _____

COMMENTS: _____

_____

_____

_____

SINGLE COMPOUND: _____

SOLVENT (CONC): _____

TEST CONCENTRATIONS: _____

DILUTION WATER: _____

CONTROL WATER: _____

TEST TEMPERATURE: _____

TEST SALINITY: _____

COMMENTS: _____

_____

_____

_____

Figure 1. Data form (1) for fertilization test using sea urchin, *Arbacia punctulata.*

TEST DATE: _____

SAMPLE: _____

_____

SPERM DILUTIONS:

HEMACYTOMETER COUNT, E: _____ x 10^4 = SPM SOLUTION E = _____

SPERM CONCENTRATIONS: SOLUTION E x 40 = SOLUTION A = _____ SPM
SOLUTION E x 20 = SOLUTION B = _____ SPM
SOLUTION E x 5 = SOLUTION D = _____ SPM

SOLUTION SELECTED FOR TEST (= 5 x 10^7 SPM):

DILUTION: SPM/(5 x 10^7) = _____ DF
[(DF) x 10) - 10 = _____ + SW, mL

FINAL SPERM COUNTS = _____

EGG DILUTIONS:

INITIAL EGG COUNT = _____
ORIGINAL EGG STOCK CONCENTRATION = 10X (INITIAL EGG COUNT) = _____
VOLUME OF SW TO ADD TO DILUTE EGG STOCK TO 2000/mL:
EGG COUNT) - 200 = _____
CONTROL WATER TO ADD EGG STOCK, mL = _____
FINAL EGG COUNT = _____

TEST TIMES:

SPERM COLLECTED: _____

EGGS COLLECTED: _____

SPERM ADDED: _____

EGGS ADDED: _____

FIXATIVE ADDED: _____

SAMPLES READ: _____

Figure 2. Data form (2) for fertilization test using sea urchin, *Arbacia punctulata.*

DATE TESTED: _____

SAMPLE: _____

TOTAL AND UNFERTILIZED EGG COUNT AT END OF TEST:

EFFLUENT	REPLICATE VIAL			
CONC (%)	1 TOTAL-UNFERT	2 TOTAL-UNFERT	3 TOTAL-UNFERT	4 TOTAL-UNFERT

STATISTICAL ANALYSIS:

ANALYSIS OF VARIANCE: _____

CONTROL: _____

DIFFERENT FROM CONTROL (P): _____

_____

COMMENTS: _____

_____

_____

_____

Figure 3. Data form (3) for fertilization test using sea urchin, *Arbacia punctulata*.

15.6.18.1 Saline test and dilution water -- the salinity of the test water must be 30‰. The salinity should vary by no more than ± 2‰ among the replicates. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.6.18.2 The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Exposure of sea urchin eggs and sperm to these effluents will require adjustments in the salinity of the test solutions. It is important to maintain a constant salinity across all treatments. In addition it may be desirable to match the test salinity with that of the receiving water. Two methods are available to adjust salinities – hypersaline brine (HSB) derived from natural seawater or artificial sea salts.

15.6.18.3 Hypersaline brine (HSB): HSB has several advantages that make it desirable for use in toxicity testing. It can be made from any high quality, filtered seawater by evaporation, and can be added to the effluent or to deionized water to increase the salinity. HSB derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and may be stored for prolonged periods without any apparent degradation. However, if 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ salinity and 70% at 30‰ salinity.

15.6.18.3.1 The ideal container for making HSB from natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a noncorrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is utilized, use only oil-free air compressors to prevent contamination.

15.6.18.3.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough deionized water rinses.

15.6.18.3.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

15.6.18.3.4 The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

15.6.18.3.5 After the required salinity is attained, the HSB should be filtered a second time through a 1 mm filter and poured directly into portable containers, (20 L cubitainers or polycarbonate water cooler jugs are suitable). The containers should be capped and labeled with the date the brine was generated and its salinity. Containers of HSB should be stored in the dark and maintained under room temperature until used.

15.6.18.3.6 If a source of HSB is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and brine before mixing in the effluent.

15.6.18.3.7 Divide the salinity of the HSB by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 30‰, 100‰ divided by 30‰ = 3.3. The proportion of brine is 1 part in 3.3 (one part brine to 2.3 parts deionized water). To make 1 L of seawater at 30‰ salinity from a HSB of 100‰, 300 mL of brine and 700 mL of deionized water are required.

15.6.18.3.8 Table 1 illustrates the preparation of test solutions at 30‰ if they are made by combining effluent (0‰), deionized water and HSB (100‰), or FORTY FATHOMS® sea salts.

15.6.18.4 Artificial sea salts: FORTY FATHOMS® brand sea salts have been used successfully at the EMSL-Cincinnati, for long-term (6-12 months) maintenance of stock cultures of sexually mature sea urchins and to perform the sea urchin fertilization test. GP2 seawater formulation (Table 2) has also been used successfully at ERL-Narragansett, RI.

15.6.18.4.1 Synthetic sea salts are packaged in plastic bags and mixed with deionized water or equivalent. The instructions on the package of sea salts should be followed carefully, and the salts should be mixed in a separate container -- not in the culture tank. The deionized water used in hydration should be in the temperature range of 21-26°C. Seawater made from artificial sea salts is conditioned (Spotte, 1973; Spotte, et al., 1984; Bower, 1983).

15.6.18.4.2 The GP2 reagent grade chemicals (Table 2) should be mixed with deionized (DI) water or its equivalent in a container other than the culture or testing tanks. The deionized water used for hydration should be between 21-26°C. The artificial seawater must be conditioned (aerated) for 24 h before use as the testing medium. If the solution is to be autoclaved, sodium bicarbonate is added after the solution has cooled. A stock solution of sodium bicarbonate is made up by dissolving 33.6 g $NaHCO_3$ in 500 mL of deionized water. Add 2.5 mL of this stock solution for each liter of the GP2 artificial seawater.

TABLE 1. PREPARATION OF TEST SOLUTIONS AT A SALINITY OF 30‰ USING NATURAL SEAWATER, HYPERSALINE BRINE, OR ARTIFICIAL SEA SALTS [1]

		Solutions To Be Combined	
Effluent Solution	Effluent Concentration (%)	Volume of Effluent Solution (mL)	Volume of Diluent Seawater (30‰) (mL)
1	100[1]	840	—
2	50	420	Solution 1 + 420
3	25	420	Solution 2 + 420
4	12.5	420	Solution 3 + 420
5	6.25	420	Solution 4 + 420
Control	0.0		420
Total			2080

[1] This illustration assumes: (1) the use of 5 mL of test solution in each of four replicates (total of 20 mL) for the control and five concentrations of effluent, (2) an effluent dilution factor of 0.5, (3) the effluent lacks appreciable salinity, and (4) 400 mL of each test concentration is used for chemical analysis. A sufficient initial volume (840 mL) of effluent is prepared by adjusting the salinity to 30‰. In this example, the salinity is adjusted by adding artificial sea salts to the 100% effluent, and preparing a serial dilution using 30‰ seawater (natural seawater, hypersaline brine, or artificial seawater). Stir solutions 1 h to ensure that the salts dissolve. The salinity of the initial 840 mL of 100% effluent is adjusted to 30‰ by adding 25.2 g of dry artificial sea salts (FORTY FATHOMS®). Test concentrations are then made by mixing appropriate volumes of salinity adjusted effluent and 30‰ salinity dilution water to provide 840 mL of solution for each concentration. If hypersaline brine alone (100‰) is used to adjust the salinity of the effluent, the highest concentration of effluent that could be tested would be 70% at 30‰ salinity.

TABLE 2. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR THE SEA URCHIN, *ARBACIA PUNCTULATA*, TOXICITY TEST[1,2,3]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
KCl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

[1] Modified GP2 from Spotte et al. (1984).
[2] The constituent salts and concentrations were taken from USEPA (l990b). The salinity is 30.89 g/L.
[3] GP2 can be diluted with deionized (DI) water to the desired test salinity.

15.6.19 TEST ORGANISMS, SEA URCHINS, *ARBACIA PUNCTULATA*

15.6.19.1 Adult sea urchins, *Arbacia punctulata*, can be obtained from commercial suppliers. After acquisition, the animals are sexed by briefly stimulating them with current from a 12 V transformer. Electrical stimulation causes the immediate release of masses of gametes that are readily identifiable by color -- the eggs are red, and the sperm are white.

15.6.19.2 The sexes are separated and maintained in 20-L, aerated fiberglass tanks, each holding about 20 adults. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or salt water prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded.

15.6.19.3 The culture unit should be maintained at 15 ± 3°C, with a water temperature control device.

15.6.19.4 The food consists of kelp, *Laminaria* sp., gathered from known uncontaminated zones or obtained from commercial supply houses whose kelp comes from known uncontaminated areas, or romaine lettuce. Fresh food is introduced into the tanks at approximately one week intervals. Decaying food is removed as necessary. Ample supplies of food should always be available to the sea urchins.

15.6.19.5 Natural or artificial seawater with a salinity of 30‰ is used to maintain the adult animals, for all washing and dilution steps, and as the control water in the tests (see Subsection 15.6.18).

15.6.19.6 Adult male and female animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, aquaria (or a single partitioned

aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to 15°C before animals are added. Healthy animals will attach to the kelp or aquarium within hours.

15.6.19.7 To successfully maintain about 25 adult animals for 7 days at a field site, a screen-partitioned, 40-L glass aquarium using aerated, recirculating, clean saline water (30‰) and a gravel bed filtration system, is housed within a water bath, such as FORTY FATHOMS® or equivalent (15°C). The inner aquarium is used to avoid contact of animals and water bath with cooling coils.

15.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

15.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sampling Preparation for Toxicity Tests.

15.8 CALIBRATION AND STANDARDIZATION

15.8.1 See Section 4, Quality Assurance.

15.9 QUALITY CONTROL

15.9.1 See Section 4, Quality Assurance.

15.10 TEST PROCEDURES

15.10.1 TEST SOLUTIONS

15.10.1.1 Receiving Waters

15.10.1.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution against a control. Using four replicate chambers per test, each containing 5 mL, and 400 mL for chemical analysis, would require approximately 420 mL or more of sample per test.

15.10.1.2 Effluents

15.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.** If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ and 70% at 30‰ salinity.

15.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%).

15.10.1.2.3 Just prior to test initiation (approximately 1 h), a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (20 ± 1°C) and maintained at that temperature during the addition of dilution water.

15.10.1.2.4 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

15.10.1.2.5 Effluent dilutions should be prepared for all replicates in each treatment in one beaker to minimize variability among the replicates. The test chambers are labeled with the test concentration and replicate number. Dispense into the appropriate effluent dilution chamber.

15.10.1.3 Dilution Water

15.10.1.3.1 Dilution water may be uncontaminated natural seawater (receiving water), HSB prepared from natural seawater, or artificial seawater FORTY FATHOMS® or GP2 sea salts (see Table 2 and Section 7, Dilution Water). Prepare 3 L of control water at 30‰ using HSB or artificial sea salts (see Table 1). This water is used in all washing and diluting steps and as control water in the test. Natural seawater and local waters may be used as additional controls.

15.10.2 COLLECTION OF GAMETES FOR THE TEST

15.10.2.1 Select four females and place in shallow bowls, barely covering the shell with seawater. Stimulate the release of eggs by touching the shell with steel electrodes connected to a 10-12 volt transformer (about 30 seconds each time). Collect the eggs from each female using a 10 mL disposable syringe fitted with an 18-gauge, blunt-tipped needle (tip cut off). Remove the needle from the syringe before adding the eggs to a conical centrifuge tube. Pool the eggs. The egg stock may be held at room temperature for several hours before use. Note: Eggs should be collected first to eliminate possibility of pre-fertilization.

15.10.2.2 Select four males and place in shallow bowls, barely covering the animals with seawater. Stimulate the release of sperm as described above. Collect the sperm (about 0.25 mL) from each male, using a 1-3 mL disposable syringe fitted with an 18-gauge, blunt-tipped needle. Pool the sperm. Maintain the pooled sperm sample on ice. The sperm must be used in a toxicity test within 1 h of collection.

15.10.3 PREPARATION OF SPERM DILUTION FOR USE IN THE TEST

15.10.3.1 Using control water, dilute the pooled sperm sample to a concentration of about 5 X 10^7 sperm/mL (SPM). Estimate the sperm concentration as described below:

1. Make a sperm dilutions of 1:50, 1:100, 1:200, and 1:400, using 30‰ seawater, as follows:

    a. Add 400 µL of collected sperm to 20 mL of seawater in Vial A. Mix by gentle pipetting using a 5-mL pipettor, or by inversion;
    b. Add 10 mL of sperm suspension from Vial A to 10 mL of seawater in Vial B. Mix by gentle pipetting using a 5-mL pipettor, or by inversion;
    c. Add 10 mL of sperm suspension from Vial B to 10 mL of seawater in Vial C. Mix by gentle pipetting using a 5-mL pipettor, or by inversion;
    d. Add 10 mL of sperm suspension from Vial C to 10 mL of seawater in Vial D. Mix by gentle pipetting using a 5-mL pipettor, or by inversion;
    e. Discard 10 mL from Vial D. (The volume of all suspensions is 10 mL).

2. Make a 1:2000 killed sperm suspension and determine the SPM.

    a. Add 10 mL 10% acetic acid in seawater to Vial C. Cap Vial C and mix by inversion.
    b. Add 1 mL of killed sperm from Vial C to 4 mL of seawater in Vial E. Mix by gentle pipetting with a 4-mL pipettor.
    c. Add sperm from Vial E to both sides of the Neubauer hemacytometer. Let the sperm settle 15 min.
    d. Count the number of sperm in the central 400 squares on both sides of the hemacytometer using a compound microscope (100X). Average the counts from the two sides.
    e. SPM in Vial E = 10^4 x average count.

3. Calculate the SPM in all other suspensions using the SPM in Vial E above:

   SPM in Vial A = 40 x SPM in Vial E
   SPM in Vial B = 20 x SPM in Vial E
   SPM in Vial D = 5 x SPM in Vial E
   SPM in original sperm sample = 2000 x SPM in Vial E

4. Dilute the sperm suspension with a SPM greater than 5 x 10^7 SPM to 5 x 10^7 SPM.

   Actual SPM/(5 x 10^7) = dilution factor (DF)

   [(DF) x 10] - 10 = mL of seawater to add to vial.

5. Confirm the sperm count by sampling from the test stock. Add 0.1 mL of test stock to 9.9 mL of 10% acetic acid in seawater, and count with the hemacytometer. The count should average 50 ± 5.

15.10.4 PREPARATION OF EGG SUSPENSION FOR USE IN THE TEST Note: The egg suspension may be prepared during the 1-h sperm exposure.

15.10.4.1 Wash the pooled eggs three times using control water with gentle centrifugation (500xg for 3 minutes using a tabletop centrifuge). If the wash water becomes red, the eggs have lysed and must be discarded.

15.10.4.2 Dilute the egg stock, using control water, to about 2000 eggs/mL.

1. Transfer the eggs to a glass beaker containing 200 mL of control water ("egg stock").

2. Mix the egg stock using an air-bubbling device. Using a wide-mouth pipet tip, transfer 1 mL of eggs from the egg stock to a vial containing 9 mL of control water. (This vial contains an egg suspension diluted 1:10 from egg stock).

3. Mix the contents of the vial by inversion. Using a wide-mouth pipet tip, transfer 1 mL of eggs from the vial to a Sedgwick-Rafter counting chamber. Count all eggs in the chamber using a dissecting microscope at 24X "egg count".

4. Calculate the concentration of eggs in the stock. Eggs/mL = 10X (egg count). Dilute the egg stock to 2000 eggs/mL by the formula below.

   a. If the egg count is equal to or greater than 200:
   (egg count) - 200 = volume (mL) of control water to add to egg stock.
   b. If the egg count is less than 200, allow the eggs to settle and remove enough control water to concentrate the eggs to greater than 200, repeat the count, and dilute the egg stock as in a. above. NOTE: It requires 24 mL of a egg stock solution for each test with a control and five exposure concentrations.
   c. Transfer 1 mL of the diluted egg stock to a vial containing 9 mL of control water. Mix well, then transfer 1 mL from the vial to a Sedgwick-Rafter counting chamber. Count all eggs using a dissecting microscope. Confirm that the final egg count = 2000/mL (± 200).

15.10.5 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

15.10.5.1 The light quality and intensity should be at ambient laboratory levels 10-20 µE/m^2/s (50-100 ft-c) with a photoperiod of 16 h light and 8 h darkness. The water temperature in the test chambers should be maintained at 20 ± 1°C. The test salinity should be in the range of 28 to 32‰. The salinity should vary by no more that ± 2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.10.6 DISSOLVED OXYGEN (DO) CONCENTRATION

15.10.6.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentrations should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet, or equivalent.

15.10.7 OBSERVATIONS DURING THE TEST

15.10.7.1 Routine Chemical and Physical Observations

15.10.7.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

15.10.7.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at least at the end of the test to determine temperature variation in environmental chamber.

15.10.7.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

15.10.7.1.4 Record all the measurements on the data sheet.

15.10.7.2 Routine Biological Observations

15.10.7.2.1 Fertilization will be determined by the presence of a fertilization membrane surrounding the egg.

15.10.8 START OF THE TEST

15.10.8.1 Effluent/receiving water samples are adjusted to salinity of 30‰. Four replicates are prepared for each test concentration, using 5 mL of solution in disposable liquid scintillation vials. A 50% (0.5) concentration series can be prepared by serially diluting test concentrations with control water. Sufficient test solution is prepared at each effluent concentration to provide additional volume for chemical analyses, at the high, medium, and low test concentrations.

15.10.8.2 All test samples are equilibrated at 20°C ± 1°C before addition of sperm.

15.10.8.3 Within 1 h of collection add 100 µL of appropriately diluted sperm to each test vial. Record the time of sperm addition.

15.10.8.4 Incubate all test vials at 20 ± 1°C for 1 h.

15.10.8.5 Mix the diluted egg suspension (2000 eggs/mL), using gentle bubbling. Add 1 mL of diluted egg suspension to each test vial using a wide mouth pipet tip. Incubate 20 min at 20 ± 1°C.

15.10.9 TERMINATION OF THE TEST

15.10.9.1 Terminate the test and preserve the samples by adding 2 mL of 1% formalin in seawater to each vial.

15.10.9.2 Vials should be evaluated within 48 hours.

15.10.9.3 To determine fertilization, transfer about 1 mL eggs from the bottom of a test vial to a Sedgwick-Rafter counting chamber. Observe the eggs using a compound microscope (100X). Count between 100 and 200 eggs/sample. Record the number counted and the number unfertilized. Fertilization is indicated by the presence of a fertilization membrane surrounding the egg. NOTE: adjustment of the microscope to obtain proper contrast may be required to observe the fertilization membrane. Because samples are fixed in formalin, a ventilation hood is set up surrounding the microscope to protect the analyst from prolonged exposure to formaldehyde fumes.

15.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

15.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

15.12 ACCEPTABILITY OF TEST RESULTS

15.12.1 The sperm:egg ratio routinely employed must result in fertilization of 70%-90% of the eggs in the control chambers.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST WITH EFFLUENT AND RECEIVING WATERS (TEST METHOD 1008.0)[1]

1	Test type:	Static (required)
2.	Salinity:	30‰ (± 2‰ of the selected test salinity) (recommended)
3.	Temperature:	20 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4.	Light quality:	Ambient laboratory light during test preparation (recommended)
5.	Light intensity:	10-20 μE/m^2/s, or 50-100 ft-c (Ambient laboratory levels) (recommended)
6.	Test chamber size:	Disposable (glass) liquid scintillation vials (20 mL capacity), presoaked in control water (recommended)
7.	Test solution volume:	5 mL (recommended)
8.	No. of sea urchins:	Pooled sperm from four males and pooled eggs from four females are used per test (recommended)
9.	No. egg and sperm cells per chamber:	About 2,000 eggs and 5,000,000 sperm cells per vial (recommended)
10.	No. replicate chambers per concentration:	4 (required minimum)
11.	Dilution water:	Uncontaminated source of natural seawater; deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, GP2, or equivalent) (available options)
12.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving waters: 100% receiving water (or minimum of 5) and a control (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST WITH EFFLUENT AND RECEIVING WATERS (TEST METHOD 1008.0) (CONTINUED)

13. Dilution factor:	Effluents: ≥0.5 (recommended) Receiving waters: None or ≥0.5 (recommended)
14. Test duration:	1 h and 20 min (required)
15. Endpoint:	Fertilization of sea urchin eggs (required)
16. Test acceptability criteria:	70% - 90% egg fertilization in controls (required)
17. Sampling requirements:	For on-site tests, one sample collected at test initiation, and used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
18. Sample volume required:	1 L per test (recommended)

15.13 DATA ANALYSIS

15.13.1 GENERAL

15.13.1.1 Tabulate and summarize the data. Calculate the proportion of fertilized eggs for each replicate. A sample set of test data is listed in Table 4.

15.13.1.2 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

TABLE 4. DATA FROM SEA URCHIN, *ARBACIA PUNCTULATA,* FERTILIZATION TEST

Copper Concentration (µg/L)	Replicate	No. of Eggs Counted	No. of Eggs Fertilized	Proportion Fertilized
Control	A	100	85	0.85
	B	100	78	0.78
	C	100	87	0.87
2.5	A	100	81	0.81
	B	100	65	0.65
	C	100	71	0.71
5.0	A	100	63	0.63
	B	100	74	0.74
	C	100	78	0.78
10.0	A	100	63	0.63
	B	100	66	0.66
	C	100	51	0.51
20.0	A	100	41	0.41
	B	100	41	0.41
	C	100	37	0.37
40.0	A	100	12	0.12
	B	100	30	0.30
	C	100	26	0.26

[1] Tests performed by Dennis M. McMullen, Technology Applications, Inc., EMSL, Cincinnati, OH.

15.13.1.3 The endpoints of toxicity tests using the sea urchin are based on the reduction in proportion of eggs fertilized. The IC25 and the IC50 are calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for fecundity are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of IC25 and IC50. See the Appendices for examples of the manual computations, and examples of data input and program output.

15.13.2 EXAMPLE OF ANALYSIS OF SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION DATA

15.13.2.1 Formal statistical analysis of the fertilization data is outlined in Figure 4. The response used in the analysis is the proportion of fertilized eggs in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 and IC50 endpoints. Concentrations at which there are no eggs fertilized in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25 and IC50.

15.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and

Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

15.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

15.13.2.4 Example of Analysis of Fecundity Data

15.13.2.4.1 This example uses toxicity data from a sea urchin, *Arbacia punctulata*, fertilization test performed with copper. The response of interest is the proportion of fertilized eggs, thus each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each copper concentration and control are listed in Table 5. The data are plotted in Figure 5.

15.13.2.5 Test for Normality

15.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

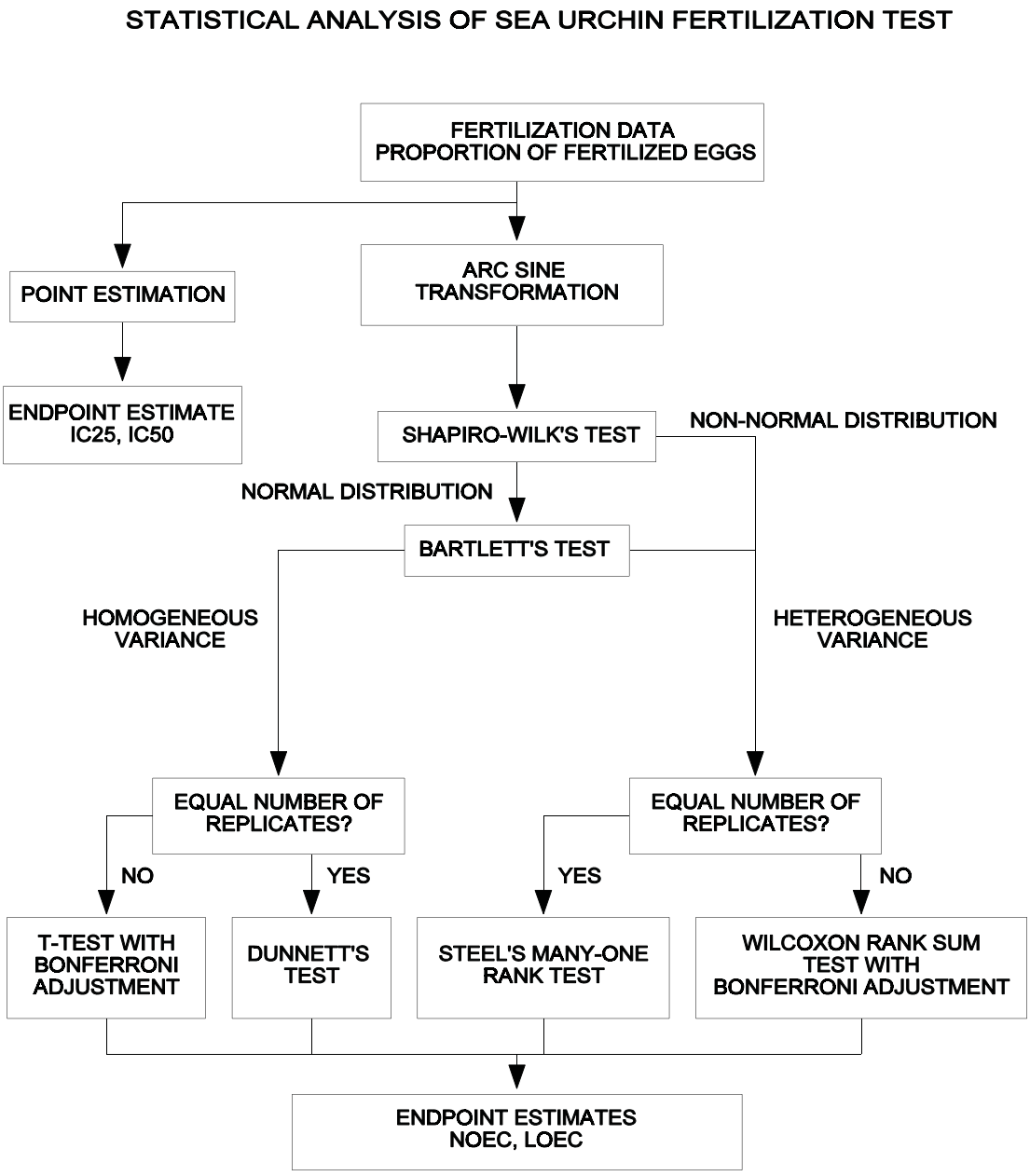

Figure 4. Flowchart for statistical analysis of sea urchin, *Arbacia punctulata*, by point estimation.

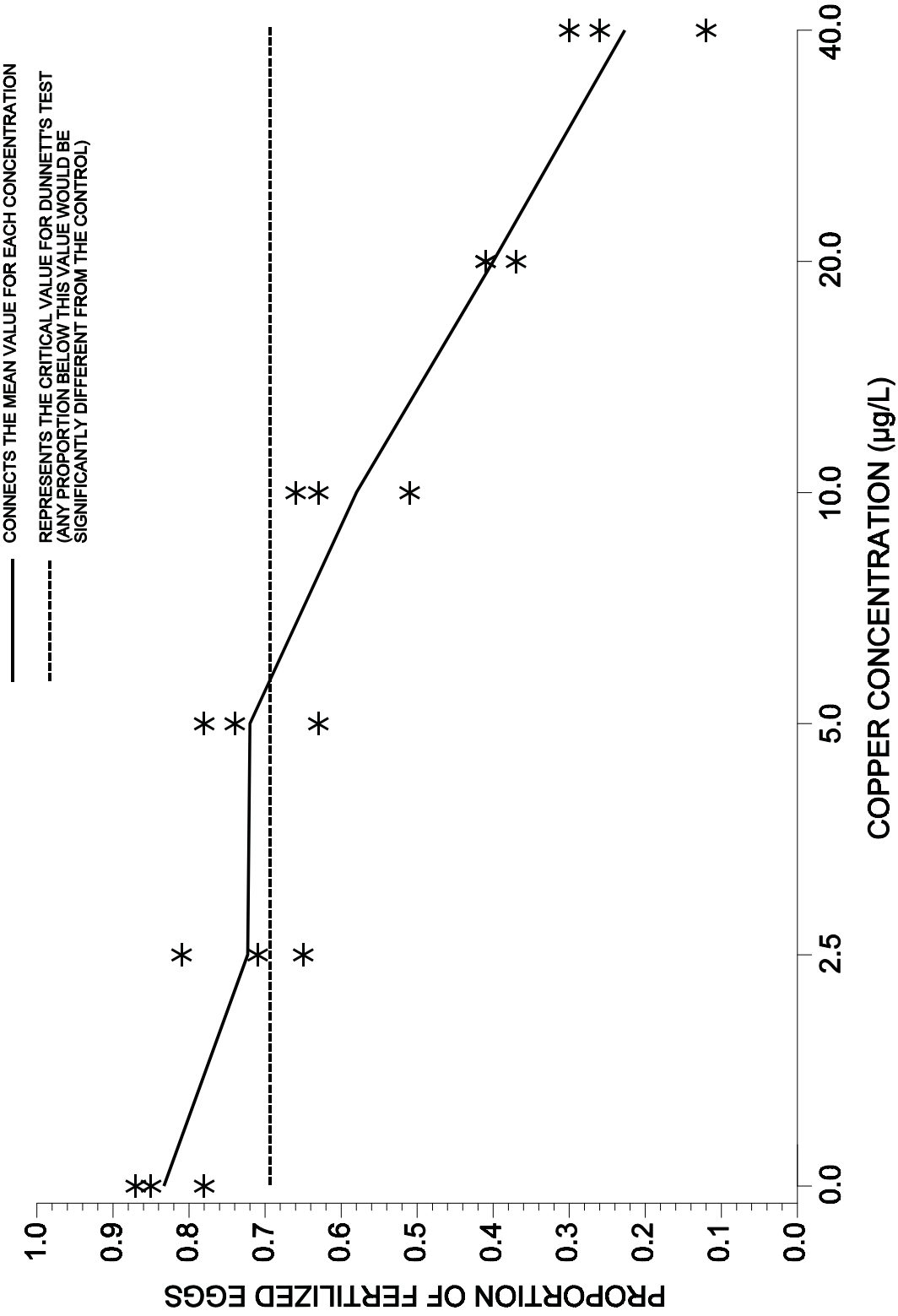

Figure 5. Plot of mean percent of fertilized sea urchin, *Arbacia punctulata*, eggs.

TABLE 5. SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION DATA

			Copper Concentration (µg/L)				
	Replicate	Control	2.5	5.0	10.0	20.0	40.0
	A	0.85	0.81	0.63	0.63	0.41	0.12
RAW	B	0.78	0.65	0.74	0.66	0.41	0.30
	C	0.87	0.71	0.78	0.51	0.37	0.26
ARC SINE	A	1.173	1.120	0.917	0.917	0.695	0.354
TRANSFORMED	B	1.083	0.938	1.036	0.948	0.695	0.580
	C	1.202	1.002	1.083	0.795	0.654	0.535
Mean ($\bar{Y}_i$)		1.153	1.020	1.012	0.887	0.681	0.490
S_i^2		0.004	0.009	0.007	0.007	0.001	0.014
i		1	2	3	4	5	6

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Copper Concentration (µg/L)				
Replicate	Control	2.5	5.0	10.0	20.0	40.0
A	0.020	0.100	-0.095	0.030	0.014	-0.136
B	-0.070	-0.082	0.024	0.061	0.014	0.090
C	0.049	-0.018	0.071	-0.092	-0.027	0.045

15.13.2.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

15.13.2.5.3 For this set of data, n = 18

$$\bar{X} = \frac{1}{18}(0) = 0$$

$$D = 0.0822$$

15.13.2.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \le X^{(2)} \le \ldots \le X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 7.

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.136	10	0.020
2	-0.095	11	0.024
3	-0.092	12	0.030
4	-0.082	13	0.045
5	-0.070	14	0.049
6	-0.027	15	0.061
7	-0.018	16	0.071
8	0.014	17	0.090
9	0.014	18	0.100

15.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 18 and k = 9. The a_i values are listed in Table 8.

15.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 8. For the data in this example:

$$W = \frac{1}{0.0822}(0.2782)^2 = 0.942$$

15.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 15.13.2.5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 18 observations is 0.858. Since W = 0.942 is greater than the critical value, conclude that the data are normally distributed.

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4886	0.236	$X^{(18)} - X^{(1)}$
2	0.3253	0.185	$X^{(17)} - X^{(2)}$
3	0.2553	0.163	$X^{(16)} - X^{(3)}$
4	0.2027	0.143	$X^{(15)} - X^{(4)}$
5	0.1587	0.119	$X^{(14)} - X^{(5)}$
6	0.1197	0.072	$X^{(13)} - X^{(6)}$
7	0.0837	0.048	$X^{(12)} - X^{(7)}$
8	0.0496	0.010	$X^{(11)} - X^{(8)}$
9	0.0163	0.006	$X^{(10)} - X^{(9)}$

15.13.2.6 Test for Homogeneity of Variance

15.13.2.6.1 The test used to examine whether the variation in the proportion of fertilized eggs is the same across all copper concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i)\ \ln \overline{S}^2 - \sum_{i=1}^{p} V_i\ \ln\ S_i^2]}{C}$$

Where: V_i = degrees of freedom for each copper concentration and control, $V_i = (n_i - 1)$

p = number of levels of copper concentration including the control

n_i = the number of replicates for concentration i.

ln = $\log_e$

i = 1,2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i^2)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{p} 1/V_i - (\sum_{i=1}^{p} V_i)^{-1}]$$

15.13.2.6.2 For the data in this example (see Table 5), all copper concentrations including the control have the same number of replicates ($n_i = 3$ for all i). Thus, $V_i = 2$ for all i.

15.13.2.6.3 Bartlett's statistic is, therefore:

$$B = [(12)\ln(0.0007) - 2\sum_{i=1}^{p} \ln(S_i^2)]/1.194$$

$$= [12(-4.962) - 2(-31.332)]/1.194$$

$$= 3.122/1.194$$

$$= 2.615$$

15.13.2.6.4 B is approximately distributed as chi-square with p-1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with 5 degrees of freedom, is 15.09. Since B = 2.615 is less than the critical value of 15.09, conclude that the variances are not different.

15.13.2.7 Dunnett's Procedure

15.13.2.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 9.

TABLE 9. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N \qquad \text{Between Sum of Squares}$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N \qquad \text{Total Sum of Squares}$$

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{p} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the proportion of fertilized eggs for upper concentration i in test chamber j)

15.13.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 3$

$N = 18$

$T_1 = Y_{11} + Y_{12} + Y_{13} = 3.458$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 3.060$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 3.036$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 2.660$
$T_5 = Y_{51} + Y_{52} + Y_{53} = 2.044$
$T_6 = Y_{61} + Y_{62} + Y_{63} = 1.469$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 15.727$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$= (43.950)/3 - (15.727)^2/18 = 0.909$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$= 14.732 - (15.727)^2/18 = 0.991$

$SSW = SST - SSB$

$= 0.991 - 0.909 = 0.082$

$S_B^2 = SSB/(p-1) = 0.909/(6-1) = 0.182$

$S_W^2 = SSW/(N-p) = 0.082/(18-6) = 0.007$

15.13.2.7.3 Summarize these calculations in the ANOVA table (Table 10).

TABLE 10. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	5	0.909	0.182
Within	12	0.082	0.007
Total	17	0.991	

15.13.2.7.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean proportion fertilized eggs for copper concentration i

$\bar{Y}_1$ = mean proportion fertilized eggs for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

Since we are looking for a decreased response from the control in the proportion of fertilized eggs, the concentration mean is subtracted from the control mean.

15.13.2.7.5 Table 11 includes the calculated t values for each concentration and control combination. In this example, comparing the 2.5 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.153 - 1.020)}{[0.084\sqrt{(1/3) + (1/3)}]} = 1.939$$

TABLE 11. CALCULATED T VALUES

Copper Concentration (µg/L)	i	t_i
2.5	2	1.939
5.0	3	2.056
10.0	4	3.878
20.0	5	6.882
40.0	6	9.667

15.13.2.7.6 Since the purpose of this test is to detect a significant decrease in the proportion of fertilized eggs, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix D. For an overall alpha level of 0.05, 12 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.50. The mean proportion of fertilized eggs for concentration i is considered significantly less than the mean proportion of fertilized eggs for the control if t_i is greater than the critical value. Therefore, the 10.0 µg/L, 20.0 µg/L and 40.0 µg/L concentrations have a significantly lower mean proportion of fertilized eggs than the control. Hence the NOEC is 5.0 µg/L and the LOEC is 10.0 µg/L.

15.13.2.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\ S_w\sqrt{(1/n_1) + (1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

15.13.2.7.8 In this example,

$$MSD = 2.50\,(0.084)\sqrt{(1/3) + (1/3)}$$

$$= 2.50\ (0.084)(0.816)$$

$$= 0.171$$

15.13.2.7.9 The MSD (0.171) is in transformed units. To determine the MSD in terms of proportion of fertilized eggs, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

$$1.153 - 0.171 = 0.982$$

2. Obtain the untransformed values for the control mean and the difference calculated in step 1 of 15.13.2.7.9.1

$$[\ \text{Sine}\ (1.153)\ ]^2 = 0.835$$

$$[\text{ Sine } (0.982)\ ]^2 = 0.692$$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from step 2 in 15.13.2.7.9.

$$MSD_u = 0.835 - 0.692 = 0.143$$

15.13.2.7.10 Therefore, for this set of data, the minimum difference in mean proportion of fertilized eggs between the control and any copper concentration that can be detected as statistically significant is 0.143.

15.13.2.7.11 This represents a 17% decrease in the proportion of fertilized eggs from the control.

15.13.2.8 Calculation of the ICp

15.13.2.8.1 The fertilization data in Table 4 are utilized in this example. Table 12 contains the mean proportion of fertilized eggs for each toxicant concentration. As can be seen, the observed means are monotonically non-increasing with respect to concentration. Therefore, it is not necessary to smooth the means prior to calculating the ICp; (see Figure 5 for a plot of the response curve).

15.13.2.8.2 An IC25 and IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of fertilized eggs, compared to the controls, would result in a mean proportion of 0.625, where $M_1(1-p/100) = 0.833(1-25/100)$. A 50% reduction in mean proportion of fertilized eggs, compared to the controls, would result in a mean proportion of 0.417. Examining the means and their associated concentrations (Table 12), the response, 0.625, is bracketed by $C_3 = 5.0$ µg/L copper and $C_4 = 10.0$ µg/L copper. The response, 0.417, is bracketed by $C_4 = 10.0$ µg/L copper and $C_5 = 20.0$ µg/L copper.

TABLE 12. SEA URCHIN, *ARBACIA PUNCTULATA*, MEAN PROPORTION OF FERTILIZED EGGS

Copper Conc. (µg/L)	i	Response Means Y_i (proportion)	Smoothed Mean M_i (proportion)
Control	1	0.833	0.833
2.5	2	0.723	0.723
5.0	3	0.717	0.717
10.0	4	0.600	0.600
20.0	5	0.397	0.397
40.0	6	0.227	0.227

15.13.2.8.3 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\ \frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 5.0 + [0.833(1 - 25/100) - 0.717]\ \frac{(10.0 - 5.0)}{(0.600 - 0.717)}$$

$$= 8.9 \text{ µg/L.}$$

15.13.2.8.4 Using the equation from Section 4.2 in Appendix L, the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j] \frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC50 = 10.0 + [0.833(1 - 50/100) - 0.600] \frac{(20.0 - 10.0)}{(0.397 - 0.600)}$$

$$= 19.0 \text{ µg/L.}$$

15.13.2.8.5 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 8.9286 µg/L. The empirical 95.0% confidence interval for the true mean was 3.3036 µg/L to 14.6025 µg/L. The computer program output for the IC25 for this data set is shown in Figure 6.

15.13.2.8.6 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 19.0164 µg/L. The empirical 95.0% confidence interval for the true mean was 16.1083 µg/L to 23.6429 µg/L. The computer program output for the IC50 for this data set is shown in Figure 7.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	2.5	5.0	10.0	20.0	40.0
Response 1	.85	.81	.63	.63	.41	.12
Response 2	.78	.65	.74	.66	.41	.3
Response 3	.87	.71	.71	.51	.37	.2

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date: Test Ending Date:
Test Species: sea urchin, Arbacia punctulata
Test Duration:
DATA FILE: urchin.icp
OUTPUT FILE: urchin.i25

Conc. ID	Number Replicates	Concentration µg/L	Response Means	Standard. Dev.	Pooled Response Means
1	3	0.000	0.833	0.047	0.833
2	3	2.500	.723	0.081	0.723
3	3	5.000	0.717	0.078	0.717
4	3	10.000	0.600	0.079	0.600
5	3	20.000	0.397	0.023	0.397
6	3	40.000	0.227	0.095	0.227

The Linear Interpolation Estimate: 8.9286 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 8.7092 Standard Deviation: 0.8973
Original Confidence Limits: Lower: 6.2500 Upper: 11.6304
Expanded Confidence Limits Lower: 3.3036 Upper: 14.6025
Resampling time in Seconds: 1.59 Random Seed: 1834854321

Figure 6. ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	2.5	5.0	10.0	20.0	40.0
Response 1	.85	.81	.63	.63	.41	.12
Response 2	.78	.65	.74	.66	.41	.3
Response 3	.87	.71	.78	.51	.37	.26

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date: Test Ending Date:
Test Species: MYSID SHRIMP
Test Duration: fecundity
DATA FILE: mysidfe.icp
OUTPUT FILE: mysidfe.i50

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Standard. Dev.	Pooled Response Means
1	8	0.000	0.934	0.127	0.934
2	7	50.000	0.426	0.142	0.426
3	7	100.000	0.317	0.257	0.317
4	8	210.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 19.0164 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 19.0013 Standard Deviation: 0.8973
Original Confidence Limits: Lower: 17.6316 Upper: 21.2195
Expanded Confidence Limits: Lower: 16.1083 Upper: 23.6492
Resampling time in Seconds: 1.65 Random Seed: -823775279

Figure 7. ICPIN program output for the IC50.

15.14 **PRECISION AND ACCURACY**

15.14.1 PRECISION

15.14.1.1 Single-Laboratory Precision

15.14.1.1.1 Single-laboratory precision data for the reference toxicants, copper (Cu) and sodium dodecyl sulfate (SDS), tested in FORTY FATHOMS® artificial seawater, GP2 artificial seawater, and natural seawater are provided in Tables 13-18. The test results were similar in the three types of seawater. The IC25 and IC50 for the reference toxicants (copper and sodium dodecyl sulfate) are reported in Tables 13-16. The coefficient of variation, based on the IC25, is 28.7% to 54.6% for natural and FORTY FATHOMS® seawater, indicating acceptable precision. The IC50 ranges from 23.3% to 48.2%, showing acceptable precision.

15.14.1.2 Multilaboratory Precision

15.14.1.2.1 No data are available on the multilaboratory precision of the test.

15.14.2 ACCURACY

15.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 13. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING GAMETES FROM ADULTS MAINTAINED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, OR OBTAINED DIRECTLY FROM NATURAL SOURCES, AND COPPER (CU) AS A REFERENCE TOXICANT[1,2,3,4,5]

Test Number	LOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)
1	5.0	8.92	29.07
2	12.5	26.35	38.96
3	<6.2	11.30	23.93
4	6.2	34.28	61.75
5	12.5	36.67	75.14
n:	4	5	5
Mean:	NA	23.51	45.77
CV(%):	NA	54.60	47.87

1 Data from USEPA (1991a)
2 Tests performed by Dennis McMullen, Technology Applications, Inc., EMSL, Cincinnati, OH.
2 All tests were performed using FORTY FATHOMS® synthetic seawater.
3 Copper test solutions were prepared with copper sulfate. Copper concentrations in Test 1 were: 2.5, 5.0, 10.0, 20.0, and 40.0 µg/L. Copper concentrations in Tests 2-5 were: 6.25, 12.5, 25.0, 50.0, and 100.0 µg/L.
4 NOEC Range: < 5.0 - 12.5 µg/L (this represents a difference of one exposure concentrations).
5 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 14. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING GAMETES FROM ADULTS MAINTAINED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, OR OBTAINED DIRECTLY FROM NATURAL SOURCES, AND SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT[1,2,3,4,5,6]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)
1	<0.9	1.11	1.76
2	0.9	1.27	1.79
3	1.8	2.26	2.87
4	0.9	1.90	2.69
5	1.8	2.11	2.78
n:	4	5	5
Mean:	NA	1.73	2.38
CV(%):	NA	29.7	23.3

1 Data from USEPA (1991a)
2 Tests performed by Dennis M. McMullen, Technology Applications, Inc., EMSL, Cincinnati, OH.
3 All tests were performed using FORTY FATHOMS® synthetic seawater.
4 NOEC Range: <0.9 - 1.8 mg/L (this represents a difference of two exposure concentration).
5 SDS concentrations for all tests were: 0.9, 1.8, 3.6, 7.2, and 14.4 mg/L.
6 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 15. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN NATURAL SEAWATER, USING GAMETES FROM ADULTS MAINTAINED IN NATURAL SEAWATER AND COPPER (CU) SULFATE AS A REFERENCE TOXICANT [1,2,3,4,5,6]

Test Number	NOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)
1	12.2	14.2	18.4
2	12.2	32.4	50.8
3	24.4	30.3	46.3
4	<6.1	26.2	34.1
5	6.1	11.2	17.2
n:	4	5	5
Mean:	NA	22.8	29.9
CV(%):	NA	41.9	48.2

1 Data from USEPA (1991a)
2 Tests performed by Ray Walsh and Wendy Greene, ERL-N, USEPA, Narragansett, RI.
3 Copper concentrations were: 6.1, 12.2, 24.4, 48.7, and 97.4 µg/L.
4 NOEC Range: < 6.1 - 24.4 µg/L (this represents a difference of two exposure concentrations).
5 Adults collected in the field.
6 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 16. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN NATURAL SEAWATER, USING GAMETES FROM ADULTS MAINTAINED IN NATURAL SEAWATER AND SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT [1,2,3,4,5,6]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)
1	1.8	2.3	2.7
2	1.8	3.9	5.1
3	1.8	2.3	2.9
4	0.9	2.1	2.6
5	1.8	2.3	2.7
n:	5	5	5
Mean:	NA	2.58	3.2
CV(%):	NA	28.7	33.3

1 Data from USEPA (1991a).
2 Tests performed by Ray Walsh and Wendy Greene, ERL-N, USEPA, Narragansett, RI.
3 SDS concentrations were: 0.9, 1.8, 3.6, 7.3, and 14.5 mg/L.
4 NOEC Range: 0.9 - 1.8 mg/L (this represents a difference of one exposure concentration).
5 Adults collected in the field.
6 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 17. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN GP2, USING GAMETES FROM ADULTS MAINTAINED IN GP2 ARTIFICIAL SEAWATER AND COPPER (CU) SULFATE AND SODIUM DODECYL SULFATE (SDS) AS REFERENCE TOXICANTS[1,2,3,4,5]

	Cu (µg/L)				SDS (mg/L)			
Test	LC50	CI	NOEC	LOEC	LC50	CI	NOEC	LOEC
1	29.1	27.3-31.1	6.3	12.5	2.1	2.0-2.1	1.3	2.5
2	47.6	44.6-50.8	25.0	50.0	1.8	1.8-1.9	1.3	2.5
3	32.7	29.8-35.8	6.3	12.5	2.2	2.1-2.2	1.3	2.5
4	78.4	73.3-83.9	50.0	100.0	2.3	2.2-2.4	1.3	2.5
5	45.6	41.0-50.7	12.5	25.0	1.8	1.7-2.8	1.3	2.5
Mean	46.7				2.0			
SD	19.5				0.2			
CV	41.8				10.0			

1 Tests performed by Pamela Comeleo, Science Application International Corp., ERL-N, USEPA, Narragansett, RI.
2 All tests were performed using GP2 artificial seawater.
3 Copper concentrations were: 6.25, 12,5, 25.0, 50.0 and 100 µg/L.
4 SDS concentrations were: 0.6, 1.25, 2.5, 5.0, and l0.0 mg/L. SDS stock (14.645 mg/mL) provided by EMSL, USEPA, Cincinnati, OH.
5 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 18. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN NATURAL SEAWATER, USING GAMETES FROM ADULTS MAINTAINED IN NATURAL SEAWATER AND COPPER (CU) SULFATE AND SODIUM DODECYL SULFATE (SDS) AS REFERENCE TOXICANTS[1,2,3,4]

	Cu (µg/L)				SDS (mg/L)			
Test	LC50	CI	NOEC	LOEC	LC50	CI	NOEC	LOEC
1	28.6	26.7-30.6	6.3	12.5	12.5	2.1-2.2	1.3	2.5
2	13.0	11.9-14.2	6.3	12.5	12.5	1.9-2.0	1.3	2.5
3	67.8	63.2-72.6	6.3	12.5	12.5	2.1-2.3	1.3	2.5
4	36.7	33.9-398	< 6.3	6.3	6.3	3.3-3.4	< 0.6	0.6
5	356	33.6-37.7	< 6.3	6.3	6.3	2.8-3.1	< 0.6	0.6
Mean	36.3				2.5			
SD	20.0				0.58			
CV	55.1				23.2			

[1] Tests performed by Anne Kuhn-Hines, Catherine Sheehan, Glen Modica, and Pamela Comeleo, Science Application International Corp., ERL-N, USEPA, Narragansett, RI.

[2] Copper concentrations were prepared with copper sulfate. Concentrations were 6.25, 12.5, 25.0, 50.0, and l00 µg/L.

[3] SDS concentrations were: 0.6, 1.25, 2.5, 5.0, and 10.0 mg/L. SDS stock (14.64 mg/mL) provided by EMSL, USEPA, Cincinnati, OH.

[4] For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

SECTION 16

TEST METHOD

RED MACROALGA, *CHAMPIA PARVULA,* SEXUAL REPRODUCTION TEST METHOD 1009.0

16.1 SCOPE AND APPLICATION

16.1.1 CAUTION: The Red Macroalga, *Champia parvula*, Reproduction Test Method 1009.0 is not listed at 40 CFR Part 136 for nationwide use.

16.1.2 This method, adapted in part from USEPA (1987f) measures the effects of toxic substances in effluents and receiving water on the sexual reproduction of the marine red macroalga, *Champia parvula*. The method consists of exposing male and female plants to test substances for two days, followed by a 5-7 day recovery period in control medium, during which the cystocarps mature.

16.1.3 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

16.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, highly volatile and highly degradable toxicants present in the source may not be detected in the test.

16.1.5 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

16.2 SUMMARY OF METHOD

16.2.1 Sexually mature male and female branches of the red macroalga, *Champia parvula*, are exposed in a static system for 2 days to different concentrations of effluent, or to receiving water, followed by a 5 to 7 day recovery period in control medium. The recovery period allows time for the development of cystocarps resulting from fertilization during the exposure period. The test results are reported as the concentration of the test substance which causes a statistically significant reduction in the number of cystocarps formed.

16.3 INTERFERENCES

16.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

16.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

16.3.3 Adverse effects of high concentrations of suspended and/or dissolved solids, and extremes of pH, may mask the presence of toxic substances.

16.3.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

16.4 SAFETY

16.4.1 See Section 3, Safety and Health.

16.5 APPARATUS AND EQUIPMENT

16.5.1 Facilities for holding and acclimating test organisms.

16.5.2 Laboratory red macroalga, *Champia parvula*, culture unit -- see culturing methods below. To test effluent or receiving water toxicity, sufficient numbers of sexually mature male and female plants must be available.

16.5.3 Samplers -- automatic samplers, preferably with sample cooling capability, that can collect a 24-h composite sample of 1 L.

16.5.4 Environmental chamber or equivalent facility with temperature control (23 ± 1°C).

16.5.5 Water purification system -- Millipore Milli-Q®, deionized water (DI) or equivalent.

16.5.6 Air pump -- for oil-free air supply.

16.5.7 Air lines, and air stones -- for aerating cultures.

16.5.8 Balance -- Analytical, capable of accurately weighing to 0.00001 g.

16.5.9 Reference weights, Class S -- for checking performance of balance.

16.5.10 Meter, pH -- for routine physical and chemical measurements.

16.5.11 Dissecting (stereoscope) microscope -- for counting cystocarps.

16.5.12 Compound microscope -- for examining the condition of plants.

16.5.13 Count register, 2-place -- for recording cystocarp counts.

16.5.14 Rotary shaker -- for incubating exposure chambers (hand-swirling twice a day can be substituted).

16.5.15 Drying oven -- to dry glassware.

16.5.16 Filtering apparatus -- for use with membrane filters (47 mm).

16.5.17 Refractometer -- for determining salinity.

16.5.1 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

16.5.19 Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.

16.5.20 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, l979b) -- to calibrate laboratory thermometers.

16.5.21 Beakers -- Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

16.5.22 Erlenmeyer flasks, 250 mL, or 200 mL disposable polystyrene cups, with covers -- for use as exposure chambers.

16.5.23 Bottles -- borosilicate glass or disposable polystyrene cups (200-400 mL) for use as recovery vessels.

16.5.24 Wash bottles -- for deionized water, for rinsing small glassware and instrument electrodes and probes.

16.5.25 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

16.5.26 Micropipettors, digital, 200 and 1000 µL – to make dilutions.

16.5.27 Pipets, volumetric -- Class A, 1-100 mL.

16.5.28 Pipettor, automatic -- adjustable, 1-100 mL.

16.5.29 Pipets, serological -- 1-10 mL, graduated.

16.5.30 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

16.5.31 Forceps, fine-point, stainless steel -- for cutting and handling branch tips.

16.6 REAGENTS AND CONSUMABLE MATERIALS

16.6.1 Mature red macroalga, *Champia parvula*, plants -- see Subsection 16.6.14 below.

16.6.2 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

16.6.3 Petri dishes, polystyrene -- to hold plants for cystocarp counts and to cut branch tips. Other suitable containers may be used.

16.6.4 Disposable tips for micropipettors.

16.6.5 Aluminum foil, foam stoppers, or other closures -- to cover culture and test flasks.

16.6.6 Tape, colored -- for labeling test chambers.

16.6.7 Markers, waterproof -- for marking containers, etc.

16.6.8 Data sheets (one set per test) -- for data recording.

16.6.9 Buffers, pH 4, pH 7, and pH l0 (or as per instructions of instrument manufacturer) for standards and calibration check (see USEPA Method 150.1, USEPA, 1979b).

16.6.10 Laboratory quality assurance samples and standards for the above methods.

16.6.11 Reference toxicant solutions see Section 4, Quality Assurance.

16.6.12 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

16.6.13 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

16.6.13.1 Saline test and dilution water -- the use of natural seawater is recommended for this test. A recipe for the nutrients that must be added to the natural seawater is given in Table 1. The salinity of the test water must be 30‰, and vary no more than ± 2‰ among the replicates. If effluent and receiving water tests are conducted concurrently, the salinity of these tests should be similar.

16.6.13.2 The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Therefore, exposure of the red macroalga, *Champia parvula*, to effluents will usually require adjustments in the salinity of the test solutions. Although the red macroalga, *Champia parvula*, cannot be cultured in 100% artificial seawater, 100% artificial seawater can be used during the two day exposure period. This allows 100% effluent to be tested. It is important to maintain a constant salinity across all treatments. The salinity of the effluent can be adjusted by adding hypersaline brine (HSB) prepared from natural seawater (100‰), concentrated (triple strength) salt solution (GP2 described in Table 2), or dry GP2 salts (Table 2), to the effluent to provide a salinity of 30‰. Control solutions should be prepared with the same percentage of natural seawater and at the same salinity (using deionized water adjusted with dry salts, or brine) as used for the effluent dilutions.

16.6.13.3 Artificial seawater -- A slightly modified version of the GP2 medium (Spotte, et al, 1984) has been used successfully to perform the red macroalga sexual reproduction test. The preparation of artificial seawater (GP2) is described in Table 2.

TABLE 1. NUTRIENTS TO BE ADDED TO NATURAL SEAWATER AND TO ARTIFICIAL SEAWATER (GP2) DESCRIBED IN TABLE 2. THE CONCENTRATED NUTRIENT STOCK SOLUTION IS AUTOCLAVED FOR 15 MINIMUM (VITAMINS ARE AUTOCLAVED SEPARATELY FOR 2 MINIMUM AND ADDED AFTER THE NUTRIENT STOCK SOLUTION IS AUTOCLAVED). THE pH OF THE SOLUTION IS ADJUSTED TO APPROXIMATELY pH 2 BEFORE AUTOCLAVING TO MINIMIZE THE POSSIBILITY OF PRECIPITATION

	Amount of Reagent Per Liter of Concentrated Nutrient Stock Solution	
	Stock Solution For Culture Medium	Stock Solution For Test Medium
Nutrient Stock Solution[1]		
$NaNO_3$	6.35 g	1.58 g
$NaH_2P0_4 \cdot H_2O$	0.64 g	0.16 g
$Na_2EDTA \cdot 2\ H_2O$	133 mg	--
$Na_3C_6H_5O_7 \cdot 2\ H_2O$	51 mg	12.8 mg
Iron[2]	9.75 mL	2.4 mL
Vitamins[3]	10 mL	2.5 mL

1 Add 10 mL of appropriate nutrient stock solution per liter of culture or test medium.

2 A stock solution of iron is made that contains 1 mg iron/mL. Ferrous or ferric chloride can be used.

3 A vitamin stock solution is made by dissolving 4.88 g thiamine HCl, 2.5 mg biotin, and 2.5 mg B_{12} in 500 mL deionized water. Adjust approximately pH 4 before autoclaving 2 min. It is convenient to subdivide the vitamin stock into 10 mL volumes in test tubes prior to autoclaving.

TABLE 2. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR USE IN CONJUNCTION WITH NATURAL SEAWATER FOR THE RED MACROALGA, *CHAMPIA PARVULA*, CULTURING AND TOXICITY TESTING[1,2,3,4,5,6,7]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
KCl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

1 Modified GP2 from Spotte et al. (1984).
2 The constituent salts and concentrations were taken from USEPA (l990b).
3 The original formulation calls for autoclaving anhydrous and hydrated salts separately to avoid precipitation. However, if the sodium bicarbonate is autoclaved separately (dry), all of the other salts can be autoclaved together. Since no nutrients are added until needed, autoclaving is not critical for effluent testing. To minimize microalgal contamination, the artificial seawater should be autoclaved when used for stock cultures. Autoclaving (120°C) should be for a least 10 minimum for 1-L volumes, and 20 minimum for 10-to-20-L volumes.
4 Prepare in 10-L to 20-L batches.
5 A stock solution of 68 mg/mL sodium bicarbonate is prepared by autoclaving it as a dry powder, and then dissolving it in sterile deionized water. For each liter of GP2, use 2.5 mL of this stock solution.
6 Effluent salinity adjustment to 30‰ can be made by adding the appropriate amount of dry salts from this formulation, by using a triple-strength brine prepared from this formulation, or by using a 100‰ salinity brine prepared from natural seawater.
7 Nutrients listed in Table 1 should be added to the artificial seawater in the same concentration described for natural seawater.

16.6.14 TEST ORGANISMS RED MACROALGA, *CHAMPIA PARVULA*

16.6.14.1 Cultures

16.6.14.1.1 Mature plants are illustrated in Figure 1. The adult plant body (thallus) is hollow, septate, and highly branched. New cultures can be propagated asexually from excised branches, making it possible to maintain clonal material indefinitely.

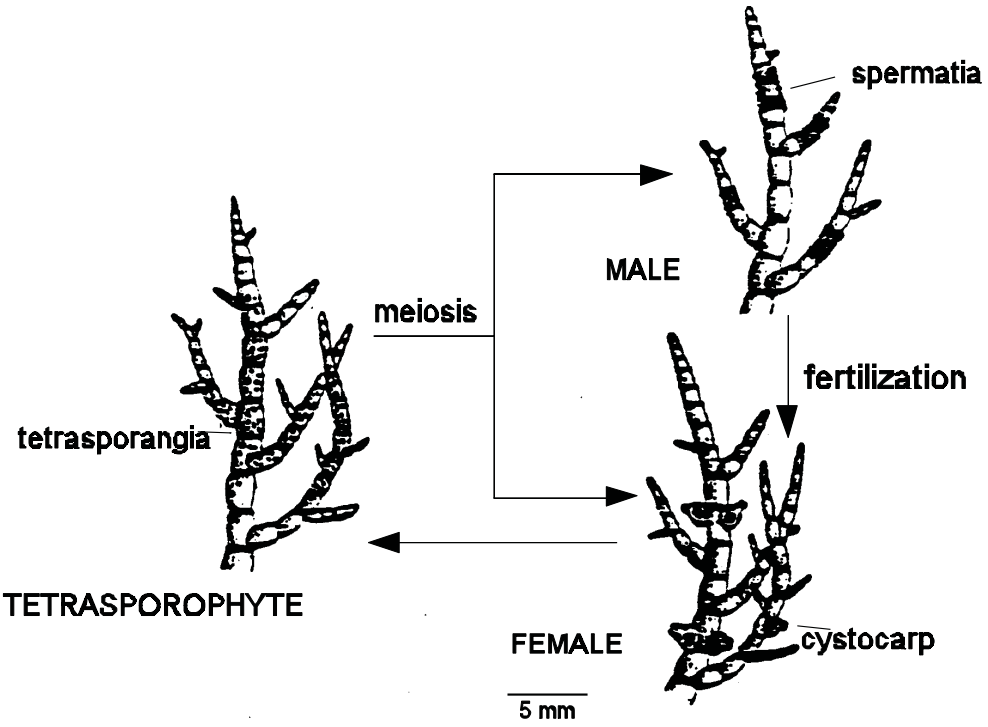

Figure 1. Life history of the red macroalga, *Champia parvula*. Upper left: Size and degree of branching in female branch tips used for toxicity tests. From USEPA (1987f).

16.6.14.1.2 Unialgal stock cultures of both males and females are maintained in separate, aerated 1000 mL Erlenmeyer flasks containing 800 mL of the culture medium. All culture glass must be acid-stripped in 15% HCl and rinsed in deionized water after washing. This is necessary since some detergents can leave a residue that is toxic to the red macroalga, *Champia parvula*. Periodically (at least every 6 months) culture glassware should be baked in a muffle furnace to remove organic material that may build up on its surface. Alternately, a few mL of concentrated sulfuric acid can be rolled around the inside of wet glassware. CAUTION: the addition of acid to the wet glassware generates heat.

16.6.14.1.3 The culture medium is made from natural seawater to which additional nutrients are added. The nutrients added are listed in Table 1. Almost any nutrient recipe can be used for the red macroalga, *Champia parvula*, cultured in either natural seawater or a 50-50 mixture of natural and artificial seawaters. Healthy, actively growing plants are the goal, not a standard nutrient recipe for cultures.

16.6.14.1.4 Several cultures of both males and females should be maintained simultaneously to keep a constant supply of plant material available. To maintain vigorous growth, initial stock cultures should be started periodically with about twenty 0.5 to l.0 cm branch tips. Cultures are gently aerated through sterile, cotton-plugged, disposable, polystyrene l mL pipettes. Cultures are capped with foam plugs and aluminum foil and illuminated with ca. 75 $\mu E/m^2/s$ (500 ft-c) of cool-white fluorescent light on a 16:8 h light:dark cycle. Depending on the type of culture chamber or room used, i.e.,

the degree of reflected light, the light levels may have to be adjusted downward. The temperature is 22 to 24°C and the salinity 28-30‰. Media are changed once a week.

16.6.14.1.5 Prior to use in toxicity tests, stock cultures should be examined to determine their condition. Females can be checked by examining a few branch tips under a compound microscope (100 X or greater). Several trichogynes (reproductive hairs to which the spermatia attach) should be easily seen near the apex (Figure 2).

16.6.14.1.6 Male plants should be visibly producing spermatia. This can be checked by placing some male tissue in a petri dish, holding it against a dark background and looking for the presence of spermatial sori. Mature sori can also be easily identified by looking along the edge of the thallus under a compound microscope (Figures 3 and 4).

16.6.14.1.7 A final, quick way to determine the relative "health" of the male stock culture is to place a portion of a female plant into some of the water from the male culture for a few seconds. Under a compound microscope numerous spermatia should be seen attached to both the sterile hairs and the trichogynes (Figure 5).

16.6.14. Culture medium prepared from natural seawater is preferred (Table 1). However, as much as 50% of the natural seawater may be replaced by the artificial seawater (GP2) described in Table 2.

16.6.14.2.1 Seawater for cultures is filtered at least to 0.45 µm to remove most particulates and then autoclaved for 30 minute at 15 psi (120°C). Carbon stripping the seawater may be necessary before autoclaving to enhance its water quality (USEPA, 1990b). This is done by adding 2 g activated carbon per liter of seawater and stirring on a stir plate for 2 h. After stirring filter through a Whatman number 2 filter, then through a 0.45 membrane filter. The culture flasks are capped with aluminum foil and autoclaved dry, for 10 minute. Culture medium is made up by dispensing seawater into sterile flasks and adding the appropriate nutrients from a sterile stock solution.

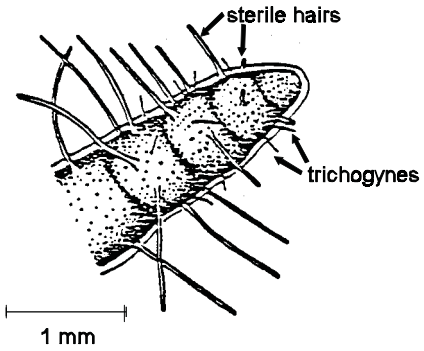

Figure 2. Apex of branch of female plant, showing sterile hairs and reproductive hairs (trichogynes). Sterile hairs are wider and generally much longer than trichogynes, and appear hollow except at the tip. Both types of hairs occur on the entire circumference of the thallus, but are seen easiest at the "edges." Receptive trichogynes occur only near the branch tips. From USEPA (1987f).

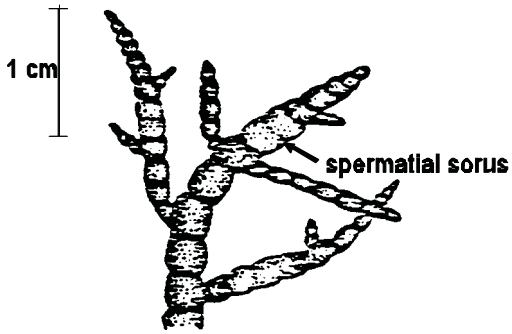

Figure 3. A portion of the male thallus showing spermatial sori. The sorus areas are generally slightly thicker and somewhat lighter in color. From USEPA (1987f).

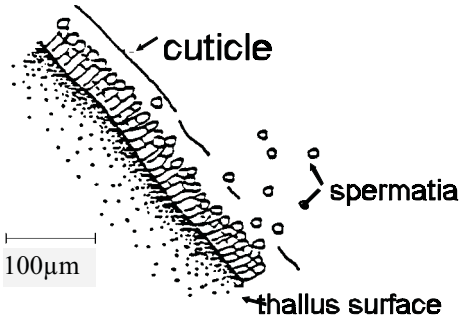

Figure 4. A magnified portion of a spermatial sorus. Note the rows of cells that protrude from the thallus surface. From USEPA (1987f).

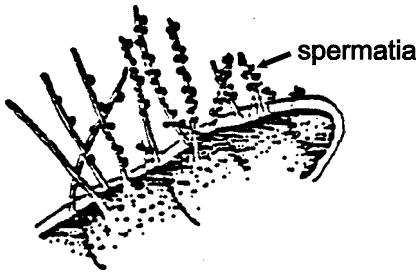

Figure 5. Apex of a branch on a mature female plant that was exposed to spermatia from a male plant. The sterile hairs and trichogynes are covered with spermatia. Note that few or no spermatia are attached to the older hairs (those more than l mm from the apex). From USEPA (1987f).

16.6.14.2.2 Alternately, 1-L flasks containing seawater can be autoclaved. Sterilization is used to prevent microalgal contamination, and not to keep cultures bacteria free.

16.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

16.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

16.8 CALIBRATION AND STANDARDIZATION

16.8.1 See Section 4, Quality Assurance.

16.9 QUALITY CONTROL

16.9.1 See Section 4, Quality Assurance.

16.10 TEST PROCEDURES

16.10.1 TEST SOLUTIONS

16.10.1.1 Receiving Waters

16.10.1.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 100 mL, and 400 mL for chemical analysis, would require approximately 800 mL or more of sample per test.

16.10.1.2 Effluents

16.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and allows for testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.**

16.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%).

16.10.1.2.3 The volume of effluent required for the test using a 0.5 dilution series is approximately 1800 mL. Prepare enough test solution at each effluent concentration (approximately 800 mL) to provide 100 mL of test solution for each of four (minimum of three) replicate test chambers and 400 mL for chemical analyses and record data (Figure 6).

16.10.1.2.4 Effluents can be tested at 100%. A 100% concentration of effluent can be achieved if the salinity of the effluent is adjusted to 30‰ by adding the GP2 dry salt formulation described in Table 2.

16.10.1.2.5 Just prior to test initiation (approximately 1 h), the temperature of sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (25 ± 1°C) and maintained at the temperature during the addition of dilution water.

SITE: _____

COLLECTION DATE: _____

TEST DATE: _____

LOCATION	INITIAL SALINITY	FINAL SALINITY	SOURCE OF SALTS FOR[1] SALINITY ADJUSTMENT

[1]Natural seawater, GP2 brine, GP2 salts, etc. (include some indication of amount)

COMMENTS:

Figure 6. Data form for the red macroalga, *Champia parvula*, sexual reproduction test. Receiving water summary sheet. From USEPA (1987f).

16.10.1.2.6 Effluent dilutions should be prepared for all replicated in each treatment in one beaker to minimize variability among the replicates. The test chambers are labeled with the test concentration and replicate number. Dispense into the appropriate effluent dilution chamber.

16.10.1.3 Dilution Water

16.10.1.3.1. The formula for the enrichment for natural seawater is listed in Table 1. Both EDTA and trace metals have been omitted. This formula should be used for the 2-day exposure period, but it is not critical for the recovery period. Since natural seawater quality can vary among laboratories, a more complete nutrient medium (e.g., the addition of EDTA) may result in faster growth (and therefore faster cystocarp development) during the recovery period.

16.10.2 PREPARATION OF PLANTS FOR TEST

16.10.2.1 Once cultures are determined to be usable for toxicity testing (have trichogynes and sori with spermatia), plant cuttings should be prepared for the test, using fine-point forceps, with the plants in a little seawater in a petri dish. For female plants, five cuttings, severed 7-10 mm from the ends of the branch, should be prepared for each treatment chamber. Try to be consistent in the number of branch tips on each cutting. For male plants, one cutting, severed 2.0 to 3.0 cm from the end of the branch, is prepared for each test chamber. Prepare the female cuttings first, to minimize the chances of contaminating them with water containing spermatia from the male stock cultures.

16.10.3 START OF TEST

16.10.3.1 Tests should begin as soon as possible after sample collection, preferably within 24 h. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test, Subsection 8.5.4).

16.10.3.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solution should be adjusted to the test temperature (23 ± 1°C) and maintained at that temperature during the addition of dilution water.

16.10.3.3 Label the test chambers with a marking pen. Use of color coded tape to identify each treatment and replicate is helpful. A minimum of five effluent concentrations and a control are used for each effluent test Each treatment (including controls) should have four (minimum of three) replicates.

16.10.3.4 Randomize the position of test chambers at the beginning of the test.

16.10.3.5 Prepare test solutions and add to the test chambers.

16.10.3.6 Add five female branches and one male branch to each test chamber. The toxicant must be present before the male plant is added.

16.10.3.7 Gently hand swirl the chambers twice a day, or shake continuously at 100 rpm on a rotary shaker.

16.10.3.8 If desired, the media can be changed after 24 h.

16.10.4 LIGHT, PHOTOPERIOD, SALINITY, AND TEMPERATURE

16.10.4.1 The light quality and intensity should be at 75 $\mu E/m^2/s$, or 500 foot candles (ft-c) with a photoperiod of 16 h light and 8 h darkness. The water temperature in the test chambers should be maintained at 23 ± 1°C. The test salinity should be in the range of 28 to 32‰. The salinity should vary by no more than ± 2‰ among the chambers

on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

16.10.5 DISSOLVED OXYGEN (DO) CONCENTRATION

16.10.5.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentrations should be measured on new solutions at the start of the test (Day 0) and should be measured before renewal of the test solution after 24 h. The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests) If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1mL KIMAX® serological pipet, or equivalent. Care should be taken to ensure that turbulence resulting from the aeration does not occur.

16.10.6 OBSERVATIONS DURING THE TEST

16.10.6.1 Routine Chemical and Physical Observations

16.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period in one test chamber at each concentration and in the control.

16.10.6.1.2 Temperature, pH, and salinity are measured at the end of each 24-h exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously, observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at least at the end of the test to determine temperature variation in environmental chamber.

16.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

16.10.6.1.4 Record all the measurements on the data sheet.

16.10.6.2 Routine Biological Observations

16.10.6.2.1 Protect the red macroalga from unnecessary disturbance during the test by carrying out the daily test observations and solution renewals carefully.

16.10.7 TRANSFER OF PLANTS TO CONTROL WATER AFTER 48 H

16.10.7.1 Label the recovery vessels. These vessels can be almost any type of container or flask containing 100 to 200 mL of seawater and nutrients (see Tables 1 and 2). Smaller volumes can be used, but should be checked to make sure that adequate growth will occur without having to change the medium.

16.10.7.2 With forceps, gently remove the female branches from test chambers and place into recovery bottles. Add aeration tubes and foam stoppers.

16.10.7.3 Place the vessels under cool white light (at the same irradiance as the stock cultures) and aerate for the 5-7 day recovery period. If a shaker is used, do not aerate the solutions (this will enhance the water motion).

16.10.8 TERMINATION OF THE TEST

16.10.8.1 At the end of the recovery period, count the number of cystocarps (Figures 7, 8, and 9) per female and record the data (Figure 10). Cystocarps may be counted by placing females between the inverted halves of a polystyrene petri dish or other suitable containers with a small amount of seawater (to hold the entire plant in one focal plane). Cystocarps can be easily counted under a stereomicroscope, and are distinguished from young branches because they possess an apical opening for spore release (ostiole) and darkly pigmented spores.

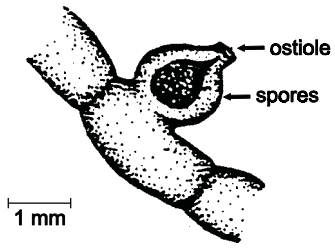

Figure 7. A mature cystocarp. In the controls and lower effluent concentrations, cystocarps often occur in clusters of 10 or 12. From USEPA (1987f).

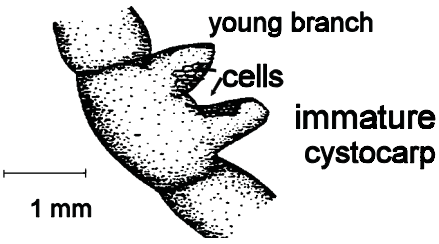

Figure 8. Comparison of a very young branch and an immature cystocarp. Both can have sterile hairs. Trichogynes might or might not be present on a young branch, but are never present on an immature cystocarp. Young branches are more pointed at the apex and are made up of larger cells than immature cystocarps, and never have ostioles. From USEPA (1987f).

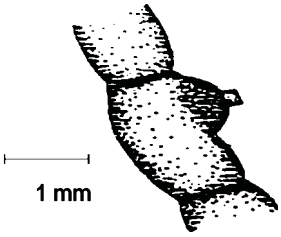

Figure 9. An aborted cystocarp. A new branch will eventually develop at the apex. From USEPA (1987f).

16.10.8.2 One advantage of this test procedure is that if there is uncertainty about the identification of an immature cystocarp, it is necessary only to aerate the plants a little longer in the recovery bottles. Within 24 to 48 h, the presumed cystocarp will either look more like a mature cystocarp or a young branch, or will have changed very little, if at all (i.e., an aborted cystocarp). No new cystocarps will form since the males have been removed, and the plants will only get larger. Occasionally, cystocarps will abort, and these should not be included in the counts. Aborted cystocarps are easily identified by their dark pigmentation and, often, by the formation of a new branch at the apex.

16.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

16.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

16.12 ACCEPTABILITY OF TEST RESULTS

16.12.1 The test is acceptable if (1) control survival equals or exceeds 80% and (2) control plants average 10 or more cystocarps per plant.

16.12.2 If plants fragment in the controls or lower exposure concentrations, it may be an indication that they are under stress.

16.13 DATA ANALYSIS

16.13.1 GENERAL

16.13.1.1 Tabulate and summarize the data. A sample set of reproduction data is listed in Table 4.

16.13.1.2 The endpoints of the red macroalga, *Champia parvula*, toxicity test are based on the adverse effects on sexual reproduction as the mean number of cystocarps. The LC50, the IC25, and the IC50 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). NOEC and LOEC values are obtained using a hypothesis testing approach, such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the IC25 and IC50. See the Appendices for examples of the manual computations, program listing, and example of data input and program output.

16.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

COLLECTION DATE_____ RECOVERY BEGAN (date) _____

EXPOSURE BEGAN (date) _____ COUNTED (date) _____

EFFLUENT OR TOXICANT _____

TREATMENT (% EFFLUENT, mG/L, or RECEIVING WATER SITES)

REPLICATES	CONTROL						
A 1							
2							
3							
4							
MEAN							
B 1							
2							
3							
4							
MEAN							
C 1							
2							
3							
4							
MEAN							
OVERALL MEAN							

Temperature _____

Salinity _____

Light _____

Source of Dilution Water _____

Figure 10. Data form for the red macroalga, *Champia parvula*, sexual reproduction test. Cystocarp data sheet. From USEPA (1987f).

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE RED MACROALGA, *CHAMPIA PARVULA*, SEXUAL REPRODUCTION TEST WITH EFFLUENTS AND RECEIVING WATERS

CAUTION: This method is not listed at 40 CFR Part 136 for nationwide use.

1. Test type:	Static, non-renewal
2. Salinity:	30‰ (± 2‰ of the selected test salinity)
3. Temperature:	23 ± 1°C
4. Light quality:	Cool-white fluorescent lights
5. Light intensity:	75 µE/m^2/s (500 ft-c)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	200 mL polystyrene cups, or 250 mL Erlenmeyer flasks
8. Test solution volume:	100 mL (minimum)
9. No. organisms per test chamber:	5 female branch tips and 1 male plant
10. No. replicate per concentration:	4 (minimum of 3)
11. No. organisms per concentrations:	24 (minimum of 18)
12. Dilution water:	30‰ salinity natural seawater, or a combination of 50% of 30‰ salinity natural seawater and 50% of 30‰ salinity GP2 artificial seawater (see Section 7, Dilution Water)
13. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water or minimum of 5 and a control
14. Dilution factor:	Effluents: ≥ 0.5 Receiving waters: None or≥ 0.5

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE RED MACROALGA, *CHAMPIA PARVULA*, SEXUAL REPRODUCTION TEST WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

15. Test duration:	2 day exposure to effluent, followed by 5 to 7-day recovery period in control medium for cystocarp development
16. Endpoints:	Reduction in cystocarp production compared to controls
17. Test acceptability criteria	80% or greater survival, and an average of 10 cystocarps per plant in controls
18. Sampling requirements:	For on-site tests, one sample collected at test initiation, and used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sampling Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4)
19. Sample volume required:	2 L per test

16.13.2 EXAMPLE OF ANALYSIS OF THE RED MACROALGA, *CHAMPIA PARVULA*, REPRODUCTION DATA

16.13.2.1 Formal statistical analysis of the data is outlined in Figure 11. The response used in the analysis is the mean number of cystocarps per replicate chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint and the IC50 endpoint. Concentrations that have exhibited no sexual reproduction (less than 5% of controls) are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the IC endpoints.

16.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test. The assumptions of Dunnett's Procedures, normality and homogeneity of variance are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. Tests for normality and homogeneity of variance are included in Appendix B. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

TABLE 4. DATA FROM THE RED MACROALGA, *CHAMPIA PARVULA*, EFFLUENT TOXICITY TEST. CYSTOCARP COUNTS FOR INDIVIDUAL PLANTS AND MEAN COUNT PER TEST CHAMBER FOR EACH EFFLUENT CONCENTRATION[1]

Effluent Concentration (%)	Replicate Test Chamber	Plant 1	2	3	4	5	Mean Cystocarp Count
Control	A	19	20	24	7	18	17.60
	B	19	12	21	11	23	17.20
	C	17	25	18	20	16	19.20
0.8	A	10	16	11	12	11	12.00
	B	12	10	6	9	10	9.40
	C	12	9	9	13	8	10.20
1.3	A	10	0	3	5	4	4.40
	B	6	4	4	8	4	5.20
	C	4	4	2	6	4	4.00
2.2	A	1	2	5	4	0	2.40
	B	7	9	9	4	6	7.00
	C	3	2	2	0	0	1.40
3.6	A	2	1	1	5	0	1.80
	B	3	4	6	4	2	3.80
	C	0	4	3	1	3	2.20
6.0	A	1	0	0	0	0	0.20
	B	1	2	1	0	0	0.80
	C	0	4	3	1	3	2.20
10.0	A	0	0	0	0	-	0.00
	B	1	0	0	0	0	0.20
	C	2	1	0	0	0	0.60

[1] Data provided by the ERL-N, USEPA, Narragansett, RI.

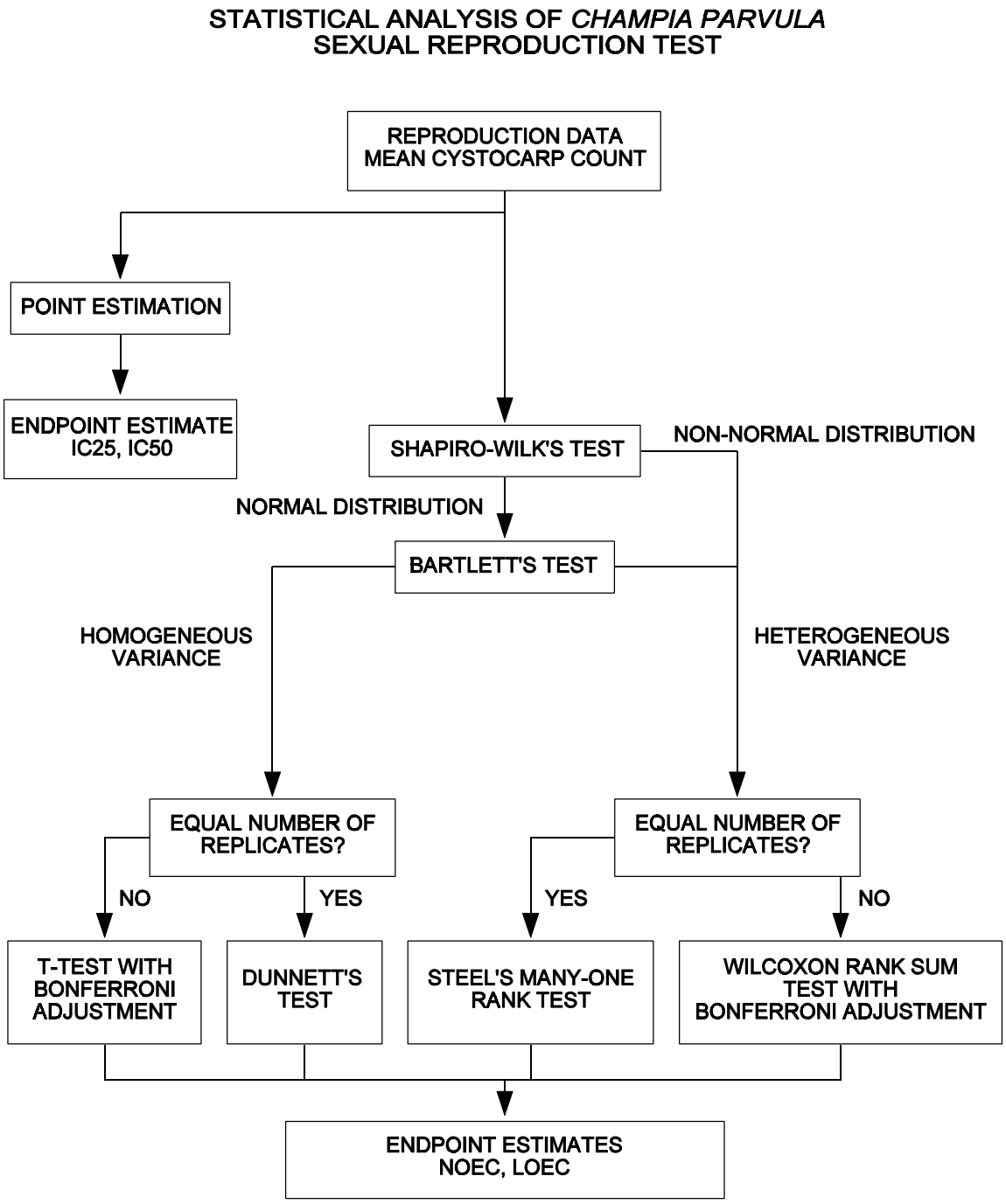

Figure 11. Flowchart for statistical analysis of the red macroalga, *Champia parvula*, data

6.13.2.3 If unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

16.13.2.4 Example of Analysis of Reproduction Data

16.13.2.4.1 In this example, the data, mean and standard deviation of the observations at each concentration including the control are listed in Table 5. The data are plotted in Figure 12. As can be seen from the data in the table, mean reproduction per chamber in the 10% effluent concentration is less than 5% of the control. Therefore the 10% effluent concentration is not included in the subsequent analysis.

TABLE 5. RED MACROALGA, *CHAMPIA PARVULA*, SEXUAL REPRODUCTION DATA

		Effluent Concentration (%)					
Replicate	Control	0.8	1.3	2.2	3.6	6.0	10.0
A	17.60	12.00	4.40	2.40	1.80	0.20	0.00
B	17.20	9.40	5.20	7.00	3.80	0.80	0.20
C	19.20	10.20	4.00	1.40	2.20	2.20	0.60
Mean($\bar{Y}_i$)	18.00	10.53	4.53	3.60	2.60	1.07	0.27
S_i^2	1.12	1.77	0.37	8.92	1.12	1.05	0.09
i	1	2	3	4	5	6	7

16.13.2.5 Test for Normality

16.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

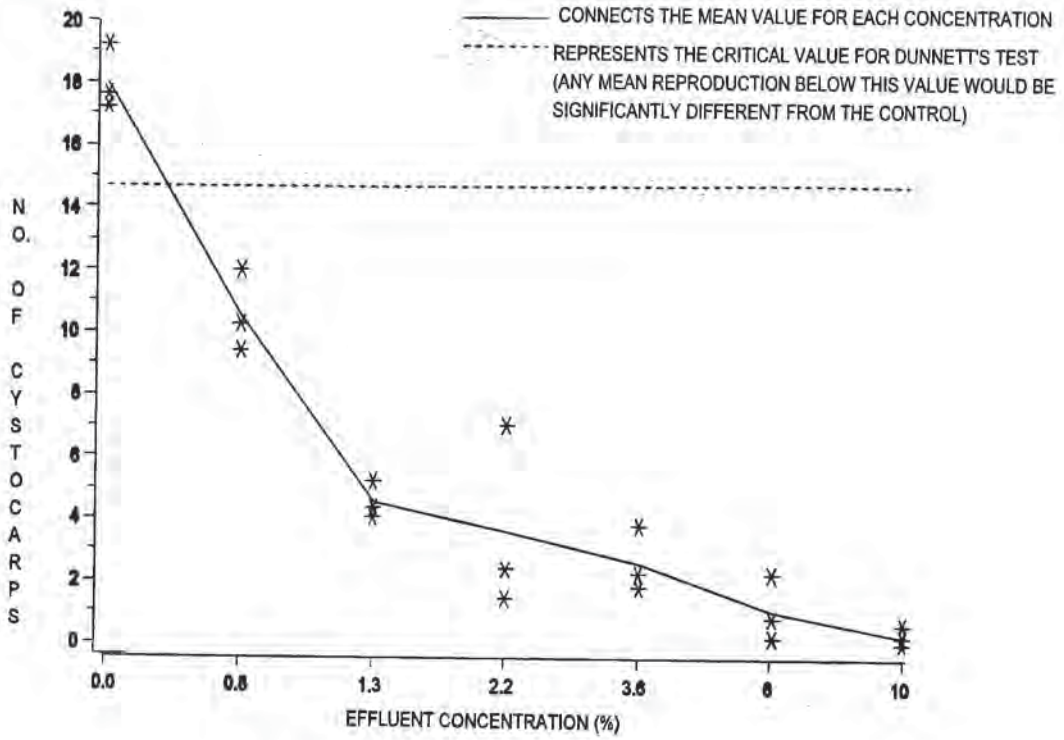

Figure 12. Plot of the number of cystocarps per plant.

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Effluent Concentration (%)				
Replicate	Control	0.8	1.3	2.2	3.6	6.0
A	-0.40	1.47	-0.13	-1.20	-0.80	-0.87
B	-0.80	-1.13	0.67	3.40	1.20	-0.27
C	1.20	-0.33	-0.53	-2.20	-0.40	1.13

16.13.2.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (x_i - \bar{X})^2$$

Where: X_i = the ith centered observation

x = the overall mean of the centered observations

n = the total number of centered observations.

16.13.2.5.3 For this set of data, n = 18

$$\overline{X} = \frac{1}{8}(0.01) = 0.00$$

$$D = 28.7201$$

16.13.2.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 7.

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

I	$X^{(i)}$	i	$X^{(i)}$
1	-2.20	10	-0.33
2	-1.20	11	-0.27
3	-1.13	12	-0.13
4	-0.87	13	0.67
5	-0.80	14	1.13
6	-0.80	15	1.20
7	-0.53	16	1.20
8	-0.40	17	1.47
9	-0.40	18	3.40

16.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 18 and k = 9. The a_i values are listed in Table 8.

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4886	5.60	$X^{(18)} - X^{(1)}$
2	0.3253	2.67	$X^{(17)} - X^{(2)}$
3	0.2553	2.33	$X^{(17)} - X^{(3)}$
4	0.2027	2.07	$X^{(15)} - X^{(4)}$
5	0.1587	1.93	$X^{(14)} - X^{(5)}$
6	0.1197	1.47	$X^{(13)} - X^{(6)}$
7	0.0837	0.40	$X^{(12)} - X^{(7)}$
8	0.0496	0.13	$X^{(11)} - X^{(8)}$
9	0.0163	0.07	$X^{(10)} - X^{(9)}$

16.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}[\sum_{i=1}^{k} a_i(X^{(n-i+1)} - X^{(i)}]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 8. For the data,

$$W = \frac{1}{28.7201}(5.1425)^2 = 0.921$$

16.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 16.3.2.5.6 with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 18 observations (n) is 0.858. Since W = 0.921 is greater than the critical value, conclude of the test is that the data are normally distributed.

16.13.2.6 Test for Homogeneity of Variance

16.13.2.6.1 The test used to examine whether the variation in mean cystocarp production is the same across all effluent concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i)\ \ln\overline{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, $V_i = (n_i - 1)$

p = number of levels of effluent concentration including the control

n_i = the number of replicates for concentration i

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations

$$\overline{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i^2)}{\sum_{i=1}^{p} V_i}$$

16.13.2.6.2 For the data in this example (See Table 5) all effluent concentrations including the control have the same number of replicates ($n_i = 3$ for all i). Thus, $V_i = 2$ for all i.

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{p} 1/V_i - (\sum_{i=1}^{p} V_i)^{-1}]$$

16.13.2.6.3 Bartlett's statistic is therefore:

$$B = [(12)\ln(2.3917) - 2\sum_{i=1}^{p} \ln(S_i^2)]/1.194$$

$$= [12(0.8720) - 2(1n(1.12)+\ln(1.77)+...+\ln(1.05))]/1.1944$$

$$= (10.4640 - 4.0809)/1.1944$$

$$= 5.34$$

16.13.2.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with five degrees of freedom, is 15.09. Since B = 5.34 is less than the critical value of 15.09, conclude that the variances are not different.

16.13.2.7 Dunnett's Procedure

16.13.2.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 9.

TABLE 9. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p - 1)
Within	N - p	SSW	S_W^2 = SSW/(N - p)
Total	N - 1	SST	

Where: p = number effluent concentrations including the control

N = total number of observations $n_1 + n_2 ... + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N \qquad \text{Between Sum of Squares}$$

$$SST = \sum_{i=1}^{p}\sum_{j=1}^{n_j} Y_{ij}^2 - G^2/N \qquad \text{Total Sum of Squares}$$

$$SSW = SST - SSB \qquad \text{Within Sum of Squares}$$

G = the grand total of all sample observations,

$$G = \sum_{i=1}^{p} T_i$$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean (across plants) number of cystocarps for effluent concentration i in test chamber j)

16.13.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 3$

$N = 18$
$T_1 = Y_{11} + Y_{12} + Y_{13} = 17.6 + 17.2 + 19.2 = 54$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 12.0 + 9.4 + 10.2 = 31.6$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 4.4 + 5.2 + 4.0 = 13.6$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 2.4 + 7.0 + 1.4 = 10.8$
$T_5 = Y_{51} + Y_{52} + Y_{53} = 1.8 + 3.8 + 2.2 = 7.8$
$T_6 = Y_{61} + Y_{62} + Y_{63} = 0.2 + 0.8 + 2.2 = 3.2$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 121.0$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= \frac{1}{3}(4287.24) - \frac{(121.0)^2}{18} = 615.69$$

$$SST = \sum_{i=1}^{p}\sum_{j=1}^{n_j} Y_{ij}^2 - G^2/N$$

$$= 1457.8 - \frac{(121.0)^2}{18} = 644.41$$

$$SSW = SST - SSB$$

$$= 644.41 - 615.69 = 28.72$$

S_B^2 = SSB/(p-1) = 615.69/(6-1) = 123.14

S_W^2 = SSW/(N-p) = 28.72/(18-6) = 2.39

16.13.2.7.3 Summarize these calculations in the ANOVA table (Table 10).

TABLE 10. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	5	615.69	123.14
Within	12	28.72	2.39
Total	17	644.41	

16.13.2.7.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_W\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean number of cystocarps for effluent concentration i

$\bar{Y}_1$ = mean number of cystocarps for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

16.13.2.7.5 Table 11 includes the calculated t values for each concentration and control combination. In this example, comparing the 0.8% concentration with the control the calculation is as follows:

$$t_2 = \frac{(18 - 10.53)}{[1.55\sqrt{(1/3) + (1/3)}]} = 5.9$$

16.13.2.7.6 Since the purpose of this test is to detect a significant reduction in cystocarp production, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 12 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.50. Mean cystocarp production for concentration i is considered significantly less control if t_i is greater than the critical value. Therefore, mean cystocarp productions for all effluent concentrations in this example have significantly lower cystocarp production than the control. Hence the NOEC is 0.8% and the LOEC is 0.8%.

TABLE 11. CALCULATED T VALUES

Effluent Concentration(%)	i	t_i
0.8	2	5.90
1.3	3	10.64
2.2	4	11.38
3.6	5	12.17
6.0	6	13.38

16.13.2.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = dS_W\sqrt{(1/n_1)+(1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration
(this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

16.13.2.7.8 In this example,

$$MSD = 2.50(1.55)\sqrt{(1/3)+(1/3)}$$

$$= 2.50\ (1.55)(.8165)$$

$$= 3.16$$

16.13.2.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 3.16 cystocarps.

16.13.2.7.10 This represents a 17.6% reduction in cystocarp production from the control.

16.13.2.8 Calculation of the ICp

16.13.2.8.1 The sexual reproduction data in Table 5 are utilized in this example. Table 12 contains the mean number of cystocarps for each effluent concentration. As can be seen, the observed means are monotonically non-increasing with respect to concentration. Therefore, it is not necessary to smooth the means prior to calculating the ICp. Refer to Figure 12 for a plot of the response curve.

TABLE 12. RED MACROALGA, *CHAMPIA PARVULA*, MEAN NUMBER OF CYSTOCARPS

Effluent Conc. (%)	i	Response Means $\bar{Y}_i$ (mg)	Smoothed Means M_i (mg)
Control	1	18.00	18.00
0.8	2	10.53	10.53
1.3	3	4.53	4.53
2.2	4	3.60	3.60
3.6	5	2.60	2.60
6.0	6	1.07	1.07
10.0	7	0.27	0.27

16.13.2.8.2 An IC25 and IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in mean number of cystocarps, compared to the controls, would result in a mean number of 13.50 cystocarps, where $M_1(1-p/100) = 18.00(1-25/100)$. A 50% reduction in mean number of cystocarps, compared to the controls, would result in a mean number of 9.00 cystocarps. Examining the means and their associated concentrations (Table 12), the response, 13.50, is bracketed by $C_1 = 0.0\%$ effluent and $C_2 = 0.8\%$ effluent. The response, 9.00, is bracketed by $C_2 = 0.8\%$ effluent and $C_3 = 1.3\%$ effluent.

16.13.2.8.3 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j] \frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 0.0 + [18.00(1 - 25/100) - 18.00] \frac{(0.8 - 0.0)}{(10.53 - 18.00)}$$

$$= 0.5\%.$$

16.13.2.8.4 Using the equation from Section 4.2 from Appendix L, the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j] \frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC50 = 0.8 + [18.00(1 - 50/100) - 10.53] \frac{(1.3 - 0.8)}{(4.53 - 10.53)}$$

$$= 0.9\%$$

16.13.2.8.5 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 0.4821%. The empirical 95.0% confidence interval for the true mean was 0.4013% to 0.6075%. The computer program output for the IC25 for this data set is shown in Figure 13.

16.13.2.8.6 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 0.9278%. The empirical 95.0% confidence interval for the true mean was 0.7893% and 1.0576%. The computer program output for the IC50 for this data set is shown in Figure 14.

16.14 PRECISION AND ACCURACY

16.14.1 PRECISION

16.14.1.1 Single-Laboratory Precision

16.14.1.1.1 The single-laboratory precision data from six tests with copper sulfate (Cu) and six tests with sodium dodecyl sulfate (SDS) are listed in Tables 13-16. The NOECs with Cu differed by only one concentration interval (factor of two), showing good precision. The precision of the first four tests with SDS was somewhat obscured by the choice of toxicant concentrations, but appeared similar to that of Cu in the last two tests. The IC25 and IC50 are indicated in Tables 13-16. The coefficient of variation, based on the IC25 for these two reference toxicants in natural seawater and a mixture of natural seawater and GP2, ranged from 59.6% to 69.0%, and for the IC50, ranged from 22.9% to 43.7%.

16.14.1.1.2 EPA evaluated single-laboratory (within-laboratory) precision of the Red Macroalga, *Champia parvula*, Reproduction Test using a database of routine reference toxicant test results from two laboratories (USEPA, 2000b). The database consisted of 23 reference toxicant tests conducted in 2 laboratories using reference toxicants including: copper and sodium dodecyl sulfate. The within-laboratory CVs calculated for routine reference toxicant tests at these 2 laboratories were 58% and 59% for the IC25 reproduction endpoint.

16.14.1.2 Multilaboratory Precision

16.14.1.2.1 The multilaboratory precision of the test has not yet been determined.

16.14.2 ACCURACY

16.14.2.1 The accuracy of toxicity tests cannot be determined.

Conc. ID	1	2	3	4	5	6	7
Conc. Tested	0	.8	1.3	2.2	3.6	6	10
Response 1	19	10	10	1	2	1	0
Response 2	20	16	0	2	1	0	0
Response 3	24	11	3	5	1	0	0
Response 4	7	12	5	4	5	0	0
Response 5	18	11	4	0	0	0	1
Response 6	19	12	6	7	3	1	0
Response 7	12	10	4	9	4	2	0
Response 8	21	6	4	9	6	1	0
Response 9	11	9	8	4	4	0	0
Response 10	23	10	4	6	2	0	2
Response 11	17	12	4	3	0	0	1
Response 12	25	9	4	2	4	4	0
Response 13	18	9	2	2	3	3	0
Response 14	20	13	6	0	1	1	0
Response 15	16	8	4	0	3	3	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: effluent Test Start Date: Test Ending Date:
Test Species: RED MACROALGA, Champia parvula
Test Duration: DATA FILE: champia.icp OUTPUT FILE: champia.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Standard. Dev.	Pooled Response Means
1	15	0.000	18.000	4.928	18.000
2	15	0.800	10.533	2.356	10.533
3	15	1.300	4.533	2.356	4.533
4	15	2.200	3.600	3.066	3.600
5	15	3.600	2.600	1.805	2.600
6	15	6.000	1.067	1.335	1.067
7	15	10.000	0.267	0.594	0.267

The Linear Interpolation Estimate: 0.4821 Entered P Value: 25

Number of Resamplings: 80 The Bootstrap Estimates Mean: 0.4947 Standard Deviation: 0.0616
Original Confidence Limits: Lower: 0.4013 Upper: 0.6075
Resampling time in Seconds: 3.68 Random Seed: 703617166

Figure 13. ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6	7
Conc. Tested	0	.8	1.3	2.2	3.6	6	10
Response 1	19	10	10	1	2	1	0
Response 2	20	16	0	2	1	0	0
Response 3	24	11	3	5	1	0	0
Response 4	7	12	5	4	5	0	0
Response 5	18	11	4	0	0	0	1
Response 6	19	12	6	7	3	1	0
Response 7	12	10	4	9	4	2	0
Response 8	21	6	4	9	6	1	0
Response 9	11	9	8	4	4	0	0
Response 10	23	10	4	6	2	0	2
Response 11	17	12	4	3	0	0	1
Response 12	25	9	4	2	4	4	0
Response 13	18	9	2	2	3	3	0
Response 14	20	13	6	0	1	1	0
Response 15	16	8	4	0	3	3	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: effluent Test Start Date: Test Ending Date:
Test Species: RED MACROALGA, Champia parvula
Test Duration: DATA FILE: champia.icp OUTPUT FILE: champia.i50

Conc. ID	Number Replicates	Concentration %	Response Means	Standard. Dev.	Pooled Response Means
1	15	0.000	18.000	4.928	18.000
2	15	0.800	10.533	2.356	10.533
3	15	1.300	4.533	2.356	4.533
4	15	2.200	3.600	3.066	3.600
5	15	3.600	2.600	1.805	2.600
6	15	6.000	1.067	1.335	1.067
7	15	10.000	0.267	0.594	0.267

The Linear Interpolation Estimate: 0.9278 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 0.9263 Standard Deviation: 0.0745
Original Confidence Limits: Lower: 0.7893 Upper: 1.0576
Resampling time in Seconds: 3.63 Random Seed: -1255453122

Figure 14. ICPIN program output for the IC50.

TABLE 13. SINGLE-LABORATORY PRECISION OF THE RED MACROALGA, *CHAMPIA PARVULA*, REPRODUCTION TEST PERFORMED IN A 50/50 MIXTURE OF NATURAL SEAWATER AND GP2 ARTIFICIAL SEAWATER, USING GAMETES FROM ADULTS CULTURED IN NATURAL SEAWATER. THE REFERENCE TOXICANT USED WAS COPPER (CU) SULFATE[1,2,3,4,5]

Test Number	NOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)
1	1.0	1.67	2.37
2	1.0	1.50	1.99
3	1.0	0.69	1.53
4	1.0	0.98	1.78
5	0.5	0.38	0.76
6	0.5	0.38	0.75
n:	6	6	6
Mean:	NA	0.93	1.5
CV(%):	NA	59.6	43.7

1 Data from USEPA (1991a).
2 Tests performed by Glen Thursby and Mark Tagliabue, ERL-N, USEPA, Narragansett, RI. Tests were conducted at 22°C, in 50/50 GP2 and natural seawater at a salinity of 30‰.
3 Copper concentrations were: 0.5, 1.0, 2.5, 5.0, 7.5, and 1.0 µg/L.
4 NOEC Range: 0.5 - 1.0 µg/L (this represents a difference of one exposure concentration).
5 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 14. SINGLE-LABORATORY PRECISION OF THE RED MACROALGA, *CHAMPIA PARVULA*, REPRODUCTION TEST PERFORMED IN A 50/50 MIXTURE OF NATURAL SEAWATER AND GP2 ARTIFICIAL SEAWATER, USING GAMETES FROM ADULTS CULTURED IN NATURAL SEAWATER. THE REFERENCE TOXICANT USED WAS SODIUM DODECYL SULFATE (SDS)[1,2,3,4,5]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)
1	< 0.80	0.6	0.3
2	0.48	0.7	0.6
3	< 0.48	0.4	0.2
4	< 0.48	0.2	0.4
5	0.26	0.2	0.5
6	0.09	0.1	0.3
7	0.16	0.2	0.3
8	0.09	0.1	0.2
9	< 0.29	0.3	0.4
n:	5	9	9
Mean:	NA	0.31	0.36
CV(%):	NA	69.0	37.0

1 Data from USEPA (1991a).

2 Tests performed by Glen Thursby and Mark Tagliabue, ERL-N, USEPA, Narragansett, RI. Tests were conducted at 22°C, in 50/50 GP2 and natural seawater at a salinity of 30‰.

3 SDS concentrations for Test 1 were: 0.8, 1.3, 2.2, 3.6, 6.0, and 10.0 mg/L. SDS concentrations for Tests 2, 3, and 4 were: 0.48, 0.8, 1.3, 2.2, 3.6, and 6.0 mg/L. SDS concentrations for Tests 5 and 6 were: 0.09, 0.16, 2.26, 0.43, 0.72, and 1.2 mg/L.

4 NOEC Range: 0.09 - 0.48 mg/L (this represents a difference of two exposure concentrations).

5 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 15. SINGLE-LABORATORY PRECISION OF THE RED MACROALGA, *CHAMPIA PARVULA*, REPRODUCTION TEST IN NATURAL SEAWATER (30‰ SALINITY). THE REFERENCE TOXICANT USED WAS COPPER (CU) SULFATE[1,2,3]

	Cu (µg/L)		
Test	NOEC	IC25	IC50
1	1.00	2.62	4.02
2	0.50	0.71	1.66
3	0.50	2.83	3.55
4	0.50	0.99	4.15
n:	4	4	4
Mean:	NA	1.79	3.35
CV(%):	NA	61.09	34.45

1 Data from USEPA (1991a).
2 Copper concentrations were 0.5, 1.0, 2.5, 5.0, 7.5, and 10 µg/L. Concentrations of Cu were made from a 100 µg/mL $CuSO_4$ standard obtained from Inorganic Ventures, Inc., Brick, NJ.
3 Prepared by Steven Ward and Glen Thursby, Environmental Research Laboratory, USEPA, Narragansett, RI.

TABLE 16. SINGLE-LABORATORY PRECISION OF THE RED MACROALGA, *CHAMPIA PARVULA*, REPRODUCTION TEST IN NATURAL SEAWATER (30‰ SALINITY). THE REFERENCE TOXICANT USED WAS SODIUM DODECYL SULFATE (SDS)[1,2,3]

	SDS (mg/L)		
Test	NOEC	IC25	IC50
1	0.60	0.05	0.50
2	0.60	0.48	0.81
3	0.30	0.69	0.89
4	0.15	0.60	0.81
n:	4	4	4
Mean:	NA	0.46	0.75
CV(%):	NA	62.29	22.92

1 Data from USEPA (1991a).
2 SDS concentrations were 0.0375, 0.075, 0.15, 0.03, 0.60, and 1.20 mg/L. Concentrations of SDS were made from a 44.64 ± 3.33 mg/mL standard obtained from the EMSL-USEPA, Cincinnati, OH.
3 Prepared by Steven Ward and Glen Thursby, Environmental Research Laboratory, USEPA, Narragansett, RI.

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APPENDICES

APPENDIX A

INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

1. STATISTICAL INDEPENDENCE

1.1 Dunnett's Procedure and the t test with Bonferroni's adjustment are parametric procedures based on the assumptions that (1) the observations within treatments are independent and normally distributed, and (2) that the variance of the observations is homogeneous across all toxicant concentrations and the control. Of the three possible departures from the assumptions, non-normality, heterogeneity of variance, and lack of independence, those caused by lack of independence are the most difficult to resolve (see Scheffe, 1959). For toxicity data, statistical independence means that given knowledge of the true mean for a given concentration or control, knowledge of the error in any one actual observation would provide no information about the error in any other observation. Lack of independence is difficult to assess and difficult to test for statistically. It may also have serious effects on the true alpha or beta level. Therefore, it is of utmost importance to be aware of the need for statistical independence between observations and to be constantly vigilant in avoiding any patterned experimental procedure that might compromise independence. One of the best ways to help insure independence is to follow proper randomization procedures throughout the test.

2. RANDOMIZATION

2.1 Randomization of the distribution of test organisms among test chambers, and the arrangement of treatments and replicate chambers is an important part of conducting a valid test. The purpose of randomization is to avoid situations where test organisms are placed serially into test chambers, or where all replicates for a test concentration are located adjacent to one another, which could introduce bias into the test results.

2.2 An example of randomization of the distribution of test organisms among test chambers, and an example of randomization of arrangement of treatments and replicate chambers are described using the Sheepshead Minnow Larval Survival and Growth test. For the purpose of the example, the test design is as follows: Five effluent concentrations are tested in addition to the control. The effluent concentrations are as follows: 6.25%, 12.5%, 25.0%, 50.0%, and 100.0%. There are four replicate chambers per treatment. Each replicate chamber contains ten fish.

2.3 RANDOMIZATION OF FISH TO REPLICATE CHAMBERS EXAMPLE

2.3.1 Consider first the random assignment of the fish to the replicate chambers. The first step is to label each of the replicate chambers with the control or effluent concentration and the replicate number. The next step is to assign each replicate chamber four double-digit numbers. An example of this assignment is provided in Table A.1. Note that the double digits 00 and 97 through 99 were not used.

TABLE A.1. RANDOM ASSIGNMENT OF FISH TO REPLICATE CHAMBERS
EXAMPLE ASSIGNED NUMBERS FOR EACH REPLICATE CHAMBER

Assigned Numbers	Replicate Chamber
01, 25, 49, 73	Control, replicate chamber 1
02, 26, 50, 74	Control, replicate chamber 2
03, 27, 51, 75	Control, replicate chamber 3
04, 28, 52, 76	Control, replicate chamber 4
05, 29, 53, 77	6.25% effluent, replicate chamber 1
06, 30, 54, 78	6.25% effluent, replicate chamber 2
07, 31, 55, 79	6.25% effluent, replicate chamber 3
08, 32, 56, 80	6.25% effluent, replicate chamber 4
09, 33, 57, 81	12.5% effluent, replicate chamber 1
10, 34, 58, 82	12.5% effluent, replicate chamber 2
11, 35, 59, 83	12.5% effluent, replicate chamber 3
12, 36, 60, 84	12.5% effluent, replicate chamber 4
13, 37, 61, 85	25.0% effluent, replicate chamber 1
14, 38, 62, 86	25.0% effluent, replicate chamber 2
15, 39, 63, 87	25.0% effluent, replicate chamber 3
16, 40, 64, 88	25.0% effluent, replicate chamber 4
17, 41, 65, 89	50.0% effluent, replicate chamber 1
18, 42, 66, 90	50.0% effluent, replicate chamber 2
19, 43, 67, 91	50.0% effluent, replicate chamber 3
20, 44, 68, 92	50.0% effluent, replicate chamber 4
21, 45, 69, 93	100.0% effluent, replicate chamber 1
22, 46, 70, 94	100.0% effluent, replicate chamber 2
23, 47, 71, 95	100.0% effluent, replicate chamber 3
24, 48, 72, 96	100.0% effluent, replicate chamber 4

2.3.2 The random numbers used to carry out the random assignment of fish to replicate chambers are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double digit number. The first number read identifies the replicate chamber for the first fish taken from the tank. For the example, the first entry in row 2 was chosen as the starting position. The first number in this row is 37. According to Table A.1, this number corresponds to replicate chamber 1 of the 25.0% effluent concentration. Thus, the first fish taken from the tank is to be placed in replicate chamber 1 of the 25.0% effluent concentration.

TABLE A.2. TABLE OF RANDOM NUMBERS (Dixon and Massey, 1983)

10 09 73 25 33	76 52 01 35 86	34 67 35 43 76	80 95 90 91 17	39 29 27 49 45
37 54 20 48 05	64 89 47 42 96	24 80 52 40 37	20 63 61 04 02	00 82 29 16 65
08 42 26 89 53	19 64 50 93 03	23 20 90 25 60	15 95 33 47 64	35 08 03 36 06
99 01 90 25 29	09 37 67 07 15	38 31 13 11 65	88 67 67 43 97	04 43 62 76 59
12 80 79 99 70	80 15 73 61 47	64 03 23 66 53	98 95 11 68 77	12 27 17 68 33
66 06 57 47 17	34 07 27 68 50	36 69 73 61 70	65 81 33 98 85	11 19 92 91 70
31 06 01 08 05	45 57 18 24 06	35 30 34 26 14	86 79 90 74 39	23 40 30 97 32
85 26 97 76 02	02 05 16 56 92	68 66 57 48 18	73 05 38 52 47	18 62 38 85 79
63 57 33 21 35	05 32 54 70 48	90 55 35 75 48	28 46 82 87 09	83 49 12 56 24
73 79 64 57 53	03 52 96 47 78	35 80 83 42 82	60 93 52 03 44	35 27 38 84 35
98 52 01 77 67	14 90 56 86 07	22 10 94 05 58	60 97 09 34 33	50 50 07 39 98
11 80 50 54 31	39 80 82 77 32	50 72 56 82 48	29 40 52 42 01	52 77 56 78 51
83 45 29 96 34	06 28 89 80 83	13 74 67 00 78	18 47 54 06 10	68 71 17 78 17
88 68 54 02 00	86 50 75 84 01	36 76 66 79 51	90 36 47 64 93	29 60 91 10 62
99 59 46 73 48	87 51 76 49 69	91 82 60 89 28	93 78 56 13 68	23 47 83 41 13
65 48 11 76 74	17 46 85 09 50	58 04 77 69 74	73 03 95 71 86	40 21 81 65 44
80 12 43 56 35	17 72 70 80 15	45 31 82 23 74	21 11 57 82 53	14 38 55 37 63
74 35 09 98 17	77 40 27 72 14	43 23 60 02 10	45 52 16 42 37	96 28 60 26 55
69 91 62 68 03	66 25 22 91 48	36 93 68 72 03	76 62 11 39 90	94 40 05 64 18
09 89 32 05 05	14 22 56 85 14	46 42 75 67 88	96 29 77 88 22	54 38 21 45 98
91 49 91 45 23	68 47 92 76 86	46 16 28 35 54	94 75 08 99 23	37 08 92 00 48
80 33 69 45 98	26 94 03 68 58	70 29 73 41 35	53 14 03 33 40	42 05 08 23 41
44 10 48 19 49	85 15 74 79 54	32 97 92 65 75	57 60 04 08 81	22 22 20 64 13
12 55 07 37 42	11 10 00 20 40	12 86 07 46 97	96 64 48 94 39	28 70 72 58 15
63 60 64 93 29	16 50 53 44 84	40 21 95 25 63	43 65 17 70 82	07 20 73 17 90
61 19 69 04 46	26 45 74 77 74	51 92 43 37 29	65 39 45 95 93	42 58 26 05 27
15 47 44 52 66	95 27 07 99 53	59 36 78 38 48	82 39 61 01 18	33 21 15 94 66
94 55 72 85 73	67 89 75 43 87	54 62 24 44 31	91 19 04 25 92	92 92 74 59 73
42 48 11 62 13	97 34 40 87 21	16 86 84 87 67	03 07 11 20 59	25 70 14 66 70
23 52 37 83 17	73 20 88 98 37	68 93 59 14 16	26 25 22 96 63	05 52 28 25 62
04 49 35 24 94	75 24 63 38 24	45 86 25 10 25	61 96 27 93 35	65 33 71 24 72
00 54 99 76 54	64 05 18 81 59	96 11 96 38 96	54 69 28 23 91	23 28 72 95 29
35 96 31 53 07	26 89 80 93 45	33 35 13 54 62	77 97 45 00 24	90 10 33 93 33
59 80 80 83 91	45 42 72 68 42	83 60 94 97 00	13 02 12 48 92	78 56 52 01 06
46 05 88 52 36	01 39 09 22 86	77 28 14 40 77	93 91 08 36 47	70 61 74 29 41
32 17 90 05 97	87 37 92 52 41	05 56 70 70 07	86 74 31 71 57	85 39 41 18 38
69 23 46 14 06	20 11 74 52 04	15 95 66 00 00	18 74 39 24 23	97 11 89 63 38
19 56 54 14 30	01 75 87 53 79	40 41 92 15 85	66 67 43 68 06	84 96 28 52 07
45 15 51 49 38	19 47 60 72 46	43 66 79 45 43	59 04 79 00 33	20 82 66 95 41
94 86 43 19 94	36 16 81 08 51	34 88 88 15 53	01 54 03 54 56	05 01 45 11 76
98 08 62 48 26	45 24 02 84 04	44 99 90 88 96	39 09 47 34 07	35 44 13 18 80
33 18 51 62 32	41 94 15 09 49	89 43 54 85 81	88 69 54 19 94	37 54 87 30 43
80 95 10 04 06	96 38 27 07 74	20 15 12 33 87	25 01 62 52 98	94 62 46 11 71
79 75 24 91 40	71 96 12 82 96	69 86 10 25 91	74 85 22 05 39	00 38 75 95 79
18 63 33 25 37	98 14 50 65 71	31 01 02 46 74	05 45 56 14 27	77 93 89 19 36
74 02 94 39 02	77 55 73 22 70	97 79 01 71 19	52 52 75 80 21	80 81 45 17 48
54 17 84 56 11	80 99 33 71 43	05 33 51 29 69	56 12 71 92 55	36 04 09 03 24
11 66 44 98 83	52 07 98 48 27	59 38 17 15 39	09 97 33 34 40	88 46 12 33 56
48 32 47 79 28	31 24 96 47 10	02 29 53 68 70	32 30 75 75 46	15 02 00 99 94
69 07 49 41 38	87 63 79 19 76	35 58 40 44 01	10 51 82 16 15	01 84 87 69 38

2.3.3 The next step is to read the double digit number to the right of the first one. The second number identifies the replicate chamber for the second fish taken from the tank. Continuing the example, the second number read in row 2 of Table A.2 is 54. According to Table A.1, this number corresponds to replicate chamber 2 of the 6.25% effluent concentration. Thus, the second fish taken from the tank is to be placed in replicate chamber 2 of the 6.25% effluent concentration.

2.3.4 Continue in this fashion until all the fish have been randomly assigned to a replicate chamber. In order to fill each replicate chamber with ten fish, the assigned numbers will be used more than once. If a number is read from the table that was not assigned to a replicate chamber, then ignore it and continue to the next number. If a replicate chamber becomes filled and a number is read from the table that corresponds to it, then ignore that value and continue to the next number. The first ten random assignments of fish to replicate chambers for the example are summarized in Table A.3.

TABLE A.3. EXAMPLE OF RANDOM ASSIGNMENT OF FIRST TEN FISH TO REPLICATE CHAMBERS

Fish		Assignment	
First	fish taken from tank	25.0% effluent,	replicate chamber 1
Second	fish taken from tank	6.25% effluent,	replicate chamber 2
Third	fish taken from tank	50.0% effluent,	replicate chamber 4
Fourth	fish taken from tank	100.0% effluent,	replicate chamber 4
Fifth	fish taken from tank	6.25% effluent,	replicate chamber 1
Sixth	fish taken from tank	25.0% effluent,	replicate chamber 4
Seventh	fish taken from tank	50.0% effluent,	replicate chamber 1
Eighth	fish taken from tank	100.0% effluent,	replicate chamber 3
Ninth	fish taken from tank	50.0% effluent,	replicate chamber 2
Tenth	fish taken from tank	100.0% effluent,	replicate chamber 4

2.3.5 Four double-digit numbers were assigned to each replicate chamber (instead of one, two, or three double-digit numbers) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each replicate chamber: the first column of assigned numbers in Table A.1. Whenever the numbers 00 and 25 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

2.4 RANDOMIZATION OF REPLICATE CHAMBERS TO POSITIONS EXAMPLE

2.4.1 Next consider the random assignment of the 24 replicate chambers to positions within the water bath (or equivalent). Assume that the replicate chambers are to be positioned in a four row by six column rectangular array. The first step is to label the positions in the water bath. Table A.4 provides an example layout.

TABLE A.4. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE LABELING THE POSITIONS WITHIN THE WATER BATH

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24

2.4.2 The second step is to assign each of the 24 positions four double-digit numbers. An example of this assignment is provided in Table A.5. Note that the double digits 00 and 97 through 99 were not used.

TABLE A.5. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE ASSIGNED NUMBERS FOR EACH POSITION

Assigned Numbers	Position
01, 25, 49, 73	1
02, 26, 50, 74	2
03, 27, 51, 75	3
04, 28, 52, 76	4
05, 29, 53, 77	5
06, 30, 54, 78	6
07, 31, 55, 79	7
08, 32, 56, 80	8
09, 33, 57, 81	9
10, 34, 58, 82	10
11, 35, 59, 83	11
12, 36, 60, 84	12
13, 37, 61, 85	13
14, 38, 62, 86	14
15, 39, 63, 87	15
16, 40, 64, 88	16
17, 41, 65, 89	17
18, 42, 66, 90	18
19, 43, 67, 91	19
20, 44, 68, 92	20
21, 45, 69, 93	21
22, 46, 70, 94	22
23, 47, 71, 95	23
24, 48, 72, 96	24

2.4.3 The random numbers used to carry out the random assignment of replicate chambers to positions are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double-digit number. The first number read identifies the position for the first replicate chamber of the control. For the example, the first entry in row 10 of Table A.2 was chosen as the starting position. The first number in this row was 73. According to Table A.5, this number corresponds to position 1. Thus, the first replicate chamber for the control will be placed in position 1.

2.4.4 The next step is to read the double-digit number to the right of the first one. The second number identifies the position for the second replicate chamber of the control. Continuing the example, the second number read in row 10 of Table A.2 is 79. According to Table A.5, this number corresponds to position 7. Thus, the second replicate chamber for the control will be placed in position 7.

2.4.5 Continue in this fashion until all the replicate chambers have been assigned to a position. The first four numbers read will identify the positions for the control replicate chambers, the second four numbers read will identify the positions for the lowest effluent concentration replicate chambers, and so on. If a number is read from the table that was not assigned to a position, then ignore that value and continue to the next number. If a number is repeated in Table A.2, then ignore the repeats and continue to the next number. The complete randomization of replicate chambers to positions for the example is displayed in Table A.6.

TABLE A.6. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE ASSIGNMENT OF ALL 24 POSITIONS

Control	100.0%	6.25%	6.25%	6.25%	12.5%
Control	12.5%	Control	25.0%	12.5%	25.0%
100.0%	50.0%	100.0%	Control	100.0%	25.0%
50.0%	50.0%	25.0%	50.0%	12.5%	6.25%

2.4.6 Four double-digit numbers were assigned to each position (instead of one, two, or three) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each position: the first column of assigned numbers in Table A.5. Whenever the numbers 00 and 25 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

3. **OUTLIERS**

3.1 An outlier is an inconsistent or questionable data point that appears unrepresentative of the general trend exhibited by the majority of the data. Outliers may be detected by tabulation of the data, plotting, and by an analysis of the residuals. An explanation should be sought for any questionable data points. Without an explanation, data points should be discarded only with extreme caution. If there is no explanation, the analysis should be performed both with and without the outlier, and the results of both analyses should be reported.

3.2 Gentleman-Wilk's A statistic gives a test for the condition that the extreme observation may be considered an outlier. For a discussion of this, and other techniques for evaluating outliers, see Draper and John (1981).

APPENDIX B

VALIDATING NORMALITY AND HOMOGENEITY OF VARIANCE ASSUMPTIONS

1. INTRODUCTION

1.1 Dunnett's Procedure and the t test with Bonferroni's adjustment are parametric procedures based on the assumptions that the observations within treatments are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using these tests, to determine if they have been met. Tests for validating the assumptions are provided in the following discussion. If the tests fail (if the data do not meet the assumptions), a nonparametric procedure such as Steel's Many-one Rank Test may be more appropriate. However, the decision on whether to use parametric or nonparametric tests may be a judgement call, and a statistician should be consulted in selecting the analysis.

2. TEST FOR NORMAL DISTRIBUTION OF DATA

2.1 SHAPIRO-WILK'S TEST

2.1.1 One formal test for normality is the Shapiro-Wilk's Test (Conover, 1980). The test statistic is obtained by dividing the square of an appropriate linear combination of the sample order statistics by the usual symmetric estimate of variance. The calculated W must be greater than zero and less than or equal to one. This test is recommended for a sample size of 50 or less. If the sample size is greater than 50, the Kolmogorov "D" statistic (Stephens, 1974) is recommended. An example of the Shapiro-Wilk's test is provided below.

2.2 The example uses growth data from the Sheepshead Minnow Larval Survival and Growth Test. The same data are used in the discussion of the homogeneity of variance determination in Paragraph 3 and Dunnett's Procedure in Appendix C. The data, the mean and variance of the observations at each concentration, including the control, are listed in Table B.1.

TABLE B.1. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL GROWTH DATA (WEIGHT IN MG) FOR THE SHAPIRO-WILK'S TEST

		Effluent Concentration (%)			
Replicate	Control	6.25	12.5	25.0	50.0
1	1.017	1.157	0.998	0.837	0.715
2	0.745	0.914	0.793	0.935	0.907
3	0.862	0.992	1.021	0.839	1.044
Mean(Y_i)	0.875	1.021	0.937	0.882	0.889
S_i^2	0.019	0.015	0.016	0.0031	0.027
i	1	2	3	4	5

2.3 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table B.2.

TABLE B.2. EXAMPLE OF SHAPIRO-WILK'S TEST: CENTERED OBSERVATIONS

		Effluent Concentration (%)			
Replicate	Control	6.25	12.5	25.0	50.0
1	0.142	0.136	0.061	- 0.009	- 0.174
2	- 0.130	- 0.107	- 0.144	0.053	0.018
3	- 0.013	- 0.029	0.084	- 0.043	0.155

2.4 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

Where: X_i = the centered observations, $\overline{X}$ is the overall mean of the centered observations, and n is the total number of the centered observations. For this set of data, $\overline{X} = 0$, and D = 0.1589.

2.4.1 For this set of data,

n = 15

$\overline{X}$ = 1/50 (0) = 0.0

D = 0.1589

2.5 Order the centered observations from smallest to largest,

$X^{(i)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$

where $X^{(i)}$ denote the ith order statistic. The ordered observations are listed in Table B.3.

TABLE B.3. EXAMPLE OF THE SHAPIRO-WILK'S TEST: ORDERED OBSERVATIONS

i	$X^{(i)}$
1	- 0.174
2	- 0.144
3	- 0.130
4	- 0.107
5	- 0.043
6	- 0.029
7	- 0.013
8	- 0.009
9	0.018
10	0.053
11	0.061
12	0.084
13	0.136
14	0.142
15	0.155

2.6 From Table B.4,for the number of observations, n, obtain the coefficients a_1, a_2, ..., a_k, where k is n/2 if n is even, and (n-1)/2 if n is odd. For the data in this example, n = 15, k = 7, and the a_i values are listed in Table B.5. The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table B.5.

2.7 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (Conover, 1980)

i \ n	Number of Observations								
	2	3	4	5	6	7	8	9	10
1	0.7071	0.7071	0.6872	0.6646	0.6431	0.6233	0.6052	0.5888	0.5739
2	-	0.0000	0.1667	0.2413	0.2806	0.3031	0.3164	0.3244	0.3291
3	-	-	-	0.0000	0.0875	0.1401	0.1743	0.1976	0.2141
4	-	-	-	-	-	0.0000	0.0561	0.0947	0.1224
5	-	-	-	-	-	-	-	0.0000	0.0399

i \ n	Number of Observations									
	11	12	13	14	15	16	17	18	19	20
1	0.5601	0.5475	0.5359	0.5251	0.5150	0.5056	0.4968	0.4886	0.4808	0.4734
2	0.3315	0.3325	0.3325	0.3318	0.3306	0.3209	0.3273	0.3253	0.3232	0.3211
3	0.2260	0.2347	0.2412	0.2460	0.2495	0.2521	0.2540	0.2553	0.2561	0.2565
4	0.1429	0.1586	0.1707	0.1802	0.1878	0.1939	0.1988	0.2027	0.2059	0.2085
5	0.0695	0.0922	0.1099	0.1240	0.1353	0.1447	0.1524	0.1587	0.1641	0.1686
6	0.0000	0.0303	0.0539	0.0727	0.0880	0.1005	0.1109	0.1197	0.1271	0.1334
7	-	-	0.0000	0.0240	0.0433	0.0593	0.0725	0.0837	0.0932	0.1013
8	-	-	-	-	0.0000	0.0196	0.0359	0.0496	0.0612	0.0711
9	-	-	-	-	-	-	0.0000	0.0163	0.0303	0.0422
10	-	-	-	-	-	-	-	-	0.0000	0.0140

i \ n	Number of Observations									
	21	22	23	24	25	26	27	28	29	30
1	0.4643	0.4590	0.4542	0.4493	0.4450	0.4407	0.4366	0.4328	0.4291	0.4254
2	0.3185	0.3156	0.3126	0.3098	0.3069	0.3043	0.3018	0.2992	0.2968	0.2944
3	0.2578	0.2571	0.2563	0.2554	0.2543	0.2533	0.2522	0.2510	0.2499	0.2487
4	0.2119	0.2131	0.2139	0.2145	0.2148	0.2151	0.2152	0.2151	0.2150	0.2148
5	0.1736	0.1764	0.1787	0.1807	0.1822	0.1836	0.1848	0.1857	0.1864	0.1870
6	0.1399	0.1443	0.1480	0.1512	0.1539	0.1563	0.1584	0.1601	0.1616	0.1630
7	0.1092	0.1150	0.1201	0.1245	0.1283	0.1316	0.1346	0.1372	0.1395	0.1415
8	0.0804	0.0878	0.0941	0.0997	0.1046	0.1089	0.1128	0.1162	0.1192	0.1219
9	0.0530	0.0618	0.0696	0.0764	0.0923	0.0876	0.0923	0.0965	0.1002	0.1036
10	0.0263	0.0368	0.0459	0.0539	0.0610	0.0672	0.0728	0.0778	0.0822	0.0862
11	0.0000	0.0122	0.0228	0.0321	0.0403	0.0476	0.0540	0.0598	0.0650	0.0697
12	-	-	0.0000	0.0107	0.0200	0.0284	0.0358	0.0424	0.0483	0.0537
13	-	-	-	-	0.0000	0.0094	0.0178	0.0253	0.0320	0.0381
14	-	-	-	-	-	-	0.0000	0.0084	0.0159	0.0227
15	-	-	-	-	-	-	-	-	0.0000	0.0076

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (CONTINUED)

i \ n	Number of Observations									
	31	32	33	34	35	36	37	38	39	40
1	0.4220	0.4188	0.4156	0.4127	0.4096	0.4068	0.4040	0.4015	0.3989	0.3964
2	0.2921	0.2898	0.2876	0.2854	0.2834	0.2813	0.2794	0.2774	0.2755	0.2737
3	0.2475	0.2462	0.2451	0.2439	0.2427	0.2415	0.2403	0.2391	0.2380	0.2368
4	0.2145	0.2141	0.2137	0.2132	0.2127	0.2121	0.2116	0.2110	0.2104	0.2098
5	0.1874	0.1878	0.1880	0.1882	0.1883	0.1883	0.1883	0.1881	0.1880	0.1878
6	0.1641	0.1651	0.1660	0.1667	0.1673	0.1678	0.1683	0.1686	0.1689	0.1691
7	0.1433	0.1449	0.1463	0.1475	0.1487	0.1496	0.1505	0.1513	0.1520	0.1526
8	0.1243	0.1265	0.1284	0.1301	0.1317	0.1331	0.1344	0.1356	0.1366	0.1376
9	0.1066	0.1093	0.1118	0.1140	0.1160	0.1179	0.1196	0.1211	0.1225	0.1237
10	0.0899	0.0931	0.0961	0.0988	0.1013	0.1036	0.1056	0.1075	0.1092	0.1108
11	0.0739	0.0777	0.0812	0.0844	0.0873	0.0900	0.0924	0.0947	0.0967	0.0986
12	0.0585	0.0629	0.0669	0.0706	0.0739	0.0770	0.0798	0.0824	0.0848	0.0870
13	0.0435	0.0485	0.0530	0.0572	0.0610	0.0645	0.0677	0.0706	0.0733	0.0759
14	0.0289	0.0344	0.0395	0.0441	0.0484	0.0523	0.0559	0.0592	0.0622	0.0651
15	0.0144	0.0206	0.0262	0.0314	0.0361	0.0404	0.0444	0.0481	0.0515	0.0546
16	0.0000	0.0068	0.0131	0.0187	0.0239	0.0287	0.0331	0.0372	0.0409	0.0444
17	-	-	0.0000	0.0062	0.0119	0.0172	0.0220	0.0264	0.0305	0.0343
18	-	-	-	-	0.0000	0.0057	0.0110	0.0158	0.0203	0.0244
19	-	-	-	-	-	-	0.0000	0.0053	0.0101	0.0146
20	-	-	-	-	-	-	-	-	0.0000	0.0049

i \ n	Number of Observations									
	41	42	43	44	45	46	47	48	49	50
1	0.3940	0.3917	0.3894	0.3872	0.3850	0.3830	0.3808	0.3789	0.3770	0.3751
2	0.2719	0.2701	0.2684	0.2667	0.2651	0.2635	0.2620	0.2604	0.2589	0.2574
3	0.2357	0.2345	0.2334	0.2323	0.2313	0.2302	0.2291	0.2281	0.2271	0.2260
4	0.2091	0.2085	0.2078	0.2072	0.2065	0.2058	0.2052	0.2045	0.2038	0.2032
5	0.1876	0.1874	0.1871	0.1868	0.1865	0.1862	0.1859	0.1855	0.1851	0.1847
6	0.1693	0.1694	0.1695	0.1695	0.1695	0.1695	0.1695	0.1693	0.1692	0.1691
7	0.1531	0.1535	0.1539	0.1542	0.1545	0.1548	0.1550	0.1551	0.1553	0.1554
8	0.1384	0.1392	0.1398	0.1405	0.1410	0.1415	0.1420	0.1423	0.1427	0.1430
9	0.1249	0.1259	0.1269	0.1278	0.1286	0.1293	0.1300	0.1306	0.1312	0.1317
10	0.1123	0.1136	0.1149	0.1160	0.1170	0.1180	0.1189	0.1197	0.1205	0.1212
11	0.1004	0.1020	0.1035	0.1049	0.1062	0.1073	0.1085	0.1095	0.1105	0.1113
12	0.0891	0.0909	0.0927	0.0943	0.0959	0.0972	0.0986	0.0998	0.1010	0.1020
13	0.0782	0.0804	0.0824	0.0842	0.0860	0.0876	0.0892	0.0906	0.0919	0.0932
14	0.0677	0.0701	0.0724	0.0745	0.0765	0.0783	0.0801	0.0817	0.0832	0.0846
15	0.0575	0.0602	0.0628	0.0651	0.0673	0.0694	0.0713	0.0731	0.0748	0.0764
16	0.0476	0.0506	0.0534	0.0560	0.0584	0.0607	0.0628	0.0648	0.0667	0.0685
17	0.0379	0.0411	0.0442	0.0471	0.0497	0.0522	0.0546	0.0568	0.0588	0.0608
18	0.0283	0.0318	0.0352	0.0383	0.0412	0.0439	0.0465	0.0489	0.0511	0.0532
19	0.0188	0.0227	0.0263	0.0296	0.0328	0.0357	0.0385	0.0411	0.0436	0.0459
20	0.0094	0.0136	0.0175	0.0211	0.0245	0.0277	0.0307	0.0335	0.0361	0.0386
21	0.0000	0.0045	0.0087	0.0126	0.0163	0.0197	0.0229	0.0259	0.0288	0.0314
22	-	-	0.0000	0.0042	0.0081	0.0118	0.0153	0.0185	0.0215	0.0244
23	-	-	-	-	0.0000	0.0039	0.0076	0.0111	0.0143	0.0174
24	-	-	-	-	-	-	0.0000	0.0037	0.0071	0.0104
25	-	-	-	-	-	-	-	-	0.0000	0.0035

TABLE B.5. EXAMPLE OF THE SHAPIRO-WILK'S TEST: TABLE OF COEFFICIENTS AND DIFFERENCES

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.181	$X^{(20)} - X^{(1)}$
2	0.3211	0.128	$X^{(19)} - X^{(2)}$
3	0.2565	0.105	$X^{(18)} - X^{(3)}$
4	0.2085	0.097	$X^{(17)} - X^{(4)}$
5	0.1686	0.076	$X^{(16)} - X^{(5)}$
6	0.1334	0.048	$X^{(15)} - X^{(6)}$
7	0.1013	0.034	$X^{(14)} - X^{(7)}$
8	0.0711	0.025	$X^{(13)} - X^{(8)}$
9	0.0422	0.008	$X^{(12)} - X^{(9)}$
10	0.0140	0.005	$X^{(11)} - X^{(10)}$

TABLE B.6. QUANTILES OF THE SHAPIRO WILK'S TEST STATISTIC (Conover, 1980)

n	0.01	0.02	0.05	0.10	0.50	0.90	0.95	0.98	0.99
3	0.753	0.756	0.767	0.789	0.959	0.998	0.999	1.000	1.000
4	0.687	0.707	0.748	0.792	0.935	0.987	0.992	0.996	0.997
5	0.686	0.715	0.762	0.806	0.927	0.979	0.986	0.991	0.993
6	0.713	0.743	0.788	0.826	0.927	0.974	0.981	0.986	0.989
7	0.730	0.760	0.803	0.838	0.928	0.972	0.979	0.985	0.988
8	0.749	0.778	0.818	0.851	0.932	0.972	0.978	0.984	0.987
9	0.764	0.791	0.829	0.859	0.935	0.972	0.978	0.984	0.986
10	0.781	0.806	0.842	0.869	0.938	0.972	0.978	0.983	0.986
11	0.792	0.817	0.850	0.876	0.940	0.973	0.979	0.984	0.986
12	0.805	0.828	0.859	0.883	0.943	0.973	0.979	0.984	0.986
13	0.814	0.837	0.866	0.889	0.945	0.974	0.979	0.984	0.986
14	0.825	0.846	0.874	0.895	0.947	0.975	0.980	0.984	0.986
15	0.835	0.855	0.881	0.901	0.950	0.975	0.980	0.984	0.987
16	0.844	0.863	0.887	0.906	0.952	0.976	0.981	0.985	0.987
17	0.851	0.869	0.892	0.910	0.954	0.977	0.981	0.985	0.987
18	0.858	0.874	0.897	0.914	0.956	0.978	0.982	0.986	0.988
19	0.863	0.879	0.901	0.917	0.957	0.978	0.982	0.986	0.988
20	0.868	0.884	0.905	0.920	0.959	0.979	0.983	0.986	0.988
21	0.873	0.888	0.908	0.923	0.960	0.980	0.983	0.987	0.989
22	0.878	0.892	0.911	0.926	0.961	0.980	0.984	0.987	0.989
23	0.881	0.895	0.914	0.928	0.962	0.981	0.984	0.987	0.989
24	0.884	0.898	0.916	0.930	0.963	0.981	0.984	0.987	0.989
25	0.888	0.901	0.918	0.931	0.964	0.981	0.985	0.988	0.989
26	0.891	0.904	0.920	0.933	0.965	0.982	0.985	0.988	0.989
27	0.894	0.906	0.923	0.935	0.965	0.982	0.985	0.988	0.990
28	0.896	0.908	0.924	0.936	0.966	0.982	0.985	0.988	0.990
29	0.898	0.910	0.926	0.937	0.966	0.982	0.985	0.988	0.990
30	0.900	0.912	0.927	0.939	0.967	0.983	0.985	0.988	0.990
31	0.902	0.914	0.929	0.940	0.967	0.983	0.986	0.988	0.990
32	0.904	0.915	0.930	0.941	0.968	0.983	0.986	0.988	0.990
33	0.906	0.917	0.931	0.942	0.968	0.983	0.986	0.989	0.990
34	0.908	0.919	0.933	0.943	0.969	0.983	0.986	0.989	0.990
35	0.910	0.920	0.934	0.944	0.969	0.984	0.986	0.989	0.990
36	0.912	0.922	0.935	0.945	0.970	0.984	0.986	0.989	0.990
37	0.914	0.924	0.936	0.946	0.970	0.984	0.987	0.989	0.990
38	0.916	0.925	0.938	0.947	0.971	0.984	0.987	0.989	0.990
39	0.917	0.927	0.939	0.948	0.971	0.984	0.987	0.989	0.991
40	0.919	0.928	0.940	0.949	0.972	0.985	0.987	0.989	0.991
41	0.920	0.929	0.941	0.950	0.972	0.985	0.987	0.989	0.991
42	0.922	0.930	0.942	0.951	0.972	0.985	0.987	0.989	0.991
43	0.923	0.932	0.943	0.951	0.973	0.985	0.987	0.990	0.991
44	0.924	0.933	0.944	0.952	0.973	0.985	0.987	0.990	0.991
45	0.926	0.934	0.945	0.953	0.973	0.985	0.988	0.990	0.991
46	0.927	0.935	0.945	0.953	0.974	0.985	0.988	0.990	0.991
47	0.928	0.936	0.946	0.954	0.974	0.985	0.988	0.990	0.991
48	0.929	0.937	0.947	0.954	0.974	0.985	0.988	0.990	0.991
49	0.929	0.937	0.947	0.955	0.974	0.985	0.988	0.990	0.991
50	0.930	0.938	0.947	0.955	0.974	0.985	0.988	0.990	0.991

2.8 The decision rule for this test is to compare the critical value from Table B.6 to the computed W. If the computed value is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 15 observations (n) is 0.835. The calculated value, 0.9516, is not less than the critical value. Therefore conclude that the data are normally distributed.

2.9 In general, if the data fail the test for normality, a transformation such as to log values may normalize the data. After transforming the data, repeat the Shapiro Wilk's Test for normality.

3. **TEST FOR HOMOGENEITY OF VARIANCE**

3.1 For Dunnett's Procedure and the t test with Bonferroni's adjustment, the variances of the data obtained from each toxicant concentration and the control are assumed to be equal. Bartlett's Test is a formal test of this assumption. In using this test, it is assumed that the data are normally distributed.

3.2 The data used in this example are growth data from a Sheepshead Minnow Larval Survival and Growth Test, and are the same data used in Appendices C and D. These data are listed in Table B.7, together with the calculated variance for the control and each toxicant concentration.

3.3 The test statistic for Bartlett's Test (Snedecor and Cochran, 1980) is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i) \ln \overline{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, ($V_i = n_i - 1$)

p = number of levels of toxicant concentration including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i = the number of replicates for concentration i.

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

TABLE B.7. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR BARTLETT'S TEST FOR HOMOGENEITY OF VARIANCE

		Effluent Concentration (%)			
Replicate	Control	6.25	12.5	25.0	50.0
1	1.017	1.157	0.998	0.873	0.715
2	0.745	0.914	0.793	0.935	0.907
3	0.862	0.992	1.021	0.839	1.044
Mean	0.875	1.021	0.937	0.882	0.889
S_i^2	0.019	0.015	0.016	0.0024	0.027
i	1	2	3	4	5

3.4 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value is obtained from a table of the chi-square distribution for p - 1 degrees of freedom and a significance level of 0.01. If B is less than the critical value then the variances are assumed to be equal.

3.5 For the data in this example, $V_i = 2$, $p = 5$, $\overline{S}^2 = 0.0158$, and $C = 1.2$. The calculated B value is:

$$B = \frac{2[5(\ln\ 0.0158) - \sum_i \ln(S_i^2)]}{1.2}$$

$$= \frac{2[5(-\ 4.1477)\ -\ (-22.1247)]}{1.2}$$

$$= 2.3103$$

3.6 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value for the test is 13.3 for a significance level of 0.01. Since B is less than 13.3, the conclusion is that the variances are not different.

4. TRANSFORMATIONS OF THE DATA

4.1 When the assumptions of normality and/or homogeneity of variance are not met, transformations of the data may remedy the problem, so that the data can be analyzed by parametric procedures, rather than nonparametric technique such as Steel's Many-one Rank Test or Wilcoxon's Rank Sum Test. Examples of transformations include log, square root, arc sine square root, and reciprocals. After the data have been transformed, the Shapiro-Wilk's and Bartlett's tests should be performed on the transformed observations to determine whether the assumptions of normality and/or homogeneity of variance are met.

4.2 ARC SINE SQUARE ROOT TRANSFORMATION (USEPA, 1993).

4.2.1 For data consisting of proportions from a binomial (response/no response; live/dead) response variable, the variance within the ith treatment is proportional to $P_i (1 - P_i)$, where P_i is the expected proportion for the treatment. This clearly violates the homogeneity of variance assumption required by parametric procedures such as Dunnett's Procedure or the t test with Bonferroni's adjustment, since the existence of a treatment effect implies different values of P_i for different treatments, i. Also, when the observed proportions are based on small samples, or when P_i is close to zero or one, the normality assumption may be invalid. The arc sine square root (arc sine $\sqrt{P}$) transformation is commonly used for such data to stabilize the variance and satisfy the normality requirement.

4.2.2 Arc sine transformation consists of determining the angle (in radians) represented by a sine value. In the case of arc sine square root transformation of mortality data, the proportion of dead (or affected) organisms is taken as the sine value, the square root of the sine value is determined, and the angle (in radians) for the square root of the sine value is determined. Whenever the proportion dead is 0 or 1, a special modification of the arc sine square root transformation must be used (Bartlett, 1937). An explanation of the arc sine square root transformation and the modification is provided below.

4.2.3 Calculate the response proportion (RP) at each effluent concentration, where:

RP = (number of surviving or unaffected organisms)/(number exposed).

Example: If 12 of 20 animals in a given treatment replicate survive:

RP = 12/20

= 0.60

4.2.4 Transform each RP to its arc sine square root, as follows:

4.2.4.1 For RPs greater than zero or less than one:

Angle (radians) = arc sine $\sqrt{RP}$

Example: If RP = 0.60:

Angle = arc sine $\sqrt{0.60}$

= arc sine 0.7746

= 0.8861 radians

4.2.4.2 Modification of the arc sine square root when RP = 0.

Angle (in radians) = arc sine $\sqrt{1/4N}$

Where: N = Number of animals/treatment replicate

Example: If 20 animals are used:

Angle = arc sine $\sqrt{1/80}$

= arc sine 0.1118

= 0.1120 radians

4.2.4.3 Modification of the arc sine square root when RP = 1

Angle = 1.5708 radians - (radians for RP = 0)

Example: Using above value:

Angle = 1.5708 - 0.1120

= 1.4588 radians

APPENDIX C

DUNNETT'S PROCEDURE

1. MANUAL CALCULATIONS

1.1 Dunnett's Procedure (Dunnett, 1955; Dunnett, 1964) is used to compare each concentration mean with the control mean to decide if any of the concentrations differ from the control. This test has an overall error rate of alpha, which accounts for the multiple comparisons with the control. It is based on the assumptions that the observations are independent and normally distributed and that the variance of the observations is homogeneous across all concentrations and control. (See Appendix B for a discussion on validating the assumptions). Dunnett's Procedure uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance. Dunnett's Procedure can only be used when the same number of replicate test vessels have been used at each concentration and the control. When this condition is not met, the t test with Bonferroni's adjustment is used (see Appendix D).

1.2 The data used in this example are growth data from a Sheepshead Minnow Larval Survival and Growth Test, and are the same data used in Appendices B and D. These data are listed in Table C.1.

TABLE C.1. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR DUNNETT'S PROCEDURE

Effluent Conc (%)	i	Replicate Test Vessel 1	2	3	Total (T_i)	Mean ($\overline{Y}_i$)
Control	1	1.017	0.745	0.862	2.624	0.875
6.25	2	1.157	0.914	0.992	3.063	1.021
12.5	3	0.998	0.793	1.021	2.812	0.937
25.0	4	0.873	0.935	0.839	2.647	0.882
50.0	5	0.715	0.907	1.044	2.666	0.889

1.3 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, using the following formulas:

Where: p = number of effluent concentrations including the control:

N = the total sample size; $N=\sum_i n_i$

n_i = the number of replicates for concentration "i"

$SST=\sum_{ij} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSB = \sum_i T_i^2/n_i - G^2/N$ Between Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations; $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration i

Y_{ij} = the jth observation for concentration i

1.4 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 3$

N = 20

$T_1 = Y_{11} + Y_{12} + Y_{13} = 2.624$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 3.063$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 2.812$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 2.647$
$T_5 = Y_{51} + Y_{52} + Y_{53} = 2.666$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 13.812$

$SST = \sum_{ij} Y_{ij}^2 - G^2/N$

$= 12.922 - (13.812)^2/15$

$= 0.204$

$= 12.763 - (13.812)^2/15$

$= 0.045$

$SSW = SST - SSB$

$= 0.204 - 0.045$

= 0.159

1.5 Summarize these data in the ANOVA table (Table C.2).

TABLE C.2. ANOVA TABLE FOR DUNNETT'S PROCEDURE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

1.6 Summarize data for ANOVA (Table C.3).

TABLE C.3. COMPLETED ANOVA TABLE FOR DUNNETT'S PROCEDURE

Source	df	SS	Mean Square
Between	5 - 1 = 4	0.045	0.011
Within	15 - 5 = 10	0.159	0.016
Total	14	0.204	

1.7 To perform the individual comparisons, calculate the t statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\overline{Y}_1 - \overline{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\overline{Y}_i$ = mean for each concentration i.

$\overline{Y}_1$ = mean for the control

S_w = square root of the within mean square

n_1 = number of replicates in the control.

n_i = number of replicates for concentration i.

1.8 Table C.4 includes the calculated t values for each concentration and control combination.

TABLE C.4. CALCULATED T VALUES

Effluent Concentration (%)	i	t_i
6.25	2	- 1.414
12.5	3	- 0.600
25.0	4	- 0.068
50.0	5	- 0.136

1.9 Since the purpose of the test is only to detect a decrease in growth from the control, a one-sided test is appropriate. The critical value for the one-sided comparison (2.47), with an overall alpha level of 0.05, 10 degrees of freedom and four concentrations excluding the control is read from the table of Dunnett's "T" values (Table C.5; this table assumes an equal number of replicates in all treatment concentrations and the control). Comparing each of the calculated t values in Table C.4 with the critical value, no decreases in growth from the control were detected. Thus the NOEC is 50.0%.

1.10 To quantify the sensitivity of the test, the minimum significant difference (MSD) may be calculated. The formula is as follows:

$$MSD = d\, S_w \sqrt{(1/n_1) + (1/n)}$$

Where: d = critical value for the Dunnett's Procedure

S_w = the square root of the within mean square

n = the number of replicates at each concentration, assuming an equal number of replicates at all treatment concentrations

n_1 = number of replicates in the control

For example:

$$MSD = 2.47(0.126)[\sqrt{(1/3)+(1/3)}] = 2.47(0.126)(\sqrt{2/3})$$

$$= 2.47\ (0.126)(0.816)$$
$$= 0.254$$

TABLE C.5. DUNNETT'S "T" VALUES (Miller, 1981)

(One-tailed) d^{α}_{k}																		
v k	α = .05									α = 0.1								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
5	2.02	2.44	2.58	2.85	2.98	3.08	3.16	3.24	3.30	3.37	3.90	4.21	4.43	4.50	4.73	4.85	4.94	5.03
6	1.94	2.34	2.56	2.71	2.83	2.92	3.00	3.07	3.12	3.14	3.61	4.88	4.07	4.21	4.33	4.43	4.51	4.39
7	1.89	2.27	2.48	2.62	2.73	2.82	2.89	2.95	3.01	3.00	3.42	3.56	3.83	3.96	4.07	4.15	4.23	4.30
8	1.86	2.22	2.42	2.55	2.66	2.74	2.81	2.87	2.92	2.90	3.20	3.51	3.67	3.79	3.88	3.96	4.03	4.09
9	1.83	2.18	2.37	2.50	2.60	2.68	2.75	2.81	2.86	2.82	3.19	3.40	3.55	3.64	3.75	3.82	3.89	3.94
10	1.81	2.15	2.34	2.47	2.56	2.64	2.70	2.76	2.81	2.76	3.11	3.31	3.45	3.56	3.64	3.71	3.78	3.83
11	1.80	2.13	2.31	2.44	2.53	2.60	2.67	2.72	2.77	2.72	3.06	3.25	3.38	3.46	3.56	3.63	3.69	3.74
12	1.78	2.11	2.29	2.41	2.50	2.58	2.64	2.69	2.74	2.68	3.01	3.19	3.32	3.42	3.50	3.56	3.62	3.67
13	1.77	2.09	2.27	2.39	2.48	2.55	2.61	2.68	2.71	2.65	2.97	3.15	3.27	3.37	3.44	3.91	3.56	3.61
14	1.76	2.08	2.25	2.37	2.46	2.53	2.59	2.64	2.69	2.62	2.94	3.11	3.23	3.32	3.40	3.46	3.51	3.56
15	1.75	2.07	2.24	2.36	2.44	2.51	2.57	2.62	2.67	2.60	2.91	3.08	3.20	3.29	3.36	3.42	3.47	3.52
16	1.75	2.06	2.23	2.34	2.43	2.50	2.56	2.61	2.65	2.58	2.38	3.05	3.17	3.28	3.33	3.39	3.44	3.48
17	1.74	2.05	2.22	2.33	2.42	2.49	2.54	2.59	2.64	2.57	2.86	3.03	3.14	3.23	3.30	3.36	3.41	3.45
18	1.73	2.04	2.21	2.32	2.41	2.48	2.53	2.58	2.62	2.55	2.84	3.01	3.12	3.21	3.27	3.33	3.38	3.42
19	1.73	2.03	2.20	2.31	2.40	2.47	2.52	2.57	2.61	2.54	2.83	2.99	3.10	3.18	3.25	3.31	3.36	3.40
20	1.72	2.03	2.19	2.30	2.38	2.46	2.51	2.56	2.60	2.53	2.81	2.97	3.08	3.17	3.23	3.29	3.34	3.38
24	1.71	2.01	2.17	2.28	2.36	2.43	2.48	2.53	2.57	2.40	2.77	2.92	3.03	3.11	3.17	3.22	3.27	3.31
30	1.70	1.99	2.15	2.25	2.33	2.40	2.45	2.50	2.54	2.46	2.72	2.87	2.97	3.05	3.11	3.16	3.21	3.24
40	1.68	1.97	2.13	2.23	2.31	2.37	2.42	2.47	2.51	2.42	2.68	2.82	2.92	2.99	3.06	3.10	3.14	3.18
60	1.67	1.95	2.10	2.21	2.28	2.35	2.39	2.44	2.48	2.39	2.64	2.78	2.87	2.94	3.08	3.04	3.06	3.12
120	1.86	1.93	2.08	2.18	2.26	2.32	2.37	2.41	2.45	2.36	2.60	2.73	2.82	2.90	2.94	2.90	3.03	3.06
α	1.64	1.92	2.06	2.16	2.23	2.29	2.34	2.33	2.42	2.33	2.56	2.68	2.77	2.34	2.90	2.83	2.97	3.00

1.11 For this set of data, the minimum difference between the control mean and a concentration mean that can be detected as statistically significant is 0.254 mg. This represents a decrease in growth of 29% from the control.

1.11.1 If the data have not been transformed, the MSD (and the percent decrease from the control mean that it represents) can be reported as is.

1.11.2 In the case where the data have been transformed, the MSD would be in transformed units. In this case carry out the following conversion to determine the MSD in untransformed units.

1.11.2.1 Subtract the MSD from the transformed control mean. Call this difference D. Next, obtain untransformed values for the control mean and the difference, D.

$$MSD_u = \text{control}_u - D_u$$

Where: MSD_u = the minimum significant difference for untransformed data

$Control_u$ = the untransformed control mean

D_u = the untransformed difference

1.11.2.2 Calculate the percent reduction from the control that MSD_u represents as:

$$\text{Percent Reduction} = \frac{MSD_u}{Control_u} \times 100$$

1.11.3 An example of a conversion of the MSD to untransformed units, when the arc sine square root transformation was used on the data, follows.

Step 1. Subtract the MSD from the transformed control mean. As an example, assume the data in Table C.1 were transformed by the arc sine square root transformation. Thus:

0.875 - 0.254 = 0.621

Step 2. Obtain untransformed values for the control mean (0.875) and the difference (0.621) obtained in Step 1, above.

$[\text{Sine } (0.875)]^2 = 0.589$

$[\text{Sine } (0.621)]^2 = 0.339$

Step 3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values obtained in Step 2.

$MSD_u = 0.589 - 0.339 = 0.250$

In this case, the MSD would represent a 42% decrease in survival from the control [(0.250/0.589)(100)].

2. COMPUTER CALCULATIONS

2.1 This computer program incorporates two analyses: an analysis of variance (ANOVA), and a multiple comparison of treatment means with the control mean (Dunnett's Procedure). The ANOVA is used to obtain the error value. Dunnett's Procedure indicates which toxicant concentration means (if any) are statistically different from the control mean at the 5% level of significance. The program also provides the minimum difference between the control and treatment means that could be detected as statistically significant, and tests the validity of the homogeneity of variance assumption by Bartlett's Test. The multiple comparison is performed based on procedures described by Dunnett (1955).

2.2 The source code for the Dunnett's program is structured into a series of subroutines, controlled by a driver routine. Each subroutine has a specific function in the Dunnett's Procedure, such as data input, transforming the data, testing for equality of variances, computing p values, and calculating the one-way analysis of variance.

2.3 The program compares up to seven toxicant concentrations against the control, and can accommodate up to 50 replicates per concentration.

2.4 If the number of replicates at each toxicant concentration and control are not equal, a t test with the Bonferroni adjustment is performed instead of Dunnett's Procedure (see Appendix D).

2.5 The program was written in IBM-PC FORTRAN by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled version of the program can be obtained from EMSL-Cincinnati by sending a diskette with a written request.

2.6 DATA INPUT AND OUTPUT

2.6.1 Data on the number of surviving mysids, *Mysidopsis bahia*, from a survival, growth and fecundity test (Table C.6) are used to illustrate the data input and output for this program.

2.6.2 Data Input

2.6.2.1 When the program is entered, the user is asked to select the type of data to be analyzed:

1. Response proportions, like survival or fertilization proportions data.
2. Counts and measurements, like offspring counts, cystocarp and algal cell counts, weights, chlorophyll measurements or turbidity measurements.

2.6.2.2 After the type of analysis for the data is chosen, the user has the following options:

1. Create a data file
2. Edit a data file
3. Perform analysis on existing data set
4. Stop

2.6.2.3 When Option 1 (Create a data file) is selected for response proportions, the program prompts the user for the following information:

1. Number of concentrations, including control
2. For each concentration and replicate:
   - number of organisms exposed per replicate
   - number of organisms responding per replicate (organisms surviving, eggs fertilized, etc.)

2.6.2.4 After the data have been entered, the user may save the file on a disk, and the program returns to the main menu (see below).

2.6.2.5 Sample data input is shown in Figure C.1.

2.6.3. Program Output

2.6.3.1 When Option 3 (perform analysis on existing data set) is selected from the menu, the user is asked to select the transformation desired, and indicate whether they expect the means of the test groups to be less or greater than the mean for the control group (see Figure C.2)

2.6.3.2 Summary statistics (Figure C.3) for the raw and transformed data, if applicable, the ANOVA table, results of Bartlett's Test, the results of the multiple comparison procedure, and the minimum detectable difference are included in the program output.

TABLE C.6. SAMPLE DATA FOR DUNNETT'S PROGRAM FOR SURVIVING MYSIDS, *MYSIDOPSIS BAHIA*

Treatment	Replicate Chamber	Total Mysids	No. Alive
1 Control	1	5	4
	2	5	4
	3	5	5
	4	5	5
	5	5	5
	6	5	5
	7	5	5
	8	5	4
2 50 ppb	1	5	4
	2	5	5
	3	5	4
	4	5	4
	5	5	5
	6	5	5
	7	5	4
	8	5	5
3 100 ppg	1	5	3
	2	5	5
	3	5	5
	4	5	5
	5	5	5
	6	5	3
	7	5	4
	8	4	4
4 210 ppb	1	5	5
	2	5	4
	3	5	1
	4	5	4
	5	5	3
	6	5	4
	7	5	4
	8	5	4
5 450 ppb	1	5	0
	2	5	1
	3	5	0
	4	5	1
	5	5	0
	6	5	0
	7	5	0
	8	5	2

EMSL Cincinnati Dunnett Software
Version 1.5

1) Create a data file
2) Edit a data file
3) Analyze an existing data set
4) Stop

Your choice ? 3

Number of concentrations, including control ? 5

Number of replicates for conc. 1 (the control) ? 8

replicate	number of organisms exposed	number of organisms responding (organisms surviving, eggs fertilized, etc.)
1	5	4
2	5	4
3	5	5
4	5	5
5	5	5
6	5	5
7	5	5
8	5	4

Number of replicates for conc. 2 ? 8
Do you wish to save the data on disk ? y

Disk file for output ? mysidsur.dat

Figure C.1. Sample Data Input for Dunnett's Program for Survival Data from Table C.6.

EMSL Cincinnati: Dunnett Software
Version 1.5

1) Create a data file
2) Edit a data file
3) Analyze an existing data set
4) Stop

Your choice ? 3
File name ? mysidsur.dat

Available Transformations

1) no transform
2) square root
3) log10
4) arcsine square root

Your choice ? 4
Dunnett's test as implemented in this program is a one-sided test. You must specify the direction the test is to be run; that is, do you expect the means for the test concentrations to be less than or greater than the mean for the control concentration.

Direction for Dunnetts test : L=less than, G=greater than ? l

Summary Statistics for Raw Data

Conc.	n	Mean	s.d.	cv%
1 = control	8	.9250	.1035	11.2
2	8	.9000	.1069	11.9
3	8	.8500	.1773	20.9
4	8	.7250	.2375	32.8
5	8	.1000	.1512	151.2

Mysid Survival Example with Data in Table C.6

Figure C.2. Example of Choosing Option 3 from the Main Menu of the Dunnett Program.

Mysid Survival Example with Data in Table C.6

Summary Statistics and ANOVA

Transformation = Arcsine Square Root

Conc.	n	Mean	s.d.	cv%
1 = control	8	1.2560	.1232	9.8
2	8	1.2262	.1273	10.4
3	8	1.1709	.2042	17.4
4*	8	1.0288	.2593	25.2
5*	8	.3424	.1752	51.2

*) the mean for this conc. is significantly less than
the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.208074
This corresponds to a difference of -.153507 in original units
This difference corresponds to -16.98 percent of control

Between concentrations
sum of squares = 4.632112 with 4 degrees of freedom.

Error mean square = .034208 with 35 degrees of freedom.

Bartlett's test p-value for equality of variances = .257

Do you wish to restart the program ?

Figure C.3. Example of Program Output for the Dunnett's Program Using the Survival Data in Table C.6.

APPENDIX D

T TEST WITH BONFERRONI'S ADJUSTMENT

1. The t test with Bonferroni's adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus, Dunnett's Procedure is a more powerful test.

2. The t test with Bonferroni's adjustment is based on the same assumptions of normality of distribution and homogeneity of variance as Dunnett's Procedure (See Appendix B for testing these assumptions), and, like Dunnett's Procedure, uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance.

3. An example of the use of the t test with Bonferroni's adjustment is provided below. The data used in the example are the same as in Appendix C, except that the third replicate from the 50% effluent treatment is presumed to have been lost. Thus, Dunnett's Procedure cannot be used. The weight data are presented in Table D.1.

TABLE D.1. SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR THE T TEST WITH BONFERRONI'S ADJUSTMENT

Effluent Conc (%)		Replicate Test Vessel			Total	Mean
	i	1	2	3	(T_i)	($\overline{Y}_i$)
Control	1	1.017	0.745	0.862	2.624	0.875
6.25	2	1.157	0.914	0.992	3.063	1.021
12.5	3	0.998	0.793	1.021	2.812	0.937
25.0	4	0.873	0.935	0.839	2.647	0.882
50.0	5	0.715	0.907	(Lost)	1.622	0.811

3.1 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, using the following formulas:

Where: p = number of effluent concentrations including the control

N = the total sample size; $N=\sum_i n_i$

n_i = the number of replicates for concentration i

$SST=\sum_{ij} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSB = \sum_i T_i^2/n_i - G^2/N$ Between Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

Where: G = The grand total of all sample observations; $G = \sum_{i=1}^{P} T_i$

T_i = The total of the replicate measurements for concentration i

Y_{ij} = The jth observation for concentration i

3.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = 3$

$N = 20$

$T_1 = Y_{11} + Y_{12} + Y_{13} = 2.624$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 3.063$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 2.812$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 2.647$
$T_5 = Y_{51} + Y_{52} + Y_{53} = 1.622$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 12.768$

$SSB = \sum_i T_i^2/n_i - G^2/N$

$= 11.709 - (12.768)^2/14$

$= 0.064$

$SST = \sum_{ij} Y_{ij}^2 - G^2/N$

$= 11.832 - (12.768)^2/14$

$= 0.188$

$SSW = SST - SSB$

$= 0.188 - 0.064$

$= 0.124$

3.3 Summarize these data in the ANOVA table (Table D.2).

TABLE D.2. ANOVA TABLE FOR BONFERRONI'S ADJUSTMENT

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

3.4 Summarize these calculations in the ANOVA table (Table D.3):

TABLE D.3. COMPLETED ANOVA TABLE FOR THE T-TEST WITH BONFERRONI'S ADJUSTMENT

Source	df	SS	Mean Square
Between	5 - 1 = 4	0.064	0.016
Within	14 - 5 = 9	0.124	0.014
Total	13	0.188	

3.5 To perform the individual comparisons, calculate the t statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\overline{Y}_1 - \overline{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\overline{Y}_i$ = mean for concentration i

$\overline{Y}_1$ = mean for the control

S_w = square root of the within mean square

n_1 = number of replicates in the control.

n_i = number of replicates for concentration i.

3.6 Table D.4 includes the calculated t values for each concentration and control combination.

TABLE D.4. CALCULATED T VALUES

Effluent Concentration (%)	i	t_i
6.25	2	- 1.511
12.5	3	- 0.642
25.0	4	- 0.072
50.0	5	0.592

3.7 Since the purpose of the test is only to detect a decrease in growth from the control, a one-sided test is appropriate. The critical value for the one-sided comparison (2.686), with an overall alpha level of 0.05, nine degrees of freedom and four concentrations excluding the control, was obtained from Table D.5. Comparing each of the calculated t values in Table D.4 with the critical value, no decreases in growth from the control were detected. Thus the NOEC is 50.0%.

TABLE D.5. CRITICAL VALUES FOR "T" FOR THE T TEST WITH BONFERRONI'S ADJUSTMENT
P = 0.05 CRITICAL LEVEL, ONE TAILED

df	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
1	6.314	12.707	19.002	25.452	31.821	38.189	44.556	50.924	57.290	63.657
2	2.920	4.303	5.340	6.206	6.965	7.649	8.277	8.861	9.408	9.925
3	2.354	3.183	3.741	4.177	4.541	4.857	5.138	5.392	5.626	5.841
4	2.132	2.777	3.187	3.496	3.747	3.961	4.148	4.315	4.466	4.605
5	2.016	2.571	2.912	3.164	3.365	3.535	3.681	3.811	3.927	4.033
6	1.944	2.447	2.750	2.969	3.143	3.288	3.412	3.522	3.619	3.708
7	1.895	2.365	2.642	2.842	2.998	3.128	3.239	3.336	3.422	3.500
8	1.860	2.307	2.567	2.752	2.897	3.016	3.118	3.206	3.285	3.356
9	1.834	2.263	2.510	2.686	2.822	2.934	3.029	3.111	3.185	3.250
10	1.813	2.229	2.406	2.634	2.764	2.871	2.961	3.039	3.108	3.170
11	1.796	2.301	2.432	2.594	2.719	2.821	2.907	2.981	3.047	3.106
12	1.783	2.179	2.404	2.561	2.681	2.730	2.863	2.935	2.998	3.055
13	1.771	2.161	2.380	2.533	2.651	2.746	2.827	2.897	2.950	3.013
14	1.762	2.145	2.360	2.510	2.625	2.718	2.797	2.864	2.924	2.977
15	1.754	2.132	2.343	2.490	2.603	2.694	2.771	2.837	2.895	2.947
16	1.746	2.120	2.329	2.473	2.584	2.674	2.749	2.814	2.871	2.921
17	1.740	2.110	2.316	2.459	2.567	2.655	2.729	2.793	2.849	2.899
18	1.735	2.101	2.305	2.446	2.553	2.640	2.712	2.775	2.830	2.879
19	1.730	2.094	2.295	2.434	2.540	2.626	2.697	2.759	2.813	2.861
20	1.725	2.086	2.206	2.424	2.528	2.613	2.684	2.745	2.798	2.846
21	1.721	2.080	2.278	2.414	2.518	2.602	2.672	2.732	2.785	2.832
22	1.718	2.074	2.271	2.406	2.509	2.592	2.661	2.721	2.773	2.819
23	1.714	2.069	2.264	2.398	2.500	2.583	2.651	2.710	2.762	2.808
24	1.711	2.064	2.258	2.391	2.493	2.574	2.642	2.701	2.752	2.797
25	1.709	2.060	2.253	2.385	2.486	2.566	2.634	2.692	2.743	2.788
26	1.706	2.056	2.248	2.379	2.479	2.559	2.627	2.684	2.734	2.779
27	1.704	2.052	2.243	2.374	2.473	2.553	2.620	2.677	2.727	2.771
28	1.702	2.049	2.239	2.369	2.468	2.547	2.613	2.670	2.720	2.764

TABLE D.5. CRITICAL VALUES FOR "T" FOR THE T TEST WITH BONFERRONI'S ADJUSTMENT
P = 0.05 CRITICAL LEVEL, ONE TAILED (CONTINUED)

df	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
29	1.700	2.046	2.235	2.364	2.463	2.541	2.607	2.664	2.713	2.757
30	1.698	2.043	2.231	2.360	2.458	2.536	2.602	2.658	2.707	2.750
31	1.696	2.040	2.228	2.356	2.453	2.531	2.597	2.652	2.701	2.745
32	1.694	2.037	2.224	2.352	2.449	2.527	2.592	2.647	2.696	2.739
33	1.693	2.035	2.221	2.349	2.445	2.523	2.587	2.643	2.691	2.734
34	1.691	2.033	2.219	2.346	2.442	2.519	2.583	2.638	2.686	2.729
35	1.690	2.031	2.216	2.342	2.438	2.515	2.579	2.634	2.682	2.724
36	1.689	2.029	2.213	2.340	2.435	2.512	2.575	2.630	2.678	2.720
37	1.688	2.027	2.211	2.337	2.432	2.508	2.572	2.626	2.674	2.716
38	1.686	2.025	2.209	2.334	2.429	2.505	2.568	2.623	2.670	2.712
39	1.685	2.023	2.207	2.332	2.426	2.502	2.565	2.619	2.667	2.708
40	1.684	2.022	2.205	2.329	2.424	2.499	2.562	2.616	2.663	2.705
50	1.676	2.009	2.189	2.311	2.404	2.478	2.539	2.592	2.638	2.678
60	1.671	2.001	2.179	2.300	2.391	2.463	2.324	2.576	2.621	2.661
70	1.667	1.995	2.171	2.291	2.381	2.453	2.513	2.564	2.609	2.648
80	1.665	1.991	2.166	2.285	2.374	2.446	2.505	2.556	2.600	2.639
90	1.662	1.987	2.162	2.280	2.369	2.440	2.499	2.549	2.593	2.632
100	1.661	1.984	2.158	2.276	2.365	2.435	2.494	2.544	2.588	2.626
110	1.659	1.982	2.156	2.273	2.361	2.432	2.490	2.540	2.583	2.622
120	1.658	1.980	2.153	2.270	2.358	2.429	2.487	2.536	2.580	2.618
Infinite	1.645	1.960	2.129	2.242	2.327	2.394	2.450	2.498	2.540	2.576

d.f. = Degrees of freedom for MSE (Mean Square Error) from ANOVA.
K = Number of concentrations to be compared to the control.

APPENDIX E

STEEL'S MANY-ONE RANK TEST

1. Steel's Many-one Rank Test is a nonparametric test for comparing treatments with a control. This test is an alternative to Dunnett's Procedure, and may be applied to data when the normality assumption has not been met. Steel's Test requires equal variances across the treatments and the control, but it is thought to be fairly insensitive to deviations from this condition (Steel, 1959). The tables for Steel's Test require an equal number of replicates at each concentration. If this is not the case, use Wilcoxon's Rank Sum Test, with Bonferroni's adjustment (See Appendix F).

2. For an analysis using Steel's Test, for each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to the observation. (Extensive ties would invalidate this procedure). The sum of the ranks within each concentration and within the control is then calculated. To determine if the response in a concentration is significantly different from the response in the control, the minimum rank sum for each concentration and control combination is compared to the significant values of rank sums given later in the section. In this table, k equals the number of treatments excluding the control and n equals the number of replicates for each concentration and the control.

3. An example of the use of this test is provided below. The test employs survival data from a mysid 7-day, chronic test. The data are listed in Table E.1. Throughout the test, the control data are taken from the site water control. Since there is 0% survival for all eight replicates for the 50% concentration, it is not included in this analysis and is considered a qualitative mortality effect.

4. For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, 3, ..., 16) to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to each tied observation.

5. An example of assigning ranks to the combined data for the control and 3.12% effluent concentration is given in Table E.2. This ranking procedure is repeated for each control and concentration combination. The complete set of rankings is listed in Table E.3. The ranks are then summed for each effluent concentration, as shown in Table E.4.

6. For this set of data, determine if the survival in any of the effluent concentrations is significantly lower than the survival of the control organisms. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for the survival at each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the survival would be considered to be significantly lower than the control. At a probability level of 0.05, the critical rank sum in a test with four concentrations and eight replicates per concentration, is 47 (see Table E.5).

7. Of the rank sums in Table E.4, none are less than 47. Therefore, due to the qualitative effect at the 50% effluent concentration, the NOEC is 25% effluent and the LOEC is 50% effluent.

TABLE E.1. EXAMPLE OF STEEL'S MANY-ONE RANK TEST: DATA FOR MYSID, *MYSIDOPSIS BAHIA*, 7-DAY CHRONIC TEST

Effluent Concentration	Replicate Chamber	Number of Mysids at Start of Test	Number of Live Mysids at End of Test
Control (Site Water)	1	5	4
	2	5	4
	3	5	5
	4	5	4
	5	5	5
	6	5	4
	7	5	4
	8	5	5
Control (Brine & Dilution Water)	1	5	3
	2	5	5
	3	5	3
	4	5	3
	5	5	4
	6	5	4
	7	5	3
	8	5	3
3.12%	1	5	4
	2	5	4
	3	5	4
	4	5	5
	5	5	4
	6	5	4
	7	5	5
	8	5	3
6.25%	1	5	3
	2	5	4
	3	5	5
	4	5	4
	5	5	4
	6	5	4
	7	5	5
	8	5	5
12.5%	1	5	5
	2	5	4
	3	5	5
	4	5	3
	5	5	5
	6	5	4
	7	5	4
	8	5	3
25.0%	1	5	5
	2	5	5
	3	5	5
	4	5	5
	5	5	3
	6	5	5
	7	5	4
	8	5	4
50.0%	1	5	0
	2	5	0
	3	5	0
	4	5	0
	5	5	0
	6	5	0
	7	5	0
	8	5	0

TABLE E.2. EXAMPLE OF STEEL'S MANY-ONE RANK TEST: ASSIGNING RANKS TO THE CONTROL AND 3.12% EFFLUENT CONCENTRATIONS

Rank	Number of Live Mysids, *Mysidopsis bahia*	Control or % Effluent
1	3	3.12
6.5	4	Control
6.5	4	Control
6.5	4	Control
6.5	4	Control
6.5	4	Control
6.5	4	3.12
6.5	4	3.12
6.5	4	3.12
6.5	4	3.12
6.5	4	3.12
14	5	Control
14	5	Control
14	5	Control
14	5	3.12
14	5	3.12

TABLE E.3. TABLE OF RANKS

Replicate Chamber	Control[1]	Effluent Concentration (%) 3.12	6.25	12.5	25.0
1	4 (6.5,6,6.5,5)	4 (6.5)	3 (1)	5 (13.5)	5 (12.5)
2	4 (6.5,6,6.5,5)	4 (6.5)	4 (6)	4 (6.5)	5 (12.5)
3	5 (14,13.5,13.5,12.5)	4 (6.5)	5 (13.5)	5 (13.5)	5 (12.5)
4	4 (6.5,6,6.5,5)	5 (14)	4 (6)	3 (1.5)	5 (12.5)
5	5 (14,13.5,13.5,12.5)	4 (6.5)	4 (6)	5 (13.5)	3 (1)
6	4 (6.5,6,6.5,5)	4 (6.5)	4 (6)	4 (6.5)	5 12.5)
7	4 (6.5,6,6.5,5)	5 (14)	5 (13.5)	4 (6.5)	4 (5)
8	5 (14,13.5,13.5,12.5)	3 (1)	5 (13.5)	3 (1.5)	4 (5)

[1] Control ranks are given in the order of the concentration with which they were ranked.

TABLE E.4. RANK SUMS

Effluent Concentration (%)	Rank Sum
3.12	61.5
6.25	65.5
12.50	63.0
25.00	73.5

TABLE E.5. SIGNIFICANT VALUES OF RANK SUMS: JOINT CONFIDENCE COEFFICIENTS OF 0.95 (UPPER) and 0.99 (LOWER) FOR ONE-SIDED ALTERNATIVES (Steel, 1959)

	k = number of treatments (excluding control)							
n	2	3	4	5	6	7	8	9
4	11	10	10	10	10	--	--	--
	--	--	--	--	--	--	--	--
5	18	17	17	16	16	16	16	15
	15	--	--	--	--	--	--	--
6	27	26	25	25	24	24	24	23
	23	22	21	21	--	--	--	--
7	37	36	35	35	34	34	33	33
	32	31	30	30	29	29	29	29
8	49	48	47	46	46	45	45	44
	43	42	41	40	40	40	39	39
9	63	62	61	60	59	59	58	58
	56	55	54	53	52	52	51	51
10	79	77	76	75	74	74	73	72
	71	69	68	67	66	66	65	65
11	97	95	93	92	91	90	90	89
	87	85	84	83	82	81	81	80
12	116	114	112	111	110	109	108	108
	105	103	102	100	99	99	98	98
13	138	135	133	132	130	129	129	128
	125	123	121	120	119	118	117	117
14	161	158	155	154	153	152	151	150
	147	144	142	141	140	139	138	137
15	186	182	180	178	177	176	175	174
	170	167	165	164	162	161	160	160
16	213	209	206	204	203	201	200	199
	196	192	190	188	187	186	185	184
17	241	237	234	232	231	229	228	227
	223	219	217	215	213	212	211	210
18	272	267	264	262	260	259	257	256
	252	248	245	243	241	240	239	238
19	304	299	296	294	292	290	288	287
	282	278	275	273	272	270	268	267
20	339	333	330	327	325	323	322	320
	315	310	307	305	303	301	300	299

APPENDIX F

WILCOXON RANK SUM TEST

1. Wilcoxon's Rank Sum Test is a nonparametric test, to be used as an alternative to Steel's Many-one Rank Test when the number of replicates are not the same at each concentration. A Bonferroni's adjustment of the pairwise error rate for comparison of each concentration versus the control is used to set an upper bound of alpha on the overall error rate, in contrast to Steel's Many-one Rank Test, for which the overall error rate is fixed at alpha. Thus, Steel's Test is a more powerful test.

2. The use of this test may be illustrated with fecundity data from the mysid test in Table F.1. The site water control and the 12.5% effluent concentration each have seven replicates for the proportion of females bearing eggs, while there are eight replicates for each of the remaining three concentrations.

3. For each concentration and control combination, combine the data and arrange the values in order of size, from smallest to largest. Assign ranks to the ordered observations (a rank of 1 to the smallest, 2 to the next smallest, etc.). If ties in rank occur, assign the average rank to each tied observation.

4. An example of assigning ranks to the combined data for the control and effluent concentration 3.12% is given in Table F.2. This ranking procedure is repeated for each of the three remaining control versus test concentration combinations. The complete set of ranks is listed in Table F.3. The ranks are then summed for each effluent concentration, as shown in Table F.4.

5. For this set of data, determine if the fecundity in any of the test concentrations is significantly lower than the fecundity in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum. Thus, compare the rank sums for fecundity of each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the fecundity would be considered to be significantly lower than the control. At a probability level of 0.05, the critical rank in a test with four concentrations and seven replicates in the control is 44 for those concentrations with eight replicates, and 34 for those concentrations with seven replicates (see Table F.5, for K = 4).

6. Comparing the rank sums in Table F.4 to the appropriate critical rank, only the 25% effluent concentration does not exceed its critical value of 44. Thus, the NOEC and LOEC for fecundity are 12.5% and 25%, respectively.

TABLE F.1. EXAMPLE OF WILCOXON'S RANK SUM TEST: FECUNDITY DATA FOR MYSID, *MYSIDOPSIS BAHIA*, 7-DAY CHRONIC TEST

Effluent Concentration	Replicate Chamber	Number of Mysids at Start of Test	Number of Live Mysids at End of Test	Proportion of Females with Eggs
Control (Site Water)	1	5	4	0.50
	2	5	4	----
	3	5	5	0.75
	4	5	4	0.67
	5	5	5	0.67
	6	5	4	0.50
	7	5	4	1.00
	8	5	5	1.00
Control (Brine & Dilution Water)	1	5	3	1.00
	2	5	5	1.00
	3	5	3	1.00
	4	5	3	1.00
	5	5	4	1.00
	6	5	4	0.50
	7	5	3	0.50
	8	5	3	0.50
3.12%	1	5	4	1.00
	2	5	4	0.50
	3	5	4	0.67
	4	5	5	1.00
	5	5	4	0.50
	6	5	4	1.00
	7	5	5	1.00
	8	5	3	0.00
6.25%	1	5	3	0.50
	2	5	4	0.00
	3	5	5	0.75
	4	5	4	1.00
	5	5	4	1.00
	6	5	4	1.00
	7	5	5	0.67
	8	5	5	0.67
12.5%	1	5	5	0.33
	2	5	4	0.50
	3	5	5	1.00
	4	5	3	----
	5	5	5	1.00
	6	5	4	0.00
	7	5	4	0.33
	8	5	3	0.50
25.0%	1	5	5	0.00
	2	5	5	0.50
	3	5	5	0.13
	4	5	5	0.00
	5	5	3	0.50
	6	5	5	0.00
	7	5	4	0.50
	8	5	4	0.50
50.0%	1	5	0	----
	2	5	0	----
	3	5	0	----
	4	5	0	----
	5	5	0	----
	6	5	0	----
	7	5	0	----
	8	5	0	----

TABLE F.2. EXAMPLE OF WILCOXON'S RANK SUM TEST: ASSIGNING RANKS TO THE CONTROL AND 3.12% EFFLUENT CONCENTRATIONS

Rank	Proportion of Females W/Eggs	Site Water Control or Effluent %
1	0.00	3.12
3.5	0.50	Control
3.5	0.50	Control
3.5	0.50	3.12
3.5	0.50	3.12
7	0.67	Control
7	0.67	Control
7	0.67	3.12
9	0.75	Control
12.5	1.00	Control
12.5	1.00	Control
12.5	1.00	3.12
12.5	1.00	3.12
12.5	1.00	3.12
12.5	1.00	3.12

TABLE F.3. TABLE OF RANKS[1]

Rep	Proportion	Site Water Control Rank	Effluent Concentration (%) 3.12	6.25	12.5	25.0
1	0.50	(3.5,3,5.5,7.5)	1.00 (12.5)	0.50 (3)	0.33 (2.5)	0.00 (2)
2		- - - -	0.50 (3.5)	0.00 (1)	0.50 (5.5)	0.50 (7.5)
3	0.75	(9,9.5,10,13)	0.67 (7)	0.75 (9.5)	1.00 (12.5)	0.33 (4)
4	0.67	(7,6.5,8.5,11.5)	1.00 (12.5)	1.00 (13)	--	0.00 (2)
5	0.67	(7,6.5,8.5,11.5)	0.50 (3.5)	1.00 (13)	1.00 (12.5)	0.50 (7.5)
6	0.50	(3.5,3,5.5,7.5)	1.00 (12.5)	1.00 (13)	0.00 (1)	0.00 (2)
7	1.00	(12.5,13,12.5,14.5)	1.00 (12.5)	0.67 (6.5)	0.33 (2.5)	0.50 (7.5)
8	1.00	(12.5,13,12.5,12.5)	0.00 (1)	0.67 (6.5)	0.50 (5.5)	0.50 (7.5)

[1]Control ranks are given in the order of the concentration with which they were ranked.

TABLE F.4. RANK SUMS

Effluent Concentration (%)	Rank Sum	No. of Replicates	Critical Rank Sum
3.12	65	8	44
6.25	65.5	8	44
12.50	42	7	34
25.00	40	8	44

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
1	3	6	10	16	23	30	39	49	59
	4	6	11	17	24	32	41	51	62
	5	7	12	19	26	34	44	54	66
	6	8	13	20	28	36	46	57	69
	7	8	14	21	29	39	49	60	72
	8	9	15	23	31	41	51	63	72
	9	10	16	24	33	43	54	66	79
	10	10	17	26	35	45	56	69	82
2	3	--	--	15	22	29	38	47	58
	4	--	10	16	23	31	40	49	60
	5	6	11	17	24	33	42	52	63
	6	7	12	18	26	34	44	55	66
	7	7	13	20	27	36	46	57	69
	8	8	14	21	29	38	49	60	72
	9	8	14	22	31	40	51	62	75
	10	9	15	23	32	42	53	65	78
3	3	--	--	--	21	29	37	46	57
	4	--	10	16	22	30	39	48	59
	5	--	11	17	24	32	41	51	62
	6	6	11	18	25	33	43	53	65
	7	7	12	19	26	35	45	56	68
	8	7	13	20	28	37	47	58	70
	9	7	13	21	29	39	49	61	73
	10	8	14	22	31	41	51	63	76

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
4	3	--	--	--	21	28	37	46	56
	4	--	--	15	22	30	38	48	59
	5	--	10	16	23	31	40	50	61
	6	6	11	17	24	33	42	52	64
	7	6	12	18	26	34	44	55	67
	8	7	12	19	27	36	46	57	69
	9	7	13	20	28	38	48	60	72
	10	7	14	21	30	40	50	62	75
5	3	--	--	--	--	28	36	46	56
	4	--	--	15	22	29	38	48	58
	5	--	10	16	23	31	40	50	61
	6	--	11	17	24	32	42	52	63
	7	6	11	18	25	34	43	54	66
	8	6	12	19	27	35	45	56	68
	9	7	13	20	28	37	47	59	71
	10	7	13	21	29	39	49	61	74
6	3	--	--	--	--	28	36	45	56
	4	--	--	15	21	29	38	47	58
	5	--	10	16	22	30	39	49	60
	6	--	11	16	24	32	41	51	63
	7	6	11	17	25	33	43	54	65
	8	6	12	18	26	35	45	56	68
	9	6	12	19	27	37	47	58	70
	10	7	13	20	29	38	49	60	73
7	3	--	--	--	--	--	36	45	56
	4	--	--	--	21	29	37	47	58
	5	--	--	15	22	30	39	49	60
	6	--	10	16	23	32	41	51	62
	7	--	11	17	25	33	43	53	65
	8	6	11	18	26	35	44	55	67
	9	6	12	19	27	36	46	58	70
	10	7	13	20	28	38	48	60	72

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicate Per Effluent Concentration							
		3	4	5	6	7	8	9	10
8	3	--	--	--	--	--	36	45	55
	4	--	--	--	21	29	37	47	57
	5	--	--	15	22	30	39	49	59
	6	--	10	16	23	31	40	51	62
	7	--	11	17	24	33	42	53	64
	8	6	11	18	25	34	44	55	67
	9	6	12	19	27	36	46	57	69
	10	6	12	19	28	37	48	59	72
9	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	30	39	48	59
	6	--	10	16	23	31	40	50	62
	7	--	10	17	24	33	42	52	64
	8	--	11	18	25	34	44	55	66
	9	6	11	18	26	35	46	57	69
	10	6	12	19	28	37	47	59	71
10	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	29	38	48	59
	6	--	10	16	23	31	40	50	61
	7	--	10	16	24	32	42	52	64
	8	--	11	17	25	34	43	54	66
	9	6	11	18	26	35	45	56	68
	10	6	12	19	27	37	47	58	71

APPENDIX G

SINGLE CONCENTRATION TOXICITY TEST - COMPARISON OF CONTROL WITH 100% EFFLUENT OR RECEIVING WATER

1. To statistically compare a control with one concentration, such as 100% effluent or the instream waste concentration, a t test is the recommended analysis. The t test is based on the assumptions that the observations are independent and normally distributed and that the variances of the observations are equal between the two groups.

2. Shapiro-Wilk's test may be used to test the normality assumption (See Appendix B for details). If the data do not meet the normality assumption, the nonparametric test, Wilcoxon's Rank Sum Test, may be used to analyze the data. An example of this test is given in Appendix F. Since a control and one concentration are being compared, the K = 1 section of Table F.5 contains the needed critical values.

3. The F test for equality of variances is used to test the homogeneity of variance assumption. When conducting the F test, the alternative hypothesis of interest is that the variances are not equal.

4. To make the two-tailed F test at the 0.01 level of significance, put the larger of the two variances in the numerator of F.

$$F = \frac{S_1^2}{S_2^2} \text{ where } S_1^2 > S_2^2$$

5. Compare F with the 0.005 level of a tabled F value with n_1 - 1 and n_2 - 1 degrees of freedom, where n_1 and n_2 are the number of replicates for each of the two groups.

6. A set of mysid growth data from an effluent (single concentration) test will be used to illustrate the F test. The raw data, mean and variance for the control and 100% effluent are given in Table G.1.

7. Since the variability of the 100% effluent is greater than the variability of the control, S^2 for the 100% effluent concentration is placed in the numerator of the F statistic and S^2 for the control is placed in the denominator.

$$F = \frac{0.00131}{0.000861} = 1.52$$

8. There are 8 replicates for the effluent concentration and 8 replicates for the control. Thus, both numerator and denominator degrees of freedom are equal to 7. For a two-tailed test at the 0.01 level of significance, the critical F value is obtained from a table of the F distribution (Snedecor and Cochran, 1980). The critical F value for this test is 8.89. Since 1.52 is not greater than 8.89, the conclusion is that the variances of the control and 100% effluent are homogeneous.

TABLE G.1. MYSID, *MYSIDOPSIS BAHIA,* GROWTH DATA FROM AN EFFLUENT (SINGLE CONCENTRATION) TEST

	Replicate									
	1	2	3	4	5	6	7	8	$\bar{X}$	S^2
Control	0.183	0.148	0.216	0.199	0.176	0.243	0.213	0.180	0.195	0.000861
100% Effluent	0.153	0.117	0.085	0.153	0.086	0.193	0.137	0.129	0.132	0.00131

9. Equal Variance T Test.

9.1 To perform the t test, calculate the following test statistic:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{S_p\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where: $\bar{Y}_1$ = mean for the control

$\bar{Y}_2$ = mean for the effluent concentration

$$S_p = \frac{\sqrt{(n_1 - 1)\ S_1^2 + (n_2 - 1)\ S_2^2}}{n_1 + n_2 - 2}$$

S_1^2 = estimate of the variance for the control

S_2^2 = estimate of the variance for the effluent concentration

n_1 = number of replicates for the control

n_2 = number of replicates for the effluent concentration

9.2 Since we are usually concerned with a decreased response from the control, such as a decrease in survival or a decrease in reproduction, a one-tailed test is appropriate. Thus, you would compare the calculated t with a critical t, where the critical t is at the 5% level of significance with $n_1 + n_2 - 2$ degrees of freedom. If the calculated t exceeds the critical t, the mean responses are declared different.

9.3 Using the data from Table G.1 to illustrate the t test, the calculation of t is as follows:

$$t = \frac{0.195 0.132}{0.0329\sqrt{\frac{1}{8} + \frac{1}{8}}} = 3.83$$

Where:

$$S_p = \frac{\sqrt{(8-1)0.000861 + (8-1)0.00131}}{8+8-2} = 0.0329$$

9.4 For an 0.05 level of significance test with 14 degrees of freedom, the critical t is 1.762 (Note: Table D.5 for K = 1 includes the critical t values for comparing two groups). Since 3.83 is greater than 1.762, the conclusion is that the growth for the 100% effluent concentration is significantly lower than growth for the control.

10. UNEQUAL VARIANCE T TEST.

10.1 If the F test for equality of variance fails, the t test is still a valid test. However, the denominator of the t statistic is adjusted as follows:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where: $\bar{Y}_1$ = mean for the control

$\bar{Y}_2$ = mean for the effluent concentration

S_1^2 = estimate of the variance for the control

S_2^2 = estimate of the variance for the effluent concentration

n_1 = number of replicates for the control

n_2 = number of replicates for the effluent concentration

10.2 Additionally, the degrees of freedom for the test are adjusted using the following formula:

$$df' = \frac{(n_1 - 1)\ (n_2 - 1)}{(n_2 - 1)\ C^2 + (1 - C)^2 (n_1 - 1)}$$

Where:

$$C = \frac{\frac{S_1^2}{n_1}}{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

10.3 The modified degrees of freedom is usually not an integer. Common practice is to round down to the nearest integer.

10.4 The t test is then conducted as the equal variance t test. The calculated t is compared to the critical t at the 0.05 significance level with the modified degrees of freedom. If the calculated t exceeds the critical t, the mean responses are found to be statistically different.

APPENDIX H

PROBIT ANALYSIS

1. This program calculates the EC1 and EC50 (or LC1 and LC50), and the associated 95% confidence intervals.

2. The program is written in IBM PC Basic for the IBM compatible PC by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled, executable version of the program and supporting documentation can be obtained from EMSL-Cincinnati by sending a written request to EMSL at 3411 Church Street, Cincinnati, OH 45244.

2.1 Data input is illustrated by a set of mortality data (Figure H.1) from a sheepshead minnow embryo-larval survival and teratogenicity test. The program begins with a request for the following information:

1. Desired output of abbreviated (A) or full (F) output? (Note: only abbreviated output is shown below.)
2. Output designation (P = printer, D = disk file).
3. Title for the output.
4. The number of exposure concentrations.
5. Toxicant concentration data.

2.2 The program output for the abbreviated output includes the following:

1. A table of the observed proportion responding and the proportion responding adjusted for the controls (see Figure H.2)
2. The calculated chi-square statistic for heterogeneity and the tabular value. This test is one indicator of how well the data fit the model. The program will issue a warning when the test indicates that the data do not fit the model.
3. The estimated LC1 and LC50 values and associated 95% confidence intervals (see Figure H.2).

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

Do you wish abbreviated (A) or full (F) input/output? A
Output to printer (P) or disk file (D)? P
Title ? Example of Probit Analysis

Number responding in the control group = ? 17
Number of animals exposed in the concurrent control group = ? 100
Number of exposure concentrations, exclusive of controls ? 5

Input data starting with the lowest exposure concentration

Concentration = ? 6.25
Number responding = ? 14
Number exposed = ? 100

Concentration = ? 12.5
Number responding = ? 16
Number exposed = ? 102

Concentration = ? 25.0
Number responding = ? 35
Number exposed = ? 100

Concentration = ? 50.0
Number responding = ? 72
Number exposed = ? 99

Concentration = ? 100
Number responding = ? 99
Number exposed = ? 99

Number	Number Conc.	Number Resp.	Exposed
1	6.2500	14	100
2	12.5000	16	102
3	25.0000	35	100
4	50.0000	72	99
5	100.0000	99	99

Do you wish to modify your data ? N

The number of control animals which responded = 17
The number of control animals exposed = 100
Do you wish to modify these values ? N

Figure H.1. Sample Data Input for USEPA Probit Analysis Program, Version 1.5.

Example of Probit Analysis

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	100	17	0.1700	0.0000
6.2500	100	14	0.1400	0.0201
12.5000	102	16	0.1569	0.0001
25.0000	100	35	0.3500	0.2290
50.0000	99	72	0.7273	0.6765
100.0000	99	99	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 3.472
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 7.815

Example of Probit Analysis

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
LC/EC 1.00	12.917	8.388	16.888
LC/EC 50.00	37.667	32.898	42.081

Figure H.2. USEPA Probit Analysis Program used for Calculating LC/EC Values, Version 1.5.

APPENDIX I

SPEARMAN-KARBER METHOD

1. The Spearman-Karber Method is a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978). The Spearman-Karber Method estimates the mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Spearman-Karber Method is recommended when partial mortalities occur in the test solutions, but the data do not fit the Probit model.

4. To calculate the LC50 using the Spearman-Karber Method, the following must be true: 1) the smoothed adjusted proportion mortality for the lowest effluent concentration (not including the control) must be zero, and 2) the smoothed adjusted proportion mortality for the highest effluent concentration must be one.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed adjusted proportion mortalities must be between zero and one.

6. The Spearman-Karber Method is illustrated below using a set of mortality data from a Sheepshead Minnow Larval Survival and Growth test. These data are listed in Table I.1.

7. Let $p_0, p_1, ..., p_k$ denote the observed response proportion mortalities for the control and k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \le p_1 \le ... \le p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \le p_1 \le ... \le p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i-1}^{s} = p_i^{s} = (p_i + p_{i-1})/2$$

Where: p_i^2 = the smoothed observed proportion mortality for effluent concentration i.

7.1 For the data in this example, because the observed mortality proportions for the control and the 6.25% effluent concentration are greater than the observed response proportions for the 12.5% and 25.0% effluent concentrations, the responses for these four groups must be averaged:

$$p_0^{s} = p_1^{s} = p_2^{s} = \frac{0.05 + 0.05 + 0.00 + 0.00}{4} = \frac{0.10}{4} = 0.025$$

TABLE I.1. EXAMPLE OF SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A SHEEPSHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25	2	0.05
12.5	0	0.00
25.0	0	0.00
50.0	26	0.65
100.0	40	1.00

7.2 Since $p_4 = 0.65$ is larger than p_3^s, set $p_4^s = 0.65$. Similarly, $p_5 = 1.00$ is larger than p_5 so set $p_4 = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table I.2.

TABLE I.2. EXAMPLE OF SPEARMAN-KARBER METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A SHEEPSHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration %	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.05	0.025	0.000
6.25	0.05	0.025	0.000
12.5	0.00	0.025	0.000
25.0	0.00	0.025	0.000
50.0	0.65	0.650	0.641
100.0	1.00	1.000	1.000

8. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_o^2)/(1 - p_o^s)$$

Where : p_o^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

8.1 For the data in this example, the data for each effluent concentration must be adjusted for control mortality using Abbott's formula, as follows:

$$p_o^a = p_1^a = p_2^a = p_3^a = \frac{p_1^s - p_o^s}{1 - p_o^s} = \frac{0.025 - 0.025}{1 - 0.025} = \frac{0.0}{0.975} = 0.0$$

$$p_4^a = \frac{p_4^s - p_o^s}{1 - p_o^s} = \frac{0.650 - 0.025}{1 - 0.025} = \frac{0.0625}{0.975} = 0.641$$

$$p_5^a = \frac{p_5^s - p_o^s}{1 - p_o^s} = \frac{1.000 - 0.025}{1 - 0.025} = \frac{0.975}{0.975} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table I.2. A plot of the smoothed, adjusted data is shown in Figure I.1.

9. Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k} -1 \frac{(p_{i+1}^a)\ (X_i + X_{i+1})}{2}$$

Where: p_i^a = the smoothed adjusted proportion mortality at concentration i

X_i = the $\log_{10}$ of concentration i

k = the number of effluent concentrations tested, not including the control.

9.1 For this example, the $\log_{10}$ of the estimated LC50, m, is calculated as follows:

m = [(0.000 - 0.000) (0.7959 + 1.0969)]/2 +
[(0.000 - 0.000) (1.0969 + 1.3979)]/2 +
[(0.641 - 0.000) (1.3979 + 1.6990)]/2 +
[(1.000 - 0.641) (1.6990 + 2.0000)]/2

= 1.656527

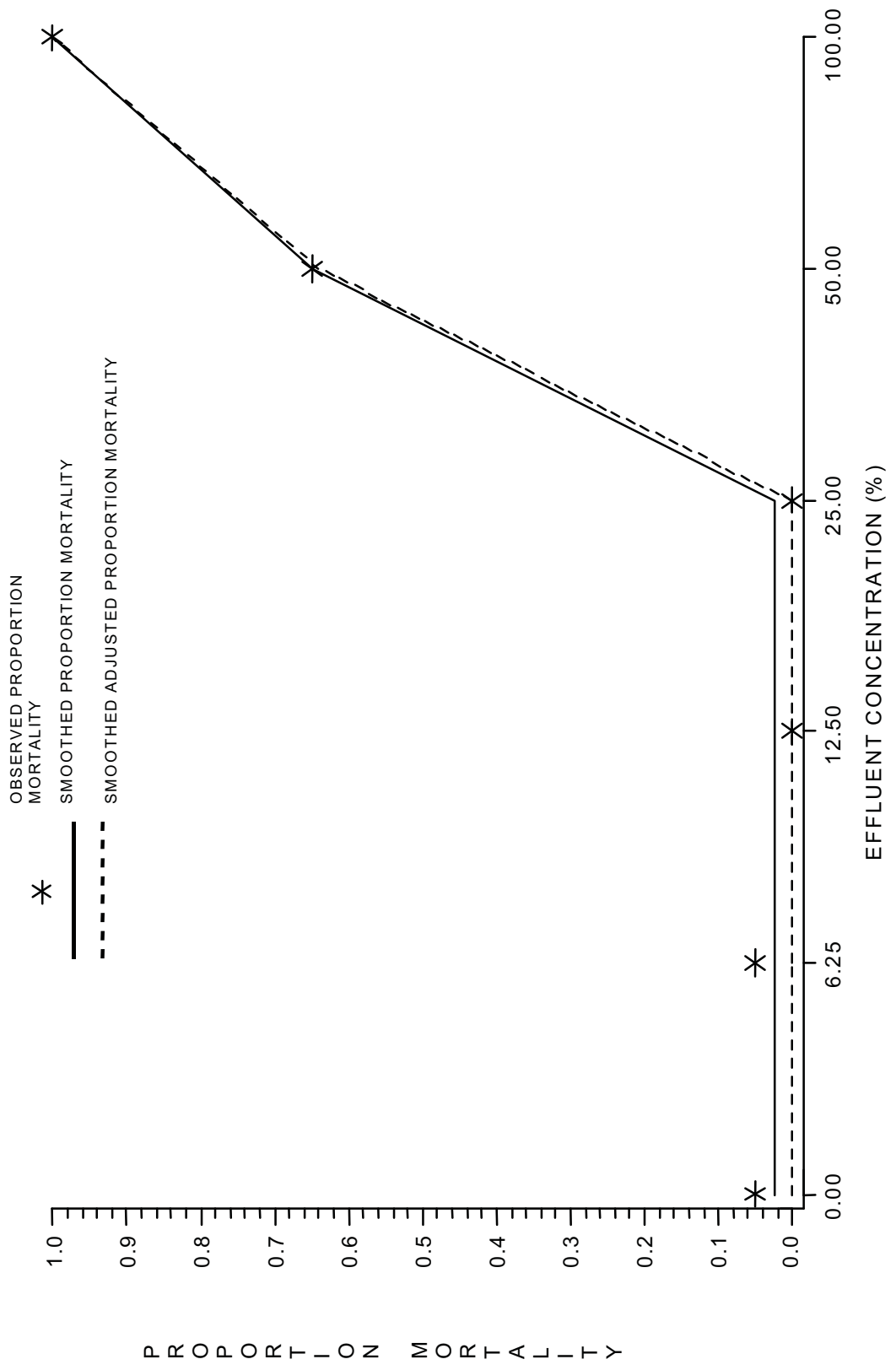

Figure I.1. Plot of observed, smoothed, and adjusted response proportions for sheepshead minnow, *Cyprinodon variegatus*, survival data.

10. Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k} -1 \frac{p_i^a(1-p_i^a)\ (X_{i+1}+X_{i-1})^2}{4(n_i-1)}$$

Where: X_i = the $\log_{10}$ of concentration i

n_i = the number of organisms tested at effluent concentration i

p_i^a = the smoothed adjusted observed proportion mortality at effluent concentration i

k = the number of effluent concentrations tested, not including the control.

10.1 For this example, the estimated variance of m, V(m), is calculated as follows:

V(m) = (0.000)(1.000)(1.3979 - 0.7959)^2/4(39) +
(0.000)(1.000)(1.6990 - 1.0969)^2/4(39) +
(0.641)(0.359)(2.0000 - 1.3979)^2/4(39)

= 0.00053477

11. Calculate the 95% confidence interval for m: $m \pm 2.0\sqrt{V(m)}$

11.1 For this example, the 95% confidence interval for m is calculated as follows:

$$1.656527 \pm 2\sqrt{0.00053477} = (1.610277,\ 1.702777)$$

12. The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking base_{10} antilogs of the above values.

12.1 For this example, the estimated LC50 is calculated as follows:

LC50 = antilog(m) = antilog(1.656527) = 45.3%.

12.2 The limits of the 95% confidence interval for the estimated LC50 are calculated by taking the antilogs of the upper and lower limits of the 95% confidence interval for m as follows:

lower limit: antilog(1.610277) = 40.8%

upper limit: antilog(1.702777) = 50.4%

APPENDIX J

TRIMMED SPEARMAN-KARBER METHOD

1. The Trimmed Spearman-Karber Method is a modification of the Spearman-Karber Method, a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Hamilton, et al, 1977). The Trimmed Spearman-Karber Method estimates the trimmed mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the trimmed mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Trimmed Spearman-Karber Method is recommended only when the requirements for the Probit Analysis and the Spearman-Karber Method are not met.

4. To calculate the LC50 using the Trimmed Spearman-Karber Method, the smoothed, adjusted, observed proportion mortalities must bracket 0.5.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed, adjusted, observed proportion mortalities must be between zero and one.

6. Let $p_0, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \leq p_1 \leq ... \leq p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \leq p_1 \leq ... \leq p_k$, with their average. For example, if p_i is less than p_{i-1} then:

Where: $pi^{s}-1^{s} = p_i^{s} = (p_i + p_{i-1})/2$

p_i^{s} = the smoothed observed proportion mortality for effluent concentration i.

7. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

Where: $p_i^{a} = (p_i^{s} - p_o^{s})/(1 - p_o^{s})$

p_o^{s} = the smoothed observed proportion mortality for the control

p_i^{s} = the smoothed observed proportion mortality for effluent concentration i.

8. Calculate the amount of trim to use in the estimation of the LC50 as follows:

Where: Trim = maximum $p_1^{a}, (1 - p_k^{a})$

p_1^{a} = the smoothed, adjusted proportion mortality for the lowest effluent concentration, exclusive of the control

p_k^{a} = the smoothed, adjusted proportion mortality for the highest effluent concentration

k = the number of effluent concentrations, exclusive of the control.

The minimum trim should be calculated for calculated for each data set rather than using a fixed amount of trim for each data set.

9. Due to the intensive nature of the calculation for the estimated LC50 and the calculation of the associated 95% confidence interval using the Trimmed Spearman-Karber Method, it is recommended that the data be analyzed by computer.

10. A computer program which estimates the LC50 and associated 95% confidence interval using the Trimmed Spearman-Karber Method, can be obtained through the EMSL, 3411 Church Street, Cincinnati, OH 45244. The program can be obtained from EMSL-Cincinnati by sending a written request to the above address.

11. The Trimmed Spearman-Karber program automatically performs the following functions:

a. Smoothing.
b. Adjustment for mortality in the control.
c. Calculation of the necessary trim.
d. Calculation of the LC50.
e. Calculation of the associated 95% confidence interval.

12. To illustrate the Trimmed Spearman-Karber method using the Trimmed Spearman-Karber computer program, a set of data from a Sheepshead Minnow Larval Survival and Growth test will be used. The data are listed in Table J.1.

12.1 The program requests the following input (Figure J.1):

a. Output destination (D = disk file or P = printer).
b. Control data.
c. Data for each toxicant concentration.

12.2 The program output includes the following (Figure J.2):

a. A table of the concentrations tested, number of organisms exposed, and the mortalities.
b. The amount of trim used in the calculation.
c. The estimated LC50 and the associated 95% confidence interval.

TABLE J.1. EXAMPLE OF TRIMMED SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A SHEEPSHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25	0	0.00
12.5	2	0.05
25.0	0	0.00
50.0	0	0.00
100.0	32	0.80

```
A:>TSK

TRIMMED SPEARMAN-KARBER METHOD.  VERSION 1.5
ENTER DATE OF TEST:
1
ENTER TEST NUMBER:
2
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
L
ENTER TEST SPECIES NAME:
Sheepshead minnow
ENTER TOXICANT  NAME:
effluent
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
%
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
40
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
2
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL;  MAXIMUM = 10):
5
ENTER THE  5 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
6.25  12.5  25  50  100
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
40
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
Days
ENTER DURATION OF TEST:
7
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:
0 2 0 0 32
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
y
```

Figure J.1. Example input for Trimmed Spearman-Karber Method.

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 1 TEST NUMBER: 2 DURATION: 7 Days TOXICANT: effluent
SPECIES: sheepshead minnow

RAW DATA:	Concentration	Number (%)	Mortalities Exposed
---	----		
	.00	40	2
	6.25	40	0
	12.50	40	2
	25.00	40	0
	50.00	40	0
	100.00	40	32

SPEARMAN-KARBER TRIM: 20.41%

SPEARMAN-KARBER ESTIMATES: LC50: 77.28
95% CONFIDENCE LIMITS ARE NOT RELIABLE.

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING. ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

Figure J.2. Example output for Trimmed Spearman-Karber Method.

APPENDIX K

GRAPHICAL METHOD

1. The Graphical Method is used to calculate the LC50. It is a mathematical procedure which estimates the LC50 by linearly interpolating between points of a plot of observed percent mortality versus the base 10 logarithm ($\log_{10}$) of percent effluent concentration. This method does not provide a confidence interval for the LC50 estimate and its use is only recommended when there are no partial mortalities. The only requirement for the Graphical Method is that the observed percent mortalities bracket 50%.

2. For an analysis using the Graphical Method the data must first be smoothed and adjusted for mortality in the control replicates. The procedure for smoothing and adjusting the data is detailed in the following steps.

3. The Graphical Method is illustrated below using a set of mortality data from an Inland Silverside Larval Survival and Growth test. These data are listed in Table K.1.

TABLE K.1. EXAMPLE OF GRAPHICAL METHOD: MORTALITY DATA FROM AN INLAND SILVERSIDE LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25	0	0.00
12.5	0	0.00
25.0	0	0.00
50.0	40	1.00
100.0	40	1.00

4. Let $p_0, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \le p_1 \le ... \le p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \le p_1 \le ... \le p_k$ with their average. For example, if p_i is less than p_{i-1} then:

Where: $p_{s-1}^{s} = p_i^{s} = (p_i + p_{i-1})/2$

p_i^{s} = the smoothed observed proportion mortality for effluent concentration i.

4.1 For the data in this example, because the observed mortality proportions for the 6.25%, 12.5%, and 25.0% effluent concentrations are less than the observed response proportion for the control, the values for these four groups must be averaged:

$$p_o^s = p_i^s = p_2^s = p_3^s = \frac{0.05+0.00+0.00+0.00}{4} = \frac{0.05}{4} = 0.0125$$

4.2 Since $p_4 = p_5 = 1.00$ are larger then 0.0125, set $p_4^s = p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table K.2.

5. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

Where: $p_1^a = (p_i^s - p_o^s)/(1 - p_o^s)$

p_o^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

5.1 Because the smoothed observed proportion mortality for the control group is greater than zero, the responses must be adjusted using Abbott's formula, as follows:

$$p_o^a = p_1^a = p_2^a = p_3^a = \frac{P_1^s - P_o^s}{1 - p_o^s} = \frac{0.0125 - 0.125}{1 - 0.0125} = \frac{0.0}{0.9875} = 0.0$$

$$p_4^a = p_5^a = \frac{P_4^s - p_o^s}{1 - p_o^s} = \frac{1.00 - 0.0125}{1 - 0.0125} = \frac{0.9875}{0.9875} = 1.00$$

A table of the smoothed, adjusted response proportions for the effluent concentrations are shown in Table K.2.

5.2 Plot the smoothed, adjusted data on 2-cycle semi-log graph paper with the logarithmic axis (the y axis) used for percent effluent concentration and the linear axis (the x axis) used for observed percent mortality. A plot of the smoothed, adjusted data is shown in Figure K.1.

TABLE K.2. EXAMPLE OF GRAPHICAL METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM AN INLAND SILVERSIDE LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration %	Mortality Proportion	Smoothed Mortality Proportion	Smoothed Adjusted Mortality Proportion
Control	0.05	0.0125	0.00
6.25	0.00	0.0125	0.00
12.5	0.00	0.0125	0.00
25.0	0.00	0.0125	0.00
50.0	1.00	1.0000	1.00
100.0	1.00	1.0000	1.00

6. Locate the two points on the graph which bracket 50% mortality and connect them with a straight line.

7. On the scale for percent effluent concentration, read the value for the point where the plotted line and the 50% mortality line intersect. This value is the estimated LC50 expressed as a percent effluent concentration.

7.1 For this example, the two points on the graph which bracket the 50% mortality line (0% mortality at 25% effluent, and 100% mortality at 50% effluent) are connected with a straight line. The point at which the plotted line intersects the 50% mortality line is the estimated LC50. The estimated LC50 = 35% effluent.

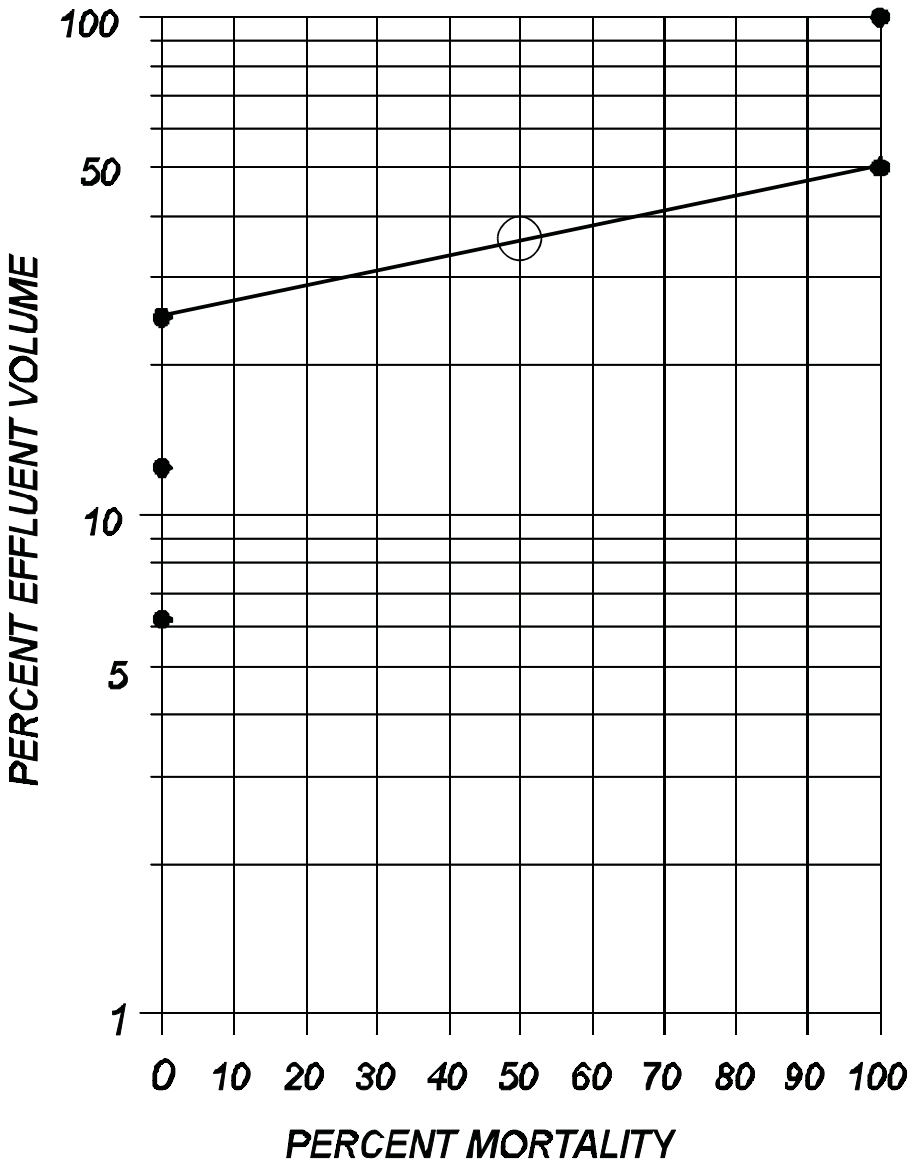

Figure K.1. Plot of the smoothed adjusted response proportions for inland silverside, *Menidia beryllina*, survival data.

APPENDIX L

LINEAR INTERPOLATION METHOD

1. GENERAL PROCEDURE

1.1 The Linear Interpolation Method is used to calculate a point estimate of the effluent or other toxicant concentration that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms (Inhibition Concentration, or IC). The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests, and the generation of an endpoint from a continuous model that allows a traditional quantitative assessment of the precision of the endpoint, such as confidence limits for the endpoint of a single test, and a mean and coefficient of variation for the endpoints of multiple tests.

1.2 The Linear Interpolation Method assumes that the responses (1) are monotonically non-increasing, where the mean response for each higher concentration is less than or equal to the mean response for the previous concentration, (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. If the data are not monotonically non-increasing, they are adjusted by smoothing (averaging). In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean. Also, no assumption is made about the distribution of the data except that the data within a group being resampled are independent and identically distributed.

2. DATA SUMMARY AND PLOTS

2.1 Calculate the mean responses for the control and each toxicant concentration, construct a summary table, and plot the data.

3. MONOTONICITY

3.1 If the assumption of monotonicity of test results is met, the observed response means ($\bar{Y}_i$) should stay the same or decrease as the toxicant concentration increases. If the means do not decrease monotonically, the responses are "smoothed" by averaging (pooling) adjacent means.

3.2 Observed means at each concentration are considered in order of increasing concentration, starting with the control mean ($\bar{Y}_1$). If the mean observed response at the lowest toxicant concentration ($\bar{Y}_2$) is equal to or smaller than the control mean ($\bar{Y}_1$), it is used as the response. If it is larger than the control mean, it is averaged with the control, and this average is used for both the control response (M_1) and the lowest toxicant concentration response (M_2). This mean is then compared to the mean observed response for the next higher toxicant concentration ($\bar{Y}_3$). Again, if the mean observed response for the next higher toxicant concentration is smaller than the mean of the control and the lowest toxicant concentration, it is used as the response. If it is higher than the mean of the first two, it is averaged with the first two, and the mean is used as the response for the control and two lowest concentrations of toxicant. This process is continued for data from the remaining toxicant concentrations. A numerical example of smoothing the data is provided below. (Note: Unusual patterns in the deviations from monotonicity may require an additional step of smoothing). Where $\bar{Y}_i$ decrease monotonically, the $\bar{Y}_i$ become M_i without smoothing.

4. LINEAR INTERPOLATION METHOD

4.1 The method assumes a linear response from one concentration to the next. Thus, the ICp is estimated by linear interpolation between two concentrations whose responses bracket the response of interest, the (p) percent reduction from the control.

4.2 To obtain the estimate, determine the concentrations C_J and C_{J+1} which bracket the response M_1 (1 - p/100), where M_1 is the smoothed control mean response and p is the percent reduction in response relative to the control response. These calculations can easily be done by hand or with a computer program as described below. The linear interpolation estimate is calculated as follows:

$$ICp = C_J + [M_1(1 - 1p/100) - M_J'] \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

Where: C_J = tested concentration whose observed mean response is greater than $M_1(1 - p/100)$.

C_{J+1} = tested concentration whose observed mean response is less than $M_1(1 - p/100)$.

M_1 = smoothed mean response for the control.

M_J = smoothed mean response for concentration J.

M_{J+1} = smoothed mean response for concentration J + 1.

p = percent reduction in response relative to the control response.

IC*p* = estimated concentration at which there is a percent reduction from the smoothed mean control response. The IC*p* is reported for the test, together with the 95% confidence interval calculated by the ICPIN.EXE program described below.

4.3 If the C_J is the highest concentration tested, the IC*p* would be specified as *greater than* C_J. If the response at the lowest concentration tested is used to extrapolate the ICp value, the ICp should be expressed as a *less than the lowest test concentration.*

5. CONFIDENCE INTERVALS

5.1 Due to the use of a linear interpolation technique to calculate an estimate of the ICp, standard statistical methods for calculating confidence intervals are not applicable for the ICp. This limitation is avoided by use a technique known as the bootstrap method as proposed by Efron (1982) for deriving point estimates and confidence intervals.

5.2 In the Linear Interpolation Method, the smoothed response means are used to obtain the ICp estimate reported for the test. The bootstrap method is used to obtain the 95% confidence interval for the true mean. In the bootstrap method, the test data Y_{ji} is randomly resampled with replacement to produce a new set of data Y_{ji}*, that is statistically equivalent to the original data, but a new and slightly different estimate of the IC*p* (IC*p**) is obtained. This process is repeated at least 80 times (Marcus and Holtzman, 1988) resulting in multiple "data" sets, each with an associate ICp* estimate. The distribution of the ICp* estimates derived from the sets of resampled data approximates the sampling distribution of the ICp estimate. The standard error of the ICp is estimated by the standard deviation of the individual ICp* estimates. Empirical confidence intervals are derived from the quantiles of the ICp* empirical distribution. For example, if the test data are resampled a minimum of 80 times, the empirical 2.5% and the 97.5% confidence limits are approximately the second smallest and second largest ICp* estimates (Marcus and Holtzman, 1988).

5.3 The width of the confidence intervals calculated by the bootstrap method is related to the variability of the data. When confidence intervals are wide, the reliability of the IC estimate is in question. However, narrow intervals do

not necessarily indicate that the estimate is highly reliable, because of undetected violations of assumptions and the fact that the confidence limits based on the empirical quantiles of a bootstrap distribution of 80 samples may be unstable.

5.4 The bootstrapping method of calculating confidence intervals is computationally intensive. For this reason, all of the calculations associated with determining the confidence intervals for the ICp estimate have been incorporated into a computer program. Computations are most easily done with a computer program such as the revision of the BOOTSTRP program (USEPA, 1988; USEPA, 1989) which is now called "ICPIN" which is described below in Subsection 7.

6. MANUAL CALCULATIONS

6.1 DATA SUMMARY AND PLOTS

6.1.1 The data used in this example are the mysid growth data used in the example in Section 14. The data is presented as the mean weight per original number of organisms. Table L.1 includes the raw data and the mean growth for each concentration. A plot of the data is provided in Figure L.1.

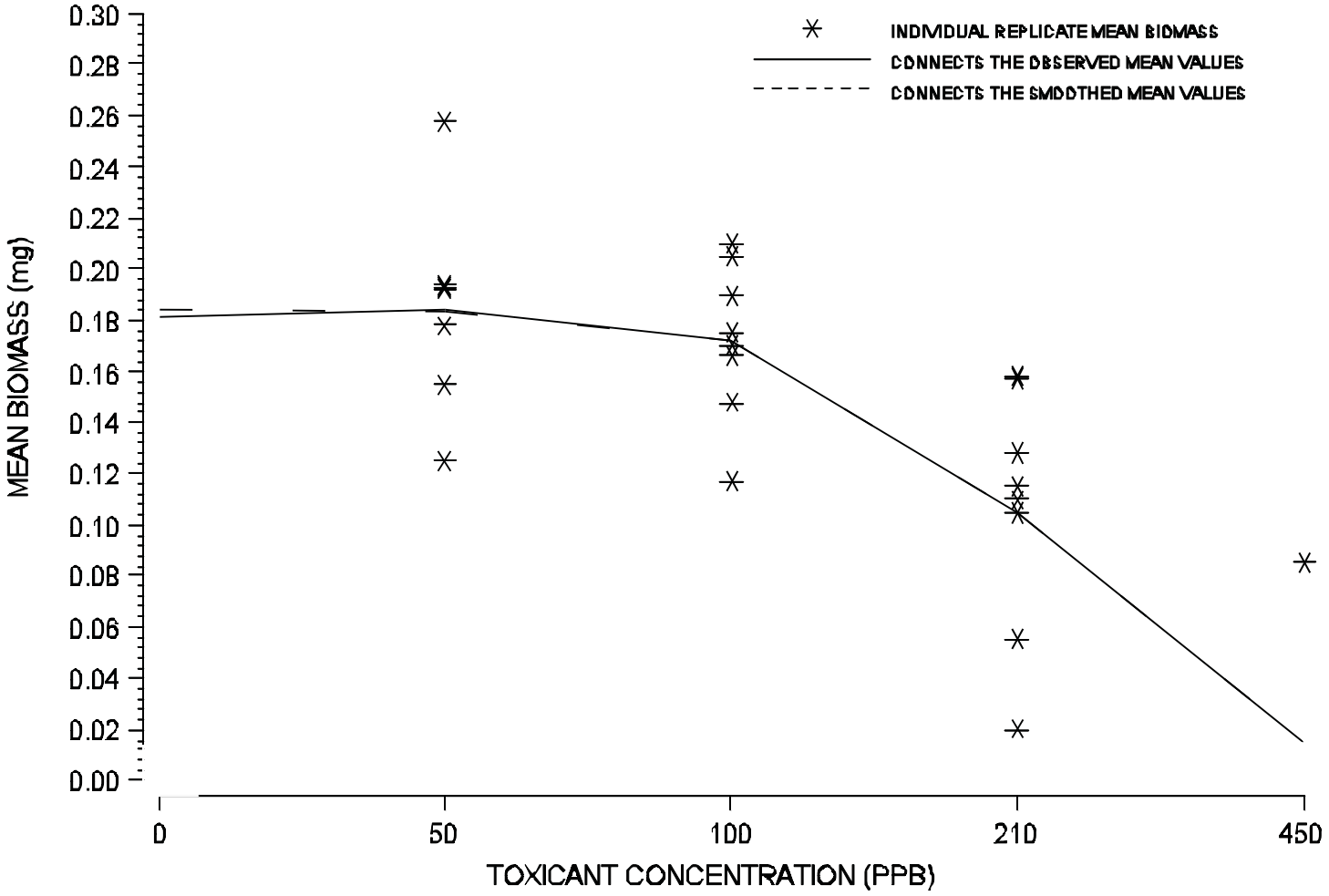

Figure L.1. Plot of raw data, observed means, and smoothed means for the mysid, *Mysidopsis bahia,* growth data.

TABLE L.1. MYSID, *MYSIDOPSIS BAHIA*, GROWTH DATA

Replicate	Control	Toxicant Concentration (ppb) 50	100	210	450
1	0.146	0.154	0.114	0.153	0
2	0.118	0.193	0.172	0.094	0.012
3	0.216	0.190	0.160	0.017	0
4	0.199	0.190	0.199	0.122	0.002
5	0.176	0.256	0.165	0.052	0
6	0.243	0.191	0.145	0.154	0
7	0.213	0.122	0.207	0.110	0
8	0.144	0.177	0.186	0.103	0.081
Mean ($\bar{Y}_i$)	0.182	0.184	0.168	0.101	0.012
i	1	2	3	4	5

6.2 MONOTONICITY

6.2.1 As can be seen from the plot in Figure L.1, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

6.2.2 Starting with the control mean $\bar{Y}_1 = 0.186$ and $\bar{Y}_2 = 0.184$, we see that $\bar{Y}_1 < \bar{Y}_2$. Calculate the smoothed means:

$$M_1 = M_2 = (\bar{Y}_1 + \bar{Y}_2)/2 = 0.193$$

6.2.3 Since $\bar{Y}_5 = 0.025 < \bar{Y}_4 = 0.101 < \bar{Y}_3 = 0.168 < M_2$, *set* $M_3 = 0.168$ and $M_4 = 0.101$, and $M_5 = 0.025$. Table L.2 contains the smoothed means and Figure L.1 gives a plot of the smoothed response curve.

6.3 LINEAR INTERPOLATION

6.3.1 Estimates of the IC25 and IC50 can be calculated using the Linear Interpolation Method. A 25% reduction in mean weight, compared to the controls, would result in a mean weight of 0.139, where $M_1(1\text{-}p/100) = 0.185(1\text{-}25/100)$. A 50% reduction in mean weight, compared to the controls, would result in a mean weight of 0.093 mg. Examining the smoothed means and their associated concentrations (Table L.2), the two effluent concentrations bracketing the mean weight per original of 0.139 mg are $C_3 = 100$ ppb and $C_4 = 210$ ppb. The two effluent concentrations bracketing a response of 0.093 mg per total original number of organisms are $C_4 = 210$ ppb and $C_5 = 450$ ppb.

TABLE L.2. MYSID, *MYSIDOPSIS BAHIA*, MEAN GROWTH RESPONSE AFTER SMOOTHING

Toxicant Conc. (ppb)	i	Smoothed Mean M_i (mg)
Control	1	0.183
50	2	0.183
100	3	0.168
210	4	0.101
450	5	0.025

6.3.2 Using the equation from section 4.2, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - 1p/100) - M_j'] \frac{(C_{j+1} - C_j)}{(M_{j+1} - M_j)}$$

$$IC25 = 100 + [0.93(1 - 25/100) - 0.164] \frac{(210 - 100)}{(0.101 - 0.164)}$$

$$= 151 \text{ ppb}$$

6.3.3 Using Equation 1 from 4.2, the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [M_1(1 - 1p/100) - M_j'] \frac{(C_{j+1} - C_j)}{(M_{j+1} - M_j)}$$

$$IC50 = 210 + [210 + [0.193(1 - 50/100) - 0.101] \frac{(450 - 210)}{(0.028 - 0.101)}$$

$$= 239 \text{ ppb}$$

6.4 CONFIDENCE INTERVALS

6.4.1 Confidence intervals for the ICp are derived using the bootstrap method. As described above, this method involves randomly resampling the individual observations and recalculating the ICp at least 80 times, and determining the mean ICp, standard deviation, and empirical 95% confidence intervals. For this reason, the confidence intervals are calculated using a computer program called ICPIN. This program is described below and is available to carry out all the calculations of both the interpolation estimate (ICp) and the confidence intervals.

7. COMPUTER CALCULATIONS

7.1 The computer program, ICPIN, prepared for the Linear Interpolation Methods was written in TURBO PASCAL for IBM compatible PCS. The program (version 2.0) has been modified by Computer Science Corporation, Duluth, MN with funding provided by the Environmental Research Laboratory, Duluth, MN (Norberg-King, 1993). The program was originally developed by Battelle Laboratories, Columbus, OH through a government contract supported by the Environmental Research Laboratory, Duluth, MN (USEPA, 1988). A compiled, executable version of the program and supporting documentation can be obtained by sending a written request to EMSL-Cincinnati, 3411 Church Street, Cincinnati, OH 45244.

7.2 The ICPIN.EXE program performs the following functions: 1) it calculates the observed response means ($_I$) (response means); 2) it calculates the standard deviations; 3) checks the responses for monotonicity; 4) calculates smoothed means (M_i) (pooled response means) if necessary; 5) uses the means, M_i, to calculate the initial ICp of choice by linear interpolation; 6) performs a user-specified number of bootstrap resamples between 80 and 1000 (as multiples of 40); 7) calculates the mean and standard deviation of the bootstrapped ICp estimates; and 8) provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven (Norberg-King, 1993).

7.3 For the IC*p* calculation, up to twelve treatments can be input (which includes the control). There can be up to 40 replicates per concentration, and the program does not require an equal number of replicates per concentration. The value of p can range from 1% to 99%.

7.4 DATA INPUT

7.4.1 Data is entered directly into the program onscreen. A sample data entry screen in shown in Figure L.2. The program documentation provides guidance on the entering and analysis of data for the Linear Interpolation Method.

7.4.2 The user selects the ICp estimate desired (e.g., IC25 or IC50) and the number of resamples to be taken for the bootstrap method of calculating the confidence intervals. The program has the capability of performing any number of resamples from 80 to 1000 as multiples of 40. However, Marcus and Holtzman (1988) recommend a minimum of 80 resamples for the bootstrap method be used and at least 250 resamples are better (Norberg-King, 1993).

ICp Data Entry/Edit Screen Current File:

Conc. ID	1	2	3	4	5	6
Conc. Tested						
Response 1						
Response 2						
Response 3						
Response 4						
Response 5						
Response 6						
Response 7						
Response 8						
Response 9						
Response 10						
Response 11						
Response 12						
Response 13						
Response 14						
Response 15						
Response 16						
Response 17						
Response 18						
Response 19						
Response 20						

F10 for Command Menu Use Arrow Keys to Switch Fields

Figure L.2. ICp data entry/edit screen. Twelve concentration identifications can be used. Data for concentrations are entered in columns 1 through 6. For concentrations 7 through 12 and responses 21-40 the data is entered in additional fields of the same screen.

7.5 DATA OUTPUT

7.5.1 The program output includes the following (Figures L.3 and L.4)

1. A table of the concentration identification, the concentration tested and raw data response for each replicate and concentration.
2. A table of test concentrations, number of replicates, concentration (units), response means (Y_i), standard deviations for each response mean, and the pooled response means (smoothed means; M_i).
3. The linear interpolation estimate of the ICp using the means (M_i). *Use this value for the ICp estimate.*
4. The mean ICp and standard deviation from the bootstrap resampling.
5. The confidence intervals calculated by the bootstrap method for the ICp. Provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven.

7.6 ICPIN program output for the analysis of the mysid growth data in Table L.1 is provided in Figures L.3 and L.4.

7.6.1 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 133.5054 (ppb). The empirical 95% confidence intervals for the true mean was 96.8623 to 186.6383 (ppb).

7.6.2 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 234.6761 (ppb). The empirical 95% confidence intervals for the true mean were 184.8692 to 283.3965 (ppb).

Conc. ID	1	2	3	4.	5
Conc. Tested	0	50	100	210	450
Response 1	.146	.154	.114	.153	0
Response 2	.118	.193	.172	.094	.012
Response 3	.216	.190	.160	.017	0
Response 4	.199	.190	.199	.122	.002
Response 5	.176	.256	.165	.052	0
Response 6	.243	.191	.145	.154	0
Response 7	.213	.122	.207	.110	0
Response 8	.144	.177	.186	.103	.081

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent:
Test Start Date: Test Ending Date:
Test Species: MYSID SHRIMP, Mysidopsis bahia
Test Duration: growth test
DATA FILE: mysidwt.icp
OUTPUT FILE: mysid.i25

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Standard. Dev.	Pooled Response Means
1	8	0.000	0.182	0.043	0.183
2	8	50.000	0.184	0.038	0.183
3	8	100.000	0.168	0.030	0.168
4	8	210.000	0.101	0.047	0.101
5	8	450.000	0.012	0.028	0.012

The Linear Interpolation Estimate: 133.5054 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 147.1702 Standard Deviation: 23.7984
Original Confidence Limits: Lower: 96.8623 Upper: 186.6383
Resampling time in Seconds: 0.16 Random Seed: -1623038650

Figure L.3. Example of ICPIN program output for the IC25.

Conc. ID	1	2	3	4.	5
Conc. Tested	0	50	100	210	450
Response 1	.146	.154	.114	.153	0
Response 2	.118	.193	.172	.094	.012
Response 3	.216	.190	.160	.017	0
Response 4	.199	.190	.199	.122	.002
Response 5	.176	.256	.165	.052	0
Response 6	.243	.191	.145	.154	0
Response 7	.213	.122	.207	.110	0
Response 8	.144	.177	.186	.103	.081

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent:
Test Start Date: Test Ending Date:
Test Species: MYSID SHRIMP, Mysidopsis bahia
Test Duration: growth test
DATA FILE: mysidwt.icp
OUTPUT FILE: mysidwt.i50

Conc. ID	Number Replicates	Concentration µg/L	Response Means	Standard. Dev.	Pooled Response Means
1	8	0.000	0.182	0.043	0.183
2	8	50.000	0.184	0.038	0.183
3	8	100.000	0.168	0.030	0.168
4	8	210.000	0.101	0.047	0.101
5	8	450.000	0.012	0.028	0.012

The Linear Interpolation Estimate: 234.6761 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 233.3311 Standard Deviation: 28.9594
Original Confidence Limits: Lower: 184.8692 Upper: 283.3965
Resampling time in Seconds: 0.11 Random Seed: 1103756486

Figure L.4. Example ICPIN program output for the IC50.

CITED REFERENCES

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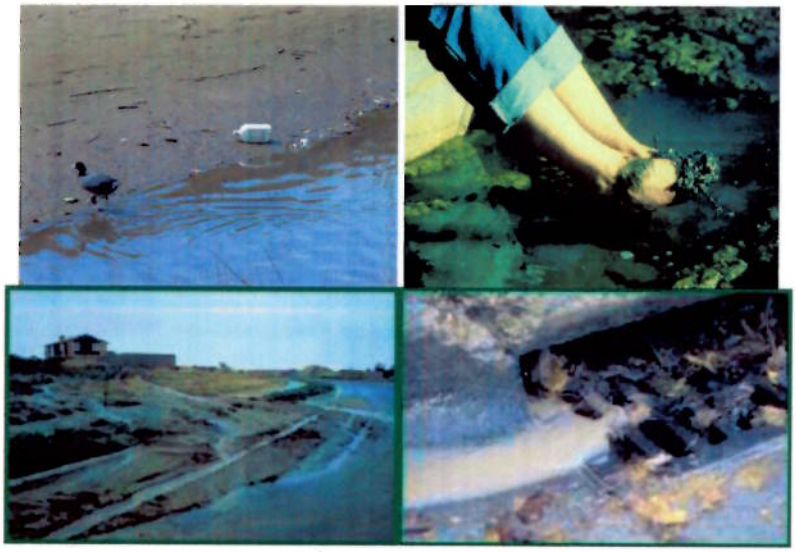
STATE OF CALIFORNIA

CALIFORNIA REGIONAL WATER QUALITY CONTROL BOARD
LOS ANGELES REGION

MONITORING PROGRAM - No. CI 7388
FOR
ORDER R4-2010-0108
NPDES PERMIT NO. CAS004002
WASTE DISCHARGE REQUIREMENTS

MUNICIPAL SEPARATE STORM SEWER SYSTEM DISCHARGES WITHIN THE VENTURA COUNTY WATERSHED PROTECTION DISTRICT, COUNTY OF VENTURA AND THE INCORPORATED CITIES THEREIN.

July 8, 2010



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MONITORING PROGRAM

1. The primary objectives of the Monitoring Program include, but are not limited to:
   (a) Assessing the chemical, physical, and biological impacts of municipal storm water sewer system discharges on receiving waters.
   (b) Assessing the overall health and evaluating long-term trends in receiving water quality.
   (c) Assessing compliance with TMDL targets and water quality objectives.
   (d) Characterization of the quality of storm water discharges.
   (e) Identifying sources of pollutants.
   (f) Measuring and improving the effectiveness of measures implemented under this Order.

2. The results of the monitoring requirements outlined below shall be used to refine BMPs for the reduction of pollutant loading and the protection and enhancement of the beneficial uses of the receiving waters in Ventura County.

3. The Permittees shall implement the Monitoring Program as follows:

CORE MONITORING

A. Mass Emissions

I. The Principal Permittee shall monitor mass emissions to accomplish the following objectives:
   i. Estimate the mass emissions from the MS4 to the watershed.
   ii. Assess trends in the mass emissions over time.
   iii. Determine if the MS4 is contributing to exceedances of water quality objectives by comparing results to applicable water quality objectives in the Water Quality Control Plan Los Angeles Region (Basin Plan) and the California Toxics Rule (CTR).

1. The Principal Permittee shall monitor mass emissions from the following 3 mass emission stations:
   (a) ME-VR2 for Ventura River
   (b) ME-SCR for Santa Clara River
   (c) ME-CC for Calleguas Creek

2. The Principal Permittee shall monitor the 3 mass emission stations on an annual basis as per A.3. below.

3. The Principal Permittee shall monitor each mass emission station each year as follows:
   (a) The first storm event of the wet season that produces a 20% or greater increase in base stream flow, and 2 additional storm events; all storm events shall be separated by 7 days of dry weather (less than 0.1 inch of rainfall) from the previously measurable storm event (0.25 inches of rain).
   (b) A total of 4 monitoring events (3 wet-weather storm events, 1 dry-weather) per mass emission station.

4. Samples for mass emission monitoring may be taken with the same type of automatic sampler used under Order 00-108. Sampling shall be in accordance with USEPA "NPDES Storm Water Sampling Guidance Document, EPA 833-8-92-001, July 1992" or other protocol approved by the Executive Officer.

5. Samplers shall be set to monitor storms that produce a 20% or greater increase in base stream flow.

6. Samples shall be flow-weighted composites, collected during the first 24 hours or for the duration of the storm if it is less than 24 hours.

7. Samples shall be collected from the discharge resulting from a storm event that is 0.25 inches or greater, samples may be analyzed if a predicted storm event produces between 0.15 and 0.24 inches of rain.

8. The flow-weighted composite sample for a storm water discharge shall be taken with a continuous sampler, or it shall be taken as a combination of a minimum of 3 sample aliquots, taken in each hour of discharge for the first 24 hours of the discharge or for the entire discharge if the storm event is less than 24 hours, with each aliquot being separated by a minimum of 15 minutes within each hour of discharge, unless the Regional Water Board Executive Officer approves an alternate protocol.

9. Flow may be estimated using U.S. EPA methods at sites where flow measurement devices are not in place.

10. Grab samples shall be taken only for pathogen indicators, oil & grease, cyanide, and volatile organics. Field measurements shall be taken for pH, temperature, and DO.

11. Each mass emission shall analyze for all of the Pollutants of Concern (POC) in its specific watershed listed in Attachment "B" (Calleguas Creek Watershed, Santa Clara River Watershed, and Ventura River Watershed Pollutants of Concern).

12. Each mass emission station shall screen for all constituents listed in Attachment "G" (Storm Water Monitoring Program's Constituents with Associated Minimum Levels), during the first storm event of the wet season for each year sampled. If a constituent is not detected at the Method Detection Limit (MDL) for its respective test method it need not be further analyzed unless the observed occurrence shows concentrations greater than the state water quality objective, and/ or the California Toxics Rule (CTR) for chronic criteria. If a constituent is detected exceeding a Basin Plan objective, and/ or CTR criteria then the constituent shall be analyzed for the remainder of the Order, at the mass emission station where it was detected.

13. At a minimum, a sufficient sample volume must be collected to perform all of the required biological and chemical tests.

14. When monitoring can not be performed to comply with the requirements of this Order due to circumstances beyond the Permittee's control, then within two working days the following shall be submitted to the Regional Water Board Executive Officer:
    (a) Statement of situation.
    (b) Explanation of circumstance(s) with documentation.
    (c) Statement of corrective action for the future.

15. Monitoring results submitted to the Regional Water Board shall include:
    (a) Rain totals and hydrographs for monitoring events in both narrative and graphic formats.
    (b) A narrative description of the date and duration of the storm event(s) sampled, rainfall estimates of the storm event which generated the sampled discharge and the duration between the storm event sampled and the end of the previous measurable storm event.
    (c) All applicable Standard Monitoring Provisions listed in part "K".

16. Results of monitoring from each mass emission station conducted in accordance with the Standard Operating Procedure submitted under Standard Provision 14 of this Attachment shall be sent electronically to the Regional Water Board's Storm Water site at MS4stormwaterRB4@waterboards.ca.gov, no later than 90 days from sample collection date, highlighting exceedances (Pollutants of Concern, POC) to the Basin Plan objectives for all test results, and the CTR for acute criteria with corresponding sampling dates per mass emission station. The sample data transmitted shall be in the most recent update of the Southern California Municipal Storm Water Monitoring Coalition's (SMC) Standardized Data Transfer Formats (SDTFs).

17. A summary of the annual mass emission monitoring results highlighting exceedances (POC) of the Basin Plan objectives and the CTR for acute criteria, with corresponding sampling dates per mass emission station, shall be included with the Annual Storm Water Report.

B. Major Outfalls

I. The Principal Permittee shall monitor major storm drain outfalls to accomplish the following objectives:
   i. Estimate the annual pollutant load of the cumulative discharges to waters of the State.
   ii. Estimate the event mean concentration of the cumulative discharges to waters of the State.
   iii. Assess trends in the major outfalls over time.
   iv. Estimate the annual pollutant load of discharges to Waters of the U.S.
   v. Estimate the event mean concentration of discharges to Waters of the U.S.
   vi. Assess trends in the major outfalls over time.
   vii. Determine if the MS4 is contributing to exceedences of water quality objectives in the Water Quality Control Plan Los Angeles Region (Basin Plan), and the California Toxics Rule (CTR).

1. The Principal Permittee shall monitor:
   (a) End-of-pipe of major outfalls, identified in Attachment I, transporting representative discharges from each Permittee's Municipal drainage area to:
      (1) Major outfalls listed in Attachment "I" (Storm Water Monitoring Program's Major Outfall Stations).
   (b) The first storm event of the wet season that produces at least 0.25 inches of rain, and 2 additional storm events per year, all storm events shall be separated by 7 days of dry weather (less than 0.1 inch) from the previously measurable storm event (0.25 inches).
   (c) A total of 4 monitoring events (3 wet-weather storm events, 1 dry-weather) shall be sampled per identified major outfall.
   (d) In the first year after permit adoption, 4 major outfall stations shall be monitored. Thereafter, all major outfall stations listed in Attachment "I" are to be monitored annually according to the schedule above.

2. If an identified monitoring site is found to be unworkable due to immitigable factors the sampling location may be relocated upon Executive Officer's approval of another location. Best professional judgment shall be used to balance the site selection rationale and criteria to determine the most appropriate site. Due to limited potential locations of urban outfalls to be monitored, there may be no sites that satisfy all criteria and rationale. Sites will be selected to satisfy the following criteria:

(a) Maximize urban runoff contribution;
(b) Greater than 60% of catchment shall be Permittee's MS4;
(c) Attempt shall be made to avoid outfalls that contain discharge from extra-jurisdictional areas (e.g. agriculture land and other NPDES discharges).
(d) Drainage area should contain representative land uses in a ratio of use as similar as reasonably possible to that found in the Permittee's jurisdiction.
(e) Drainage areas with a higher percentage of the Permittee's MS4 are preferred;
(f) Ability to accurately measure flow
(g) Safety of monitoring personnel is the highest priority. Specific location of sampling collection may be upstream of the actual outfall if field safety or accurate flow measurement require it.

3. Samples shall be collected from the discharge resulting from a storm event that is 0.25 inches or greater, samples may be analyzed if a predicted storm event produces between 0.15 inches and 0.24 inches of rain.

4. Samples shall be collected during the first 24 hours of storm water discharge or for the entire storm water discharge if it is less than 24 hours.

5. Samples shall be flow-weighted composites and can be collected automatically or manually (see subparts A.7 and A.8) in accordance with U.S. EPA protocol or other procedure approved by the Executive Officer.

6. Grab samples shall be taken only for pathogen indicators, oil & grease, cyanide, and volatile organics. Field measurements shall be taken for pH, temperature, and DO.

7. Major outfall samples taken within a subwatershed shall be analyzed for the biological and chemical parameters listed in the preceding subpart B.6.

8. Each major outfall station shall screen for all constituents listed in Attachment "G" (Storm Water Monitoring Program's Constituents with Associated Minimum Levels) twice per wet season, per year, (1st storm event of the wet season and one other storm event of the wet season). If a constituent is not detected at the Method Detection Limit (MDL) for its respective test method it need not be further analyzed unless the observed occurrence shows concentrations greater than the state water quality objective, and/ or the California Toxics Rule (CTR) acute criteria. If a constituent is detected exceeding a Basin Plan objective, and/or chronic CTR criteria then the constituent shall be sampled for the remainder of the Order, at the major outfall station where it was detected.

9. At a minimum, a sufficient sample volume must be collected to perform all of the required biological and chemical tests. Sampling shall be in accordance with USEPA "NPDES Storm Water Sampling Guidance Document, EPA 833-8-92-001, July 1992" or other protocol approved by the Executive Officer.

10. When monitoring can not be performed to comply with the requirements of this Order due to circumstances beyond the Permittee's control, then within 2 working days the following shall be submitted to the Regional Water Board Executive Officer:
    (a) Statement of situation
    (b) Explanation of circumstance(s) with documentation
    (c) Statement of corrective action for the future

11. Monitoring results submitted to the Regional Water Board shall include:
    (a) Rain totals and hydrographs for monitoring events in both narrative and graphic formats.
    (b) A narrative description of the date and duration of the storm event(s) sampled, rainfall estimates of the storm event which generated the sampled discharge and the duration between the storm event sampled and the end of the previous measurable storm event.
    (c) All applicable Standard Monitoring Provisions listed in part "K".

12. Results of monitoring from each major outfall station conducted in accordance with the Standard Operating Procedure submitted under Standard Provision 14 of this Attachment shall be sent electronically to the Regional Water Board's Storm Water Site at MS4stormwaterRB4@waterboards.ca.gov, no later than 90 days from sample collection date, highlighting exceedances to the Basin Plan objectives for all test results, and the CTR for acute criteria with corresponding sampling dates per major outfall station. The sample data transmitted shall be in the most recent update of the Southern California Municipal Storm Water Monitoring Coalition's (SMC) Standardized Data Transfer Formats (SDTFs).

13. A summary of the annual major outfall monitoring results, highlighting exceedences (pollutants of concern POC) to the Basin Plan objectives, and the CTR for acute criteria with corresponding sampling dates per major outfall station, shall be included with the Annual Storm Water Report.

C. Dry Weather Analytical Monitoring

I. The Principal Permittee shall develop and implement a monitoring program to characterize pollutant discharges from representative MS4 outfalls in each municipality and in the unincorporated County area during dry weather. This monitoring program shall be implemented within each jurisdiction and shall begin within the 2010-2011 monitoring year.

1. Dry weather analytical monitoring shall include:
   (a) Analytical monitoring, field measurements and observations at selected stations.
   (b) Reports of analytical data in a SWAMP comparable format.

2. Selection of Dry Weather Analytical Monitoring stations: Based upon a review program data, the storm drain system and land uses, the Co-Permittees shall select dry weather analytical monitoring stations within their jurisdiction. At least 5 dry weather analytical monitoring stations need to be identified per Co-Permittee. The dry weather analytical monitoring stations shall be established using the following guidelines and criteria:
   (a) Stations should be located downstream of municipal land uses where illegal or illicit activity may occur;
   (b) Stations shall be located at accessible downstream locations within the storm drain system of each municipality or at major outfalls;
   (c) Hydrological conditions, total drainage area of the site, traffic density, age of the structures or buildings in the area, history of the area, and land use types shall be considered in locating stations;
   (d) Each Co-Permittee shall determine a primary station and at least 4 alternate stations to be sampled in case primary stations do not have flow in dry weather. The dry weather monitoring may utilize the same outfalls as those used for wet weather monitoring, if such outfalls are found to discharge during dry weather.
   (e) Fact sheets of general information such as site descriptions (i.e., conveyance type, dominant watershed land uses) shall be created.

3. The Principal and Co-Permittees shall develop and/or update written procedures for dry weather analytical monitoring (these procedures must be consistent with 40 CFR part 136), including field observations, monitoring, and analyses to be conducted. At a minimum, the procedures must meet the following guidelines and criteria:
   (a) Dry weather analytical monitoring shall be conducted at each identified station at least once between May 1st and September 30th of each year.
   (b) If flow or ponded runoff is observed at a dry weather analytical monitoring station and there has been at least seventy-two (72) hours of dry weather, make observations and collect at least one (1) grab sample.
   (c) Record general information such as site descriptions (i.e., conveyance type, dominant watershed land uses), flow estimation (i.e., width of water surface, approximate depth of water, approximate flow velocity, flow rate), and visual observations (i.e., odor, color, clarity, floatables, deposits/stains, vegetation condition, structural condition, and biology).

4. At a minimum, collect samples for analytical laboratory analysis of the following constituents:
   (a) Total Hardness
   (b) Total Organic Carbon or Oil and Grease
   (c) Lead (Dissolved)
   (d) Zinc (Dissolved)
   (e) Copper (Dissolved)
   (f) Total Coliform bacteria
   (g) E. Coli bacteria

5. Other required field observations include:
   (a) Flow Estimation
   (b) Temperature
   (c) pH
   (d) Odor
   (e) Color
   (f) Turbidity
   (g) Floatables (foam, oil sheen)
   (h) Staining
   (i) Algal growth

6. If the station is dry (no flowing or ponded runoff), make and record all applicable observations and select another station from the list of alternate stations for monitoring.

7. Visually assess the presence of trash in receiving waters and urban runoff. Assessments of trash shall provide information on the spatial extent and amount of trash present, as well as the nature of the types of trash present.

8. Develop and/or update procedures for source identification follow up investigations in the event elevated levels are found. These procedures shall be consistent with procedures required in IC/ID section.

D. Aquatic Toxicity Monitoring

I. The objective of aquatic toxicity monitoring is to evaluate if storm water (wet weather) discharges are causing or contributing to chronic toxic impacts on aquatic life by the following:
   i. Toxicity testing at mass emission and major outfall stations to assess impacts on the marine and freshwater environments.

1. The Principal Permittee shall collect and analyze mass emission and major outfall samples for toxicity to evaluate the extent and causes of toxicity in receiving waters. Permittees shall utilize documents such as: Ventura County's

Technical Guidance Manual for Storm Water Quality Control Measures and U.S. EPA's National Management Measures to Control Nonpoint Source Pollution from Urban Areas to implement measures to eliminate or reduce sources of toxicity in storm water.

2. Toxicity samples may be flow-weighted composite samples or grab samples for both wet and dry event sampling (see subparts A.7 and A.8).

3. Volume of sample shall be determined by specific test methods to be used. At a minimum it is suggested to collect 5 gallons for baseline testing, and an additional 5 gallons for TIE studies. Sufficient sample volume shall be collected to perform the required toxicity tests.

4. All toxicity tests shall be conducted as soon as possible following sample collection. The 36-hour sample holding time for test initiation shall be targeted. However, no more than 72 hours shall elapse before initial use of a sample.

5. When toxicity tests can not be performed to comply with the requirements of this Order due to circumstances beyond the Permittee's control, then the following shall be submitted to the Regional Water Board Executive Officer within 2 working days:
   (a) Statement of situation
   (b) Explanation of circumstance(s) with documentation
   (c) Statement of corrective action for the future

6. The Principal Permittee shall conduct critical life stage chronic toxicity tests on undiluted samples in accordance with:
   (a) U.S. EPA's Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to *West Coast* Marine and Estuarine Organisms, (EPA/600/R-95/136, 1995) for all mass emission stations, and for major outfalls discharging to marine and estuarine environments, or
   (b) U.S. EPA's Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, October 2002 (EPA/821/R-02/013, 2002) or current version for major outfalls discharging to freshwater environments.

7. The Principal Permittee shall analyze samples for chronic toxicity according to the schedule below:
   (a) During the first year of the Order, 2 storm events shall be monitored at each mass emission and major outfall station. The first storm event of the wet season that produces at least 0.25 inches of rain, and 1 additional storm event. All storm events shall be separated by 7 days of dry weather (less than 0.1 inch of rain) from the previously measurable storm event.

(1) During the first year of the Order, all 3 test species shall be used for their respective chronic toxicity test method for the 2 storm events monitored, to determine the most sensitive test species for each monitoring station (see subparts D.8 and D.9 below).

(b) During the next 4 years of the Order, the first storm event of the wet season that produces at least 0.25 inches of rain shall be monitored for each mass emission and major outfall station. .

(1) During the next 4 years of the Order, the most sensitive test species determined from the first year of testing at each mass emission and major outfall station shall be used for its respective chronic toxicity test method (see subpart D.6).

8. Marine and Estuarine Species and Test Methods.

(a) Marine and estuarine species and short-term test methods for estimating the chronic toxicity of NPDES effluents shall be used and are found in the first edition of *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA/600/R-95/136, 1995) and applicable water quality standards; also see 40 CFR Parts 122.41(j)(4) and 122.44(d)(1)(iv).

(1) The Permittee shall conduct:

(A) A static renewal toxicity test with the topsmelt, *Atherinops affinis* (Larval Survival and Growth Test Method 1006.01)

(B) A static non-renewal toxicity test with the giant kelp *Macrocystis pyrifera* (Germination and Growth Test Method 1009.0); and

(C) A static non-renewal toxicity test with the purple sea urchin, *Strongylocentrotus purpuratus*, (Fertilization Test Method 1008.0)

(b) In no case shall the preceding toxicity test species be substituted with another organism unless written authorization from the Regional Water Board Executive Officer is received.

9. Freshwater Species and Test Methods.

(a) Species and short-term test methods for estimating the chronic toxicity of NPDES effluent shall be used and are found in the fourth edition of *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* (EPA/821/R-02/013, 2002; Table IA, 40 CFR Part 136).

(1) The Permittee shall conduct

(A) A static renewal toxicity test with the fathead minnow, *Pimephales promelas* (Larval Survival and Growth Test Method 1000.0[1])

[1] Daily observations for mortality make it possible to calculate acute toxicity for desired exposure periods (i.e., 7-day LC50, 96-hour LC50, etc.).

(B) A static renewal toxicity test with the daphnid, *Ceriodaphnia dubia* (Survival and Reproduction Test Method 1002.0[1]); and

(C) A static renewal toxicity test with the green alga, *Selenastrum capricornutum* (also named *Raphidocelis subcapitata*) (Growth Test Method 1003.0)

(b) In no case shall the preceding toxicity test species be substituted with another organism unless written authorization from the Regional Water Board Executive Officer is received.

10. The test endpoint data is analyzed using a standard t-test approach. Statistical analysis methods shall be consistent with U.S. EPA test method manuals.

11. If significant toxicity is found then according to paragraph 10.2.6.2 of the U.S. EPA freshwater test methods manual, all chronic toxicity test results from the multi-concentration tests required by this Order must be reviewed and reported according to U.S. EPA guidance on the evaluation of concentration-response relationships found in *Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing (40 CFR 136)* (EPA/821/B-00-004, 2000).

12. Toxic samples shall be immediately subjected to Toxicity Identification Evaluation (TIE) procedures to identify the toxic chemical(s) if toxicity is demonstrated by the standard t-test.

13. A TIE is to be performed to identify the causes of toxicity using the same species and test method and, as guidance, U.S. EPA test method manuals: *Toxicity Identification Evaluation: Characterization of Chronically Toxic Effluents, Phase I* (EPA/600/6-91/005F, 1992); *Methods for Aquatic Toxicity Identification Evaluations, Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA/600/R-92/080, 1993); *Methods for Aquatic Toxicity Identification Evaluations, Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA/600/R-92/081, 1993); and *Marine Toxicity Identification Evaluation (TIE): Phase I Guidance Document* (EPA/600/R-96-054, 1996).

14. The Principal Permittee shall complete chronic Phase I (Toxicity Characterization Procedures) TIEs for all sites showing significant toxicity. For the purpose of triggering TIE (Toxicity Characterization Procedures), significant toxicity is defined as at least 50% mortality. The 50% mortality threshold is consistent with the approach recommended in guidance published by USEPA for conducting TIEs (USEPA, 1996), which recommends a minimum threshold of 50% mortality because the probability of completing a successful TIE decreases rapidly for samples with less than this level of toxicity.

(a) The TIE shall be conducted on test species, demonstrating the most sensitive toxicity response at a sampling station. However, a TIE(s) may be conducted on an additional test species with the caveat that once the toxicant(s) has been identified then the most sensitive test species triggering the TIE event needs to be tested additionally to verify that the toxicant has been identified and addressed.

15. A TIE Prioritization Metric may be utilized to rank sites for TIEs.[2]

16. Toxicity Reduction Evaluation (TRE) when toxicity is identified
    (a) When the same pollutant or class of pollutants is identified through 2 consecutive TIE evaluations, a TRE shall be performed for that identified toxic pollutant.
    (b) The TRE development shall be performed by a neutral third party (retained by the Permittees), in consultation with the Regional Water Board staff.
    (c) The TRE shall include all reasonable steps to identify the source(s) of toxicity and discuss appropriate BMPs to eliminate the causes of toxicity. No later than 30 days after the source of toxicity and appropriate BMPs are identified, the Permittees shall submit the TRE Corrective Action Plan to the Regional Water Board Executive Officer for approval. At a minimum, the Plan shall include a discussion of the following items:
        (1) The potential sources of pollutant(s) causing toxicity.
        (2) A list of municipalities and agencies that may have jurisdiction over sources of pollutant(s) causing toxicity.
        (3) Recommended BMPs to reduce the pollutant(s) causing toxicity.
        (4) Proposed post construction control measures to reduce the pollutant(s) causing toxicity.
        (5) Follow-up monitoring to demonstrate that toxicity has been removed.
    (d) The TRE process shall be coordinated with TMDL development and implementation (i.e., If a TMDL for 4,4'-DDD is being implemented when a TRE for 4,4'-DDD is required, the efforts shall be coordinated to avoid overlap).

17. Results of Toxicity monitoring conducted in accordance with the Standard Operating Procedure under Standard Provision 14 of this Attachment shall be sent to the Regional Board's Storm Water Site at MS4stormwaterRB4@waterboards.ca.gov, no later than 90 days from sample collection date for the initial toxicity test and no more than 30 days from completion of each aspect of the analysis for TIEs/TREs. The sample data transmitted shall be in the most recent update of the Southern California Municipal Storm Water Monitoring Coalition's (SMC) Standardized Data Transfer Formats (SDTFs).

[2] Appendix 5. SMC Model Monitoring Program.

18. The Annual Storm Water Report shall include:
    (a) A full laboratory report for all toxicity testing.
    (b) A summary of the years' mass emission and major outfall monitoring station's toxicity test results reported according to the test methods manual chapter on report preparation and test review.
    (c) The dates of sample collection and initiation of each toxicity test.
    (d) All results for effluent parameters monitored concurrently with the toxicity test(s).
    (e) TIE Phase testing (Phase I, Phase II, and Phase III) that has been or is in the process of being conducted per monitoring station.
    (f) The development, implementation, and results for each TRE Corrective Action Plan in the Annual Storm Water Report, beginning the year following the identification of each pollutant or pollutant class causing toxicity.

19. When the SMC Standardized Toxicity Testing Guidance is completed, the Regional Water Board Executive Officer may direct Permittees to replace the current toxicity program with the standardized guidance procedure.

SPECIAL STUDIES

E. Pyrethroid Insecticides Study

I. The Principal Permittee shall perform a Pyrethroid Insecticides study to accomplish the following objectives:
    i. Establish baseline data for major watersheds
    ii. Evaluate whether Pyrethroid Insecticide concentrations are at or approaching levels known to be toxic to sediment-dwelling aquatic organisms.
    iii. Determine if Pyrethroids discovered are from urban sources.
    iv. Assess any trends over the permit term.

1. The Permittees shall incorporate monitoring for Pyrethroid Insecticides within the Calleguas Creek, Santa Clara River and Ventura River Watersheds according to the following:
    (a) No later than the second year of this Order, monitoring shall begin.
    (b) Quality Assurance Project Plan (QAPP) to be submitted to the Regional Board for approval 12 months prior to beginning monitoring.
    (c) In selecting sites to conduct monitoring for Pyrethroid Insecticides, Permittees shall review existing monitoring programs in the watersheds by other public and private entities, watershed coalitions, and citizen volunteers, so as to complement and not duplicate efforts.
    (d) Establish at least 2 stations along the mainstems of each major watershed river that are influenced by urban discharges.

(e) The study shall be repeated every third year following the year monitoring begins.

2. The Principal Permittee shall monitor Pyrethroid Insecticides stations according to the following:
   (a) The Principal Permittee shall monitor 1 sampling event per station per monitoring year.
      (1) Monitoring shall occur after sediment has settled within the waterbody, and safe access can be assured.
   (b) Sufficient sediment is to be collected at each station in a pre-cleaned glass jar by skimming the upper 1 cm of the sediment column with a steel scoop, and held on ice until returned to the laboratory.
   (c) Sediment shall be homogenized in the laboratory by hand mixing, then held at 4 °C (toxicity samples) or -20 °C (chemistry samples).
   (d) All samples taken shall be analyzed for the following Pyrethroids:
      (1) biefenthrin
      (2) cyfluthrin
      (3) cypermethrin
      (4) deltamethrin
      (5) esfenvalerate
      (6) lambda-cyhalothrin
      (7) permethrin
      (8) tralomethrin (if laboratory is capable of analyzing for it)
   (e) Detection limits for all Pyrethroids shall be as close to 1ng/g (dry weight) as reasonably achievable.
   (f) Each sediment sample is to measure the following:
      (1) total organic carbon (TOC).

3. All samples shall be tested for toxicity to 7 to 10 day old ***Hyalella azteca*** according to standard U.S. EPA testing methods.[3]
   (a) Use of the approach described in *Aquatic Toxicity Due to Residential Use of Pyrethroid Insecticides*[4] for toxicity testing shall be used.

4. Analysis by a laboratory that has performed sediment toxicity testing for Pyrethroid Insecticides is preferred.

5. Monitoring results from each station shall be sent electronically to the Regional Board's Storm Water Site at MS4stormwaterRB4@waterboards.ca.gov, no later than 90 days from sample collection date. The sample data transmitted shall be

[3] *U.S. EPA. Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates*; EPA Publication 600/R-99/064; U.S. Environmental Protection Agency: Washington, DC, 2000; 192 pp.
[4] *Aquatic Toxicity Due to Residential Use of Pyrethroid Insecticides*; Weston, D.P.; Holmes, R.W.; You, J.; Lydy, M.J. Environ. Sci. Technol.; (Article); 2005; 39(24); 9780 pp.

in the most recent update of the Southern California Municipal Storm Water Monitoring Coalition's (SMC) Standardized Data Transfer Formats (SDTFs).

6. If toxicity is attributed to Pyrethroids then consultation with staff at U.S. EPA, the California Department of Pesticide Regulations and the California Stormwater Quality Association's (CASQA) pesticides committee (UP3 Project web site), shall be required to obtain relevant information to use in developing the recommendations to mitigate Pyrethroids in the Final Report.

7. Final Report for the Pyrethroid Insecticides study shall contain the following:
   (a) Executive summary
   (b) Methods
   (c) Results (including map depicting monitoring stations)
   (d) Discussion
   (e) Recommendations to mitigate Pyrethroids

8. The Final Report shall be completed and submitted to the Executive Officer of the Regional Water Board no later than 8 months after completion of the study.

9. The Pyrethroid Insecticides Study requirement may be satisfied by another tributary monitoring program within the Watershed performing a sediment Pyrethroid Insecticides Study that is monitoring to assess pyrethroid concentrations and sediment toxicity, so as to complement other ongoing programs.

F. Hydromodification Control Study

1. The Principal Permittee shall conduct or participate in special studies to develop tools to predict and mitigate the adverse impacts of Hydromodification, and to comply with hydromodification control criteria. This can be achieved by the following:
   (a) Develop a mapping and classification system for streams based on their susceptibility to the effects of hydromodification.
   (b) Establish protocols for ongoing monitoring to assess the effects of hydromodification.
   (c) Develop dynamic models to assess the effects of hydromodification on stream condition.
   (d) Develop a series of tools that managers can easily apply to make recommendations or set requirements relative to hydromodification for new development and redevelopment.

2. The Principal Permittee may satisfy this requirement by participating in the 'Development of Tools for Hydromodification Assessment and Management' Project undertaken by the SMC and coordinated by the SCCWRP.

3. The Principal Permittee shall continue to partner with the SMC and collect data or sponsor its collection for the Ventura County sites to reduce statistical uncertainty and/ or improve model predictability.

4. The Principal Permittee shall submit a letter to the Regional Water Board Executive Officer stating how they will satisfy this requirement, no later than 2 months after Order adoption date.

G. Low Impact Development

1. The Principal Permittee shall conduct or participate in a special study to assess the effectiveness of low impact development techniques in semi-arid climate regimes such as in Southern California.

2. The Principal Permittee may satisfy this requirement by participating in the SMC project titled "Quantifying the Effectiveness of Site Design/ Low Impact Development Best Management Practice in Southern California".

3. The Principal Permittee shall submit a letter to the Regional Water Board Executive Officer stating how they are satisfying this requirement, no later than 2 months after deciding to either conduct or participate in special study.

H. Southern California Bight Project

1. The Principal Permittee and Permittees shall participate with other government organizations regulating discharges in southern California in the collaboration to conduct a regional monitoring survey (Southern California Bight Project (SCBP)), which was started in 2008 and to be continued in successive years. The survey's primary objective is to assess the spatial extent and magnitude of ecological disturbances on the mainland continental shelf of the SCB and to describe relative conditions among different regions of the SCBP.

2. The Principal Permittee shall participate on the Steering Committee for the bight-wide monitoring project, and assist with the estuary and nearshore sampling effort requirement of the proposed monitoring project for Ventura County as defined in the SCBP plan.

I. Bioassessment

1. The Principal Permittee consents to participate in the following regional water quality program for watershed management and planning:
   (a) SMC Regional Monitoring Program
      (1) Southern California Regional Bioassessment
         (A) Level of effort per watershed per year
            (i) Probabilistic sites per watershed
               (I) Ventura River - Six
               (II) Santa Clara River - Three
               (III) Calleguas Creek - Six
            (ii) Integrator sites per watershed
               (A) Ventura River - One
               (B) Santa Clara River - One
               (C) Calleguas Creek – One
   (b) Ventura County Bioassessment: Permittees shall conduct bioassessment at one fixed site in each of the watersheds above on an annual basis. Southern California Regional Bioassessment protocols shall be used to conduct the Ventura County Bioassessment program.

J. Volunteer Monitoring Programs

1. The Permittees shall provide limited assistance if requested in the development and implementation of volunteer monitoring programs in the Ventura watersheds. These include, but are not limited to the following:
   (a) Ventura River - (Ventura Stream Team)
   (b) Santa Clara River - (Santa Clara River Stream Team)
   (c) Calleguas Creek - (Calleguas Creek Watershed Quality Monitoring Program)
   (d) Malibu Creek - (Malibu Creek Watershed Quality Monitoring Program)

K. Standard Monitoring Provisions

I. All monitoring activities shall meet the following requirements.

1. Monitoring and Records [40 CFR 122.41(j)(1)]
   (a) Samples and measurements taken for the purpose of monitoring shall be representative of the monitored activity.

2. Monitoring and Records [40 CFR 122.41(j)(2)] [CWC §13383(a)]
   (a) The Principal Permittee and Permittees shall retain records of all monitoring information, including all calibration and maintenance of monitoring instrumentation, copies of all reports required by this Order, and records of all data used to complete the Report of Waste Discharge (ROWD) and application for this Order, for a period of at least five (5) years from the date

of the sample, measurement, report, or application. This period may be extended by request of the Regional Water Board or U.S. EPA at any time and shall be extended during the course of any unresolved litigation regarding this discharge.

3. Monitoring and Records [40 CFR 122.21(j)(3)]
   (a) Records of monitoring information shall include:
      (1) The date, time of sampling or measurements; exact place, weather conditions, and rain fall amount.
      (2) The individual(s) who performed the sampling or measurements.
      (3) The date(s) analyses were performed.
      (4) The individual(s) who performed the analyses.
      (5) The analytical techniques or methods used.
      (6) The results of such analyses.
      (7) The data sheets showing toxicity test results.

4. Monitoring and Records [40 CFR 122.21(j)(4)]
   (a) All sampling, sample preservation, and analyses must be conducted according to test procedures under 40 CFR Part 136, unless other test procedures have been specified in this Order. If a particular Minimum Level (ML) is not attainable in accordance with procedures set forth in 40 CFR 136, the lowest quantifiable concentration of the lowest calibration standard analyzed by a specific analytical procedure may be used instead.

5. Monitoring and Records [40 CFR 122.21(j)(5)]
   (a)The CWA provides that any person who falsifies, tampers with, or knowingly renders inaccurate any monitoring device or method required to be maintained under this Order shall, upon conviction, be punished by a fine of not more than $10,000, or by imprisonment for not more than 2 years, or both. If a conviction of a person is for a violation committed after a first conviction of such person under this paragraph, punishment is a fine of not more than $20,000 per day of violation, or by imprisonment of not more than four years, or both.

6. All chemical, bacteriological, and toxicity analyses shall be conducted at a laboratory:
   (a) Certified for such analyses by an appropriate governmental regulatory agency.
   (b) Participated in 'Intercalibration Studies' for storm water pollutant analysis conducted by the SMC. [5]

[5] The 'Intercalibration Studies' are conducted periodically by the SMC to establish a consensus based approach for achieving minimal levels of comparability among different testing laboratories for storm water samples to minimize analytical procedure bias. Stormwater Monitoring Coalition Laboratory Document, Technical Report 420 (2004) and subsequent revisions and augmentations.

(c) Which performs laboratory analyses consistent with the storm water monitoring guidelines as specified in, the *Stormwater Monitoring Coalition Laboratory Guidance Document,* 2nd Edition R. Gossettt and K. Schiff (2007), and its revisions.

7. For priority toxic pollutants that are identified in the CTR (65 *Fed. Reg.* 31682), the MLs published in Appendix 4 of the *Policy for Implementation of Toxics Standards for Inland Surface Waters, Enclosed Bays and Estuaries of California* (SIP) shall be used for all analyses, unless otherwise specified. The MLs from the SIP are incorporated into Attachment "G".

8. The Monitoring Report shall specify the analytical method used, the Method Detection Level (MDL) and the ML for each pollutant. For the purpose of reporting compliance with numerical limitations, performance goals, and receiving water limitations, analytical data shall be reported with 1 of the following methods, as appropriate:
   (a) An actual numerical value for sample results greater than or equal to the ML.
   (b) "Not-detected (ND)" for sample results less than the laboratory's MDL with the MDL indicated for the analytical method used.
   (c) "Detected, but Not Quantified (DNQ)" if results are greater than or equal to the laboratory's MDL but less than the ML. The estimated chemical concentration of the sample shall also be reported. This is the concentration that results from the confirmed detection of the substance by the analytical method below the ML value.

9. For priority toxic pollutants, if the Permittee can demonstrate that a particular ML is not attainable, in accordance with procedures set forth in 40 CFR 136, the lowest quantifiable concentration of the lowest calibration standard analyzed by a specific analytical procedure (assuming that all the method specified sample weights, volumes, and processing steps have been followed) may be used instead of the ML listed in Appendix 4 of the SIP. The Principal Permittee must submit documentation from the laboratory to the Regional Water Board Executive Officer for approval prior to raising the ML for any constituent.

10. Monitoring Reports [40 CFR 122.41(I)(4)(ii)]
   (a) If the Principal Permittee monitors any pollutant more frequently than required by the Order using test procedures approved under 40 CFR part 136, unless otherwise specified in the Order, the results of this monitoring shall be included in the calculation and reporting of the data submitted in the Annual Monitoring Reports.

11. Monitoring Reports [40 CFR 122.41(I)(4)(iii)]
    (a) Calculations for all limitations, which require averaging of measurements, shall utilize an arithmetic mean unless otherwise specified in this Order.

12. If no flow occurred during the reporting period, then the Monitoring Report shall, so state.

13. The Regional Water Board Executive Officer or the Regional Board, consistent with 40 CFR 122.41, may approve changes to the Monitoring Program, after providing the opportunity for public comment, either:
    (a) By petition of the Principal Permittee or by petition of interested parties after submittal of the Monitoring Report. Such petition shall be filed not later than 60 days after the Monitoring Report submittal date, or
    (b) As deemed necessary by the Regional Water Board Executive Officer following notice to the Principal Permittee.

14. The Principal Permittee must provide a copy of the Standard Operation Procedures (SOPs) for the Monitoring Program No. CI 7388 to the Regional Water Board upon request. The SOP will consist of five elements: Title page, Table of Contents, Procedures, Quality Assurance/ Quality Control (QA/ QC), and References. Briefly describe the purpose of the work or process, including any regulatory information or standards that are appropriate to the SOP process, and the scope to indicate what is covered. Denote what sequential procedures should be followed, divided into significant sections; e.g., possible interferences, equipment needed personnel qualifications, and safety considerations. Describe QA/ QC activities, and list any cited or significant references.

L. Total Maximum Daily Load (TMDL) Monitoring

1. TMDL monitoring is to determine compliance with the TMDL Waste Load Allocations (WLAs) and numeric targets for the MS4 Permittees that have been adopted by the Regional Water Board and have been approved by the Office of Administrative Law and the U.S. EPA.

2. TMDL monitoring is in accordance with approved TMDLs as discussed in part 5 of the permit. TMDL monitoring for specific watersheds is in accordance with the agreed upon monitoring plans submitted by stakeholders, including MS4 Permittees.

M. Beach Water Quality Monitoring

If funding from state and federal sources is not available for beach water quality monitoring the Principal Permittee shall conduct weekly year-round beach water quality sampling and analysis at a maximum of ten sites in accordance with the procedures and locations used in AB 411 monitoring and listed below:

1. Rincon Beach – 25 yards south of the creek mouth*
2. Oil Piers Beach – south of the drain, bottom of the wood staircase
3. Faria County Park – south of the drain at the north end of the park*
4. Solimar Beach – south (end of east gate access road)*
5. Emma Wood State Beach – 50 yards south of first drain
6. Oxnard Beach – at J Street drain
7. Surfer's Point at Seaside – end of the access path via wooden gate
8. Promenade Park – Figueroa Street
9. Surfer's Knoll – beach adjacent to the parking lot*
10. San Buenaventura Beach – south of drain at San Jon Road

* Not associated with MS4 discharges.

Ordered by:

Samuel Unger, P.E.
Interim Executive Officer

Date: July 8, 2010

Model Monitoring Program for Municipal Separate Storm Sewer Systems in Southern California

A report from the Stormwater Monitoring Coalition's
Model Monitoring Technical Committee

August 2004

Technical Report #419

EXECUTIVE SUMMARY

This report describes a model monitoring program for receiving waters affected by urban runoff in both wet and dry weather. It provides a common design framework for municipal urban runoff programs and Regional Board staff to use in developing and/or revising program requirements for monitoring receiving waters for impacts, status and trends, toxicity, mass emissions, and source identification. This effort was funded in part by the State Water Resources Control Board (SWRCB), prompted by Senate Bill 72 (Kuehl), which addressed the standardization of sampling and analysis protocols in municipal stormwater monitoring programs. The development of the model monitoring program itself was organized through the Southern California Stormwater Monitoring Coalition (SMC), which impaneled a technical committee including representatives from:

- Regional Water Quality Control Boards (Los Angeles, Santa Ana, San Diego)
- Municipal permittees (Counties of Ventura, Los Angeles, San Bernardino, Riverside, Orange, and San Diego)
- Heal the Bay
- Southern California Coastal Water Research Project (SCCWRP).

As a result of the SMC's role and the makeup of the technical committee, the model stormwater monitoring program reflects issues and contexts of paramount importance in southern California and addresses some, but not all, of the requirements of SB72. Additional technical guidance related to performance standards for laboratory analysis and data reporting formats is detailed in companion documents.

The model program is structured around five fundamental management questions, with the goal of achieving a basic degree of comparability across southern California monitoring programs, while maintaining individual programs' ability to adapt to site-specific and local concerns.

The five core management questions are:

- Question 1: Are conditions in receiving waters protective, or likely to be protective, of beneficial uses?
- Question 2: What is the extent and magnitude of the current or potential receiving water problems?
- Question 3: What is the relative urban runoff contribution to the receiving water problem(s)?
- Question 4: What are the sources to urban runoff that contribute to receiving water problem(s)?
- Question 5: Are conditions in receiving waters getting better or worse?

As illustrated in Figure Ex-1, the questions are linked in a logical progression that defines an efficient sequence of study design steps.

While there is a wide range of beneficial uses defined in the Basin Plans for southern California, the model monitoring program focuses on a subset of these beneficial uses that are common to most urban runoff management programs in the region and relate to human health and habitat protection:

- Contact Water Recreation (REC1)
- Non-contact Water Recreation (REC2)
- Warm Freshwater Habitat (WARM)
- Estuarine Habitat (EST)
- Marine Habitat (MAR)
- Wildlife Habitat (WILD).

For each category of beneficial use (i.e., human health, habitat protection) the model program defines monitoring objectives and study designs. Where adequate historical data were available, statistical analyses were used to develop detailed guidance on appropriate levels of sampling effort. Rather than define a static program, the technical committee develop several tools to serve as adaptive triggers for initiating more monitoring effort if an impact was observed, or a reduction in monitoring effort if no impact (or potential for impact) was found. These tools include triggers for toxicity identification evaluations, upstream source tracking, a prioritization scheme for special studies, and a computer program for estimating sample size based on statistical power to detect trends.

The following types of stations could be integral parts of a stormwater monitoring program that address each of the five key management questions:

- Long-term, fixed, bottom-of-watershed (but above tidal influence) stations to assess cumulative water quality and aggregate loads, with monitoring based primarily on a mass emissions model including wet weather chemistry and toxicity
- Spatially extensive, perhaps randomly sited or rotating, stations to support statistically valid comparisons across multiple watersheds, and with monitoring based primarily on the Triad approach for dry weather sampling and on chemistry and toxicity for wet weather
- Site-specific stations focused on the status of high-priority inland habitats of concern, with monitoring based primarily on the Triad approach for dry weather sampling and on chemistry and toxicity for wet weather
- High-priority inland body contact recreation areas
- Site-specific stations designed to generate information to support key program goals, such as source prioritization or BMP implementation and evaluation
- Coastal estuarine stations to assess status in these key habitats, with monitoring based primarily on the Triad approach
- Coastal ocean stations to assess stormwater plume impacts, conducted primarily as part of the periodic Bight surveys.

While the idealized monitoring design in Figure 5-2 shows each type of monitoring station separately, in practice there may be overlap among two or more types of stations.

The technical committee gave significant consideration to how the model program would be used in practice. It was well aware that stormwater monitoring has been ongoing for some time in southern California and that important basic steps, such as characterization studies, have been completed by many programs. In addition, the degree to which programs have addressed the five management questions in Figure 2-1 varies substantially, in part due to each program's history and in part due to the nature of the surface waters in different parts of the region.

Thus, the model stormwater monitoring program does not assume that each program is starting with a blank sheet of paper. Nor does the model program assume that each permittee will proceed through Figure 2-1 in a linear, stepwise fashion. Instead, the model program is intended to

improve each program's ability to build appropriate linkages among the five core management questions. This is best accomplished through the following steps:

1. Evaluate a program's ability to answer each of the five management questions
2. Identify critical gaps in knowledge (e.g., inability to document impacts, lack of knowledge about potential sources, absence of trend monitoring component) relevant to each program's circumstances
3. Use the monitoring designs in the model monitoring program as a framework for developing monitoring components suited to each program's circumstances.

The SMC's technical committee intended that the model program be used to direct an incremental process of adaptation using the three steps above, rather than one of wholesale change. This incremental change should be based on a prioritization of needs (i.e. using the triad approach in perennial streams before ephemeral streams). Through this process, the ultimate goal of developing regionally consistent programs that directly address key management questions in a scientifically rigorous and cost effective manner can be accomplished.

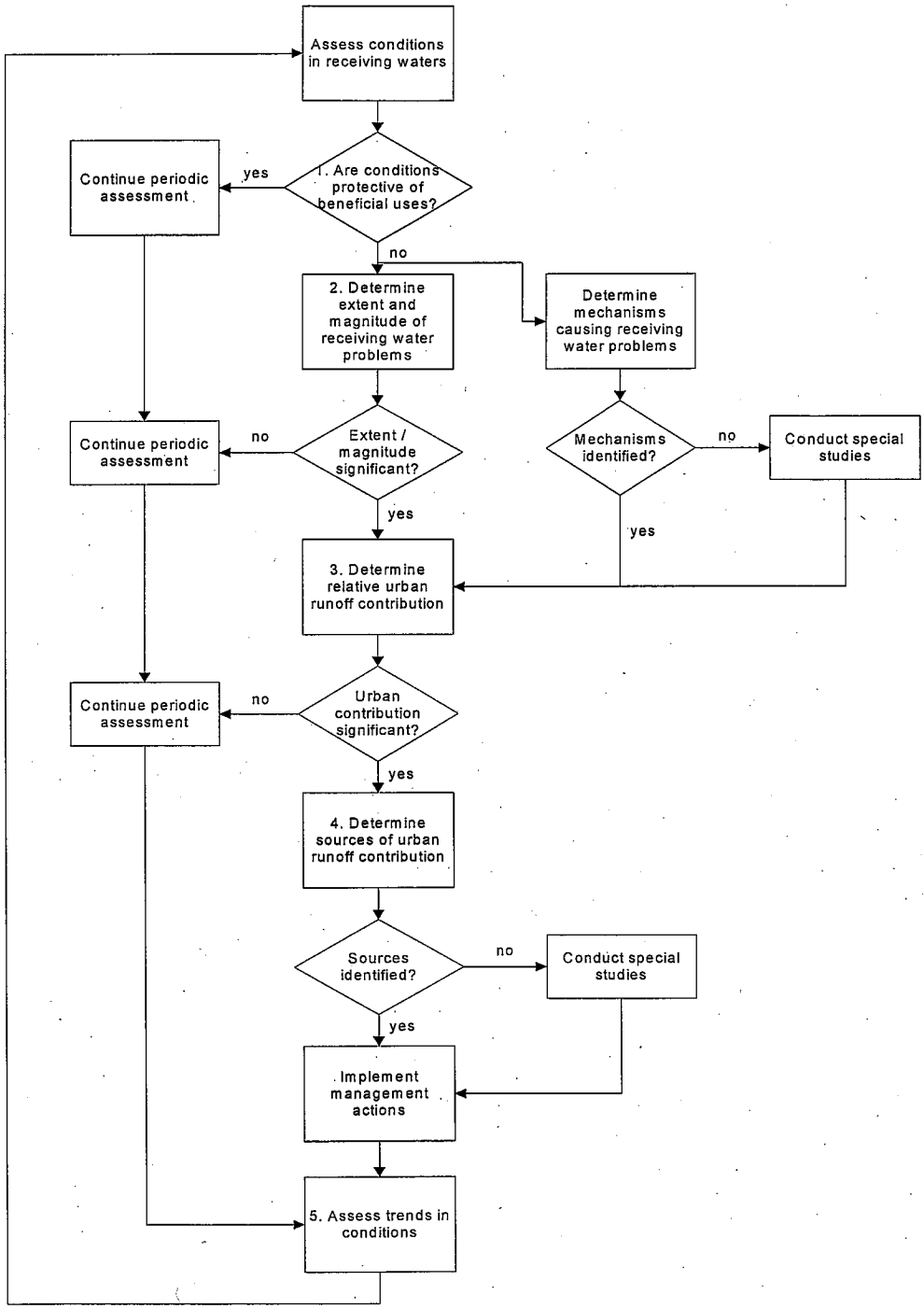

Figure Ex-1. Graphical illustration of the idealized logical flow through the five core management questions (reworded as statements to fit flowchart conventions). The answer to each question provides the basis for developing the monitoring design to answer the next. In actuality, monitoring programs may have addressed questions in parallel or out of sequence, depending on available knowledge and specific information needs.

ACKNOWLEDGEMENTS

This project was partially funded by the State Water Resources Control Board and the Southern California Stormwater Monitoring Coalition. The content of the report was developed over the course of one year a technical committee consisting of regulated, regulatory, environmental and research organizations including:

Name	Affiliation
Brock Bernstein	Facilitator
Linda Garcia	Riverside County Flood Control District
Dave Gibson	San Diego Regional Water Quality Control Board
Fred Gonzales	Los Angeles County Department of Public Works
Phil Hammer	San Diego Regional Water Quality Control Board
Karen Hauptly	Orange County Public Facilities and Resources Department
Lisa Kay	MEC Analytical (San Diego Cnty Department of Environment Health)
Eric Klein	San Diego County Department of Environment Health
Thomas Lo	Camp, Dresser & McKee (San Bernardino County Flood Control Dist)
Bruce Moore	Orange County Public Facilities and Resources Department
Ken Schiff	SCCWRP
Nate Stevenson	Los Angeles County Public of Works
Xavier Swamikannu	Los Angeles Regional Water Quality Control Board
Mitzy Taggart	Heal the Bay
Bob Whitaker	Santa Ana Regional Water Quality Control Board
Darla Wise	Ventura County Watershed Protection Division
Michael Yang	Los Angeles Regional Water Quality Control Board
Matt Yeager	San Bernardino County Department of Public Works

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1.0 INTRODUCTION

1.1 Rationale and approach

Large municipalities in southern California are required, under the Clean Water Act (CWA) and National Pollutant Discharge Elimination System (NPDES) permits from their respective Regional Water Quality Control Boards (RWQCBs), to monitor discharges of urban runoff[1] from municipal separate storm sewer systems (MS4s) and their impacts on receiving waters. However, urban runoff monitoring programs throughout southern California often focus on different monitoring questions, approach the same question in different ways, sample different sets of parameters, and use a range of field and laboratory methods to collect and analyze samples. This inconsistency makes it difficult, if not impossible, to address questions on a broader spatial scale, to compare urban runoff monitoring results across programs, and to improve efficiency by taking advantage of opportunities for exchanging data and coordinating monitoring responsibilities at regional scales.

In response to this set of circumstances, the Southern California Stormwater Monitoring Coalition (SMC) has undertaken a project to develop regionally consistent monitoring approaches and designs. The goal of the study is to produce a "model" monitoring program that will provide a foundation for each of the urban runoff monitoring programs in southern California to build on for their respective agency's needs. The development of the model monitoring program will therefore focus on developing regionally consistent management questions, efficient monitoring designs to answer those questions, creating standardized laboratory analysis protocols, and coordinating necessary quality assurance activities to ensure comparability among programs. This document focuses specifically on management questions and monitoring designs. Standardization of laboratory analysis protocols and data transfer and reporting methods are dealt with in companion documents.

This report reflects the collaborative work of a technical committee impaneled by the SMC. The technical committee included representatives from three southern California RWQCBs (the Colorado Region was not represented), the lead municipal MS4 management programs (commonly referred to as stormwater programs), SCCWRP, Heal the Bay and the State Water Resources Control Board (SWRCB). This report makes recommendations about a model urban runoff monitoring program, assesses current monitoring practice, and recommends adjustments to bring current programs more in line with the model program.

1.2 Relationship to SB72 and State Board efforts

Senate Bill 72 (Kuehl), adopted in October 2001, required the State Water Resources Control Board (SWRCB) to develop "minimum monitoring requirements for regulated municipalities that were subject to a stormwater permit on or before December 31, 2001." The SWRCB therefore has initiated efforts to develop standardized protocols for collection and analysis of stormwater samples, as well as a standardized reporting format. Working in coordination with local stormwater agencies and RWQCBs through the SMC presents an opportunity to gain consensus towards a common shared goal.

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[1] Urban runoff includes those discharges from residential, commercial, industrial and construction areas within the Permit Area and excludes discharges from feedlots, dairies, farms and open space.

There are, however, some important differences among the SMC and SWRCB goals. On one hand, the SWRCB goals are much larger than the SMC's goals. The SWRCB is mandated to develop statewide consistency while the goal of this project is development of consistency only for the southern California region. On the other hand, the SWRCB goals are more limited than the SMC's goals. The SWRCB is mandated to develop the "how to's" of stormwater monitoring (i.e. sampling, analysis, reporting), whereas this project starts with understanding the "why, where, and what" (i.e. monitoring questions and study designs) of developing an integrated stormwater monitoring program. Finally, the SWRCB and the SMC have different focal points of their monitoring programs. The SWRCB is mandated to develop standardized monitoring protocols for stormwater from all of their regulated discharges (i.e. municipal agencies and industrial facilities). This project, however, only addresses monitoring programs developed for municipal agencies, but examines monitoring designs for both wet and dry weather runoff.

1.3 A note on terminology

It is important to emphasize that the monitoring designs described in subsequent sections of this report focus explicitly on supporting the management of urban runoff to protect receiving water quality, with "receiving water" defined as surface Waters of the State, with the exception of ground water and lakes/reservoirs. While the SMC's technical committee recognized that there are other point and nonpoint sources of receiving water impact, the core focus of municipal stormwater programs is urban runoff, in both wet and dry weather. Thus, references to "stormwater" throughout the body of the report should be understood to refer to urban runoff.

It is also important to recognize that this document focuses on potential water quality problems and impacts, as opposed to water quality impairments. The technical committee opted to avoid the terminology of impairments because it has a distinct regulatory connotation that eventually leads to a Total Maximum Daily Load (see box on TMDLs). Water quality problems and impacts are more broad than impairments, which seemed more appropriate since some monitoring elements are meant to be early warning indicators and hopefully will avoid TMDLs in the future.

Total Maximum Daily Loads (TMDLs)

TMDLs are a regulatory framework for trying to restore beneficial uses in impaired waterbodies. Waterbodies sometimes have impaired water quality, even when all discharges to that waterbody are regulated under national pollutant discharge elimination system (NPDES) permits. The State will often use NPDES monitoring data to create the list of impaired waterbodies, also called the §303(d) list, which refers to the specific section in the Clean Water Act for TMDLs. Once promulgated, TMDLs typically call for additional monitoring either to refine source assessment or to determine if management actions implemented as a result of the TMDL are improving water quality. The model program described in this document is for urban runoff monitoring and, while there is some potential overlap with TMDL monitoring, the intent is to deliberately keep them separate. The reason is twofold. First, TMDLs are inherently site-specific and the goal of this document is to ensure regional applicability. Secondly, urban runoff may, or may not, be the cause of the water quality impairment that leads to a TMDL. If TMDL monitoring is called for, it is prudent to link monitoring from all NPDES dischargers to the impaired waterbody. Regardless of a §303(d) listing, urban runoff monitoring will be a necessity in order to characterize impacts, or lack of impacts, in receiving waters. Additional information on TMDLs in southern California can be found at www.swrcb.ca.gov

2.0 GOALS, OBJECTIVES AND IMPLEMENTATION

This model urban runoff monitoring program is intended as a framework to assist permittees and Regional Board staff in modifying existing monitoring programs, both wet and dry weather, with the goal of improving their ability to answer key management questions common to all programs in a cost effective and scientifically rigorous way. This is described in the following sections:

- The principals and philosophy for developing the model program are given in Section 3.0.
- A description of the key management questions, including rationale and expected data products, are given in section 4.0.
- The specific design elements, such as identifying the number of sampling sites and frequency of sampling, are given in Section 5.0.

This section, however, first addresses the basic program goals, how these goals address universal NPDES permit objectives as defined by the State and the Federal government, and describes an approach for applying the model monitoring program to an existing stormwater permit. Such modifications can occur when permits are periodically renewed and/or when permittees propose monitoring program revisions to their respective Regional Boards.

2.1 Monitoring program goals

Figure 2-1 summarizes the model monitoring program's ultimate goal, which is to ensure that each stormwater program has the ability to assess and manage its overall performance by answering five basic questions:

- Question 1: Are conditions in receiving waters protective, or likely to be protective, of beneficial uses?
- Question 2: What is the extent and magnitude of the current or potential receiving water problems?
- Question 3: What is the relative urban runoff contribution to the receiving water problem(s)?
- Question 4: What are the sources to urban runoff that contribute to receiving water problem(s)?
- Question 5: Are conditions in receiving waters getting better or worse?

These basic questions are universal to all MS4 programs in southern California and were prioritized by their program managers during the technical committees' early meetings.

2.2 Meeting permit objectives

Stormwater monitoring programs in southern California focus on meeting a set of NPDES permit objectives that, with some minor differences, are common to all programs in the region. These include the following (edited slightly for conciseness):

- Define water quality status, trends, and pollutants of concern associated with urban stormwater and non-stormwater discharges
- Evaluate impact of stormwater/urban runoff on biological species in receiving waters

- Identify those waters which cannot reasonably be expected to attain or maintain applicable water quality standards required to sustain beneficial uses
- Identify significant water quality problems related to urban stormwater and non-stormwater discharges
- Estimate annual mass emissions of pollutants discharged to surface waters through the MS4
- Evaluate water column and sediment toxicity in receiving waters
- Determine and prioritize pollutants of concern in stormwater
- Identify sources of urban runoff pollutants
- Identify other sources of pollutants in stormwater and non-stormwater runoff
- Evaluate the effectiveness of existing municipal stormwater quality management programs, including that of BMPs
- Identify and prohibit illicit connections
- Identify and prohibit illicit discharges.

The basic questions outlined in section 2.1 above, and described in detail in sections 4.0 and 5.0, will produce improved information that will help address many of these objectives (Table 2-1). The model program's structure, which moves from assessment monitoring, through source identification, and to tracking of longer-term trends, reflects the range of concerns represented in the set of common permit objectives.

2.3 Applying the model program

The technical committee's intent was to create the model program as guidance, providing sufficient detail to assure consistency in approach, but allowing for site-specific modifications and adaptations as necessary. This document serves as the starting point for negotiating a monitoring and reporting program. It is not a "copy and paste" list of static monitoring requirements, but an attempt to provide useful guidance. Therefore, this section outlines a procedure for implementing this guidance. We strongly recommend the user reread this section after reading sections 3.0, 4.0 and 5.0 in order to more fully understand this important implementation guidebook and place it in context.

The technical committee that developed the model program was well aware that stormwater monitoring has been ongoing for some time in many parts of southern California. Thus, important basic steps, such as stormwater characterization studies, have been completed by many programs (See Box on Discharge Characterization). In addition, the degree to which programs have addressed the five management questions in Figure 2-1 varies substantially, in part due to each program's history and in part due to the nature of the surface waters in different parts of the region. For example, inland programs in general have focused relatively more on identifying sources while coastal programs have allocated much more effort to assessing receiving

Stormwater Discharge Monitoring

The US EPA has published a manual (US EPA 1992) that provides detailed guidance for the basic elements of stormwater monitoring program design and implementation. This guidance is an extremely useful starting point for management programs faced with the necessity of performing initial characterization studies.

The manual describes when and where to sample, including defining storm event criteria, obtaining rainfall data, and dealing with the logistics of locating sampling sites. Alternative sampling methods (e.g., grab vs. composite, manual vs. automatic) are described and evaluated and special attention is given to the issue of measuring or estimating flow rates. In addition, the manual follows the analysis and reporting pathway once sampling is complete, providing detailed instructions on sample documentation, labeling, shipping, and chain of custody procedures.

water impacts, especially in high-use areas such as Newport or Santa Monica Bays.

The model stormwater monitoring program does not assume that each program is starting with a blank sheet of paper, nor that each program will implement the monitoring guidance in a linear, stepwise fashion. Instead, the model program is intended to improve each program's ability to build appropriate linkages among a key set of management questions (see Figure 2-1; Section 4.0). This is best accomplished through the following steps (see also Figure 2-3):

1. Evaluate a program's ability to answer each of the five management questions
2. Identify critical gaps in knowledge (e.g., inability to document impacts, lack of knowledge about potential sources, absence of trend monitoring component) relevant to each program's circumstances
3. Use the model program's monitoring guidance as a framework for developing monitoring components suited to each program's circumstances.

For Step 1, Appendix 1 summarizes current (as of June 2003) stormwater monitoring efforts in southern California, providing a first cut at assessing each program's ability to answer the five management questions. A full assessment under Step 1 would also involve a cumulative analysis of available historical monitoring data for each program. However, the variation among programs demonstrated in Appendix 1 suggests that implementing the model monitoring program would most likely involve focusing on different questions, and thus emphasizing different designs, for different programs. For example, source identification designs (Questions 3 and 4) might be needed for one program, but trend monitoring designs (Question 5) for another.

For Step 2, determining where to focus additional monitoring effort will depend on specific information on source characterization, patterns of development, hydrography and watershed structure, resources at risk, and levels and patterns of contamination. In addition, management initiatives in each program's area can influence decisions about what represents a critical knowledge gap. For example, TMDL development may require additional effort toward source identification. As another example, planned or ongoing BMP implementation may involve allocating additional effort to problem definition and/or to long-term trend monitoring to track BMP effectiveness.

For Step 3, the monitoring designs in the model monitoring program provide a starting point for developing detailed monitoring designs appropriate to the specifics of each program. The model framework is merely the foundation on which to build a permit-specific monitoring and reporting program. For example, the application of habitat monitoring designs based on bioassessment must take into account patterns of stream flow, the nature of biological communities, and the relative importance of urban runoff. The committee also considered the advisability of preparing explicit recommendations on the numbers and locations of sampling sites, and the degree of replication, but concluded that this was inappropriate given the amount of variation from program to program, as well as from place to place within each program. For example, numbers of stations will depend, among other things, on watershed size and complexity, amount and intensity of human use, severity and significance of potential impacts, known patterns of contamination, and hydrography of the study area. The degree of replication will depend on the kinds and amounts of variability in each area, as well as on the relative degree of certainty required by management agencies and the timeframe for decision making. Thus, the committee determined that each program should address the same five management questions and apply the same general monitoring design approaches, but then adapt the specifics of sampling to each individual situation. In this way, the model program will optimize comparability yet provide sufficient flexibility to address permit or site-specific needs

Table 2-1. Relationship between typical stormwater program monitoring objectives, as stated in NPDES permits, and the monitoring elements for which the model stormwater monitoring program provides design guidance. "Q" refers to the management questions described in Section 4.0 (e.g., Q1 refers to Question 1).

	Management question and type of monitoring				
Permit objective	Q1: Assessment	Q2: Extent and magnitude	Q3: Urban runoff contribution	Q4: Source identification	Q5: Trends
Define status, trends, pollutants	X				X
Evaluate impacts	X				X
Identify waters that do not attain uses	X	X			
Estimate mass emissions	X				X
Evaluate toxicity	X	X		X	
Identify polllutants of concern	X	X		X	
Identify sources of urban runoff pollutants			X	X	
Identify other sources			X	X	
Evaluate program effectiveness	X				X
Identify illicit connections				X	
Identify illicit discharges				X	

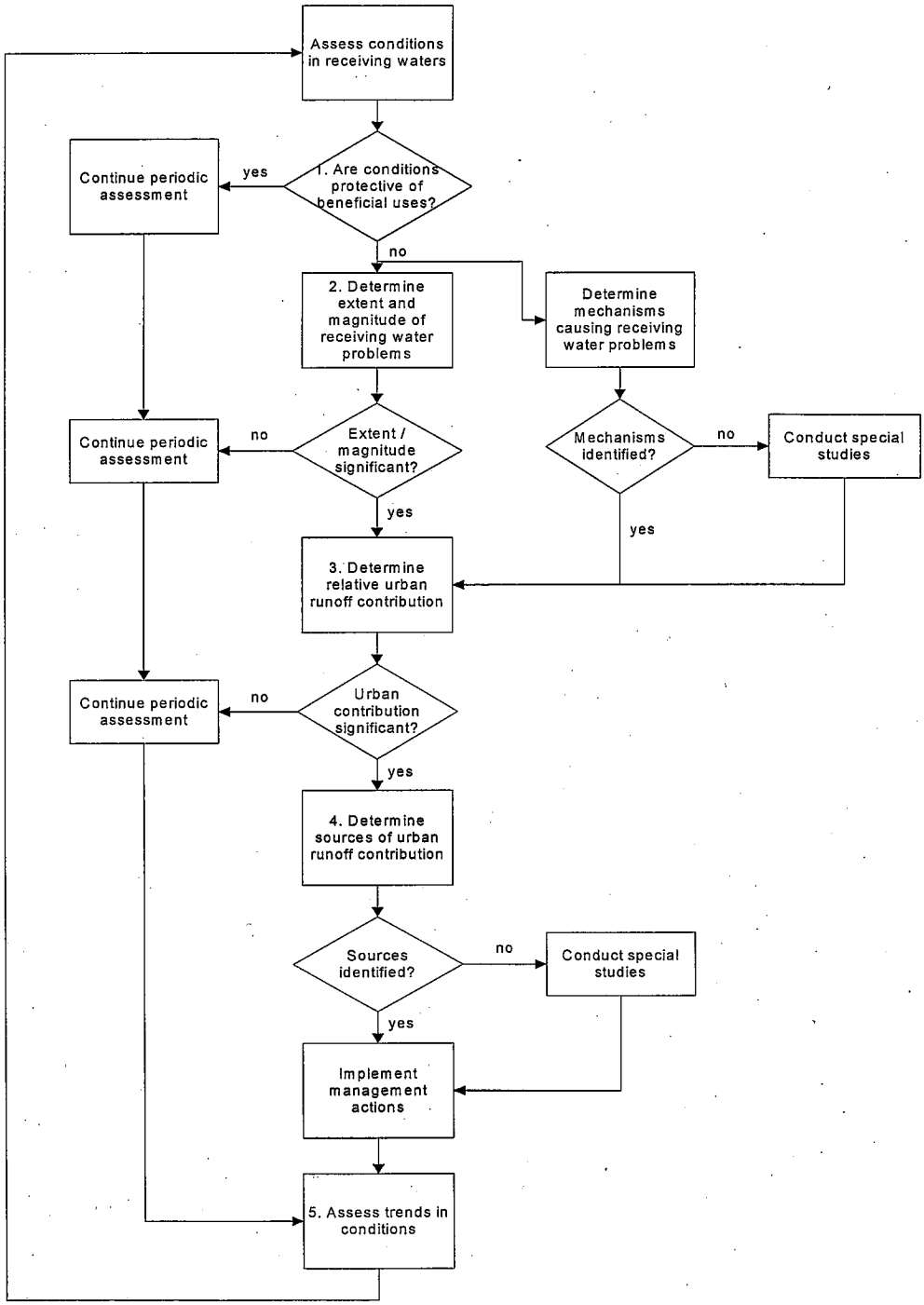


Figure 2-1. Graphical illustration of the idealized logical flow through the five core management questions (reworded as statements to fit flowchart conventions). The answer to each question provides the basis for developing the monitoring design to answer the next. In actuality, monitoring programs may have addressed questions in parallel or out of sequence, depending on available knowledge and specific information needs.

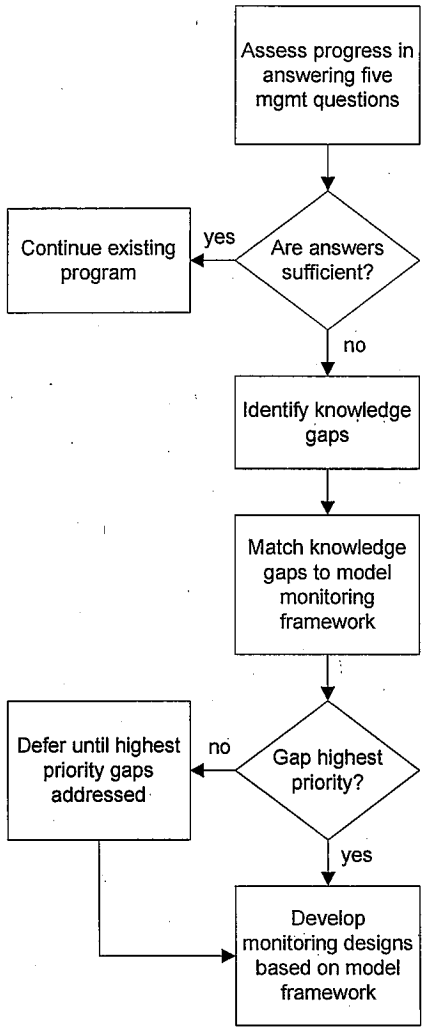


Figure 2-2. Sequence of steps involved in applying the model monitoring program framework to an existing stormwater monitoring program.

3.0 PRINCIPLES AND FRAMEWORK FOR A MODEL MONITORING PROGRAM

3.1 Principles for allocating monitoring effort

In developing the details of monitoring guidance to address the management questions and their related objectives, the committee was guided by three basic principles that provided an overall set of boundary conditions for monitoring design:

- Monitoring should be focused on decision making; data not helpful in making a decision about clearly defined regulatory, management, or technical issues should not be collected.
- The level of monitoring effort should reflect the potential for impact, with more monitoring allocated to situations where the potential impact (in terms both of the probability of an impact's occurrence and its extent and magnitude) is higher and less monitoring to situations where such potential is lower or where monitoring is not likely to provide useful information.
- Monitoring should be adaptive, in terms of its ability to both trigger follow-on studies as needed and make necessary mid-course corrections based on monitoring findings.

In addition, the committee identified three categories of monitoring activities that fulfill different types of information needs and defined them in the monitoring guidance in Section 5.0.

Core monitoring includes long-term monitoring, intended to track compliance with specific regulatory requirements or limits, to conduct ongoing assessments, or to track trends in certain important conditions over time. Thus, core monitoring generally occurs at fixed stations that are sampled routinely over time.

Regional monitoring includes cooperative studies that provide a larger-scale view of conditions in the southern California region. Regional monitoring can be used to assess the cumulative results of anthropogenic and natural effects on the environment. Regional monitoring also helps to place individual stormwater agencies' monitoring in perspective by comparing local results (i.e. core monitoring) to the breadth and depth of human impacts and natural variability found throughout southern California's watersheds. Regional monitoring requires the participation of all dischargers to the environment, not just MS4 permittees, thus potentially making this type of monitoring more cost-effective (see Box on Regional Watershed Monitoring). Finally, regional monitoring is best conducted periodically (i.e.

Regional Watershed Monitoring

There are many agencies in and around southern California in addition to municipal stormwater agencies that are interested in watershed to regional scale monitoring. For instance, the Statewide Ambient Monitoring Program (SWAMP) coordinated by the State Water Resources Control Board conducts monitoring throughout the southern California region in order to assess, among other things, the health of California's watersheds and estuaries. Another is the State's Wetlands Recovery Project, which has a similar goal as SWAMP, but is focused on wetland habitats. In a similar vein, the US Environmental Protection Agency coordinates the Environmental Monitoring and Assessment Program (EMAP), which monitors watersheds in the southern California region, but attempts to integrate these assessments nationally. Several other agencies are also monitoring in southern California's watersheds including the US Geological Survey's National Water Quality Assessment Program (NAWQA), the US Fish and Wildlife Service, numerous water reclamation plants, water districts, citizen monitoring organizations, and universities. Although each agency has a slightly different motive for monitoring, they all have at least one goal in common; to assess the health of the environment. Therefore, each one of these agencies represent an opportunity for a productive partnership in regional monitoring since they bring a different set of skills and perceptions to a meaningful collaboration.

every five years) because of its large spatial scale and integration among program types.

Special projects include specific targeted studies included as adaptive elements within core or regional monitoring designs. These are shorter-term efforts intended to extend or provide more insight into core monitoring results, for example, by investigating the specific sources of a receiving water problem. Special projects also include developmental research, designed to move monitoring science and policy forward. These can be used to demonstrate the value of particular analyses, to illustrate ways in which data can be used, or to develop new skills. These projects have a specified beginning, middle, and end. Stormwater programs may wish to conduct special studies individually or in coordination with the SMC.

3.2 Framework for developing monitoring questions and designs

The first major philosophical approach of the model urban runoff monitoring program is to help ensure that monitoring activities are:

- Linked directly to key management questions
- Integrated into a logically consistent whole
- Designed and structured for both cost effectiveness and scientific rigor.

To accomplish this set of ideals, the committee followed the philosophical framework of Bernstein et al. (1993), which outlines a series of successively more detailed levels of monitoring objectives. This philosophy includes series of logical steps that led from defining the key monitoring questions to specifying the technical detail of monitoring designs. This framework was defined as:

- Level I: broadly stated public and management core concerns (management questions)
- Level II: management and scientific objectives that include specific statements about time and space scales, reference conditions, and the monitoring approach to be used
- Level III: measurement goals that identify the types and amounts of change to be monitored for
- Level IV: specific technical plans and methods for implementing monitoring.

The second philosophical approach of the model program was to develop a framework that would provide broad consistency of approach, but can also be adapted or customized to meet local needs and conditions. One major concern of the technical committee was that, in its attempt to standardize monitoring programs regionwide, the model urban runoff monitoring program would become too inflexible to adapt to local site specific needs. Therefore, this document fully specifies Level I and Level II objectives (See Section 5.0), partially spells out Level III objectives, and provides examples, through technical guidance and brief case studies, of possible Level IV objectives (See Section 5.0). In addition, the companion documents that describe the laboratory intercalibration study and the data transfer and reporting formats do provide detailed Level IV objectives for two aspects of monitoring design that are important for ensuring comparability of data among programs. In this way, the model urban runoff monitoring program is not too restrictive, but provides sufficient guidance to make certain that managers throughout southern California have similar aims and approaches among programs.

4.0 MANAGEMENT QUESTIONS

There are five questions (or Level I objectives) that create a common foundation for monitoring design and urban runoff management in the region. These questions are not strictly independent, but are logically linked (Figure 2-1) where the answer to one question establishes the context for addressing the next. Thus, the management questions provide a means of organizing information about impacts, sources, and long-term trends in receiving water conditions into a logically consistent whole. The five management questions are:

- Question 1: Are conditions in receiving waters protective, or likely to be protective, of beneficial uses?
- Question 2: What is the extent and magnitude of the current or potential receiving water problems?
- Question 3: What is the relative urban runoff contribution to the receiving water problem(s)?
- Question 4: What are the sources to urban runoff that contribute to receiving water problem(s)?
- Question 5: Are conditions in receiving waters getting better or worse?

Each question can be addressed by one or more categories of monitoring effort, as summarized in Table 3-2. That is, some questions are best addressed using core monitoring, others questions are best addressed in a cooperative regional monitoring program, and others by directed special studies. The category(ies) of monitoring effort is identified within each question description.

The committee recognized that there are many beneficial uses enumerated in the region's Basin Plans and agreed that the five core management questions are equally applicable to the entire range of beneficial uses. However, for purposes of developing specific monitoring guidance, it chose to focus on a subset that is common to most municipal stormwater programs in the region and for which monitoring and regulatory approaches are relatively well established. These include:

- Contact Water Recreation (REC1)
- Non-contact Water Recreation (REC2)
- Warm Freshwater Habitat (WARM)
- Estuarine Habitat (EST)
- Marine Habitat (MAR)
- Wildlife Habitat (WILD).

The following subsections describe each question in more detail including background and rationale, explain how they are functionally interrelated (see also Figure 2-1), and describe the specific management and scientific objectives appropriate to each question including expected data products. For each question, the technical committee defined:

- What is the management goal?
- What monitoring strategy is suitable?
- What degree of certainty and precision is possible or required?
- What reference conditions are appropriate?
- What spatial scale is appropriate?

- What temporal scale is appropriate?

These questions and objectives then form the basis for the more detailed monitoring designs described in Section 5.0.

4.1 Question 1: Are conditions in receiving water protective, or likely to be protective, of beneficial uses?

4.1.1 Background to Question 1

Question 1 is a fundamental linch-pin for many, if not most, aspects of stormwater management. The presence of receiving water problems, or at least the potential for such problems, is the justification for a broad range of activities to better identify and reduce sources of contamination from urban runoff that may cause or contribute to such problems. In addition, detailed information about the nature of receiving water problems can greatly improve the effectiveness of a wide range of management actions. In principle, the design of any receiving water monitoring program should be based on reconnaissance and/or characterizations studies that target the likely sources and locations of receiving water problems. However, the southern California stormwater programs have already generated substantial information about where receiving water problems should be monitored for. Thus, the model monitoring program does not include a reconnaissance or characterization step for Question 1. However, where information on conditions in receiving waters is sparse or nonexistent, it may be necessary to initially conduct broad reconnaissance studies and/or evaluations of available historical data to determine the likely sources and locations of current or potential problems in receiving waters. In those cases, USEPA guidance (US EPA 1992) is available to direct the design of such studies.

In general, there are two often competing approaches to assessing whether conditions in receiving waters constitute a "problem", the compliance approach and the assessment approach. The committee described a compliance approach as one in which monitoring is used to determine if the value of an indicator is above or below a quantitative regulatory threshold. In this approach, the indicator measure would be considered as evidence of recreational water quality or habitat problem, acting as a surrogate for more detailed studies involving a larger range of measures. Exceedance of compliance standards would then provide the basis for management actions such as source identification studies, source control efforts, and further iterative monitoring and management actions. In contrast, an assessment approach would not be based primarily on comparison to specific quantitative thresholds or limitations. Rather, it would focus on better understanding actual conditions in the receiving water (i.e., the actual nature of problems) and is based on a weight of evidence approach in which chemical, biological, and ecological data are used to assess impacts. This approach emphasizes developing evidence of actual impacts in receiving waters in addition to, or instead of, evidence derived only from indicator measures.

The model monitoring committee believes that these two approaches should be complementary, rather than competitive or mutually exclusive. Thus, evidence provided by indicators could help initiate further studies to determine actual problem(s) and identify sources. Quantitative thresholds or limitations could be used to trigger or justify needed management actions, and the overall timeframe would be long enough to encompass iterations of monitoring and management efforts. The program design guidance in Section 5.0 illustrate how both approaches can be used in tandem, as in, for example, the use of mortality levels of indicator organisms in toxicity tests as a trigger for follow-up TIEs to identify the source(s) of toxicity.

4.1.2 Recreational water quality objectives

The Level II objective suggested for recreational water quality for Question 1 is described in Table 4-3 that focuses primarily on identifying conditions that may present elevated risk to humans from body contact recreation. The Level II management/monitoring objective can be stated as:

> Monitor a suite of bacterial indicators at high-priority sites selected by qualitative risk characterization and affected by urban runoff, including along beaches; in enclosed bays and estuaries; and along creeks, streams, and rivers at frequencies needed to ensure that relevant freshwater and marine standards are being met, to a moderate degree of certainty and precision.

The types of data products appropriate for answering Question 1 for recreational water quality may include:

- Frequent (daily, weekly, monthly depending on the circumstance) measures of fecal coliform or E. coli, total coliform, and Enterococcus at high-priority (defined in Section 5.1.1 as having both high use and elevated levels of indicator bacteria) beaches, coastal storm drains, lagoons, bays, estuaries, and inland creeks, streams, and rivers (Tables of individual measurements and relevant averages)
- Comparisons of bacterial indicator values with relevant standards (i.e., REC1, REC2, AB411) on spatial and temporal scales that match sampling scales as closely as possible (tables that highlight exceedances, figures that show exceedances over time)
- Summaries that identify the relative degree of contamination at monitored locations (i.e., maps, Heal the Bay's Report Card for beaches in Santa Monica Bay).

4.1.3 Habitat objectives

The Level II objective suggested for habitat health for Question 1 is described in Table 4-4. The Level II management/monitoring objective can be stated as:

> Use the Triad approach as a basis for monitoring both specific sites of high concern, as well as a set of random watershed sites, at least yearly and assess overall habitat health by comparing a suite of measurements to relevant reference conditions, to a moderate degree of certainty and precision. Use the Triad results to trigger an appropriate set of adaptive follow-up studies intended to better characterize conditions.

As might be expected, given both the inherent complexity of ecosystem monitoring and the variety of measurements included in the Triad approach, there is a range of reference conditions potentially applicable to monitoring of this question. The committee therefore recommended a structured framework for using reference conditions in the interpretation of Triad monitoring results (see Section 5.0).

The types of data products appropriate for answering Question 1 for habitat include:

- Site-by-site summaries of each sampled leg of the Triad (tables of individual measurements and relevant averages)
- Site-by-site interpretations and conclusions based on synthesized Triad results (narrative conclusions, decision trees)

- Comparisons across sites for each leg of the Triad (tables highlighting differences, maps)
- Comparisons across sites for synthesized Triad results (narrative conclusions, decision trees, maps)

4.2 Question 2: What is the extent and magnitude of the current or potential receiving water problems?

4.2.1 Background to Question 2

Question 2 is framed as the logical next step once receiving water problems related to urban runoff are found or predicted. Question 2 thus expands on the information provided by Question 1as a basis for describing the spatial and temporal extent of existing or likely impacts, as well as their relative intensity. This information is necessary for assessing the relative severity or importance of different problems, targeting source identification efforts, and planning management actions such as source reduction efforts.

In most cases, monitoring designs to answer Question 1 will include only representative sites within key recreational areas or habitats. Thus, once a receiving water problem is found, data from these sites will most often be insufficient to characterize the full extent and magnitude of the problem and additional studies will normally be called for. This is because most managers need to know the severity of a problem before proceeding with some remedial action. Impacts that cover large areas or extend over long periods of time typically require more immediate attention. The information collected to answer Question 2 is important for scoping the source identification studies that are the focus of Questions 3 and 4 (see Figure 2-1).

In some cases, the extent, magnitude, and/or severity of a receiving water problem will be immediately apparent from the core monitoring data obtained under Question 1. In such cases, for example, very high bacteria counts along a popular beach or severe toxicity in an enclosed lagoon, source identification work as described in Questions 3 and 4 should begin promptly. In addition, un-permitted dry weather discharges are specifically forbidden and such discharges should therefore also be a high priority for prompt source identification studies. In other cases, broader sampling to assess spatial and temporal extent will be required, usually as shorter-term studies that are conducted once or perhaps periodically when there is reason to believe the scale of the problem has changed. In some situations, where the problem is complex and/or covers a large area, addressing Question 2 will involve regional studies that require the cooperative efforts of several agencies. Monitoring under Question 2 would be conducted in either wet or dry weather, depending on the specific issue and in accord with the findings of Question 1.

4.2.2 Recreational water quality objectives

The Level II objective suggested for recreational water quality for Question 2 is described in Table 4-6. The Level II management/monitoring objective can be stated as:

> Monitor a suite of bacterial indicators at a spatially and temporally more intensive set of stations around sites, prioritized by risk, in order to define the extent of problems to a moderate degree of certainty and precision, and compare indicator levels to relevant marine and freshwater standards in order to define the relative severity of the problem, also to a moderate degree of certainty and precision.

The types of data products appropriate for answering Question 2 for recreational water quality include:

- Measures of the spatial extent of bacterial contamination (maps)
- Measures of the temporal patterns of bacterial contamination (figures that show temporal patterns, measures of variance)
- Measures of the relative magnitude of indicator values over space and time (graphs of concentration over time or by site).

4.2.3 Habitat objectives

The Level II objective suggested for habitat for Question 2 is described in Table 4-7. The Level II management objective can be stated as:

> Monitor specific aspects of the Triad, including adaptive elements such as additional chemistry measurements or TIEs, at a spatially and temporally more intensive set of stations where impacts have been observed in order to define the extent of problems to a moderate degree of certainty and precision, and compare measurements to relevant marine and freshwater standards in order to define the relative severity of the problem, also to a moderate degree of certainty and precision.

The types of data products appropriate for answering Question 2 for habitat include:

- Measures of the spatial extent of modified communities, chemical contamination, and/or elevated toxicity (maps)
- Measures of the temporal patterns of modified communities, chemical contamination, and/or elevated toxicity (figures that show temporal patterns, measures of variance)
- Measures of the relative magnitude of indicator values over space and time (graphs of concentration or toxicity over time or by site).

4.3 Question 3: What is the relative urban runoff contribution to the receiving water problem(s)?

4.3.1 Background to Question 3

Once monitoring or other studies demonstrate that there is a current or potential impact to receiving waters (Question 1) and describe the problem's extent and magnitude (Question 2), decisions about any management responses depend on information about the source(s) of the problem. The model monitoring framework breaks this source identification into two parts (Figure 2-1), represented by Questions 3 and 4. The purpose of this two-step process is to prioritize more detailed source identification efforts in Question 4 at only those problems for which urban runoff is a significant contributor. Question 3 begins this process by taking the information from Questions 1 and 2 and beginning to work upstream, both literally and figuratively, to better define the overall contribution of urban runoff to receiving water problems. It is important to clarify that this two-step process involving Questions 3 and 4 is not intended in any way to diminish or replace municipalities' permit requirements to reduce contaminant inputs to the maximum extent practicable. It is rather intended to help determine when additional, more detailed and extensive, upstream source identification efforts should be conducted by a municipality, with the goal of ensuring that the full burden of source identification work not be shifted to the MS4 permittees where action by them would not solve the larger problem.

The model monitoring framework assumes that, if urban runoff contributes only a very small percentage to the receiving water problem, then there would be no need for a municipal permittee to **independently** carry out substantial source identification efforts in addition to those activities usually carried out under the municipal stormwater permit. For a first-cut estimation, therefore, Question 3 requires only minimal resolution, including at least a rough estimate of the identity and magnitude of the non-urban runoff contributions. In many situations, aggregate estimates of the non-urban runoff contribution, rather than source-by-source estimates, may be adequate and may already be available from previous characterization and/or monitoring studies. Only if urban runoff is found to contribute significantly to receiving water problems would a municipality be required to take the lead on conducting further source identification studies at greater resolution (as described in Question 4).

The committee engaged in substantial discussion of criteria for prioritizing source identification work and agreed that several factors should be taken into account in each instance, including:

- The severity of the problem
- The type of pollutant(s) involved
- The potential for human health risk
- The relative certainty of the estimates of relative contribution from different sources. If the estimate of urban contribution is very low, then even high uncertainty might not be important. However, if the estimate is higher, e.g., 10%, and the uncertainty is high (e.g., could be as high as 30%) then that would be a different situation
- Whether the problem occurs during dry and/or wet weather, since dry weather problems may be more easily dealt with
- The biological resources at issue
- Regulations and other legal mechanisms that require source identification and/or control
- Stakeholder involvement such as watershed group planning priorities.

The committee agreed that source identification work should be prioritized based on the factors above, and that the threshold level for further independent source identification efforts by the permittees should be somewhere between 5 – 10%. It is important to emphasize that this threshold is intended as a guideline only in situations where the source of a receiving water problem is not known. Where the source(s) of such problems are known, then relevant permit conditions related to source reduction and cleanup would come into play. As emphasized above, this threshold is not intended to diminish or replace permit requirements to reduce contaminant inputs to the maximum extent practicable (MEP) or other regulations or legal requirements.

4.3.2 Recreational water quality and habitat objectives

The Level II objective suggested for both recreational water quality and habitat for Question 3 is described in Table 4-8. The Level II management objective can be stated as:

> Using parameters relevant to the nature of the receiving water problem, estimate the proportional contribution of urban runoff at the most downstream point of input to the receiving water, based on a loads study performed at minimal to moderate resolution, and repeated every several years as needed.

The types of data products appropriate for answering Question 3 for both recreational water quality and habitat include:

- Description of all potential sources of inputs to the receiving water (maps of potential sources)
- Rough estimates of the relative magnitude of loads from all sources (table of concentrations or loads by source)
- Rough estimate of the proportional contribution of urban runoff to total loads (pie charts or stacked bar charts).

4.4 Question 4: What are the sources of the urban runoff contribution to receiving water problems?

4.4.1 Background to Question 4

Once it has been determined, either through specific studies carried out under Question 3 or through other available data, that urban runoff is, or is likely to be, a significant source of one or more receiving water problems, then more intensive source identification efforts are called for. Question 4 thus involves more thorough source identification studies intended to provide more detailed information about the nature, location, and quantity of inputs to the receiving waters identified in Question 1. This information can help refine receiving water monitoring, improve fundamental understanding of stormwater contamination processes, and help guide management actions intended to reduce sources and their attendant impacts. It can also help focus trend monitoring on those parameters that are potentially most responsive to urban runoff source reduction efforts.

In the context of Question 4, "sources" can refer to multiple layers of sources, such as a golf course that is the source of pesticides, which are in turn the source of toxicity in the receiving water. Thus, questions about sources should be framed carefully in order to clarify both the spatial definition of "upstream source" as well as the level of causality that is the central focus of the investigation.

4.4.2 Recreational water quality and habitat objectives

The Level II objective suggested for both recreational water quality and habitat for Question 4 is described in Table 4-9. The Level II management objective can be stated as:

> Using parameters relevant to the nature of the receiving water problem, prioritize receiving water sites for upstream source identification studies and perform source identification studies at the watershed scale and to a moderate degree of resolution until the appropriate stopping rules are reached.

The types of data products appropriate for answering Question 4 for both recreational water quality and habitat include:

- Prioritization of receiving water sites in terms of severity of impact (ranked list of sites)
- Description of all potential urban runoff sources of inputs to the higher priority receiving waters (map of potential sources)
- Determination of actual sources of urban runoff and their relative magnitude (table of concentrations and flows by source with estimated levels of confidence)
- Quantitative estimates of the loads from urban runoff sources(table of loads by source with estimated levels of confidence).

4.5 Question 5: Are conditions in receiving waters getting better or worse?

4.5.1 Background to Question 5

Assuming that monitoring related to Questions 1 – 4 has resulted in improved information about the nature and source(s) of current and/or potential receiving water problems, and that this in turn has led to management actions to address such sources, Question 5 provides the logical feedback to determine if such actions are improving conditions in receiving waters. Given that changes in receiving water conditions are likely to occur over several years (at the least), Question 5 is a trends monitoring question. The trends of interest are in both discharges and receiving waters and the time frame for this question is the longer-term period needed to determine if management actions are having their intended effects.

In its simplest form, a trend monitoring design involves repeated sampling over time at the same monitoring site(s). The ability of a trend design to detect change depends on:

- The amount of change it is important or necessary to detect
- The timeframe within which decision makers need information about trends
- The variability of the indicator on different time scales, typically shorter term (weekly, monthly) and longer term (yearly)
- The resources available for sampling and analysis.

Developing the specifics of the monitoring design thus involves making a series of tradeoffs among these factors.

The statistical power of a monitoring design is its ability to detect a change of a certain size, if it in fact has occurred. Power analysis, used to estimate the power of a given design, can provide insight into the sampling effort (both in terms of the number of samples per year and the number of years) required to observe trends of different size. In addition, power analyses can reveal important inherent constraints on the ability to detect trends imposed by underlying variability in the system being monitored. This can provide a realistic basis for establishing both management and monitoring goals, as well as a basis for making tradeoffs in the monitoring design (e.g., between the number of samples collected per year and the number of years over which the trend monitoring will extend).

Figure 4-1 provides an example of how site-specific power analysis results might be used. In one instance (Figure 4-1a), trend monitoring would be futile and monitoring resources should be shifted to another site and/or issue. In a second instance (Figure 4-1b), the only way to improve the design's ability to detect a trend is to increase the number of years to be monitored. In such an instance, the length of time needed to detect a trend must be compared against both the management time horizon (i.e., how quickly is information needed?) and the timeframe over which changes are expected to occur (e.g., how rapidly are BMPs expected to reduce loads?). In a third instance (Figure 4-1c), the main way to improve the design's power is to increase the number of samples per year. However, for some questions, there is a natural constraint imposed by the relatively small number of storms per year in southern California. In such cases, the monitoring design will have an inherent limit on its ability to detect trends within a given time period. In a final example (Figure 4-1d), sampling additional times per year and monitoring for more years must be traded off against each other, since increasing both kinds of sampling intensity improves power. Such tradeoffs should be based on both the management time horizon and the timeframe over which changes are expected to occur. Thus, if an answer to Question 5 is

not immediately urgent, then the number of samples per year can be reduced and the timeframe extended into the future.

Appendix 2 uses historical data from the southern California region to provide example power analysis results for trend monitoring of bacteria and mass emissions. Sufficient data for power analyses of other data types (e.g., bioassessment, toxicity) are not yet available. Because power analysis results can vary widely from site to site and across constituents, these results should be considered only as a "starter kit" for trend monitoring designs. The committee strongly recommends that each trend monitoring program conduct its own site-specific power analyses after obtaining three years of trend data, and revise its monitoring design accordingly based on these results. To support such program-specific design efforts, the committee has developed a simple software package that automates the needed power analysis (Go to http://www.sccwrp.org to download a copy of this program). Because trend monitoring programs will typically continue for many years, this approach will enable trend monitoring to begin and then to adjust its design appropriately with little or no loss of information.

The central importance of estimates of variability in trend monitoring highlights the importance of improving our basic understanding of sources of variability in MS4s. Thus, in addition to tracking trends over time, the analysis of monitoring data under Question 5 should include efforts to examine and quantify sources and patterns of variability in monitoring data, with the overall goal of reducing any controllable variability (i.e., variability introduced through sampling techniques and laboratory analysis, or due to spatial and temporal sources that can be accounted for in the structure of the monitoring design itself).

Finally, a full answer to Question 5 should also include an assessment of changes in the extent and magnitude of impacts over time. Such an assessment can be accomplished by repeating the studies described in Question 2.

4.5.2 Recreational water quality objectives

The Level II objective suggested for recreational water quality for Question 5 is described in Table 4-10. The Level II management objective can be stated as:

> Monitor bacterial indicators at fixed stations over a number of years to determine, to a moderate degree of resolution, whether levels have increased or decreased compared to historical data and to relevant standards.

The types of data products appropriate for answering Question 5 for recreational water quality include:

- Graphs of the levels of bacterial indicators over time at each station of concern
- Periodic statistical power analysis results to confirm the power of the trend monitoring design.

4.5.3 Habitat objectives

The Level II objective suggested for habitat for Question 5 is described in Table 4-11. The Level II management objective can be stated as:

Monitor relevant habitat indicators at fixed stations over a number of years to determine, to a moderate degree of resolution, whether levels have increased or decreased compared to historical data and to relevant standards.

The types of data products appropriate for answering Question 2 for habitat include:

- Graphs of the levels of habitat indicators over time at each station of concern
- Periodic statistical power analysis results to confirm the power of the trend monitoring design.

Table 4-1. Relationship between the five key management questions and the three basic categories of monitoring activity. Core monitoring for Questions 1 and 5 typically occurs at fixed stations over a period of time. The design of regional monitoring and special projects under Questions 2, 3, and 4 is contingent on monitoring results from other core management questions. Numbers in italics (e.g., *5.1.1*) refer to report sections that detail specific design guidance for each element of the model monitoring program.

Management Questions	Core Monitoring	Regional Monitoring	Special Projects
1. Are conditions protective?	Recreational water quality assessment (wet & dry) *5.1.1* Ecosystem assessment (dry) *5.1.2*		
2. What is extent / magnitude?		One-time or periodic larger-scale assessment (depends on Question 1) *5.2*	One-time or periodic larger-scale assessment (depends on Question 1) *5.2*
3. What is urban runoff contribution?		One-time characterization, assessment (depends on Questions 1 & 2) *5.3*	One-time characterization, assessment (depends on Questions 1 & 2) *5.3*
4. What are sources of urban contribution?			Site-specific, one-time or periodic source ID studies (wet & dry) (depends on Question 3) *5.4*
5. Are conditions getting better or worse?	Long-term trends monitoring (wet & dry) of: • Bacterial indicators *5.5.1* • Habitat indicators (incl. loads) *5.5.2*		

Table 4-2. Level II objectives for recreational water quality monitoring for Question 1: Are conditions in receiving waters protective of beneficial uses? Aspects of the objective are organized around the six categories of information that make up each Level II objective.

Level II category – Q1 recreational water quality	Level II objective – Q1 recreational water quality
Management goal	Protect human health by meeting existing standards
Monitoring strategy	Allocate sampling effort with respect to overall risk (combination of use and contamination) Monitor bacteria indicators (fecal coliform (or E. coli), total coliform, Enterococcus) Use improved indicators when available and approved by health department Adaptive link to magnitude, extent, and upstream urban runoff source identification studies
Degree of certainty and precision	Moderate
Reference conditions	Freshwater standards (REC1, REC2) Marine standards (AB411)
Spatial scale	Open-coast beaches Specific coastal storm drains Bay, lagoons, estuaries Rivers and creeks
Temporal scale	Daily (for health risk) Weekly (for health risk) Seasonal (for health risk, trends)

Table 4-3. Level II objectives for habitat monitoring for Question 1: Are conditions in receiving waters protective of beneficial uses? Aspects of the objective are organized around the six categories of information that make up each Level II objective.

Level II category – Q1 habitat	Level II objective – Q1 habitat
Management goal	Protect ecosystem health by tracking the relationship of indicators to relevant reference conditions
Monitoring strategy	Triad approach Coordinated watershed and subwatershed scales Adaptive monitoring triggers depending on triad results Sites targeted at specific management issues
Degree of certainty and precision	Moderate
Reference conditions	Basin Plan Ocean Plan Regional IBI (for stream bioassessment) CTR (for chemistry) Toxicity test reference Historical reference conditions (site-specific) Local reference conditions (site-specific) Other watersheds (regional)
Spatial scale	Site-specific (e.g., Talbert Marsh) Watershed / subwatershed Jurisdictional
Temporal scale	Yearly Several years

Table 4-4. Level II objectives for recreational water quality monitoring for Question 2: What is the extent and magnitude of receiving water problems? Aspects of the objective are organized around the six categories of information that make up each Level II objective.

Level II category – Q1 recreational water quality	Level II objectives – Q1 recreational water quality
Management goal	Define the scale of impact
Monitoring strategy	Short-term sampling at broader spatial extent Sampling appropriate to define temporal patterns at weekly to seasonal scales Measure bacteria loads at MS4 discharge locations
Degree of certainty and precision	Moderate
Reference conditions	Freshwater standards (REC1, REC2) Marine standards (AB411) Comparisons across parts of the region
Spatial scale	Watershed / subwatershed Jurisdictional Regional
Temporal scale	For REC1 objective, geomean over a season Process-based (e.g., seasonal) 3 years for impairment (303d)

Table 4-5. Level II objectives for habitat monitoring for Question 2: What is the extent and magnitude of receiving water problems? Aspects of the objective are organized around the six categories of information that make up each Level II objective.

Level II category – Q2 ecosystem	Level II objectives – Q2 ecosystem
Management goal	Receiving water conditions improve (if impaired) Receiving water conditions remain the same (if not impaired)
Monitoring strategy	Triad monitoring in key receiving waters Long-term trend monitoring Adaptive toxicity testing Adaptive upstream toxicity testing
Degree of certainty and precision	Moderate
Reference conditions	Basin Plan Ocean Plan Regional IBI (for stream bioassessment) CTR (for toxicity) Toxicity test reference Comparisons across parts of the region
Spatial scale	Watershed / subwatershed Jurisdictional Regional
Temporal scale	Periodic snapshots (yearly)

Table 4-6. Level II objectives for both recreational water quality and habitat monitoring for Question 3: What is the relative urban runoff contribution to the receiving water problem(s)? Aspects of the objective are organized around the six categories of information that make up each Level II objective.

Level II category – Q3 recreational water quality & habitat	Level II objective – Q3 recreational water quality & habitat
Management goal	Estimate the proportional contribution of urban runoff to problem(s) in specific receiving water
Monitoring strategy	Loads estimation
Degree of certainty and precision	Minimal to moderate
Reference conditions	Relative severity of local receiving water problem(s) Relative contribution of urban runoff to other receiving waters in the region
Spatial scale	Point of input to receiving water (scales depending on definition of receiving water)
Temporal scale	Periodic assessment (every 5 years)

Table 4-7. Level II objectives for both recreational water quality and habitat monitoring for Question 4: What are the sources to urban runoff that contribute to receiving water problems? Aspects of the objective are organized around the six categories of information that make up each Level II objective.

Level II category – Q4 recreational water quality & ecosystem	Level II objectives – Q 4 recreational water quality & ecosystem
Management goal	Urban sources identified and resolved
Monitoring strategy	Prioritize downstream sites Upstream source ID studies
Degree of certainty and precision	Moderate to great
Reference conditions	Internal tests of "signal" strength
Spatial scale	Watershed / subwatershed Jurisdictional Regional
Temporal scale	Until stopping rules reached

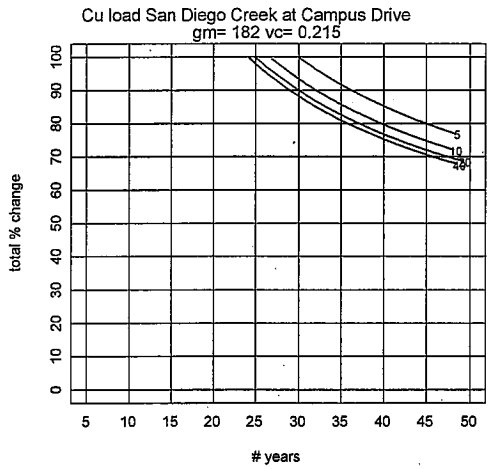
Table 4-8. Level II objectives for recreational water quality monitoring for Question 5: Are conditions in receiving waters getting better or worse? Aspects of the objective are organized around the six categories of information that make up each Level II objective.

Level II category – Q5 recreational water quality	Level II objectives – Q5 recreational water quality
Management goal	Reduction in indicator levels Identification and removal of key sources
Monitoring strategy	Repeated monitoring at specific sites over a season Long-term trend monitoring
Degree of certainty and precision	Moderate
Reference conditions	Standards Historical data as a basis of trends
Spatial scale	Specific receiving waters Where use is concentrated Watershed / subwatershed Jurisdictional
Temporal scale	For REC1 objective, geomean over a season Process-based (e.g., seasonal) 3 years for impairment (303d) Permit term (~ 5 years) for trends TMDL implementation phase (~ 10 years)

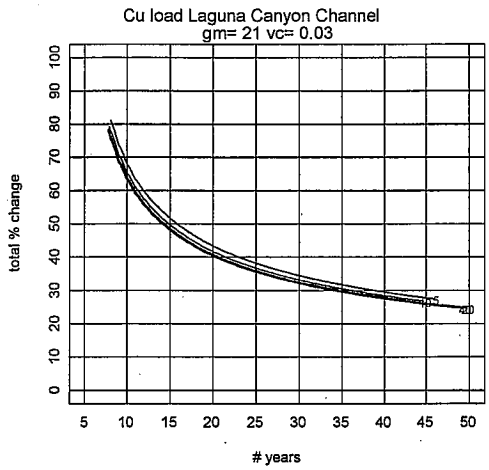
Table 4-9. Level II objectives for habitat monitoring for Question 5: Are conditions in receiving waters getting better or worse? Aspects of the objective are organized around the six categories of information that make up each Level II objective.

Level II category – Q5 habitat	Level II objective – Q5 habitat
Management goal	Conditions improve (if degraded) Conditions remain the same (if not degraded)
Monitoring strategy	Triad approach
Degree of certainty and precision	Moderate
Reference conditions	Basin Plan Ocean Plan Regional IBI (for stream bioassessment) CTR (for chemistry) Toxicity test reference
Spatial scale	Watershed / subwatershed Jurisdictional
Temporal scale	Permit cycle for overall assessment Process-based for specific components Bioassessment (greater than 5 years) Bioaccumulation (e.g., short-term for Se, long-term for DDT) BMP (based on site-specific geomorphology, BMP mechanism) Hydrology (annual) Toxicity (sporadic, seasonal)

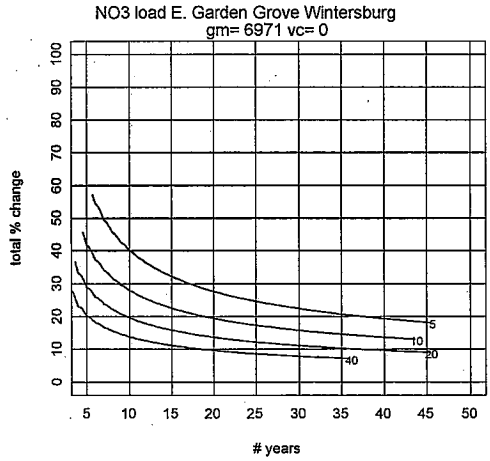
a.



b.



c.



d.

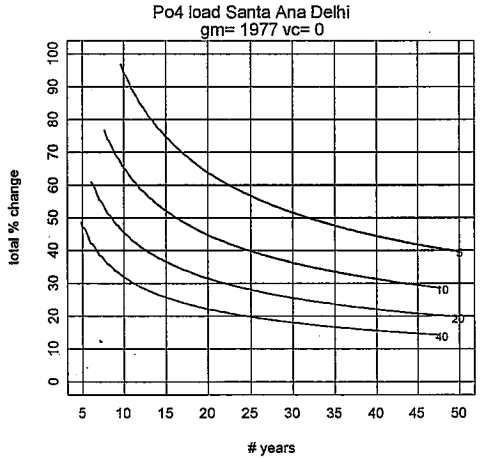


Figure 4-1. An example of the range of site-specific statistical power analysis results that can provide useful guidance for both trend monitoring design and setting management and monitoring goals. In the example figures below, the x-axis shows the number of years over which trend monitoring could continue and the y-axis the amount of change monitoring could detect. The four curves represent different amounts of sampling intensity per year. a. Even large amounts of sampling will not detect trends. b. Increasing the number of sampling events per year will not increase power because virtually all the variability is year-to-year variability. c. Increasing the number of years sampled beyond a certain point will not increase power because virtually all the variability is within-year variability. d. Both within- and between-year variability are important and increasing both kinds of sampling intensity will increase power.

5.0 MONITORING GUIDANCE

The following sections provide specific guidance for the design of core monitoring, regional monitoring, and special projects needed to address each of the core management questions (Table 4-1). The description of each monitoring design follows the same format:

- An overview that quickly summarizes the major features of the design, including a table of key design elements
- A lengthier description of specific design elements, such as station selection, monitoring frequency, indicators, triggers or thresholds for regional monitoring and/or special projects
- A discussion of design issues that describes the underlying rationale for the design and any important constraints that may affect monitoring success.

As the description of the key management questions and objectives (Section 4.0) makes clear, monitoring guidance focuses on recreational water quality and habitat issues. In some cases, distinct monitoring designs are required for each set of issues, while in other cases the same design approach is suitable for both. Where distinct monitoring designs are required, recreational water quality and habitat beneficial uses are presented separately.

5.1 Assessment monitoring

Assessment of recreational water quality and habitat conditions addresses Question 1: Are conditions in receiving waters protective, or likely to be protective, of beneficial uses? Data from such monitoring provides the basis for other aspects of the model program (Figure 3-1) intended to better characterize the extent and magnitude of any problems (Question 2), identify sources (Questions 3 and 4), and track trends in condition over time (Question 5).

Assessment monitoring effort falls exclusively into the core and regional monitoring categories (Table 4-1). Core monitoring is that conducted by individual agencies to evaluate issues related to specific sites or watershed. Regional monitoring is that conducted cooperatively by multiple agencies (see box on regional monitoring) to address issues across broader scales and time periods. In many cases, the same monitoring sites and/or approaches can meet both core and regional monitoring needs.

5.1.1 Recreational water quality assessment

5.1.1.1 Overview and philosophy

Design overview. Table 5-1 presents an overview of the technical design elements for assessment of recreational water quality conditions at beaches; bays and estuaries; and creeks, streams, and rivers. The following discussion does not include inland lakes and reservoirs, which, because they represent a special case, the committee agreed to defer until a later time.

The model monitoring framework allocates core monitoring of bacterial indicators to high-priority locations based on risk of adverse health effects. This risk is defined in terms of a combination of level of contamination and degree of human body contact use. In general, local public health departments have completed risk characterization for many waterbodies with recreational beneficial uses, particularly for marine beaches. Additionally, local health agencies have already established routine monitoring locations at many marine beaches that are sampled at

least weekly. To address the assessment of recreational water quality conditions, the model program recommends that stormwater agencies build upon the monitoring data, local knowledge, and experience of the local public health departments. Thus, the model monitoring committee explicitly assumed that local public health agencies would take the lead in specifying high priority areas based on their knowledge and experience.

Somewhat different monitoring designs are recommended for linear open-coast beaches, enclosed bays and estuaries, and creeks/streams/rivers, based on differences in their basic hydrology. The number of sampling locations is dependent on the size of an area and its level of relative risk, as is the sampling frequency. However, at all monitoring sites, monitoring should measure indicator levels in the discharge itself, as well as upcoast and downcoast (or upstream and downstream) of the discharge. In addition, where the monitoring objective is to determine whether overall conditions constitute a problem as opposed to monitoring for body contact, monitoring may be focused on that portion of the year that represents the worst-case scenario.

At the moment, there are no ongoing regional monitoring efforts focused on recreational water quality, with the exception of the Bight Program's periodic snapshots of shoreline water quality and the new regional harbors monitoring program being developed by the San Diego Regional Water Quality Control Board. Both programs rely on a probability-based design intended to support general conclusions about the relative degree of contamination in different parts of the region.

Design philosophy. The model design framework specifies somewhat different approaches for core monitoring at linear open-coast beaches; enclosed bays and estuaries; and creeks, streams, and rivers. However, all three approaches reflect the basic assumption that monitoring resources should be allocated based on risk. Where the county health departments and the State Board's Beach Water Quality Work Group (BWQWG) have established monitoring approaches and/or designs, the model stormwater program will remain consistent with these. The committee felt that, ultimately, the county health departments are one end-user for much of this data since they have the responsibility for assessing if a beach should be closed or posted for swimming.

Widely accepted risk management principles recommend allocating monitoring and management effort in proportion to the relative degree of risk. The basic design feature of the model urban runoff monitoring program for Question 1 for recreational water quality therefore is to focus effort at those places and times (whether wet or dry weather) where human health risk associated with urban runoff is the highest. The model design assumes that risk, at the population level, is directly related to exposure, and estimates exposure, in turn, as the qualitative combination of estimates of bacterial contamination and the intensity of human use. While these qualitative risk estimates may be improved by future risk assessments, this improved information should not alter the basic principle of allocating monitoring in terms of relative risk.

There are three types of situations that are relevant to stormwater monitoring programs and that require somewhat different monitoring approaches:

- Linear, open-coast beaches
- Enclosed bays and estuaries
- Inland creeks, streams, and rivers.

Both linear, open-coast beaches and enclosed bays and estuaries can have many kinds of bacteria inputs, although they differ somewhat. Open-coast beaches can receive bacteria from storm drains, river discharge, wildlife, intense beach usage, pet waste, terrestrial vegetation

decomposition, improperly maintained toilet facilities, and perhaps kelp decomposition. In addition to these potential sources, enclosed bays and estuaries can also be affected by bacteria from maintenance activities, homeless populations, groundwater, moored boats, failing septic systems, and concentrated populations of birds. The primary urban inputs of bacteria to inland creeks, streams, and rivers are through storm drain discharges, homeless encampments, unauthorized public use, and passage of domestic and wild animals. These and other differences stemming from their basic hydrology lead to a different approach for each situation. For each type of area, risk characterization should be completed in conjunction with the local health department.

The model design framework for regional monitoring adopts the probability-based design approach used by the Bight Program and assumes that, for purposes of assessment, the Bight Program does an adequate job of shoreline assessment. The Bight Program design is being used as a basis for developing a somewhat more spatially intensive regional monitoring program for enclosed bays and estuaries in the San Diego Region. Once this is completed, it can act as a design template for regional monitoring in other enclosed bays and estuaries throughout southern California. The model monitoring committee determined that, at present, concerns about recreational water quality in inland creeks, streams, and rivers were site specific enough to be dealt with by the core monitoring design. If there is a need for a regional assessment in the future, the basic stratified random sampling design used in the Bight Program would also apply here (see Section 5.1.2 Habitat assessment for more detail on regional watershed designs).

5.1.1.2 Design elements

Somewhat different monitoring designs are recommended for core monitoring at the three types of monitoring locations.

Linear open-coast beaches. Monitoring of storm drains (i.e., MS4s) discharging to beaches should conform to the prioritization framework (Table 5-2) established by the State Board's Beach Water Quality Work Group (BWQWG).

In this framework, the highest monitoring frequency of daily to five times per week is targeted at beaches with lifeguards and many potential sources of bacteria and a lower monitoring frequency (e.g., weekly to monthly) is applied to less heavily used beaches and/or beaches with only a few probable sources. Monitoring should measure indicator levels in the discharge itself, as well as upcoast and downcoast of the discharge. The basic monitoring approach includes stations situated both upcoast and downcoast of monitored storm drains because the ocean current direction in a portion of beach can frequently change, and because the dispersion of storm drain discharges in the surfzone can vary widely from place to place (due to discharge volume, bacteria concentration, beach configuration, current patterns, tidal height, and water temperature). The specific location of these stations should be determined after a characterization study of plume behavior to estimate the average seasonal range of influence of the storm drain discharge. This zone of influence will often extend further along the beach in one direction than the other, and will typically be much larger during wet weather. The upcoast and downcoast stations should then be located within the outer bounds of this influence, with a wet weather zone of influence applied to those stations that are routinely sampled during wet weather.

The model monitoring program does not include monitoring of beach coastal stations directly in front of storm drains, or "Point 0", the point in the surfzone where the storm drain discharge meets ocean water. Instead, most of the coastal beach monitoring completed by the local health departments and others is conducted at varying distances from the drain, depending on the sampling agency. Currently, county health departments do not monitor directly in front of storm

drains and freshwater outlets because all flowing outlets are posted with permanent warning signs of poor water quality. The model program follows this approach in accord with its primary intent to maximize coastal monitoring efforts by remaining consistent with the health departments' sampling protocols. However, since the ultimate management goal is to ensure that all locations at the beach are safe for swimming, stormwater monitoring agencies should be aware that the impacts to beaches directly in front of most storm drains and freshwater outlets is unknown, but are conservatively considered impacted by the health departments and the SWRCB. As stormwater monitoring and management programs progress, monitoring near drains and freshwater outlets at the beach may need to be adapted to include Point 0 monitoring. The issue of Point 0 sampling is being reevaluated by the State's Beach Water Quality Workgroup and, if the BWQWG revises the recommended health department sampling protocols, the model stormwater program should adjust to reflect this structure.

Enclosed bays and estuaries. In general, beaches in enclosed bays and estuaries (e.g., Newport Bay, Mission Bay) should be an important concern for allocating monitoring resources. This is because there is more potential for retention of bacteria, such waterbodies often have more numerous inputs, populations of birds are often denser, and children are more likely to engage in body contact recreation at these beaches. As at the open-coast beaches, monitoring effort should be allocated in proportion to relative risk, with high-use areas that have numerous inputs receiving the highest priority (Table 5-2). If all portions of an enclosed beach have equal risk, and it is not possible to monitor all urban runoff inputs, then a random subset of such inputs should be monitored, with the number of samples set based on analyses of the statistical power of alternative subsampling schemes. Monitoring should measure indicator levels in the targeted discharges themselves, as well as upcoast and downcoast of the discharge.

Creeks, streams, and rivers. As for the other two types of areas, monitoring of creeks, streams, and rivers should measure indicator levels in targeted discharges themselves, as well as upstream and downstream of the discharge. Because many inland waters in southern California are seasonal or intermittent, rather than perennial, monitoring should be prioritized with the risk-based approach described in Section 5.1.1. This approach prioritizes potential monitoring locations based on both their amount of body contact recreation and levels of bacteria contamination.

The Aliso Creek watershed in southern Orange County (which has been monitored intensively for the past two years) provides one example of how this approach can be applied. In this watershed, a recreational use survey indicated that human use "where the ingestion of water is reasonably possible" (Basin Plan definition of REC1 beneficial use) is concentrated in the lower portion of the Creek in the summer and early fall, when temperatures are warmest. An examination of two years' of monitoring data showed (Figure 5-2) that the late summer and early fall are also the period when bacterial levels are the highest. (The selection of monitoring sites was based on a field reconnaissance to identify those drains above a threshold size that typically had dry weather flow.) Given that bacterial levels are consistently elevated during this time period, if compliance with the Basin Plan REC1 objective could be demonstrated with one or two 30-day, 5-sample monitoring efforts in the late summer and early fall, which would represent the worst-case scenario, compliance is more likely during the rest of the year. This design is the most efficient approach to assessing the condition of the beneficial use; however, because large portions of the year are not monitored, it does not fulfill public health monitoring requirements.

This example from Aliso Creek illustrates the application of the criterion of allocating monitoring effort based on a qualitative risk assessment. It also demonstrates the difference between monitoring to address Question 1 and monitoring to fulfill public health requirements. Thus, even though exceedances occur during other periods of the year, the purpose of the monitoring design

in the example was to assess receiving water quality during the high-priority period. When bacteria levels in the high-priority period consistently drop to near the Basin Plan objectives in the future, it might be worthwhile at that point to expand monitoring to other parts of the year, on the assumption that indicator levels will have dropped below the objectives by that point (assuming the historical pattern stays the same, with the highest levels typically found in the late summer and early fall).

Indicators. Monitoring should use existing indicators (Table 5-1) and use comparable methods across the region. Laboratory intercalibration exercises for bacterial indicators were conducted as part of the Bight '98 and Bight '03 regional studies. Another intercalibration study, sponsored by the City of Los Angeles, will begin in early 2004 and includes most of the laboratories analyzing monitoring samples for SMC member agencies. With all indicators, the emphasis for assessment of recreational water quality conditions should be on comparison to existing standards, the Basin Plan REC1and REC2 for inland areas and AB411 for beaches.

5.1.1.3 Design issues

In general, there is currently a spatial distribution of responsibility for assessing recreational water quality conditions in the region, with county health departments having primary responsibility for beaches and for major inland water bodies (e.g., rivers, bays, lakes and reservoirs) where substantial body contact recreation occurs. In contrast, stormwater programs (i.e., MS4 permittees) tend to monitor inland storm drains and channels and, in some cases, storm drains that discharge directly to the beach. Thus, while there is some overlap between the two sets of agencies, health departments have a responsibility to protect public health while stormwater agencies focus on receiving water conditions and identifying urban runoff contributions to impaired receiving waters. As a result of their respective responsibilities, health departments typically monitor more frequently than do stormwater agencies.

To address Question 1, stormwater agencies should build upon the existing recreational water monitoring programs already implemented by local county health agencies. As a starting point, the stormwater agency should become thoroughly familiar with the existing health agencies' monitoring programs including risk characterization of beaches, monitoring locations, and sampling frequencies for both wet and dry weather. Next, with consultation from the health agency, the following types of questions should be answered to determine if additional monitoring should be conducted by the stormwater agency to answer Question 1:

- Are there urban runoff discharge points at marine beaches that are currently not monitored by the health agency?
- Are there marine beaches impacted by wet or dry urban runoff that are not monitored by the health agency?
- At marine beaches, what is the distance from the point the discharge enters the surfzone and the local health agency's monitoring location?
- What freshwater locations are frequently used for recreation? Are any of these currently monitored by the health agency or another entity? Which of these are potentially impacted by dry and wet weather urban runoff?
- Does the health agency monitor their routine sites during wet weather?

In general, data gaps in the existing health agency monitoring programs that may require new monitoring locations sampled by the stormwater agencies will likely include marine beaches where the health agencies' monitoring location is located away from the storm drain discharge point (thus, existing data may not indicate if the stormwater discharge is causing a problem on the

beach) and at freshwater locations that are currently not monitored by the health agencies. The stormwater agency should work with the health agencies to identify any new monitoring locations and to develop risk characterizations of these locations.

Stormwater agencies may be already working with local health agencies in southern California in many instances. For example:

- For the Pathogen TMDL in Newport Bay, the Orange County Health Care Agency conducts sampling while the Orange County Stormwater Program reports on the results
- In southern Orange County, the Stormwater Program samples and prioritizes coastal storm drains and reports the data to the Health Care Agency
- The Orange County Stormwater Program contracts with the Health Care Agency to conduct sampling and laboratory analyses
- The Riverside County Flood Control and Water Conservation District and San Bernardino County Flood Control District are conducting a bacterial source identification study on the Santa Ana River
- Orange County and San Diego Counties carry out routine IC/ID programs on their respective MS4s during dry weather, with a major focus on bacteria
- The City of Los Angeles conducts daily monitoring of more than two dozen beaches in Santa Monica Bay.

Such collaborative efforts formed the basis for the model monitoring committee's recommendation that such functional coordination be encouraged and expanded throughout the region, in two primary ways:

- Stormwater monitoring programs should strive to fill gaps in spatial coverage of high-priority areas not monitored by County Health Departments and characterized by the combination of elevated indicator levels and human use
- The application of adaptive triggers that would initiate upstream source identification studies by stormwater management agencies when receiving water monitoring has identified a receiving water problem.

Such a division of labor improves overall efficiency by emphasizing the respective strengths of each type of agency.

5.1.2 Habitat assessment

5.1.2.1 Overview and philosophy

Design overview. Table 5-3 presents an overview of the technical design elements for assessment of habitat status, using six distinct station types that fall into both core and regional monitoring categories (see detailed design elements in Section 5.1.2.2).

The model monitoring framework for habitat assessment is based primarily on the Triad approach, in which bioassessment, chemical, and toxicity data provide a variety of perspectives on conditions at a site. It is especially suited to situations where the primary concern is habitat or ecosystem condition and no single or simple suite of indicators afford an unambiguous measure of status (see Box on Bioassessment and Index of Biological Integrity). The framework identifies six different types of stations designed to capture the range of issues related to habitat condition, and capturing both core and regional monitoring issues. In addition to describing a decision framework for interpreting Triad results, the framework includes adaptive features intended to furnish the flexibility needed to adjust to specific local conditions and to accommodate the needs of both wet weather and dry weather sampling. However, the bioassessment leg of the Triad is best suited to perennial streams. Ephemeral stream systems may not be appropriate for routine bioassessment monitoring because they lack established biological communities except perhaps during periods in the spring.

Bioassessment and the Index of Biological Integrity

Rapid bioassessments of macrobenthic invertebrates are quickly becoming a valuable monitoring tool because biological communities are integrators of anthropogenic impacts. These organisms respond to both physical and chemical disturbances and can integrate these impacts over several storms or an entire wet season. The California department of Fish and Game (CDFG) has developed protocols for rapid bioassessments in wadeable rivers and streams and has conducted numerous surveys throughout the State. The CDFG has also developed an Index of Biological Integrity (IBI) for quantitatively assessing the status of biological communities in the San Diego Region. The Stormwater Monitoring Coalition has formed a partnership with the CDFG and the State Water Resources Control Board to build a monitoring infrastructure and standardize bioassessments throughout southern California, then refine an assessment tool, such as the IBI, for the entire region. The CDFG rapid bioassessment manual can be found at www.dfg.ca.gov/cabw/cabw/professionals.PDF

Design philosophy. The inherent complexity of watershed structure, and the variability in structure across watersheds, leads to a range of concerns about the effects of urban runoff on habitat conditions. Each concern is somewhat distinct, requiring a somewhat different monitoring approach, sampling frequency, and set of indicators. For example, some sites may be intended to measure conditions in specific, high-priority habitats (core monitoring), others to provide information about the watershed as a whole (core and/or regional monitoring), and yet others to improve knowledge about certain management issues related to urban runoff (special projects). This complexity is reflected in the several different types of habitat monitoring stations that can be established. In addition, the model monitoring design framework uses the Triad approach to organize this range of possible monitoring needs. The strength of the Triad approach (which is essentially a weight of evidence approach) is that it relies on multiple types of measures to reduce the chance of mistakenly concluding there is no impact when one in fact does exist.

5.1.2.2 Design elements

Types of monitoring sites. The committee identified several kinds of core and regional monitoring stations that could be required in assessing habitat conditions at the watershed scale:

- Long-term, fixed, bottom-of-watershed (but above tidal influence) mass emissions stations to assess cumulative water quality and aggregate loads, with monitoring based primarily on a mass emissions model and including wet weather chemistry and toxicity (core station)
- Spatially extensive, perhaps randomly sited or rotating, stations to support statistically valid comparisons across multiple watersheds, and with monitoring based primarily on the Triad

approach for dry weather sampling and on chemistry and toxicity for wet weather (regional station)

- Site-specific stations focused on the status of high-priority inland habitats of concern, with monitoring based primarily on the Triad approach for dry weather sampling and on chemistry and toxicity for wet weather (core station)
- Site-specific stations designed to generate information to support key program goals, such as source prioritization or BMP implementation and evaluation (core station, special project)
- Coastal estuarine stations to assess status in these key habitats, with monitoring based primarily on the Triad approach (core and/or regional station)
- Coastal ocean stations to assess stormwater plume impacts, conducted primarily as part of the periodic Bight surveys (regional station).

Given this potential variety of station types, monitoring within any particular watershed must be carefully integrated to achieve design efficiencies as well as an overall picture of the watershed. For example, Figure 5-1 presents an example watershed monitoring design, with a range of types of watershed monitoring stations, that illustrates how individual stations can serve more than one function within the watershed design (also see the US EPA strategy for randomized watershed sampling in US EPA 2002).

While there is an extensive body of experience in the region to support the development of core monitoring designs, this is less so for regional monitoring, or watershed-based, designs. The committee therefore outlined the following types of regional assessment designs that could be developed and implemented:

- Probability based designs, similar to the Bight Program design, in which stations are located randomly in order to provide the ability to draw statistically valid inferences about an area as a whole, rather than just the site itself. For example, the probability design used in the Bight Program permits statements about the percentage of the area that is above/below particular levels of different indicators. Such designs can allocate monitoring sites randomly throughout the entire region, or can subdivide the region into a number of strata that are relatively homogeneous. Strata can be defined on any number of grounds, depending on the questions or concerns that have motivated the program. For example, watershed strata could be based on relative amount of urbanization, general habitat type, or channel morphology, among others. Whatever the stratification scheme, the basic design principle is that samples are allocated randomly among strata, with the number of samples per stratum based on a consistent weighting factor (e.g., area of the respective strata). The level of sampling effort required in probability based designs depends, as in all designs, on the specific questions being asked, the underlying levels of variability in the data, and on the level of precision needed for decision making. The intent of the Bight Program's design, for example, is to be purely descriptive, rather than to test for conformity to a predetermined threshold or to detect a particular amount of change over time. Thus, the Program's requirement of 30 samples per stratum is based on a subjective decision by the Program's designers about the size of the confidence limit they are willing to accept in the descriptive statistics.
- Systematic designs, in which stations are located at set intervals along one or more underlying spatial or conceptual frameworks. For example, regional stations could be located on a 1-mile grid, every 1-mile along each river, creek, or stream, at every major discharge into rivers, and so on. One value of systematic designs is that they allow for more detailed mapping of indicator levels across a region. In addition, if resources permit, systematic designs can provide more thorough coverage than do probability based designs. The sampling

requirements in systematic designs are typically based on the degree of spatial resolution desired.

- Early warning designs, in which stations that are considered to be particularly vulnerable a particular impact are monitored as "canaries in the coal mine." Such monitoring can take place on a regular schedule or after the occurrence of an event thought to increase the probability of an impact past an acceptable level. The number of stations in an early warning design will depend on the number of suitable locations available and whether the potential for impact is homogeneous across the region. If the impact potential is homogeneous, then a subset of locations could adequately represent the entire region. If the impact potential is heterogeneous, then the region should be stratified in terms of impact potential and sampling within each stratum scheduled accordingly.
- Rotating designs, in which a different subset of stations in sampled during each sampling event, with the goal of sampling the entire set of stations over a certain period of time. Such designs have the virtue of maximizing the impact of limited monitoring resources because the entire suite of monitoring stations need not be sampled each time. However, because conditions change over time, rotating designs have a diminished ability to support valid comparisons between sets of stations sampled at different times in the rotation schedule. This can be compensated for to some extent by defining comparisons of interest during the design process and then ensuring that such stations are sampled during similar index periods or seasons. The location of stations in rotating designs can be random, systematic, or early warning depending on the kinds of questions being asked.

Evaluating Triad results. Once monitoring data are available, determining whether conditions are protective of beneficial uses depends on a combination of explicit definitions of reference conditions (see Table 4-4) and the ability to interpret results in the context of individual watershed conditions. Given the potential complexity of ecosystem impacts, the committee agreed that no single benchmark should be automatically used as evidence of impact. Thus, there are no hard and fast rules for determining that a receiving water impact has occurred. However, Table 5-4 provides an organized set of rules of thumb for interpreting Triad results and determining if further studies are warranted. Where the full Triad has not been sampled, Basin Plan, Ocean Plan, and other reference benchmarks listed in Table 4-4 could be applicable.

Adaptations of the basic design. Because of the range of specific situations that may occur in different watersheds, the basic design shown in Figure 5-1 may be adapted with a variety of alternative approaches. For example:

- Chemistry and toxicity could be used in wet weather when the bioassessment leg of the Triad is not feasible (e.g., in high flow conditions when biological communities. Any finding of impact could be investigated further with the complete Triad during dry weather (except in ephemeral streams, which rarely have dry-weather flow)
- Toxicity tests could be used in lieu of broader chemistry scans where historical data demonstrates no evidence of impacts at the site and there is no *a priori* reason to believe there are significant sources of chemical contamination
- Bioassessment could be used in lieu of toxicity tests and chemistry scans where the primary concern is the status of a particular habitat and historical data demonstrates no evidence of impacts from urban runoff at the site
- The spatial and temporal intensity of sampling could be adapted to match the spatial scale of the site and the temporal scale of the processes that influence habitat condition
- The suite of chemical analyses can be adjusted (see Tables 5 and 6, and following subsection) based on prior knowledge about sources of contamination.

Thus, the model framework provides an overall context for assessing and tracking habitat status, while allowing for the flexibility needed to make the best use of available information to adapt to specific management information needs.

Constituent list. While the committee emphasizes that the Triad approach (bioassessment, toxicity testing, chemistry monitoring) works best when all three legs are consistently sampled, it also recognized that certain situations may call for sampling only one or two, rather than all three, legs. Thus, the particular combination of Triad measurements to be collected at any individual site or time could be based on the season of the year (wet vs. dry), the location and purpose(s) of the station, the specific problem or question being addressed, the past history of monitoring results at that location. In general, however, sampling effort might be distributed as in Table 5-3 and Table 5-5 (core monitoring column).

The model monitoring committee gave particular attention to the suite of chemical constituents that should be measured at the watershed stations, attempting to balance a desire for regional comparability with the ability to adapt to the specifics of each situation. The committee developed a short list of common constituents (Table 5-6) to be sampled routinely by all programs and an expanded list, some of which would be sampled if needed. In addition, the full EPA priority pollutant list would be sampled once every several years in concert with the regional Bight Program.

A decision about when to add constituents from the expanded list would be dependent on both available information and the management question(s) being asked. For example, past monitoring data or data on historical land uses indicating the presence of legacy pesticide contamination could cause these constituents to be added to the program. As another example, where the focus is on total loads or trends, as at the mass emissions stations, then total metals would be the appropriate monitoring target. In contrast, where receiving water impacts are the primary concern, as at specific habitat stations, then dissolved metals should be measured. It will thus be important to consider the potential use(s) of the monitoring data when deciding which constituents to monitor. Dissolved metals might also be measured when toxicity has been found, the site is on the 303(d) list, or total metals exceeds the relevant CTR value, which is often used as a benchmark in receiving waters for stormwater effects.

Flow measurement and compositing approaches. Many field sampling methods relevant to stormwater monitoring programs are described and reviewed in BASMAA (1995), as well as in various USEPA guidance documents (e.g., USEPA 1992). Of particular interest are methods for estimating flow and compositing approaches for deriving mass emissions estimates.

In general, there are two basic methods for estimating flow. The first is based on engineering equations that use gravity, the height of water in an idealized pipe, the slope of the pipe, and a friction coefficient to derive the flow. The second is based more on direct measurement of the speed of the flow, combined with an estimate of the cross sectional area of the water (computed from the shape of the channel and the height of the water) to derive the flow. There are variations within each of these basic methods. For example, the engineering equations can use unimpeded flow in a pipe or channel or, alternatively, the height of flow over a weir. Similarly, direct flow measurements can be based on the rate of spin of a paddlewheel or on ultrasonic signals from sensors in the water. In addition, there is a range of methods for measuring the height of the water in a channel, the other key input to flow estimates. These methods range from simple staff gauges to various types of pressure transducers or ultrasonic sensors suspended over the water. As a rule of thumb, the more engineered a hydrologic system is, the easier it is to rate for flow. For

example, a concrete channel will typically have a constant cross section and the slope will be constant.

Different approaches have different strengths and weaknesses depending on the particular situation. For example, flow sensors that are mounted in the water are vulnerable to damage from debris carried in storm flows. Downward looking flow sensors can be more suited to smaller channels with space constraints, however, foam on the surface of the water can degrade the accuracy of the reading. Various pressure transducer models differ in their sensitivity and the maximum height of water they can accurately measure. Where the channel configuration permits, flow can be routed through a flume for more accurate measurements; however, flumes cannot handle large volumes of flow.

The United States Geological Survey (USGS), which is generally recognized as producing the most accurate and precise flow estimates, measures flow velocity at several points along a channel cross section at several times with different water heights. These data are then used to develop a flow rating curve specific to that channel. The rating curve can then be used to estimate flow based simply on the height of water at any given time, on the premise that water at a particular height will be moving at a specific speed. This is an empirically derived relationship as opposed to one based on modeled engineering principles. While this method can be the most accurate, it is also the most difficult to implement in terms of up-front effort and costs. In addition, changes to the channel morphology due, for example, to siltation or erosion can undermine the accuracy of a flow rating curve.

The various approaches to flow measurement also differ in terms of their relative accuracy and precision. The USGS attempts to achieve accuracy to within 5%, but that level can be difficult to achieve in channels with scouring, filling, and other sources of bias. Despite the potential drawbacks of empirical methods, they are considered to have better accuracy than the model-based engineering methods, although it can be very expensive to improve the precision of the empirical estimates. In contrast, the engineering methods can produce relatively precise estimates, but the accuracy may be less than that achievable with empirical methods, depending on the degree to which model assumptions are violated. The model monitoring committee was reluctant to propose specific performance standards for flow monitoring. The preferred approach in any particular situation will depend on site characteristics and the use(s) intended for the data.

In contrast, the committee considered that performance standards for the measurement of mass emissions, especially as part of a long-term trends monitoring program, were more relevant. There are two primary methods for estimating the concentrations of constituents of interest in urban runoff. The first, flow compositing, collects water samples for analysis at specific increments of flow. The second, time compositing, collects water samples at specific increments of time. These two approaches were described in more detail and compared in an intensive year-long sampling program (Leecaster et al., 2002) that found that flow compositing was the most efficient sampling approach to achieve a given degree of accuracy and precision. A minimum of 10 to 12 samples per composite using a flow-weighted scheme efficiently reduced bias and improved precision. Time-weighted composites can achieve similar levels of precision and bias, but required a far greater number of samples; more than 42 samples per composite were necessary.

5.1.2.3 Design issues

Ecosystem perspective. There are four major habitat types in the region:

- Ocean
- Estuaries / wetlands
- Streams, creeks, and channels
- Lakes and reservoirs.

Because they are somewhat of a special case, the committee agreed to set lakes and reservoirs aside for possible consideration at a later time. Based on the Bight '98 study in the coastal ocean, which showed that riverine effects on the benthic ecosystem are small, the committee agreed that further study of stormwater impacts in the coastal zone should be the focus of regional efforts (as in Bight '03). Such regional studies could then provide more concrete guidance to individual programs where urban runoff plume effects (perhaps on the water column) are found to be substantial. Thus, the model monitoring program framework focuses explicitly on streams, creeks, channels, and rivers, and on estuaries and wetlands.

Habitat monitoring for these habitat types can involve a wide range of methods, including:

- Water chemistry
- Sediment chemistry
- Aqueous toxicity
- Sediment toxicity
- Bioaccumulation
- Bioassessment
- Hydrology

Given this variety of potential measurements, the committee determined that monitoring should be based on an ecosystem perspective, rather than consisting of collections of functionally disconnected measurements on the one hand, or focusing on individual species or chemical parameters on the other. The Triad approach, which combines chemistry, toxicity, and bioassessment (including physical habitat measures) provides a practical means of integrating a wide range of measurements, as well as a structure on which to base adaptive follow-up monitoring. It should be noted that the bioassessment leg of the Triad may not be applicable in some situations, such as ephemeral streams, where minimum requirements are not consistently met. However, the overall watershed monitoring framework (see Figure 5-1) also provides a structure for including sites targeted at specific management issues such as problem characterization or BMP evaluation.

5.2 Extent and magnitude monitoring

Evaluation of the extent and magnitude of receiving water problems addresses Question 2: What is the extent and magnitude of the current or potential receiving water problems? Monitoring related to Question 2 provides useful information for prioritization of both source identification studies (Questions 3 and 4) and specific management actions intended to remediate the problem. Monitoring of the extent and magnitude of problems falls primarily into the special projects category (Table 4-1) because these are typically efforts targeted at specific problems and with clear beginning and ending points. However, to the extent that such studies require collaboration among multiple responsible parties and/or extend over large areas, they would also have some of the characteristics of regional monitoring.

5.2.1Extent and magnitude assessment – recreational water quality

5.2.1.1 Overview and philosophy

Table 5-7 presents an overview of the technical design elements for regional monitoring of recreational water quality monitoring focused on estimating the extent and magnitude of receiving water problems.

The model monitoring framework for assessing the extent and magnitude of recreational water quality problems assumes that the stormwater agency will work with local health departments to determine those high-priority (i.e., combination of human use and contamination) locations where extent and magnitude of a bacteria problem should be defined. Currently, coastal beach monitoring by local health agencies near urban runoff discharge points is comprised of one or two fixed stations located at various distances from the point of discharge. Thus, often the length of beach impacted by urban runoff has not been fully characterized. In most cases, even less data is likely available for inland freshwater sites. The monitoring design to determine the extent and magnitude of bacterial contamination should include estimates of bacterial loads, in addition to upcoast/downcoast (at beaches) or upstream/downstream (along creeks, streams, and rivers) arrays of samples. An estimate of temporal persistence would depend on monitoring through at least one complete year.

The extent and magnitude monitoring design is essentially the same for both regional monitoring and special projects aspects of the program, with regional monitoring encompassing a larger area and/or greater numbers and kinds of potential sources (see 5.2.1.2 Design elements).

5.2.1.2 Design elements

Regional monitoring to establish extent and magnitude is distinguished from special projects in its larger geographic scale and/or greater number and kinds of potential sources. The committee did not establish an explicit dividing line between these two categories of monitoring (i.e., regional and special projects), since real-world situations will exist on a continuum of scale and complexity. Regional monitoring will therefore most likely involve a wider range of parties and require more collaborative implementation. However, this is not a substantive design issue because the basic design approach is the same for both regional monitoring and special projects.

A monitoring design to establish the extent and magnitude of bacterial contamination must have the ability to determine:

- The degree of temporal persistence of a particular receiving water problem
- The spatial extent of a particular receiving water problem
- The relative severity of a particular receiving water problem, compared to other parts of the region.

Therefore, the monitoring design for this question should include:

- The core or regional monitoring assessment site(s) in the location of interest
- Measures of bacteria loads, which requires flow estimates
- Measures of the spatial extent of actual impact in receiving waters, which requires an array of upstream/downstream samples in creeks, and upcoast/downcoast samples, regularly spaced grids, or random arrays on the beach and in bays/estuaries
- Measures of temporal persistence or pattern, such as between wet and dry weather, which requires at a minimum samples through one calendar year.

Depending on the extent of existing knowledge, these design elements may be scaled as needed to fill data gaps. For example, where the spatial extent of contamination is well understood, additional sampling at only a few representative stations might be required to define the temporal extent of contamination. Conversely, where the spatial extent is not well understood, a survey of shorter-term but more intensive monitoring at an array of stations (either regularly spaced or random, depending on the site) might be necessary to define the boundary of contamination during periods when human use and contamination combine to create a high-priority period. Finally, if the spatial and temporal extent are well defined, a focused sampling effort during one or more representative subsets of the high-priority period might be used to determine peak loads and/or receiving water levels. A rule of thumb in such studies is to use the highest sampling frequency possible in order to better characterize the nature of variability in extent and magnitude. The key adaptive element of the bacteria monitoring design for assessing extent and magnitude thus includes the ability to modify the spatial and temporal intensity of sampling as needed, both in the discharge and the receiving waters.

Indicators in studies of the magnitude and extent of recreational water quality problems should include the levels and loads of the three main bacterial indicators (Table 5-7), along with other measures that may add useful information (e.g., stream or channel flow, patterns of human use).

5.2.13 Design issues

Existing sampling effort may be adequate in many cases to characterize the spatial and temporal extent of bacterial contamination, along with its severity. For example there already exist substantial monitoring data on levels of bacterial indicators at many coastal monitoring sites. Additional monitoring effort is being initiated along the San Diego and southern Orange County coasts, targeted at coastal storm drains, and at specific inland sites as part of these counties' dry weather reconnaissance and IC/ID programs.

In many cases, however, existing monitoring designs may not be optimal for measuring the extent, magnitude, and severity of bacterial contamination associated with urban runoff. At coastal locations near urban runoff discharges, sampling data that provides length of beach impacted by bacteria densities above the health standards may not available. Many factors can affect the length of beach impacted including the bacteria densities and flow rate of the urban runoff discharge, surfzone conditions including swell, wind and tide, and the configuration of the storm drain and beach relative to the incoming swell. Routine coastal monitoring completed by local health agencies may not capture extent. At freshwater inland sites, less routine monitoring data are collected by local health agencies and data on the extent of the problem will typically be limited to special studies. The extent and magnitude of bacteria problems along inland creeks, streams, and rivers resulting from urban runoff discharges may be particularly difficult to assess because there are often many, diffuse sources of bacteria, including natural sources. For both coastal and freshwater sites, defining extent of impact from individual discharges during wet weather may be difficult because plumes from separate discharges will often overlap and because of increased loading of bacteria from natural sources (particularly at freshwater locations). A further complication stems from the fact that extent and magnitude are likely to be very different in wet and dry weather.

In addition to these issues, data from the various bacterial monitoring programs are not aggregated, making it difficult to identify broad spatial patterns and temporal trends, and only recently has a laboratory intercalibration study for bacteria been undertaken. Further, there are growing concerns that the bacterial indicators, alone, may not provide an accurate picture of the extent and magnitude of actual human pathogen contamination. Not only do the indicators not measure pathogens directly, there is some evidence that the indicators themselves may propagate

in the MS4 system and may derive partly or entirely from animals and birds. Thus, even a data aggregation and mapping exercise that described indicator patterns in detail might not necessarily describe the extent and magnitude of actual pathogen problems associated with urban runoff.

While the model monitoring committee developed quantitative metrics to assist in prioritizing studies under other aspects of the overall program (e.g., TIEs, upstream bacterial source identification, it determined that the expert judgment of health department staff is the best source of information for triggering efforts to determine the extent and magnitude of bacterial contamination in each of the three kinds of areas (linear open-coast beaches; enclosed bays and estuaries; creeks, streams, and rivers). Thus, stormwater program staff would review monitoring data with health department staff and representatives of other potential sources to determine if they have completed additional sampling or have knowledge of data that establish the extent and magnitude of contamination, and to receive recommendations from health agency staff about which monitoring locations should be the first priority for additional efforts.

5.2.2 Extent and magnitude assessment – habitat

5.2.2.1 Overview and philosophy

The model monitoring framework for assessing the extent and magnitude of habitat problems builds on the core monitoring Triad approach, by adding repeated measurements to characterize temporal persistence, upstream sampling of the Triad components to describe spatial extent, and/or adaptive features such as TIEs or targeted upstream source identification studies to better define the magnitude of the problem (see Table 5-4). These latter two types of studies begin to merge into the kinds of special project source identification efforts described in Sections 5.3 and 5.4, illustrating the fact that real-world distinctions between monitoring categories are not always clear cut.

The extent and magnitude monitoring design is essentially the same for both regional monitoring and special projects aspects of the program, with regional monitoring encompassing a larger area and/or greater numbers and kinds of potential sources.

5.2.2.2 Design elements

Regional monitoring to establish extent and magnitude is distinguished from special projects in its larger geographic scale and/or greater number and kinds of potential sources. The committee did not establish an explicit dividing line between these two categories of monitoring (i.e., regional and special projects), since real-world situations will exist on a continuum of scale and complexity. Regional monitoring will therefore most likely involve a wider range of parties and require more collaborative implementation. However, this is not a substantive design issue because the design approach is the same for both regional monitoring and special projects.

Table 5-4 provides an overall framework for a set of adaptive monitoring and special study responses to a finding that there is or could be a receiving water problem, many of which focus on determining the magnitude, extent, and/or severity of any such problem. The type and design of any such adaptive monitoring in a particular instance will depend on the results of the Triad measurements, site-specific factors, and other types of relevant knowledge such as land use data or information on upstream sources. For example, additional toxicity tests at higher dilutions, accompanied in some instances by TIEs, can provide more information about the nature of toxicity (as described above, this is one example where assessment of the extent and magnitude of a problem would overlap somewhat with source identification special projects). Or, repeating toxicity tests with different toxicity test organisms could also improve the understanding of toxicity. In addition, repeating routine measurements over time at a specific station or group of related stations will determine the temporal extent of the problem. Similarly, extending an array

of stations upstream and downstream of the original monitoring station will help assess the spatial extent of the problem.

While Table 5-4 presents a conceptual overview of possible studies, the specific efforts required in any particular situation will depend on which leg(s) of the Triad have been sampled, on the nature of the monitoring findings, and on the characteristics of the environment. Issues that should be considered in designing adaptive studies of extent and magnitude include:

- The nature of the "signal," e.g., which leg(s) of the Triad are involved
- The strength of the "signal"
- Available information about possible causes of actual or potential problems
- The spatial and temporal extent of the habitat of concern
- Local geography and hydrology.

The preferred monitoring design, whether it be regional or a special study, is described in detail within the section on habitat assessment (Section 5.1.2). There are a variety of approaches for allocating sites including stratified random, systematic, or rotating designs depending on the specific area to be evaluated and indicators to be measured. For sure, managers will want to integrate the extent and magnitude designs into design(s) for assessment, which will maximize continuity and cost-efficiency.

5.2.2.2 Design issues

Assessing the extent and magnitude of impacts on ecosystem health will begin with an assessment of results from the suite of core watershed stations. As described in more detail in Section 5.1.2, stations should be located in receiving waters with key beneficial uses, where significant contamination problems related to urban runoff are known to exist, where the likelihood of such problems is high, in high-value habitats whose continued protection is a high priority, at core mass emissions stations, and at distributed locations that will provide a basis for comparisons among watersheds.

5.3 Urban runoff contribution assessment

Assessment of the relative contribution of urban runoff to a receiving water problem addresses Question 3: What is the relative urban runoff contribution to the receiving water problem(s)? Data from this monitoring element are useful primarily in prioritizing more extensive source identification efforts under Question 4. Assessments of the urban runoff contribution fall into the special projects category (Table 4-1) because such studies are targeted, one-time efforts. However, they may also take on the collaborative aspects of regional monitoring if they involve multiple parties and/or cover large areas (Table 4-1).

5.3.1 Overview and philosophy

The model monitoring framework for assessing the relative urban runoff contribution to both recreational water quality and habitat problems is primarily a matter of loads estimation at a fixed downstream reference point. Similar loads estimation approaches apply to both recreational water quality and habitat indicators, as described in the following sections, including expert judgment, visual reconnaissance, land use modeling, empirical tributary monitoring, the use of conservative tracers, and the evaluation of existing data (see Box on Modeling). The actual combination of methods in any particular instance will depend on the quantity and quality of historical data, the

nature of the receiving water problem, the number and types of potential sources, and the physical structure and hydrography of the watershed. In addition, many of the methods applicable to this issue are also directly applicable to the more detailed source identification special projects described in Section 5.3 and 5.4.

The extent and magnitude monitoring design is essentially the same for both regional monitoring and special projects aspects of the program, with regional monitoring encompassing a larger area and/or greater numbers and kinds of potential sources. The source identification case studies in Appendix 3 include examples of both. For example, the Contaminated Sediment Task Force study in Los Angeles Harbor was a regional study involving many participants, while the investigation of elevated total dissolved solids in Orange County was a special project conducted by the County Stormwater Program alone.

Modeling

Watershed modeling is a useful tool for estimating flow, concentrations or loads from unmonitored watersheds or unmonitored storm events. There are a variety of models available to watershed managers, from very simplistic spreadsheet-based techniques to very complex time-variable algorithms. The decision on which model to use is a function of the management questions and types of assumptions watershed managers are willing to make, as well as the availability of data for running the model. Simplistic models (i.e. rational method) answer questions at large temporal and spatial scales, make the most assumptions, and require the least data. Complex models (i.e. HSPF, SWMM, etc.) answer questions at finer temporal and spatial scales, make fewer assumptions, but require the most data. See Singh and Woolheiser (2002) for a recent review of watershed hydrology models.

5.3.2 Design elements

This section describes a general set of approaches to source identification, accompanied by a set of illustrative case studies presented in Appendix 3. The committee chose this approach because the wide variety of specific situations in which source identification studies might be required makes it impossible to define a standard approach.

Assessing the relative urban runoff contribution to a particular receiving water problem involves loads estimation at a fixed downstream point, which is in or near the affected receiving water. Depending on how the receiving water is defined in a particular instance, "downstream" may be at the point where a tributary enters a larger creek, where a creek enters a wetland or estuary, or where a river empties into the ocean. Similar loads estimation approaches apply to both recreational water quality and habitat indicators, including:

- Expert judgment
- Visual reconnaissance and observation
- Land use modeling
- Empirical tributary monitoring
- The use of unique and/or conservative tracers
- Evaluation of existing data.

These approaches can be extended with more detailed information provided in US EPA (1993), Pitt (2001), and SWRCB (2001), which describe a range of methods for identifying sources of stormwater pollution. A decision about which approach(es) to use in any particular instance will depend on the quantity and quality of historical data, the nature of the receiving water problem, the number and types of potential sources, and the physical structure and hydrography of the watershed. Thus, even a preliminary loads estimation for a high-priority bathing beach, such as in Mission Bay in San Diego, might proceed through several steps from expert judgment which provides the basis for targeting visual observation which in turn forms the basis for modeling

and/or empirical measurement. Where information from the Triad approach is available, Table 5-4 provides an example of a decision framework for interpreting monitoring results to better focus preliminary source identification efforts.

One key element of the committee's thinking is that such preliminary loads assessments should ideally be a collaborative effort, undertaken by all the parties responsible for potential inputs to the receiving water. Such regional, collaborative efforts will be more efficient and more productive because they will streamline data acquisition, integration, and evaluation. They can also provide a basis for future, more intensive, collaborative source identification efforts, should they be required.

While in general the needed accuracy and precision is only low to moderate, the degree of accuracy and precision needed will depend in part on the relative size of the urban contribution to the overall loads. For example, if the urban contribution is small (i.e., less than 5% of the cumulative load), there would probably be no need to refine the estimate any further because large variability does not change the answer to the question; urban runoff is still a small contribution. In contrast, if the urban contribution is 15% +/- 15%, there would be a need to refine the estimate to determine whether and to what extent to proceed to the more detailed source identification work described in Section 5.4. Thus, monitoring designs for this issue might proceed through multiple iterations.

When identifying and characterizing potential sources, it is important to use terminology that is consistent with standard USEPA usage. Thus:

- Urban runoff: both wet (stormwater) and dry weather (non-stormwater) runoff from urban land uses
- Dry weather runoff: runoff from urban land uses in dry weather
- Stormwater runoff: runoff from urban land uses during storms

In addition, there are other land uses and sources that discharge to MS4s but that are typically not under the jurisdiction of municipalities, including:

- Industry and POTW discharges (which are regulated by state permit)
- Other discharges permitted by the RWQCB
- State and federal facilities
- Agriculture
- Augmented water
- Open lands
- Native American lands
- Special districts, school districts, parks
- Utilities
- Aerial deposition.

5.3.3 Design issues

There are two primary design issues associated with determining the relative urban runoff contribution to a receiving water problem. The first is the fact that the wide variety of specific situations likely to be encountered makes it infeasible to recommend a standard design. The committee resolved this issue by providing general guidance on study design, referencing two reports that describe detailed monitoring methods, and including a set of representative case

studies in Appendix 3. The second is that there may be cases in which the relative urban runoff contribution is small. In such cases, the committee agreed that a municipal permittee should not be obligated to **independently** conduct detailed source identification studies beyond the activities already required in their respective NPDES permits. The committee therefore recommended a threshold level of urban runoff contribution above which permittees would be required to independently perform detailed source identification studies, and set this level at 5 – 10%. A lengthier discussion of the threshold issue is provided in Section 4.3.

5.4 Source identification studies

More detailed source identification studies address Question 4: What are the sources to urban runoff that contribute to receiving water problems? These are almost always special studies and are conducted when preliminary source identification work under Question 3 (Table 4-1) shows that urban runoff constitutes a significant portion of the source(s) of a receiving water problem. Information from these more detailed special projects can help refine receiving water monitoring, improve fundamental understanding of stormwater contamination processes, and help guide management actions intended to reduce sources and their attendant impacts.

5.4.1 Overview and philosophy

Table 5-8 presents an overview of the technical design elements for special projects monitoring of recreational water quality and habitat focused on source identification. Since the primary philosophy of the model program is not to design site-specific studies, this section provides guidance on adaptive triggers for special studies. Therefore, this section creates a series of starting and stopping rules for when to initiate detailed source identification studies and tools for prioritizing locations on where to conduct them.

The model monitoring framework for detailed source identification for both recreational water quality and habitat involves two kinds of studies. The first are studies at downstream stations to gain additional insight into the sources of the problem. For bacteria, this may include more traditional sanitary survey methods and/or more sophisticated biological testing. For habitat, this may include toxicity tests with a broader suite of test organisms, TIEs, or more detailed analyses of the pattern of impact in communities or on key organisms. The second kind of study will be upstream source tracking and source identification studies that may use a variety of methods. In general, however, they will share the same design, which will involve using a basic indicator of impact (e.g., bacterial indicator, toxicity) to trace the strength of the impact signal upstream, in either wet or dry weather, combined with more powerful and/or targeted methods (e.g., genetic source identification, TIEs, chemical reconnaissance, physical reconnaissance) to locate the specific source(s) of pollution.

5.4.2 Design elements – recreational water quality

There are two primary design elements for source identification related to recreational water quality. The first is to identify and then prioritize the upstream sites at which source identification efforts will be conducted. The second is to identify a core set of methods for bacterial source tracking at these sites. The approaches for these issues are somewhat different for beaches and for inland waters because inland waters (i.e., creeks, streams, and rivers) have a clear upstream – downstream morphology while beaches may not. Instead, contamination from a discharge can often spread out in both directions along a beach.

Open-coast and enclosed beaches. In contrast to creeks, there is no consistent and obvious upstream – downstream relationship between urban runoff inputs (typically storm drains) and the receiving water. Thus, it is not possible to estimate impact in terms of the difference between an upstream and a downstream station. The committee therefore proposed a prioritization approach based on the relationship between bacterial levels in individual storm drains and levels in the nearby receiving water.

Figure 5-3 demonstrates this approach with bacterial monitoring data from San Diego County. The figure is divided into five sections that reflect different relationships between indicator levels in the receiving water and those in the outflow of the coastal storm drain itself. In general, higher priority is given to storm drain discharges that are consistently high and receiving water densities that exceed health standards. While Figure 5-3 illustrates a prioritization approach specifically for fecal coliform, parallel methods could readily be developed for total coliforms and Enterococcus, since standards for these indicators in marine waters have been developed.

Once a subset of inputs has been identified for further source identification efforts, the well-accepted approaches described in US EPA (1993), Pitt (2001), and SWRCB (2001) are excellent sources of guidance. When implementing such approaches, it will be important to be systematic and thorough yet also have clear stopping points (Figure 5-4). In particular, the stopping rules are prioritized to focus on determining, first, whether there identifiable sources of human sewage and, second, whether there are other controllable anthropogenic sources. First and foremost, stormwater agencies need to identify and remove all sources of human inputs (See Box on Microbial Source Tracking). The committee agreed that further source identification efforts for nonhuman inputs should await the development of more powerful microbial source identification tools with the ability to more accurately distinguish among a range of specific sources (e.g., livestock, pets, birds, other wildlife). This testing is currently being conducted by the SMC and others (Griffith *et al* 2003).

Microbial Source Tracking

Microbial Source Tracking (MST) is a class of potentially powerful tools for identifying sources of bacteria in receiving waters. The traditional fecal indicator bacteria typically measured by county health departments are not human specific and can arise from any warm-blooded organism including birds, dogs, cats, livestock, horses or other mammals. Thus, the goal of most MST techniques is to determine if the measured indicator bacteria are of human origin and, if not, what was their host of origin. There are numerous MST techniques available, but all are still experimental. The Stormwater Monitoring Coalition co-sponsored a study in 2002 to test 11 MST techniques by 22 of the nations' leading researchers for their accuracy and precision in southern California. The results, which are summarized in the *Journal of Water and Health* (Volume 1, No. 4, November 2003), show that none of techniques worked perfectly and many were susceptible to false positives. As a result of the intercalibration study, research continues on refining and improving the more promising methods, but a single definitive technique(s) is still unavailable.

Creeks, streams, and rivers. Source identification studies in creeks, streams, and rivers are particularly problematic because bacteria are not conservative in the MS4, may originate from a wide range of small, diffuse sources, and can be highly variable in both space and time. However, because bacteria die off due to ultraviolet (UV) exposure as they flow downstream, there may be an upper limit on the distance bacteria can travel in longer natural creeks and streams and still impact high-priority areas of concern. (Bacteria can also be removed through sedimentation; however, during the low flow conditions characteristic of dry weather in southern California, UV exposure is the dominant factor.) Therefore, the committee developed a conceptual model (Figure 5-5; Appendix 4) to identify and prioritize inputs for upstream source identification work. This conceptual model also assumes that core monitoring has shown there is an exceedance of a bacteria water quality objective in a high-priority recreational use area, and

that either regional monitoring or special projects that it is persistent and large enough to warrant further action, and that urban runoff constitutes a substantial proportion of the source(s).

The following steps describe how to apply this conceptual model in a particular situation:

1. Locate high-priority use area
2. Define upstream boundary of high-priority use area
3. Calculate the number of days required for 95% of bacteria to die off, using the equation in Appendix 4 and an inactivation rate selected from the range presented in Appendix 4
4. Calculate average net downstream flow rate of the creek or stream in meters/day
5. Calculate the linear distance required for 95% of bacteria to die off, using the following equation:

   Days required for 95% die off x flow rate in meters/day = X meters
6. Define an upstream segment with its bottom edge at the upstream boundary of the high-priority use area and its upstream edge X meters upstream above that.

There are two constraints that would affect the application of this conceptual model. First, it may be most appropriate in dry weather in longer natural creeks and streams with relatively slow flow rates, because bacteria die off rates in creeks that are partially or fully concrete-lined may be less than transport times. For example, it would be less applicable to systems with discontinuous flow. Second, spatial and temporal variability in bacteria densities mean that deriving more than rough estimates of the upstream segment may require substantial sampling effort. However, even a somewhat rough estimate could prove valuable in focusing upstream source identification studies. Thus, this conceptual model is not directly applicable to all situations and should be applied carefully. For example, in the Santa Ana River above Prado Dam, flow during dry weather is discontinuous and consists of disinfected POTW effluent and rising groundwater. Though bacteria levels in this case exceed REC1 standards, there are no dry weather urban runoff discharges and the conceptual model would not be directly applicable.

Within this upstream portion of the drainage system, termed a "potential source segment," there may be a number of discharges or other inputs that must be prioritized for source identification study. The committee developed a unique tool for prioritizing such inputs, based on a combination of their loads and local impact on the receiving water, as explained in the following paragraph.

The influence of inputs within the potential source segment on the downstream high-priority recreational use area will result from a combination of the size of the input (bacterial load) and the effect of each input on the receiving water (impact). This is because loads alone do not reflect a discharge's potential impact on the receiving water. A large load discharged into a creek section with high flow may have little downstream effect, while a small load discharged into a creek section with low flow may have a disproportionately large downstream effect. Thus, prioritization of inputs for upstream source identification efforts, as well as monitoring of the inputs in the potential source area, should be based on both loads and impact, with impact measured as the difference between bacterial indicator levels at upstream and downstream stations. Table 5-9 demonstrates the committee's approach for combining measurements of both load and impact into a single metric for prioritizing a series of inputs. Generating this metric involves the following steps:

1. Calculate the bacterial load of each direct input to the creek within the potential source segment

2. Calculate the receiving water impact of each direct input, measured as the simple difference in bacterial concentration between stations 25 feet upstream and downstream of the input
3. Scale loads values from 0 – 1, with the lowest load assigned the value of 0 and the highest the value of 1
4. Scale impact values from 0 – 1
5. For each input, calculate the average of the scaled loads and impact values as:

$$\frac{\text{Scaled load} + \text{scaled impact}}{2}$$

6. Rank inputs within the potential source segment in terms of their average scaled value
7. Select highest ranked inputs for further source identification efforts upstream of each input.

If desired, loads and/or impact estimates could be weighted to emphasize one or the other to a greater degree. The highest ranked inputs would be selected for further source identification efforts, with the threshold established based on the pattern of average scaled values and cost and logistical constraints. It will be important to ensure that the data used for calculating this metric be gathered during that portion of the year when human health risk is the highest. It is also important to recognize that the relationship of each individual input in potential source segment to health-based water quality objectives in their immediate vicinity is not directly relevant to the prioritization exercise. There are two reasons for this. First, the prerequisite for the upstream prioritization exercise is that the downstream high-priority recreational use area has been determined to exceed water quality objectives on a regular basis. Second, a series of inputs could all contribute to a cumulative problem at the downstream use area, even if none of them individually exceeds water quality objectives.

Similar to the approach for beaches, once a set of inputs have been identified as potential sources of receiving water problems, upstream source identification studies on creeks, streams, and rivers should be conducted. The stopping rules described above for beaches and in Figure 5-4, are also directly applicable to creeks, streams, and rivers.

5.4.3 Design elements – habitat

The design elements for source identification for habitat are somewhat more complex than for recreational water quality. There are two main reasons for this. First, it is more difficult to quantitatively prioritize sites because the Triad approach involves three distinct types of data and there are no established standards or benchmarks for two of these, toxicity and bioassessment. Second, because these three data types sometimes produce inconsistent results (see Table 5-4 for examples), it can be difficult to establish clear benchmarks for when the weight of evidence calls for upstream source identification efforts. In addition, the availability of complete Triad data, as well as the interpretation of monitoring results, may be more complex in certain situations, such as ephemeral streams.

Rather than a quantitative metric, such as that shown for bacterial indicators in Table 5-9, the committee developed an overall framework for implementing a weight of evidence approach to triggering additional, targeted source identification studies (Tables 5-10 and 11). Table 5-10 provides expanded definitions of the thresholds in Table 5-4 that would trigger additional adaptive studies in response to combinations of Triad results. Table 5-11 then assigns a priority for source identification studies to each possible combination of Triad results from Table 5-4. Thus, in Table 5-11, the combination of results represented by Row 3 of Table 5-4 (persistent chemical exceedances, no toxicity, no benthic impact) would have a low priority for source identification studies. In contrast, the combination of results represented in Table 5-11 by Row 7 (no chemical exceedances, high toxicity, benthic impact) would have a high priority.

Monitoring results should be evaluated, using Table 5-4 as guidance, to determine whether the probable source(s) of impact is physical, chemical, or unknown. Upstream source identification efforts should then be initiated according to the set of priorities suggested in Table 5-21. Upstream source identification efforts should build on those performed to preliminarily assess the urban runoff contribution to receiving water problems, and should include detailed visual inspection of MS4s, water courses, and drainage areas as a first step. Visual inspections can then be followed with the water quality based source identification methods described in U.S. EPA (1993) and Pitt (2001).

As part of this overall framework, the committee did develop a quantitative method for combining toxicity testing results into a single metric (see Appendix 5) that would assist in ranking stations in terms of their aggregate toxicity. This ranking can then be used to assign priorities to stations for follow-up TIEs, as part of a source identification effort. The metric combines information about the degree of toxicity, the persistence of toxicity at a station throughout the year, and the percentage of test species found to exhibit toxicity.

Table 5-11 sets forth a set of starting rules for source identification efforts targeted at habitat impacts. Stopping rules are similar to those described for recreational water quality (Figure 5-6), with the same emphasis on identifying controllable sources.

5.4.4 Design issues

The same basic methods for detailed source identification apply to both recreational water quality and habitat. While the specific methods used in any instance will, of course, differ somewhat depending on the watershed structure and the constituents involved, they will include one or more of the following set of approaches, which are listed in order of increasing effort involved:

1. Evaluation of existing data
2. Visual reconnaissance and observation
3. Empirical tributary monitoring, which involves sampling tributary mouths upstream of the receiving water impact in order to identify the most likely point(s) of input
4. Sampling, or chemical "fingerprinting" of individual sources, including further upstream along tributaries, which can include the use of unique and/or conservative tracers.

These are similar to the methods described for the preliminary source identification in Question 3. However, Question 4 involves a more detailed focus on identifying specific sources of urban runoff and a greater degree of quantification than needed for Question 3. These methods are described more fully in US EPA (1993), Pitt (2001), and SWRCB (2001), which provide detailed descriptions of study designs, field sampling, and data analysis and interpretation appropriate for tracking sources of both bacteria and chemical pollutants.

The committee also recognized the need to supplement these methods descriptions with more explicit starting and stopping rules for detailed source identification studies. Starting rules are necessary for ensuring that source identification studies, which can be costly and time consuming, are triggered where and when monitoring data strongly suggest the presence of a persistent problem. Such rules are also needed to focus available resources on the highest priority problems. Stopping rules are essential for ensuring that source identification studies do not continue indefinitely, but end when reasonable and realistic expectations have been met. Such rules are proposed for receiving water problems associates with both recreational water quality and habitat.

5.5 Trend monitoring

Assessment of trends, for both recreational water quality and habitat, addresses Question 5: Are conditions in receiving waters getting better or worse? Question 5 provides the logical feedback to determine if management actions are having their intended effects. While this is a core monitoring element, the locations of stations and the relative emphasis on specific indicators may depend on information developed in answer to other questions (Table 4-1) related to the where problems exist (Question 1), the extent and magnitude of such problems (Question 2), and the nature and number of sources (Questions 3 and 4). Trends monitoring is a core monitoring program element (Table 4-1).

5.5.1 Recreational water quality trends

5.5.1.1 Overview

Table 5-12 presents an overview of the technical design elements for trend monitoring of recreational water quality at beaches; bays and estuaries; and creeks, streams, and rivers.

The model monitoring framework for trend monitoring of recreational water quality is based on statistical power analysis of a monitoring design that involves repeated sampling over time at fixed stations. For recreational water quality, sampling data from one inland watershed (Aliso Creek) suggests that trend monitoring might productively focus on one period of the year when both bacteria levels and human use are highest. Power analysis results from this watershed also suggest that the statistical power of the trend monitoring design can vary widely from station to station, as well as across indicators. However, comparable data were not available to support analogous conclusions for beaches and bays and estuaries. Thus, the committee recommends that programs begin trend monitoring with ten to fifteen weekly samples per year for three years, and then conduct site-specific power analyses with the software package developed by the committee and made available on the SCCWRP website. Power analyses on available data from southern California show clearly that differences across sites mean that a "one size fits all" approach to trend monitoring design will not work. The recommended approach will therefore ensure appropriate levels of both within-year replication and number of years of trend monitoring. Given that trend monitoring will most likely need to continue for a minimum or ten or fifteen years, devoting the first three years to obtaining site-specific data will not result in any substantial reduction in the longer-term power of the trend monitoring design. Any such reduction will be outweighed by gains in site-specific efficiency and statistical power.

See Section 4.5, for an expanded discussion of the use of statistical power analysis in the core trend monitoring aspects of the model monitoring design.

5.5.1.2 Design elements

The following subsections address, in turn, trend monitoring design in high-priority recreational areas where use is concentrated and then in the upstream areas that are the sources of contamination. Where such upstream source areas have been identified and are the targets of active source reduction efforts, it may be useful to monitor trends in the levels of these sources.

Conditions in high-priority recreational areas. Figure 5-2 shows that fecal coliform levels (the basis of the REC1 Basin Plan standard in this case) vary considerably among months in the high-priority area in lower Aliso Creek. While there are not equally intensive data records from other creeks throughout the region, it is reasonable to assume that similar variability would be present elsewhere. Thus, it would statistically be most efficient to stratify trend analyses by month, with separate trend analyses for each month. Lumping months that normally have highly divergent

fecal coliform counts would increase the within-year variability and make it more difficult to detect trends over time.

Power tests (see Section 4.5.1 and Figure 4-1 for a discussion of the importance of statistical power analyses as part of a trend monitoring design) on the monthly Aliso Creek data were thus conducted to estimate the number of years and number of samples within a 30-day period that might be required to detect different percentages of decrease in fecal coliform counts. Power tests were performed only at stations and for months for which more than one year was sampled because the power tests require an estimate of between-year variability. Figure A2.1 (Appendix 2), with plots for each station organized in order of increasing geomean, shows that the ideal months to sample differ from station to station. For example, the highest power for a given sampling effort occurs in August for the SOCWA treatment plant site (Figure A2.1d) but in June for the Aliso Wood Canyon Park Site (Figure A2.1c).

These results provide guidance that illustrates how the details of a trend monitoring design could be developed. Figure 5-5 shows that in the study of Aliso Creek the peak bacteria levels coincide with the period of highest recreational use in the late summer and early fall. Thus, it would be most efficient to target a trend monitoring program at one or more of the months in that portion of the year. Once a monitoring period is chosen, power analyses such as those in Figure A2.1 can be used to determine a preferred combination of reduction in indicator values, short-term sampling intensity, and length in years of the monitoring program. As mentioned above, the software package available on the SCCWRP website provides a straightforward means for each program to conduct power analysis with site-specific data.

High-priority inputs. Trend monitoring may also be useful where specific upstream inputs have been identified that contribute to contamination at a high-priority recreational use area. The key trend monitoring question for such inputs is whether the loads of bacteria, and their local impacts on the receiving water, are decreasing over time. Loads are a clear measure of the size of the input itself, and directly reflect the relative success of BMPs in the local drainage area. However, loads alone are insufficient to measure a discharge's potential impact on the receiving water. Thus, trend monitoring of high-priority inputs should include both loads and impact (measured as the difference between stations upstream and downstream of the discharge). A quantitative method for prioritizing upstream inputs for management actions and for trend monitoring is described above in Section 5.4.2, which discusses source identification approaches.

The monitoring data from Aliso Creek provide a useful illustration of how power analysis can be used to design a site-specific trend monitoring program. Figure A2.2 (Appendix 2) shows that bacterial levels in the high-priority drains in Aliso Creek, as well as at the upstream and downstream stations associated with each, are typically highest in the June – September period and lower throughout the rest of the year. The illustrative power analyses therefore focused on this period in order to reduce the within-year variability. Power analyses were performed for two measures, the load from each drain (Figure A2.3) and the impact of each drain (Figure A2.4) measured as the difference between the downstream and upstream stations. These results suggest that it will not be feasible to track loads at station J06 (Figure A2.3) nor to track impacts at station J01P08 (Figure A2.4). With the exception of these parameters at these stations, however, the power analysis also suggests that a sampling frequency of 20 samples, collected in the June – September period, would be adequate to detect an average 50% reduction in loads and an average 30% reduction in impact over a ten year period.

5.5.1.3 Design issues

Trends monitoring of existing bacterial indicators is complicated by their extreme variability in space and time. Thus, there may be limitations on our ability to detect change with current monitoring technology.

There are two aspects of trend monitoring with regards to recreational water quality. The first is related to conditions in the high-priority recreational use area(s) that are the major focus of concern. The question here is therefore whether indicator levels are trending downward toward applicable water quality objectives. The second aspect of such trend monitoring is related to whether the high-priority inputs upstream of the high-priority recreational area are improving. The question here is somewhat different, instead being whether loads and localized impacts (a measure of the direct effect on the receiving water) are declining. This is why the recommended indicators include both a measure of concentration and a measure of loads.

5.5.2 Habitat trends

5.5.2.1 Overview

Assessment of habitat trends addresses Question 5: Are conditions in receiving waters getting better or worse? Question 5 provides the logical feedback to determine if management actions are having their intended effects. While this is a core monitoring element, the locations of stations and the relative emphasis on specific indicators may depend on information developed in answer to other questions (Table 4-1) related to the where problems exist (Question 1), the extent and magnitude of such problems (Question 2), and the nature and number of sources (Questions 3 and 4).

Table 5-13 presents an overview of the technical design elements for trend monitoring of habitat conditions.

The model monitoring framework for trend monitoring of habitat conditions is based on statistical power analysis of a trend monitoring design that involves repeated sampling over time at fixed stations. For habitat, the timing of trend monitoring will differ depending on the parameters being tracked, with mass emissions monitored during wet weather and bioassessment during dry weather. Available data indicate that power analysis results can vary widely from station to station, as well as across parameters. Thus, the committee recommends that programs begin trend monitoring with two or three samples per year for three years, and then conduct site-specific power analyses with the software package developed by the committee and made available on the SCCWRP website.

5.5.2.2 Design elements

Trend monitoring is relevant to all aspects of habitat monitoring in the watershed design. Trend monitoring can occur at one or more of the core monitoring assessment stations depending on criteria such as the level of management concern or whether a receiving water problem has previously been documented.

Appendix 2 provides example statistical power analysis results for two aspects of a trend monitoring design (event mean concentration (EMC) and mass emissions) for which sufficient data currently exist for such an analysis. These results Figures A2.5 - and A2.14, which use data from several representative long-term stations in Orange County, reflect, as do the bacteria results described above, large differences in power from station to station and across parameters. (Data from other monitoring programs were not suitable for power analysis.) As a result, it is not possible to recommend levels of sampling effort that would be generally applicable across the

region. Therefore, the committee recommends that trend monitoring programs for habitat begin by collecting two or three samples per year for three years and then use these data to conduct site-specific power analyses to refine the following aspects of the design:

- The amount of change expected or desired
- The number of samples to be collected per year
- The number of years before the expected change is detected.

These analyses can be carried out with the software package available on the SCCWRP website. While results will undoubtedly vary from site to site, the committee's analyses of available historical monitoring data (Appendix 2) suggests that it is unlikely that substantial amounts of change (e.g., reductions of 50% or more) will be observable in less than ten years and that management targets and monitoring designs should be developed accordingly.

5.5.2.3 Design issues

Trends monitoring of habitat indicators is complicated by the variety of station types, the long list of monitored constituents, and the complexity of ecosystem processes that influence observed trends. Therefore, general guidance is presented for two main categories of monitoring data – mass emissions and toxicity. As for the recreational water quality aspect of Question 5, the power analysis software available on the SCCWRP website will enable each program to perform relevant site-specific power analyses as required

Table 5-1. Design overview for assessment monitoring of recreational water quality.

Type of area	Site location	Frequency	Indicator(s)
Open-coast beach	Gaps in Health Department coverage • High-priority areas • Flowing stormdrains Drain itself Upcoast and downcoast of drain Characterize dispersion of drain plume prior to siting upcoast and downcoast stations	Based on BWQWG prioritization Daily – monthly (see Table 5-2)	Total coliform Fecal coliform Enterococcus
Enclosed bays and estuaries	Gaps in Health Department coverage High priority areas Subsample of flowing stormdrains Drain itself Upcoast and downcoast of drain	Based on BWQWG prioritization Daily – monthly (see Table 5-2)	Total coliform Fecal coliform Enterococcus
Creeks, streams, and rivers	High-priority areas Drain or other input Upstream and downstream of input	Weekly in high-use season	Total coliform or E. coli Fecal coliform Enterococcus

Table 5-2. The Beach Water Quality Workgroup's risk-based approach for determining sampling frequency. The presence of lifeguards is an indicator of high-use beaches that are most likely above the 50,000 users threshold in Assembly Bill 411.

	Likelihood of Contamination			
Usage	**High**: e.g., stormdrains that flow continuously, frequently exceeding bacterial standards; pier areas	**Medium**: e.g., stormdrains that flow intermittently or continuously with infrequent exceedances of standards	**Low**: source nearby, do not expect a problem, stormdrain not flowing but if had a sewage spill it would flow to beach, if a problem it would be long term	**No known source**
High use beach: lifeguarded, high use surf/dive area	Daily or 5X per week	5X per week	Weekly or 5X per month	Weekly or 5X per month
Accessible sandy beach: low use surf/dive area or other water contact recreation area (wind surfing, kayaking)	2 – 3X per week	Weekly or 5X per month	Weekly or 5X per month	None
Other accessible shoreline: rocky coastline, small coves accessible by trails, private homes limit access	Weekly or 5X per month	Weekly or 5X per month	Monthly or other identification system	None
Inaccessible: beach area > 1 mile from access area	None	None	None	None

Table 5-3. Design overview for assessment monitoring of habitat.

Type of area	Site location	Frequency	Indicator(s)
Mass emissions	Bottoms of watersheds	3 storms / yr for 3 yrs, then modify per results of power analyses	Chemistry (see Table 5-16) Toxicity
Watershed	Random or rotating, perhaps per Bight design	Every few years, perhaps per Bight Program schedule	Triad in dry weather Chemistry, toxicity in wet weather
High-priority inland habitat	High-value habitat either impacted or threatened	1 or 2 / yr in dry weather	Triad
Program goals	As needed based on nature of specific goal(s)	Dependent upon problem, question	Dependent upon problem, question
Estuaries	Random per Bight design Key habitats and/or downstream of major inputs	Every few years per Bight design 1 or 2 / yr, in wet and/or dry, depending on problem, question	Chemistry (see Table 5-16) Toxicity
Nearshore ocean	Random and/or clustered in plumes, per Bight design	Every few years per Bight design	Chemistry (see Table 5-16) Toxicity

Table 5-4. Decision framework for interpreting triad results. Possible conclusions and actions/decisions are intended as general guidance, dependent on the specific monitoring results found and the actual relationships among chemistry, toxicity, and benthic data.

	Chemistry	Toxicity	Benthic Alteration	Example Conclusions	Example Actions or Decisions
1.	Persistent exceedance of water quality objectives	Evidence of toxicity	Indications of alteration	Strong evidence of pollution-induced degradation	Toxicity tests at higher dilutions to better quantify toxicity Use TIE to identify contaminants of concern, based on TIE metric Initiate upstream source identification as a high priority
2.	No persistent exceedances of water quality objectives	No evidence of toxicity	No indications of alteration	No evidence of current pollution-induced degradation Potentially harmful pollutants not yet concentrated enough to cause visible impact	No immediate action necessary Conduct periodic broad scans for new and/or potentially harmful pollutants
3.	Persistent exceedance of water quality objectives	No evidence of toxicity	No indications of alteration	Contaminants are not bioavailable Test organisms not sensitive to problem pollutants	TIE would not provide useful information with no evidence of toxicity Continue monitoring for toxic and benthic impacts Consider whether different or additional test organisms should be evaluated Initiate upstream source identification as a low priority
4.	No persistent exceedances of water quality objectives	Evidence of toxicity	No indications of alteration	Unmeasured contaminant(s) or conditions have the potential to cause degradation Pollutant causing toxicity at very low levels Synergistic effects of multiple chemicals at low levels causing toxicity	Recheck chemical analyses and evaluate detection limits relative to reported toxic levels Verify toxicity test results Consider additional advanced chemical analyses Toxicity tests at higher dilutions to better quantify toxicity Use TIE to identify contaminants of concern, based on TIE metric Initiate upstream source identification as a medium priority

	Chemistry	Toxicity	Benthic Alteration	Example Conclusions	Example Actions or Decisions
5.	No persistent exceedances of water quality objectives	No evidence of toxicity	Indications of alteration	Alteration may be due to physical impacts, not toxic contamination Test organisms not sensitive to problem pollutants Synergistic effects of multiple chemicals at low levels causing toxicity	No action necessary due to toxic chemicals Initiate upstream source identification (for physical sources) as a high priority Consider whether different or additional test organisms should be evaluated
6.	Persistent exceedance of water quality objectives	Evidence of toxicity	No indications of alteration	Toxic contaminants are bioavailable, but in situ effects are not demonstrable Benthic analysis not sensitive enough to detect impact Potentially harmful pollutants not yet concentrated enough to change community	Determine if chemical and toxicity tests indicate persistent degradation Recheck benthic analyses; consider additional data analyses Toxicity tests at higher dilutions to better quantify toxicity If recheck indicates benthic alteration, perform TIE to identify contaminants of concern, based on TIE metric Initiate upstream source identification as a high priority If recheck shows no effect, use TIE to identify contaminants of concern, based on TIE metric Initiate upstream source identification as a medium priority
7.	No persistent exceedances of water quality objectives	Evidence of toxicity	Indications of alteration	Unmeasured toxic contaminants are causing degradation Pollutant causing toxicity at very low levels Synergistic effects of multiple chemicals at low levels causing toxicity Benthic impact due to habitat disturbance, not toxicity	Recheck chemical analyses and consider additional advanced analyses Toxicity tests at higher dilutions to better quantify toxicity Use TIE to identify contaminants of concern, based on TIE metric Initiate upstream source identification as a high priority Consider potential role of physical habitat disturbance
8.	Exceedance of water quality objectives	No evidence of toxicity	Indications of alteration	Test organisms not sensitive to problem pollutants Benthic impact due to habitat disturbance, not toxicity	TIE would not provide useful information with no evidence of toxicity Initiate upstream source identification as a high priority Consider whether different or additional test organisms should be evaluated Consider potential role of physical habitat disturbance

Table 5-5. Example distribution of monitoring effort among the various kinds of stations in the watershed design. Special studies would be implemented when results of core or regional monitoring indicated a need for them. Specific triggers initiate adaptive monitoring and special studies designed to answer questions about the magnitude, extent, and source(s) of problems.

Station type	Core monitoring	Regional monitoring	Further monitoring / special studies
Mass emissions	Triad, including broader suite of chemistry		TIEs (Q4) Upstream source ID (Q4)
Watershed		Bioassessment Basic chemistry Some toxicity	Expanded toxicity, chemistry (Q2) TIEs (Q4) Upstream source ID (Q4)
High-priority habitat	Bioassessment Chemistry (if prior reason) *		Toxicity, chemistry (Q1, 2) TIEs (Q4) Upstream source ID (Q4)
Program goals	Dependent on question(s)		
Estuaries	Toxicity Chemistry (if prior reason) *		TIEs (Q4) Upstream source ID (Q4) Process studies (Q1 – 4) Biology (e.g., benthos, bioaccumulation) (Q1, 2)
Nearshore ocean +		Plume tracking Plume toxicity Plume chemistry	

* Chemical monitoring could be deferred until bioassessment or toxicity results suggest a potential problem.
+ Conducted as part of the periodic regional Bight program.

Table 5-6. The short list of chemical constituents that should be sampled routinely by all programs and an expanded list to be sampled where routine monitoring data or other information suggest the need for additional information and/or where appropriate to the management question being asked.

Category	Short list	Expanded list
Trace metals	total Cd, Cr, Cu, Ni, Pb, Zn	dissolved (with hardness)
Nutrients	NH_3, total Kjeldahl Nitrogen (TKN), NO_3, total P	
Bacteria	total coliform, fecal coliform or E. coli, Enterococcus	
Pesticides	diazinon, chlorpyrifos, other OP pesticides	others as necessary, e.g., legacy pesticides (DDT, chlordane, lindane), emergent pesticides (e.g., pyrethroids)
Conventionals	temperature, pH, hardness, specific conductance, dissolved oxygen	chemical oxygen demand (COD), sulfides
PAHs	if methods are available that are suitable for measuring on particles, at low detection limits	
Volatiles		dry weather only
Suspended solids	total suspended solids (TSS)	
Priority pollutants	every 5 years, with Bight Program	

Table 5-7. Design overview for extent and magnitude monitoring of recreational water quality problems. Regional monitoring is distinguished from special projects in its larger geographic scale and/or greater number and kinds of potential sources

Type of area	Site location	Frequency	Indicator(s)
Open-coast beach	Input(s) of concern Spaced array upcoast and downcoast of input of concern	One calendar year to establish basic pattern, then: Daily within representative periods (e.g., storms, dry weather, dominant current regimes)	Concentration and loads: • Total coliform • Fecal coliform • Enterococcus Dye
Enclosed bays and estuaries	Input(s) of concern Based on nature of problem, either: • Spaced array around input of concern • Regular grid throughout area of concern • Random array throughout area of concern • Gradient array downcurrent of input of concern	One calendar year to establish basic pattern, then: Daily within representative periods (e.g., storms, dry weather, dominant current regimes)	Concentration and loads: • Total coliform • Fecal coliform • Enterococcus Dye
Creeks and streams	Regular grid throughout high-priority use area	One calendar year to establish basic pattern, then: Daily within subsample of high-use period	Total coliform or E. coli Fecal coliform Enterococcus Dye

Table 5-8. Design overview for both recreational water quality and habitat source identification under Question 4: What are the sources to urban runoff that contribute to receiving water problems?

Type of area	Site location	Frequency	Indicator(s)
Recreational water quality			
Open-coast beach	Inputs that fall in upper right section of Figure 5-3	Ongoing until reach stopping rules in Figure 5-7	Total coliform Fecal coliform Enterococcus
Enclosed bays and estuaries	Inputs that fall in upper right section of Figure 5-3	Ongoing until reach stopping rules in Figure 5-7	Total coliform Fecal coliform Enterococcus
Creeks and streams	High-priority inputs as identified with conceptual model in Figure 5-3	Ongoing until reach stopping rules in Figure 5-7	Total coliform or E. coli Fecal coliform Enterococcus
Habitat			
All areas except coastal ocean	At core monitoring site Upstream of core monitoring site	Ongoing until reach stopping rules in Figure 5-8	Constituents identified in Questions 1 - 3

Table 5-9. Aliso Creek stations in order of scaled load and impact. Impact = downstream concentration – upstream concentration on each date. Load = factor x flow (csf) x concentration in pipe on each date. Scaled values are rescaled to 0 – 1. Average scaled values = average of scaled impact and load values. Stations are ranked in order of average scaled values.

Station	Avg scaled	Impact scaled	Load scaled	Impact	Load
J01P08	0.45442	0.30327	0.60558	1.09240	5.12476
J01P28	0.42830	0.16437	0.69223	0.59208	5.80725
J02P05	0.41653	0.21205	0.62102	0.76381	5.24640
J04	0.41279	0.12574	0.69984	0.45291	5.86718
J01P01	0.40517	0.22449	0.58585	0.80864	4.96939
J02TBN1	0.40237	0.23424	0.57049	0.84376	4.84841
J03P01	0.39515	0.17026	0.62004	0.61328	5.23868
J01TBN8	0.39016	0.27729	0.50303	0.99881	4.31707
J01P03	0.35542	0.10754	0.60331	0.38738	5.10688
J03P02	0.34631	0.04993	0.64270	0.17984	5.41714
J01P27	0.34413	0.05564	0.63262	0.20040	5.33780
J01P30	0.34067	0.10229	0.57906	0.36847	4.91588
J03P05	0.33684	0.04794	0.62573	0.17270	5.28352
J03TBN2	0.33178	0.19535	0.46821	0.70366	4.04286
J01P23	0.32485	0.09907	0.55062	0.35686	4.69191
J03P13	0.32480	0.09263	0.55698	0.33365	4.74199
J06	0.32433	0.09277	0.55589	0.33415	4.73343
J01P06	0.30957	0.12342	0.49573	0.44455	4.25962
J01P26	0.30500	0.05205	0.55794	0.18750	4.74956
J05	0.28978	0.05328	0.52628	0.19192	4.50018
J01P05	0.28974	0.08773	0.49175	0.31600	4.22829
J01TBN2	0.28929	0.12163	0.45695	0.43812	3.95419
J03TBN1	0.28574	0.09032	0.48116	0.32534	4.14483
J01P22	0.28419	0.06092	0.50745	0.21944	4.35194
J07P01	0.26681	0.09332	0.44031	0.33613	3.82309
J01TBN4	0.26512	0.05184	0.47840	0.18674	4.12309
J01P04	0.25812	0.06553	0.45072	0.23603	3.90509
J01P25	0.25406	0.05010	0.45801	0.18047	3.96254
J01TBN3	0.24684	0.04788	0.44580	0.17248	3.86634
J01P33	0.24246	0.05250	0.43242	0.18909	3.76094
J01P24	0.20587	0.03620	0.37553	0.13040	3.31286
J01P21	0.14696	0.04556	0.24836	0.16411	2.31128

Table 5-10. Definitions of the triggers in Table 5-4, the Triad interpretation framework, designed to initiate further adaptive studies to identify potential sources of impact. "BRI" refers to the regional Benthic Response Index for estuaries developed by the Bight Program. "IBI" refers to the Index of Biotic Integrity, a regional bioassessment index under development by the Stormwater Monitoring Coalition.

Possible trigger in Table 5-4	Definition of trigger
Persistent exceedance of water quality objectives	Exceedance of relevant Basin Plan or CTR objectives by 20% for 3 sampling periods
Evidence of toxicity	High score, in relation to other stations, on metric that combines magnitude and persistence of toxicity observed over an entire year (see Appendix 5: TIE Metric)
Evidence of benthic alteration	BRI score that indicates substantially degraded community (in estuaries) IBI score that indicates substantially degraded community (in freshwater creeks, streams, and rivers)

Table 5-11. Summary of the upstream source identification priorities from Table 4-7, based on combinations of the chemical, toxicity, and benthic components of the triad approach. "Yes" and "No" refer to whether or not data from each component exceeded the triggers described in Table 5-20.

Table 4-7 Row	Triad Component	Yes	No	Source ID Priority
1	chemistry	X		
	toxicity	X		
	benthos	X		High
2	chemistry		X	
	toxicity		X	
	benthos		X	None
3	chemistry	X		
	toxicity		X	
	benthos		X	Low[1]
4	chemistry		X	
	toxicity	X		
	benthos		X	Medium
5	chemistry		X	
	toxicity		X	
	benthos	X		High (for physical components)
6	chemistry	X		
	toxicity	X		
	benthos		X	Medium
7	chemistry		X	
	toxicity	X		
	benthos	X		High
8	chemistry	X		
	toxicity		X	
	benthos	X		High

[1] If further testing indicates appropriate and sensitive enough toxicity tests were used and analytical results suggest pollutant is not bioavailable.

Table 5-12. Design overview for trends monitoring of recreational water quality.

Type of area	Site location	Frequency	Duration	Indicator(s)
Open-coast beach	Input(s) of concern at high-priority beaches Upcoast and downcoast stations	Weekly within representative periods (e.g., storms, dry weather, dominant current regimes) Repeated yearly	10 – 15 per year for 3 years, then based on power analysis	Concentration and loads: • Total coliform • Fecal coliform • Enterococcus Dye
Enclosed bays and estuaries	Input(s) of concern at high-priority sites Stations bracketing input(s)	Weekly within representative periods (e.g., storms, dry weather, dominant current regimes) Repeated yearly	10 – 15 per year for 3 years, then based on power analysis	Concentration and loads: • Total coliform • Fecal coliform • Enterococcus Dye
Creeks and streams	High-priority use area High-priority upstream inputs	Weekly with representative periods (e.g., storms, dry weather, dominant current regimes) Repeated yearly	10 – 15 per year for 3 years, then based on power analysis	Concentration and loads: • Total coliform or E. coli • Fecal coliform • Enterococcus Dye

Table 5-13. Design overview for trends monitoring of habitat.

Type of area	Site location	Frequency	Duration	Indicator(s)
Mass emissions	Bottoms of watersheds	3 storms / yr for 3 yrs, then modify per results of power analyses	Based on power analysis	Chemistry (see Table 4-8) Toxicity
Watershed	Random or rotating, perhaps per Bight design	Every few years, perhaps per Bight Program schedule	Ongoing	Triad in dry weather Chemistry, toxicity in wet weather
High-priority habitat	High-value habitat either impacted or threatened	1 or 2 / yr in dry weather	Based on power analysis Revisit when habitat status changes	Triad
Program goals	As needed	Dependent upon problem, question	Based on power analysis Until goals met / change	Dependent upon problem, question
Estuaries	Random per Bight design Key habitats and/or downstream of major inputs	Every few years per Bight design 1 or 2 / yr, in wet and/or dry, depending on problem, question	Ongoing for Bight Based on power analysis for key habitats and downstream stations	Chemistry (see Table 4-8) Toxicity
Nearshore ocean	Random and/or clustered in plumes, per Bight design	Every few years per Bight design	Ongoing	Chemistry (see Table 4-8) Toxicity

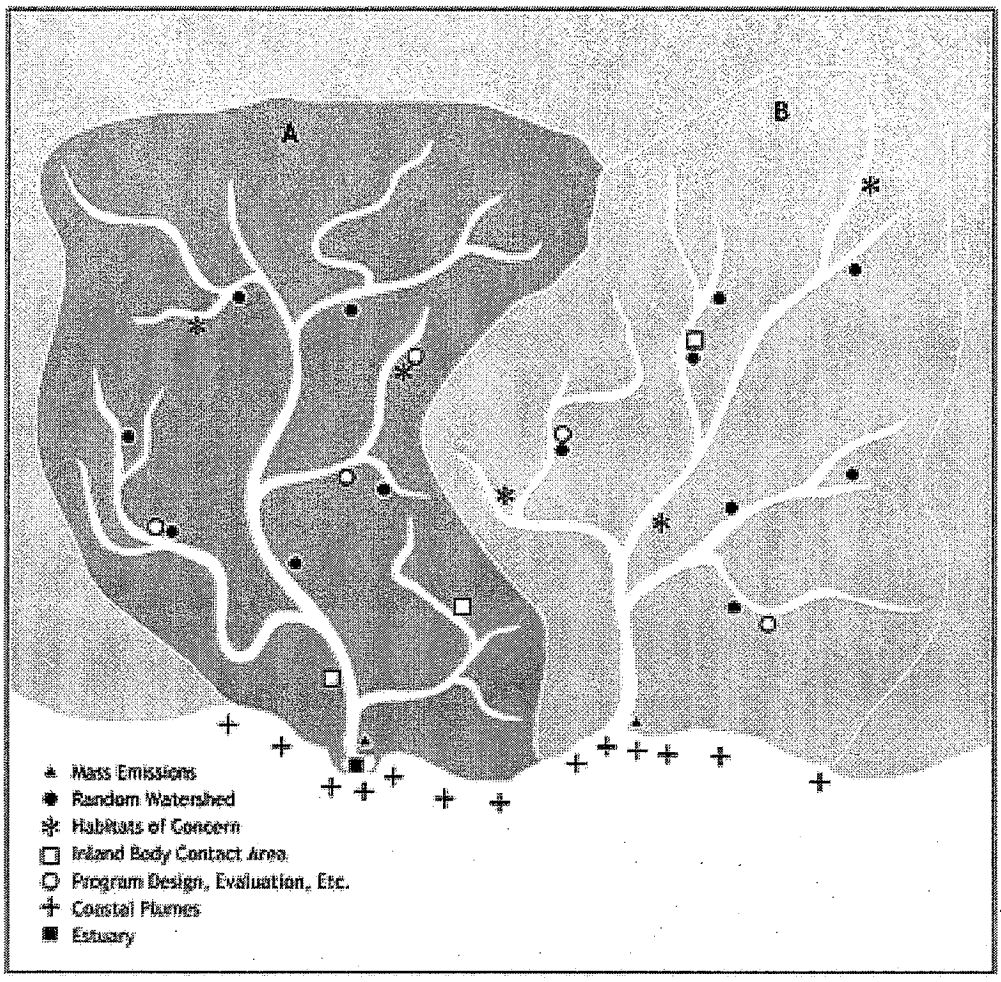


Figure 5-1. Example model monitoring program design in an idealized watershed.

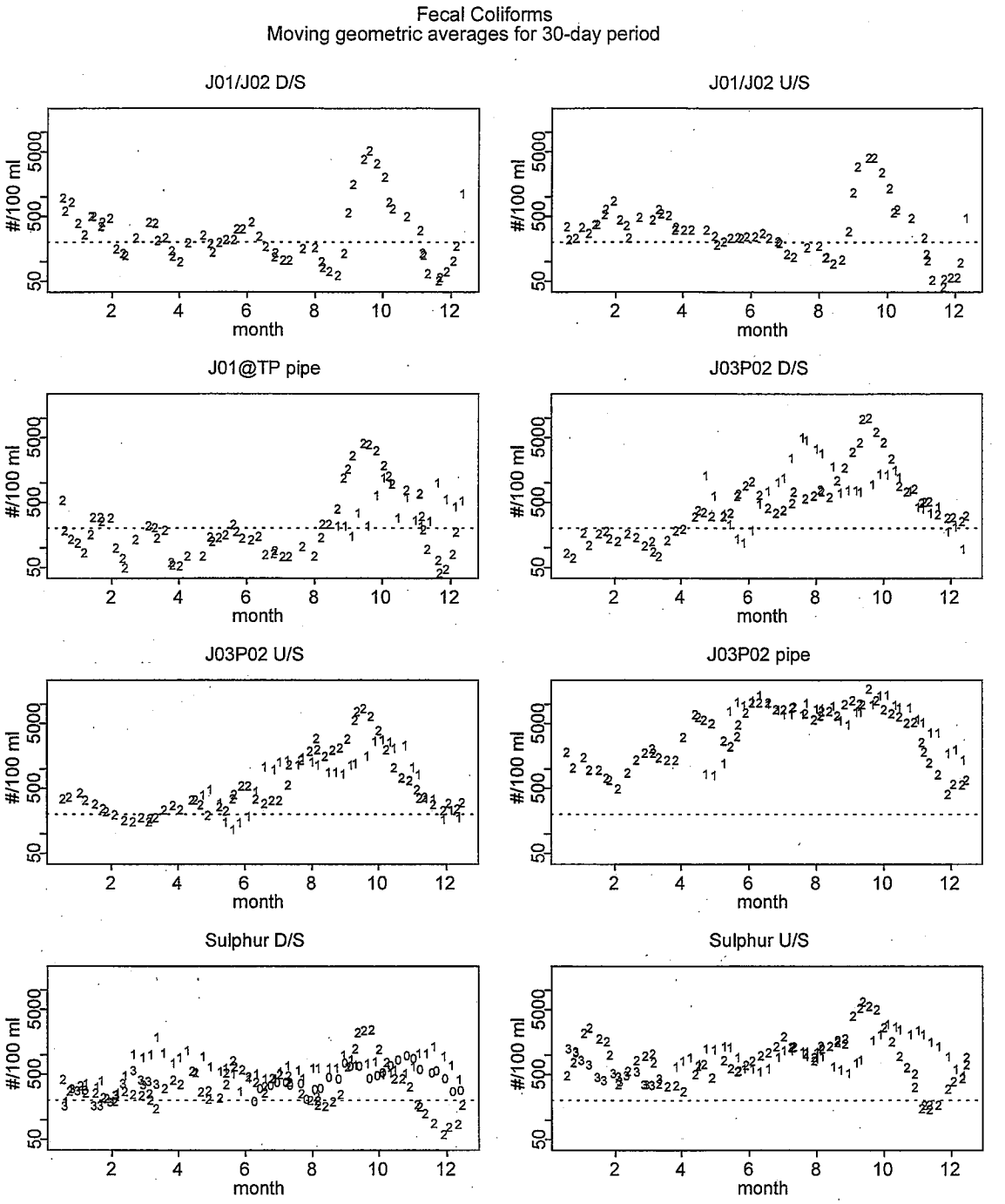


Figure 5-2a. Fecal coliform measurements at and upstream/downstream of discharge points in lower Aliso Creek. Data points are 5-sample moving geometric averages. The horizontal dashed line represents the Basin Plan REC1 objective for fecal coliforms (geomean not higher than 200/100 ml). The point symbols indicate the year of sampling (i.e., 1 for 2001, 2 for 2002).

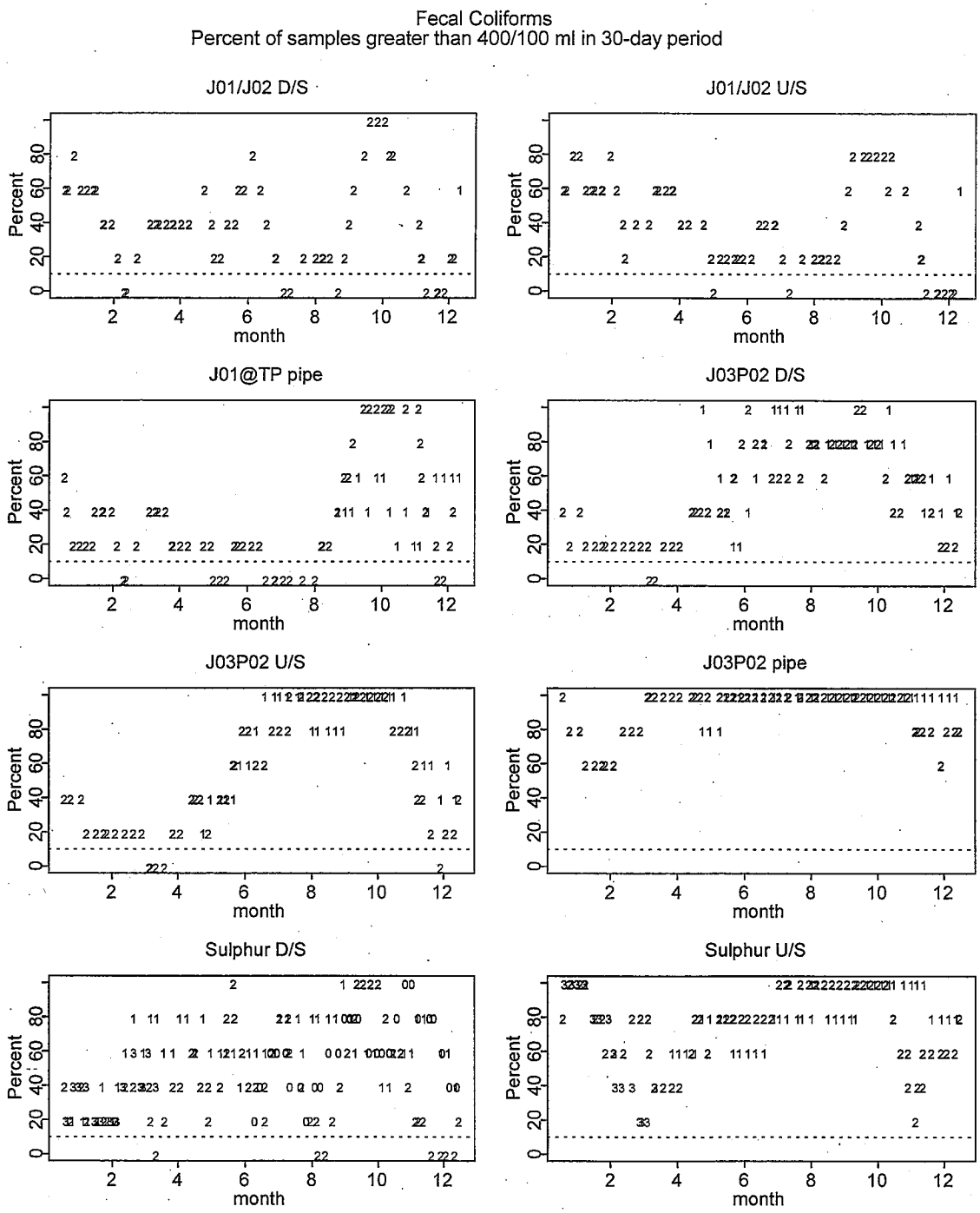


Figure 5-2b. Fecal coliform measurements at and upstream/downstream of discharge points in lower Aliso Creek. The data points are the percent of fecal coliform samples above 400/100 ml in the five most recent samples. The horizontal dashed line represents the Basin Plan REC1 objective for fecal coliforms (no more than 10% above 400/100 ml). The point symbols indicate the year of sampling (i.e., 1 for 2001, 2 for 2002).

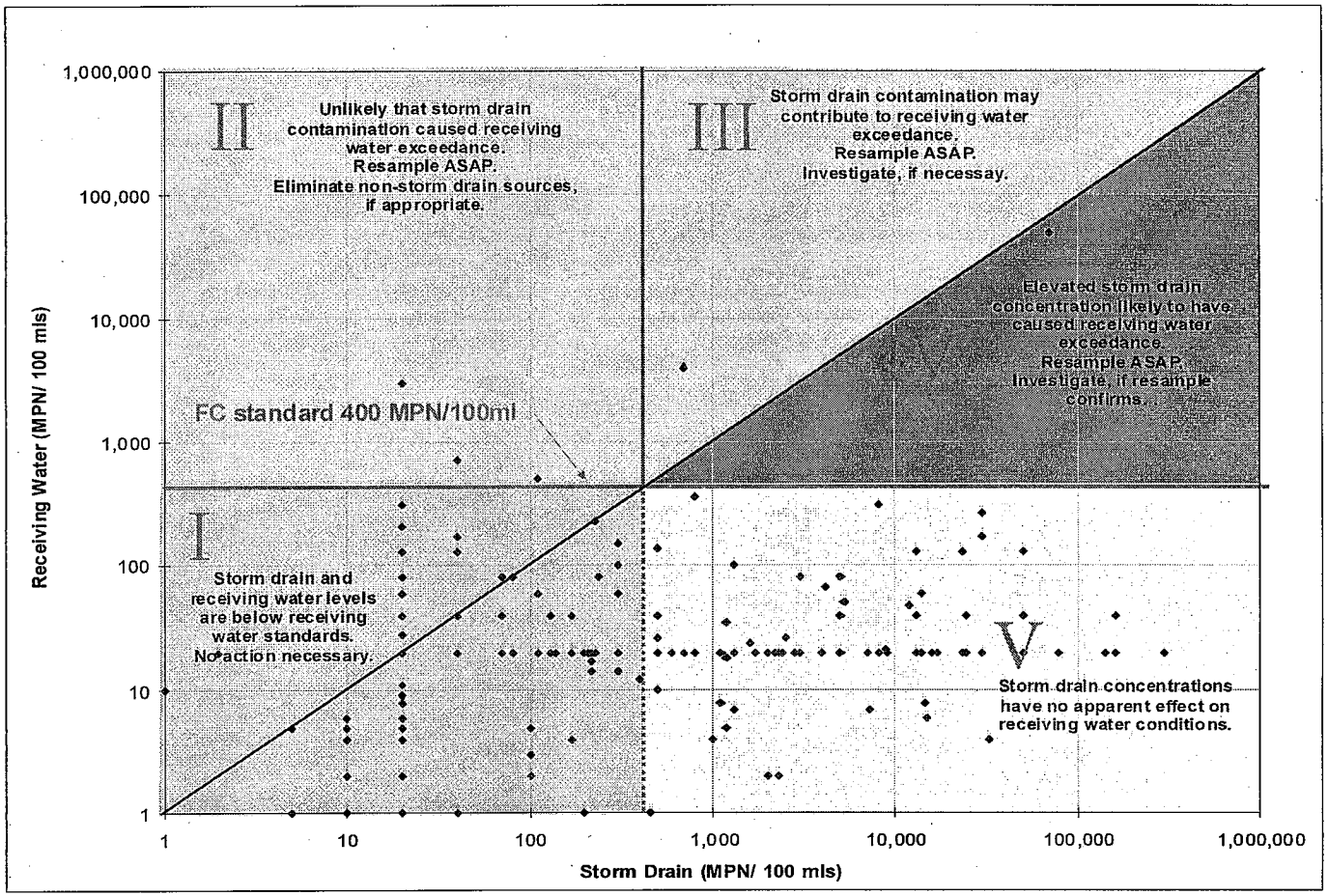


Figure 5-3. Approach for prioritizing coastal and estuarine bacterial inputs for further upstream source identification efforts. The highest priority is given to situations in which elevated bacterial indicator levels in the discharge are matched with elevated levels in the receiving water. This figure shows an example using fecal coliform, and analagous figures could be prepared with other indicator data.

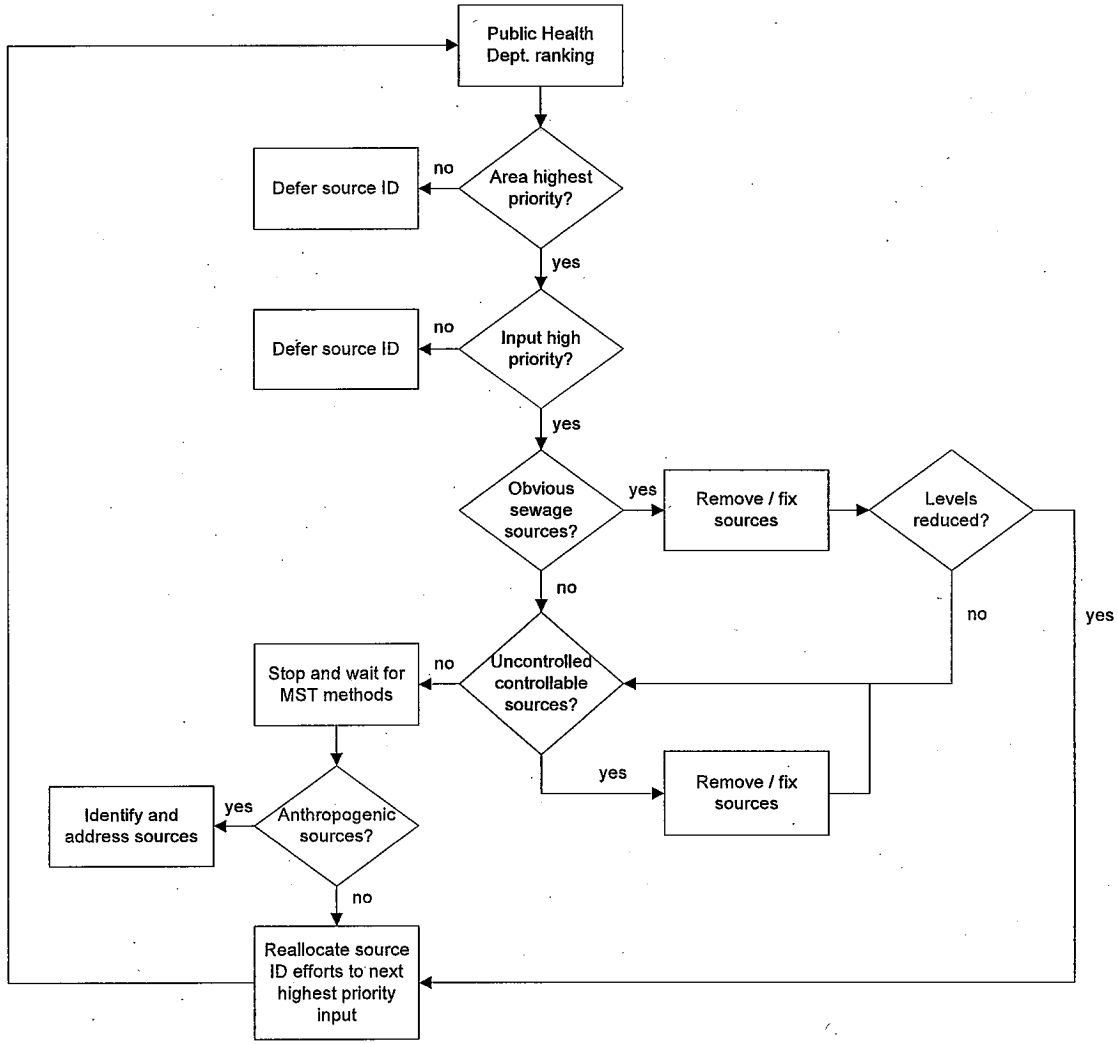


Figure 5-4. Decision tree that organizes starting and stopping rules for upstream bacterial source identification efforts. MST refers to microbial source tracking methods.

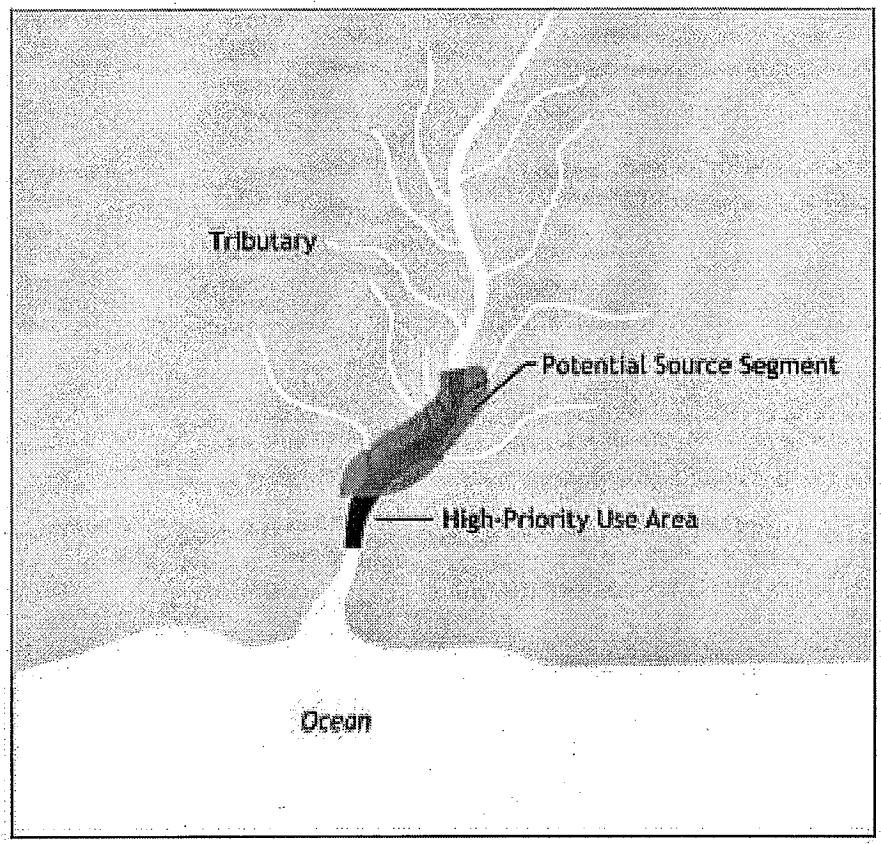


Figure 5-5. Conceptual model for determining the upstream segment of a creek or stream that should be the focus of source identification efforts for bacterial contamination. The model assumes that bacterial dieoff as water flows downstream places an upper limit on the distance bacteria can travel and still impact the high-priority use area.

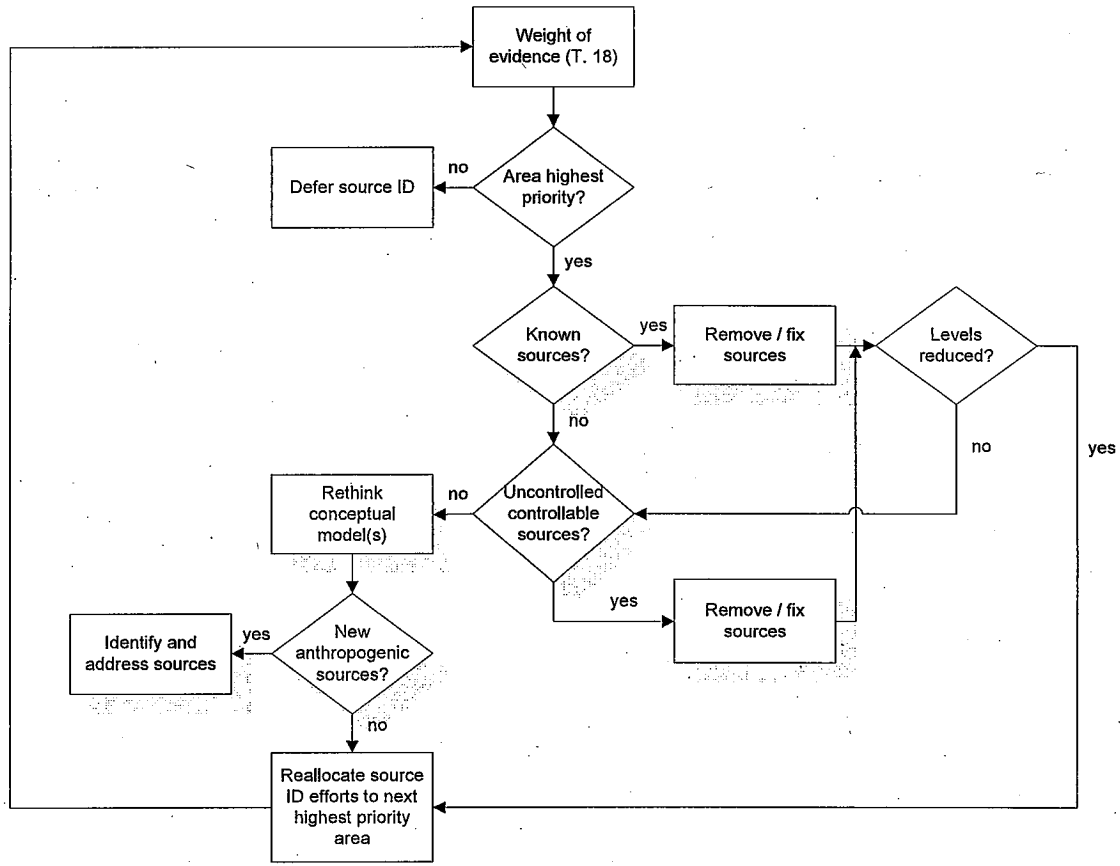


Figure 5-6. Decision tree that organizes starting and stopping rules for upstream source identification efforts targeted at habitat.

6.0 REFERENCES

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7.0 APPENDICES

Appendix 1: Summary of Existing Municipal Stormwater Monitoring Programs in Southern California

June 2003

As part of the effort to develop a model stormwater monitoring program, we have reviewed and summarized the existing monitoring designs being implemented by each of the major stormwater programs in southern California. This information will be used, in a later step of the project, as a starting point for assessing what sorts of changes might be advisable to bring existing monitoring efforts more into line with the recommendations of the model program.

Program-specific details

There are six basic types of monitoring approaches currently used in NPDES stormwater monitoring programs throughout southern California, including:

- End of watershed designs that typically measure the cumulative mass emissions from all discharges
- Dispersed watershed designs that assess overall conditions and impacts in freshwater habitats
- Site-specific watershed designs that assess conditions and trends in freshwater or estuarine habitats of particular concern
- Beach stormdrain designs that assess stormwater impacts on the surfzone
- Near-coastal designs that assess the impact of stormwater plumes on near-coastal habitats
- Dry-weather reconnaissance designs focused on identifying sources of pollution to the MS4 system.

While all of these approaches can be found in the region, not every stormwater program includes all six, as illustrated in Table A1-1.

Table A1-1. Distribution of monitoring approaches across the separate stormwater programs in southern California.

Program	End of watershed	Dispersed watershed	Site-specific watershed	Beach	Near-coastal	Recon-naissance
Ventura		X				
Los Angeles	X		X			
Long Beach	X		X			
Orange	X	X	X	X	X	X
San Bernardino	X					
Riverside	X	X				
San Diego	X	X	X	X		X

In addition, the relative attention paid to each type of monitoring varies across programs. This reflects differences in habitat types, regulatory emphasis, stage of program development, and patterns of urbanization across the region. For example, monitoring in the northern part of Orange County focuses more heavily on problems intrinsic to more urbanized areas than does monitoring in Ventura County, which has a larger proportion of agricultural and open space land uses.

The following set of tables summarizes the existing distribution of effort in each of the programs. Information was drawn from the most recent set of program documents available for each program.

Table A1-2 shows the distribution of end of watershed monitoring efforts across the region's programs. All the programs except for San Bernardino County and Riverside County have ongoing end of watershed designs focused primarily on estimating mass loads from larger watersheds. The lack of such stations from San Bernardino and Riverside Counties partly reflects the fact that these inland areas are at the upper ends of large watersheds (such as for the Santa Ana River), which is a quite different situation than in the coastal counties. However, the lack of mass emissions stations in the inland counties also hampers their ability to estimate the proportional contribution of these inland areas to cumulative loads downstream.

Table A1-2. End of watershed monitoring efforts in each stormwater program.

Program	No. Sites	No. Events/Yr	Indicators	Notes
Ventura	3	2 wet, 5 dry		
Los Angeles	7 6/trib	3 storms, 2 dry 4 storms, 1 dry	Water qual, tox, trash Water qual	Adaptive TIE Rotate among tributaries each year
Long Beach	4	4 storms, 2 dry	Water qual, tox	Adaptive TIE Additional sites in LA and San Gabriel river watersheds as decided by Reg. Board
Orange	12 * 6	3 storms, 3 dry 3 storms	Water qual, tox Water qual, tox	Adaptive tox, TIE, source ID Adaptive TIE
San Bernardino	-			
Riverside	-			
San Diego	11	3 storms	Water qual, tox	Link to bioassessment at other sites

* For Orange County, the upper set of information refers to the Santa Ana Region of the County and the lower to the San Diego Region of the County, which have somewhat different monitoring programs.

Table A1-3 shows the distribution of dispersed watershed monitoring efforts across the monitoring programs in the region. Dispersed watershed monitoring efforts are typically used to assess the extent and magnitude of impact on watersheds and their beneficial uses. All the programs except for Long Beach (which is a relatively small, heavily urbanized area) include this approach, and four of these six programs contain bioassessment sampling. This reflects a growing awareness that chemical measurements alone, or even chemical measurements combined with toxicity testing, will not necessarily capture impacts to aquatic habitats. The inclusion of bioassessment monitoring is an effort to directly measure habitat quality in areas where this is of concern. The model program will identify the types of locations where dispersed watershed monitoring should occur and define the measurement indicators, including bioassessment, that should be monitored at these sites.

Table A1-3. Dispersed watershed monitoring efforts in each stormwater program.

Program	No. Sites	No. Events/Yr	Indicators	*Notes*
Ventura	3 landuse	1 dry	Water qual	Characterize landuse
	2 rec. water	1 dry	Water qual	discharges
	14 bioass	1 dry	Bioassessment	Characterize receiving water quality in smaller tributaries
Los Angeles	20	1 dry	Bioassessment	CDFG methods
Long Beach	-			
Orange	11	2 dry	Water qual, tox, bioassessment	Includes reference sites
	15	2 dry	Water qual, tox, bioassessment	Adaptive chem, tox, TIEs, source ID
San Bernardino	5	4 storms	Water quality	
Riverside	25	1 – 5 wet	Water qual	
	25	3 dry	Water qual	
San Diego	23	2 dry	Bioassessment	Link to mass emissions, tox at other sites

Table A1-4 shows the distribution of site-specific watershed monitoring across the region. These efforts are targeted at locations that are considered of concern because of their high ecological and societal value. The uneven distribution of such effort across the region reflects the uneven distribution of high-value habitats such as lagoons and estuaries (e.g., Newport Bay), as well as the varying degree to which management agencies have addressed this issue. The effect of sample size has not been fully evaluated in southern California. Too few samples will lead to conclusions with low confidence or even erroneous conclusions while oversampling leads to wasted resources. The model program will address this issue through power analysis of historical data to assess the optimal number of samples.

Table A1-4. Site-specific watershed monitoring efforts in each stormwater program.

Program	No. Sites	No. Events/Yr	Indicators	Notes
Ventura	-			
Los Angeles	-			
Long Beach	1	4 storms, 2 dry	Water qual, tox, bacteria	Alamitos Bay receiving water
Orange	12 estuary	2 storms, 2 dry	Water qual, tox, seds, benthos	Adaptive tox, TIE, source ID, link to Bight '03
	6 channel	2 storms, 2 dry		
San Bernardino	-			
Riverside	11	4	Water qual	
San Diego	13 lagoon	1 dry	Sed chem, tox, benthos	Adaptive prioritization using triad

Table A1-5 shows the distribution across the region of monitoring efforts targeted at storm drains discharging directly to the beach or coastal zone. The absence of such sites in San Bernardino and Riverside Counties is due to their inland location. In other coastal counties, beach monitoring may be conducted by county health departments rather than by stormwater programs. The stormwater model monitoring technical workgroup has teamed up with the SWRCB's Beach Water Quality Workgroup to evaluate potential collaborative monitoring designs that would coordinate with the county health department and other shoreline monitoring efforts.

Table A1-5. Beach drain monitoring efforts in each stormwater program.

Program	No. Sites	No. Events	Indicators	Notes
Ventura	-			Co DHS
Los Angeles	26	Daily	Bacteria	City of LA conducts
Long Beach	-			City DHS
Orange	TBD	Weekly	Bacteria	In addition to HCA; monitor surfzone up- and downcoast, adaptive source ID, risk assessment
	36	Weekly	Bacteria	
San Bernardino	-			
Riverside	-			
San Diego	60	Weekly	Bacteria	Cities conduct program, monitor drain and receiving water, adaptive source ID

Table A1-6 shows the distribution of near-coastal monitoring effort across the programs in the region. This is a relatively new priority for stormwater programs, as reflected in the fact that only the Los Angeles County and Orange County programs include this component. The model monitoring program is looking to integrate this monitoring with existing near coastal monitoring through southern California Regional Monitoring.

Table A1-7 shows the distribution of dry-weather reconnaissance monitoring efforts across the region's stormwater monitoring programs. This type of monitoring is targeted specifically at source identification, and is contained only in the Orange and San Diego County programs. Source characterization monitoring is important and the model monitoring program is looking to integrate this design as an adaptive element, triggered by the extent and magnitude of impacts described in Table A1-3.

Table A1-6. Near-coastal monitoring efforts in each program.

Program	No. Sites	No. Events/Yr	Indicators	Notes
Ventura	-			
Los Angeles	50	1 / permit period	Sed chem, tox, benthic infauna	Paired sites at and beyond mouths of rivers Adaptive TIE
Long Beach	-			
Orange	0 8	2 storm, 2 dry	Water qual, tox	Adaptive drain characterization and nearshore plume tracking
San Bernardino	-			
Riverside	-			
San Diego	-			

Table A1-7. Dry-weather reconnaissance monitoring efforts in each program.

Program	No. Sites	No. Events/Yr	Indicators	Notes
Ventura	-			
Los Angeles	-			
Long Beach	-			
Orange	40 58	5 / dry 5 / dry	Water qual	Adaptive source ID
San Bernardino	-			
Riverside	-			
San Diego	90 County > 500 cities	3 / per permit period 1 / per permit period	Water qual	Adaptive source ID

Summary and Discussion

The monitoring programs described above were designed and implemented to address issues specific to each county or city. Thus, many of the differences between the programs reflect a logical amount of variety, given the variability across the entire region in factors such as degree

of urbanization, type and amount of critical habitat, design of the MS4 system, and kinds of beneficial uses. However, there are other differences that are more arbitrary in nature, for example the frequency of sampling events, the analyte list, and whether to include a dry weather reconnaissance program. In addition, some programs have moved aggressively to include adaptive elements, while others have chosen designs that remain relatively constant across sampling events throughout the permit term.

In addition, the monitoring programs currently in place in the region have to some extent accreted over time, with new elements being added as permits are renewed. Thus, programs have not all been designed with the goal of addressing, in a logical and integrated way, the core management questions the technical committee has identified:

1. Are conditions in receiving water protective of beneficial uses?
1a. What are the mechanism(s) causing receiving water problems?
2. What is the extent and magnitude of the receiving water problems?
3. What is the relative urban runoff (both storm and non-storm, wet and dry) contribution to the receiving water problem(s)?
4. What are the sources of the urban runoff contribution to receiving water problems?
5. Are conditions in receiving waters getting better or worse?

As a result, information about the extent and magnitude of impacts on receiving waters is not always available, nor are loadings estimates (Question 3) that separate out the urban runoff component always an integral part of monitoring designs. In addition, upstream source identification efforts occur in some programs but not others, and are designed to different standards of rigor.

This overview of current monitoring practice provides a concrete starting point for two distinct but complementary considerations. First, the variety across programs provides insight into the breadth and flexibility the model program needs to encompass to be applicable to programs throughout the region. Second, the overview presents information needed for assessing what adjustments could be made to individual programs to bring them more into accord with the model monitoring program, once it is fully fleshed out. The model monitoring program must balance the desire for consistency, standardization, and regional efficiency with reasonable requirements for program-specific differences in design needed to address site-specific issues.

Appendix 2: Power Analysis Results

This appendix contains results of statistical power analyses for long-term trend with several types of historical monitoring data from southern California, including:

- Bacteria indicators at a high-priority recreational use area on Aliso Creek
- Bacteria indicators at stations upstream of the high-priority use area
- Bacteria loads from discharges upstream of the high-priority use area
- Bacteria impacts from discharges upstream of the high-priority use area
- Event mean concentrations (EMC) at a series of mass emissions stations
- Loads at a series of mass emissions stations.

Bacteria loads were calculated as the difference between indicator levels 25 feet upstream and 25 feet downstream of the discharge. The specific figures included in this appendix are listed in the following table:

Figure	Content
Fig A3.1	Aliso Creek, downstream use area power analysis results
Fig A3.2	Levels of bacterial indicators at upstream Aliso Creek stations
Fig A3.3	Power analysis results for bacteria loads at upstream Aliso Creek stations
Fig A3.4	Power analysis results for bacteria impact at upstream Aliso Creek stations
Fig A3.5	Power analysis results for EMC, Anaheim Barber Channel
Fig A3.6	Power analysis results for EMC, Westminster Channel
Fig A3.7	Power analysis results for EMC, Santa Ana Delhi Channel
Fig A3.8	Power analysis results for EMC, San Diego Creek at Campus
Fig A3.9	Power analysis results for EMC, Oso Creek
Fig A3.10	Power analysis results for loads, Anaheim Barber Channel
Fig A3.11	Power analysis results for loads, Westminster Channel
Fig A3.12	Power analysis results for loads, Santa Ana Delhi Channel
Fig A3.13	Power analysis results for loads, San Diego Creek at Campus
Fig A3.14	Power analysis results for loads, Oso Creek

In each figure, the X-axis shows the number of years over which monitoring may continue, the Y-axis the cumulative percent change to be detected, and the four curves options for the number of samples per year that could be taken. Thus, each figure shows the amount of change that could be detected (at a statistical power of 80%) at each combination of within- and between-year sampling intensity.

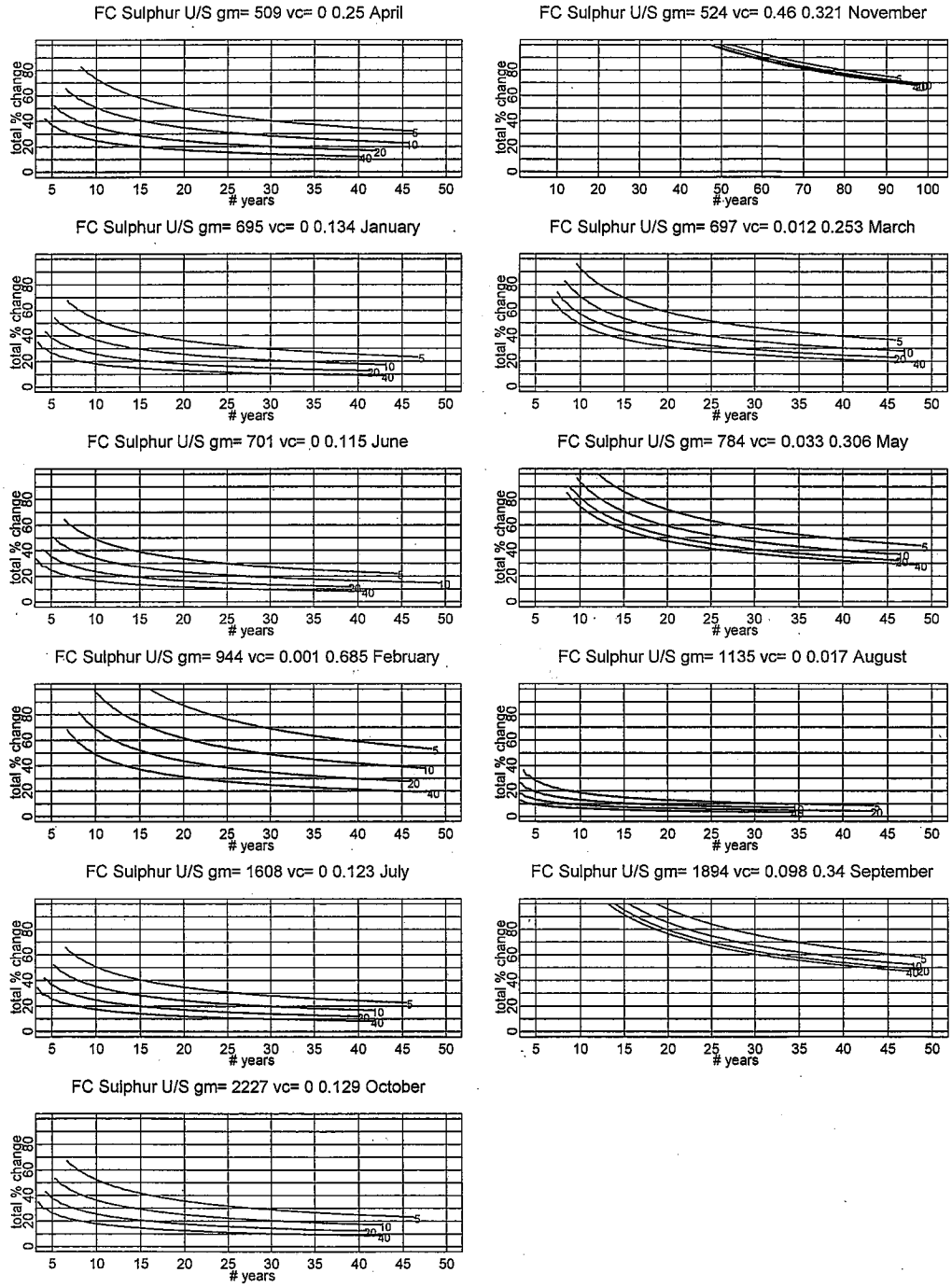


Figure A2.1a. Power analysis of a trend monitoring design at the AWMA ROAD Bridge, station Sulphur Creek upstream. The y-axis shows the amount of change detectable, the x-axis the years of sampling, and the different curves the number of samples in a given 30-day period (5, 10, 20, 40) needed for 80% power.

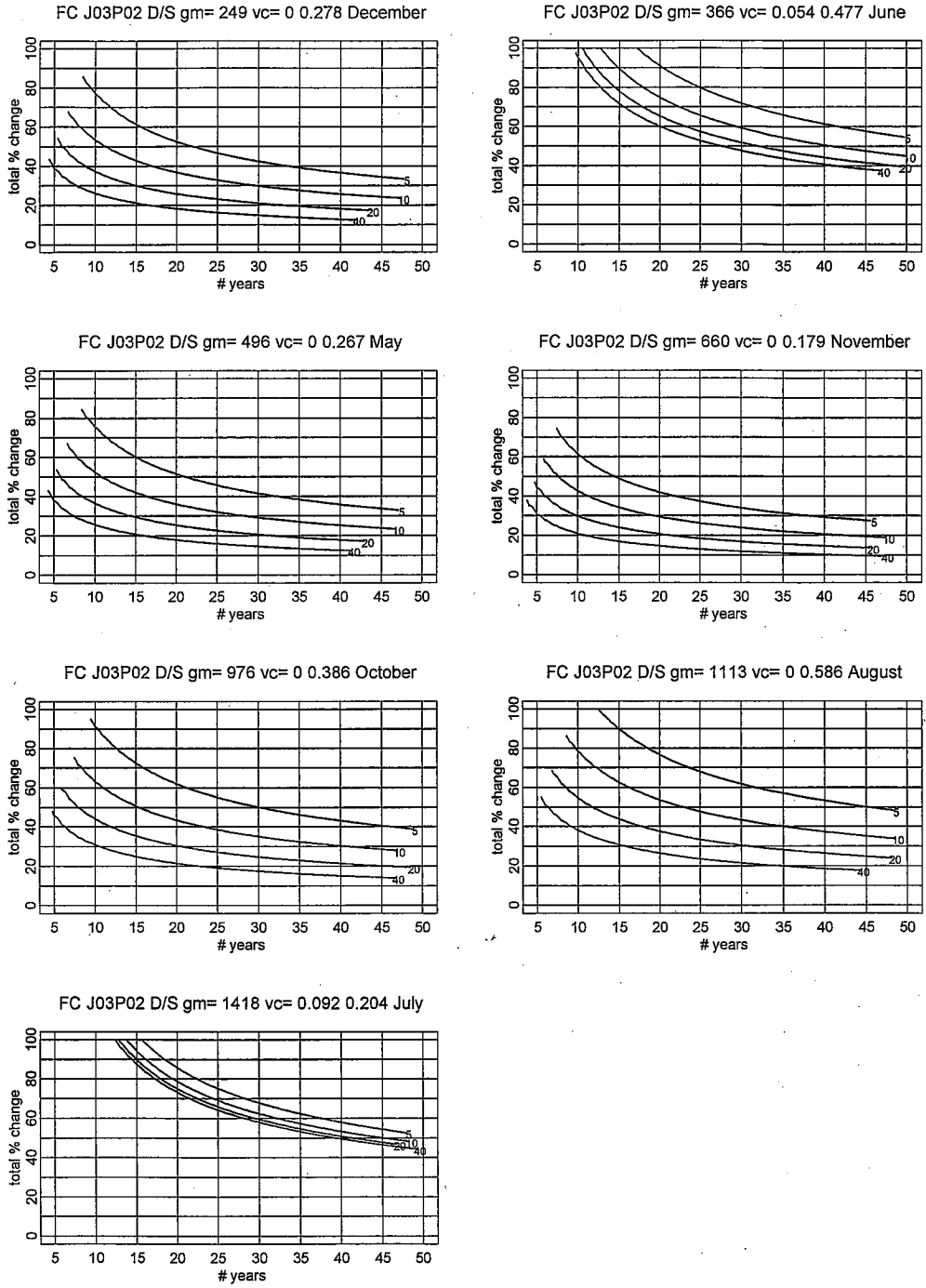


Figure A2.1b. Power analysis of a trend monitoring design at the confluence of Aliso and Sulphur Creeks, station J03P02 downstream. The y-axis shows the amount of change detectable, the x-axis the years of sampling, and the different curves the number of samples in a given 30-day period (5, 10, 20, 40) needed for 80% power.

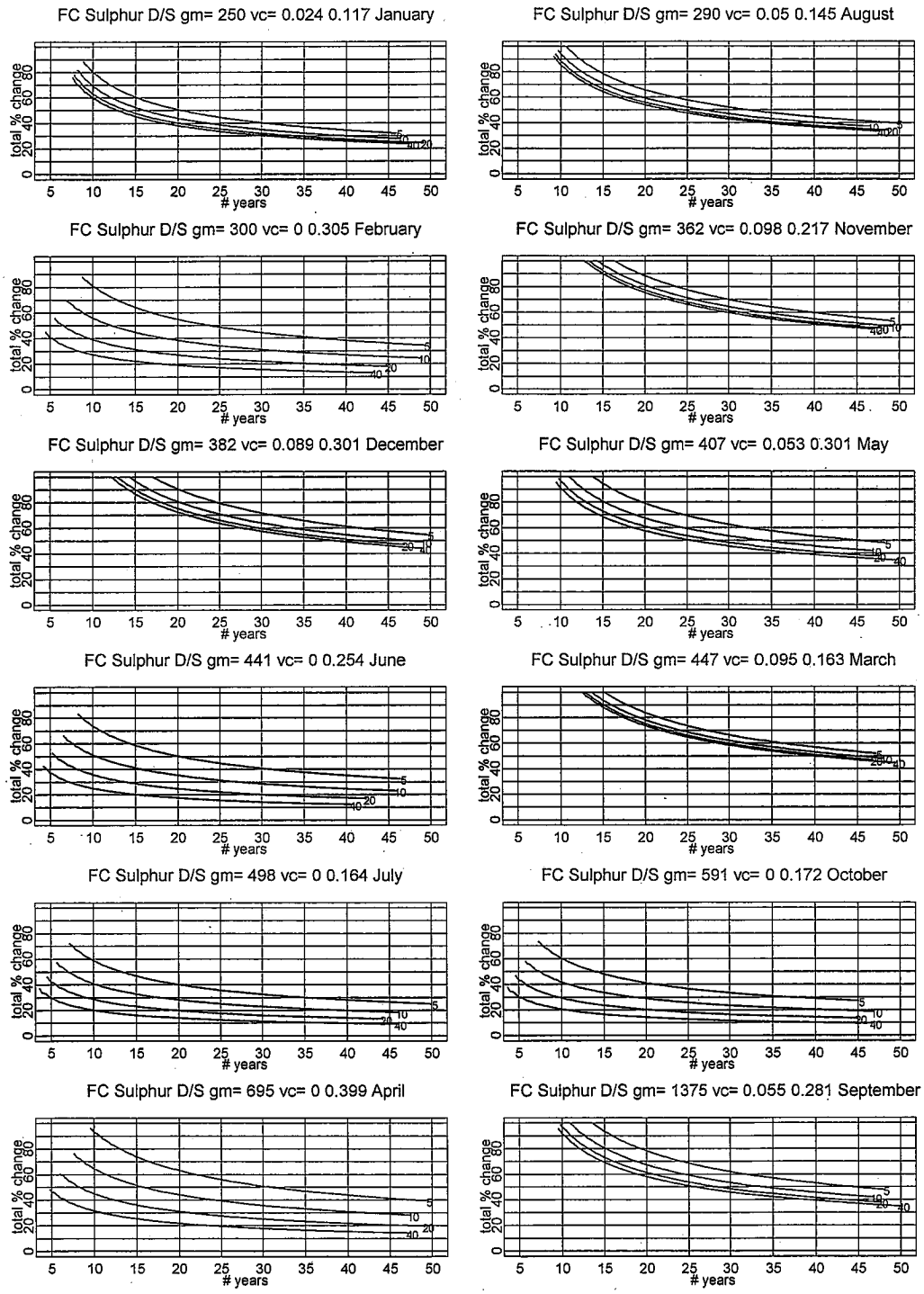


Figure A2.1c. Power analysis of a trend monitoring design at Aliso Wood Canyon Park, station Sulphur Creek downstream. The y-axis shows the amount of change detectable, the x-axis the years of sampling, and the different curves the number of samples in a given 30-day period (5, 10, 20, 40) needed for 80% power.

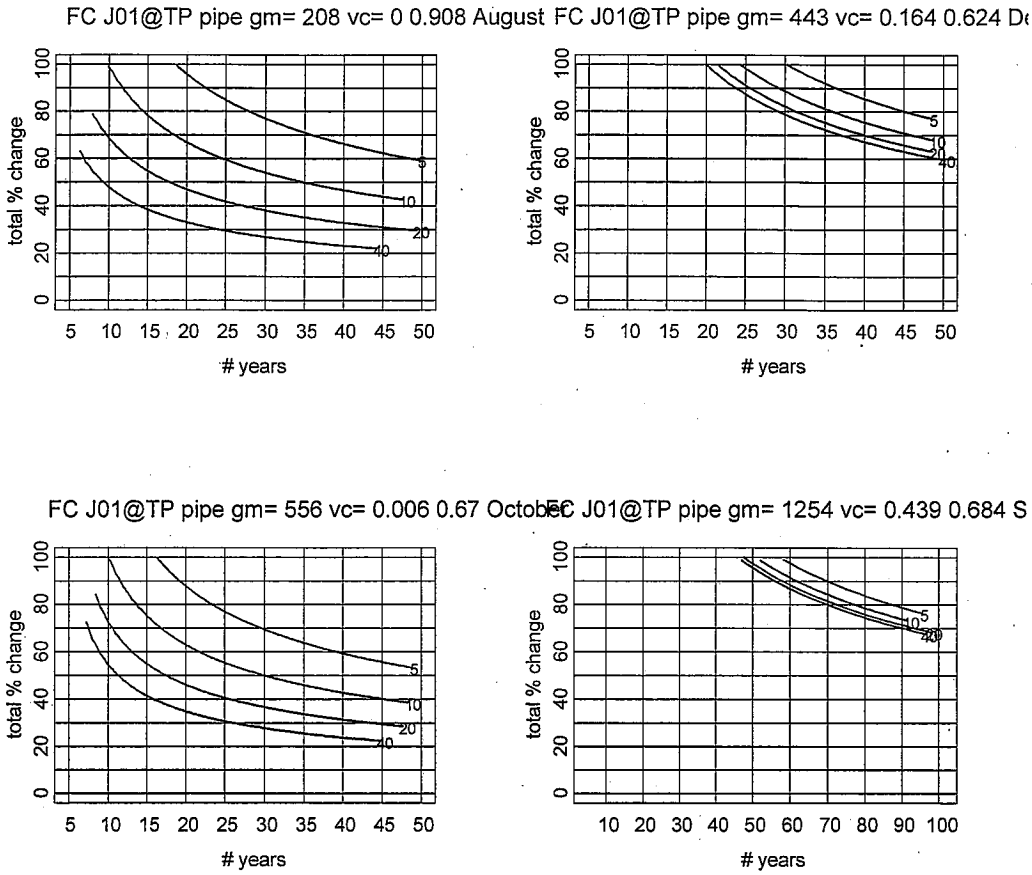


Figure A2.1d Power analysis of a trend monitoring design at the SOCWA treatment plant, station J01@TP. The y-axis shows the amount of change detectable, the x-axis the years of sampling, and the different curves the number of samples in a given 30-day period (5, 10, 20, 40) needed for 80% power.

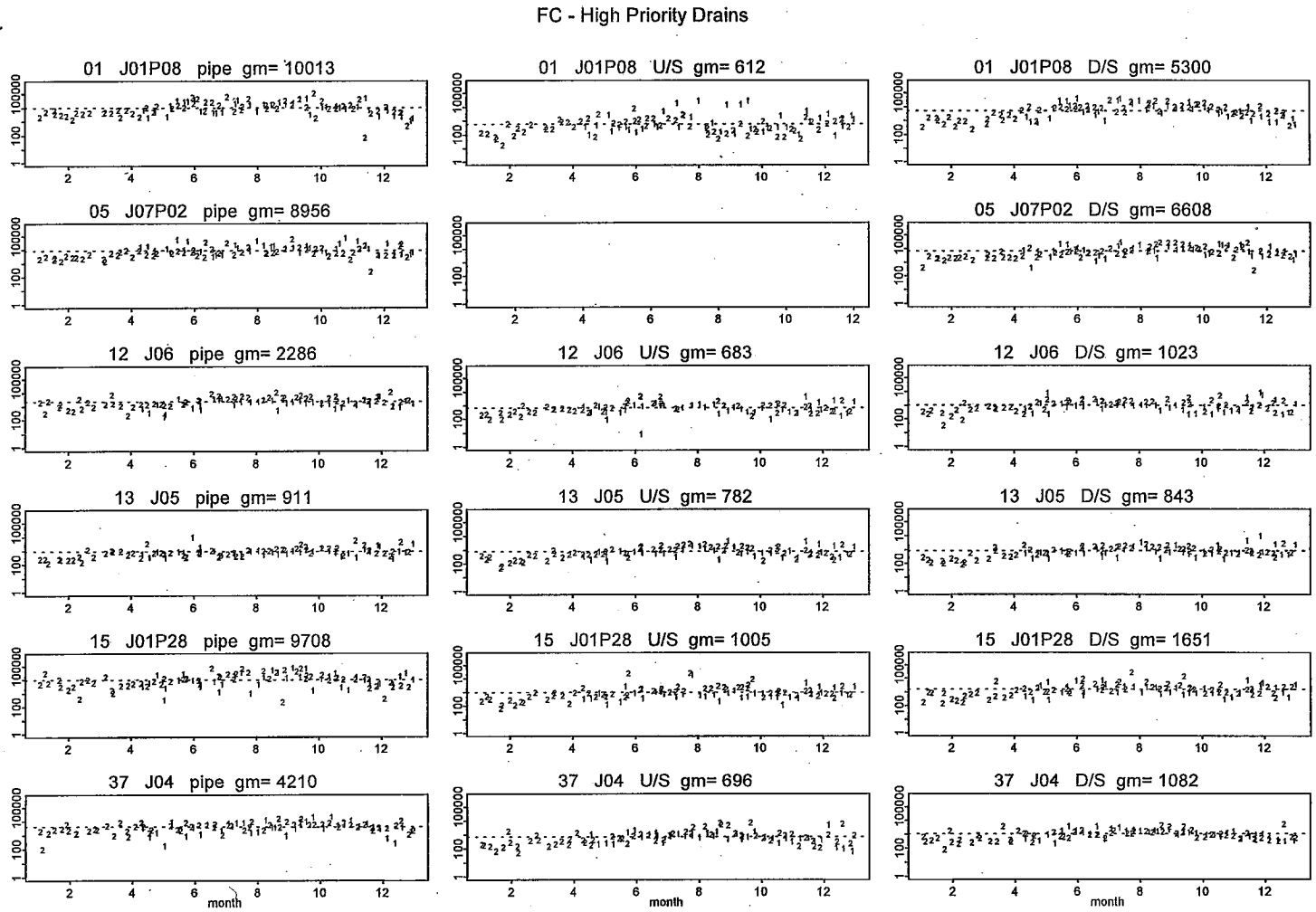


Figure A2.2. Levels of bacterial indicators at the upstream stations in Aliso Creek that are the focus of source reduction efforts.

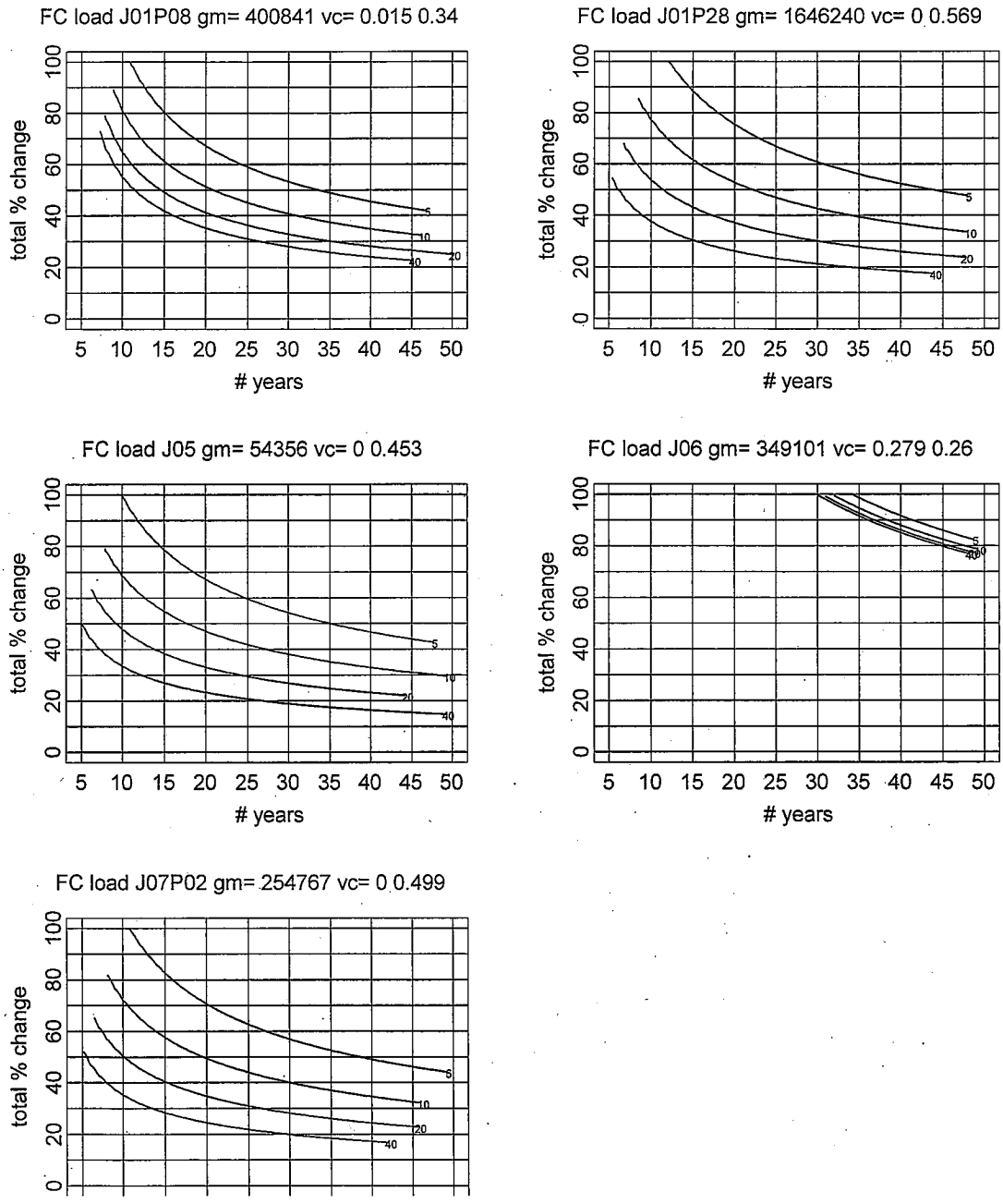


Figure A2.3. Statistical power analysis results of a trend monitoring design for bacterial loads at the upstream stations in Aliso Creek.

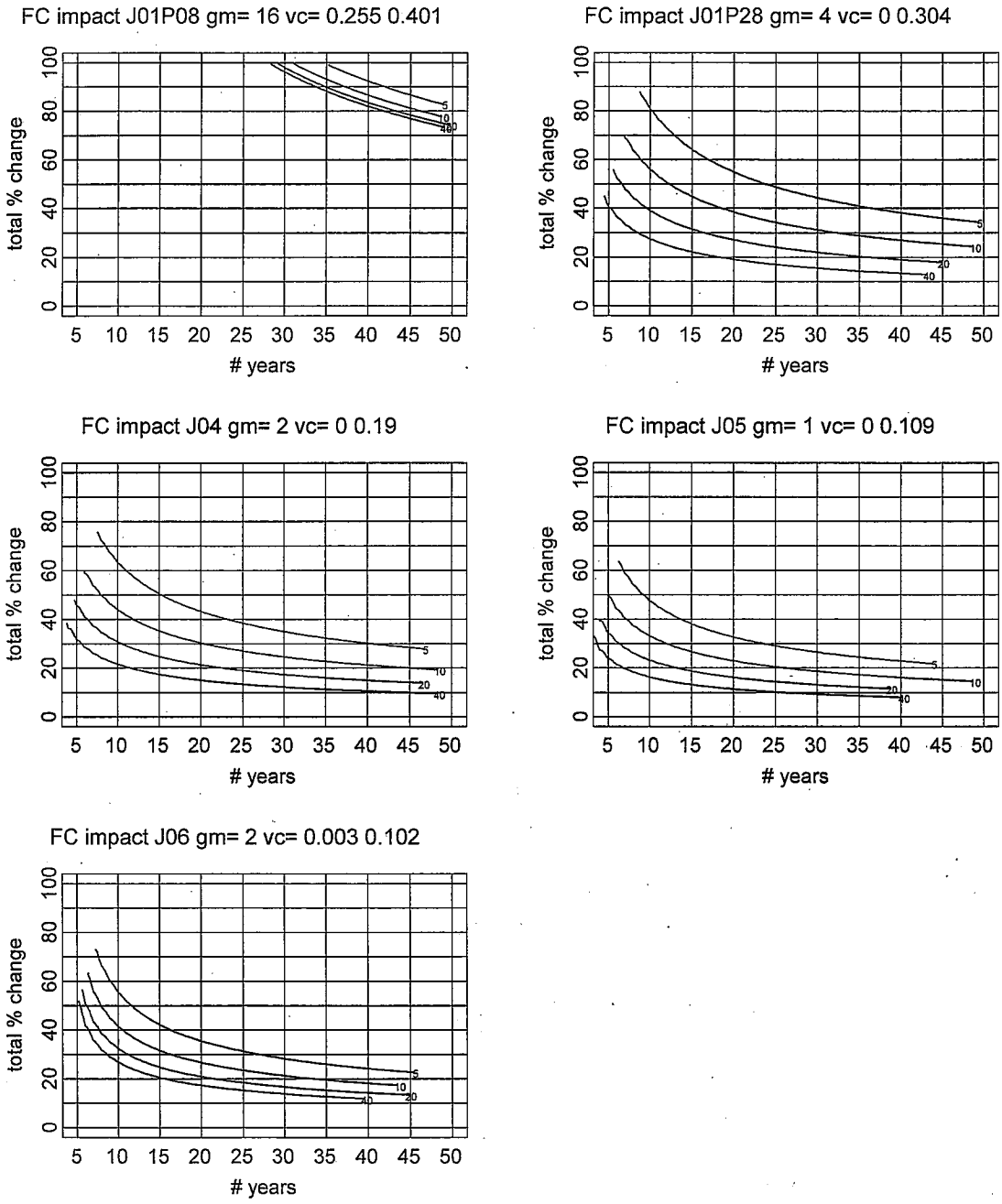


Figure A2.4. Statistical power analysis results of a trend monitoring design for receiving water impact (measured as the difference in bacterial levels between stations 25 feet upstream and downstream of the discharge point) at upstream stations in Aliso Creek.

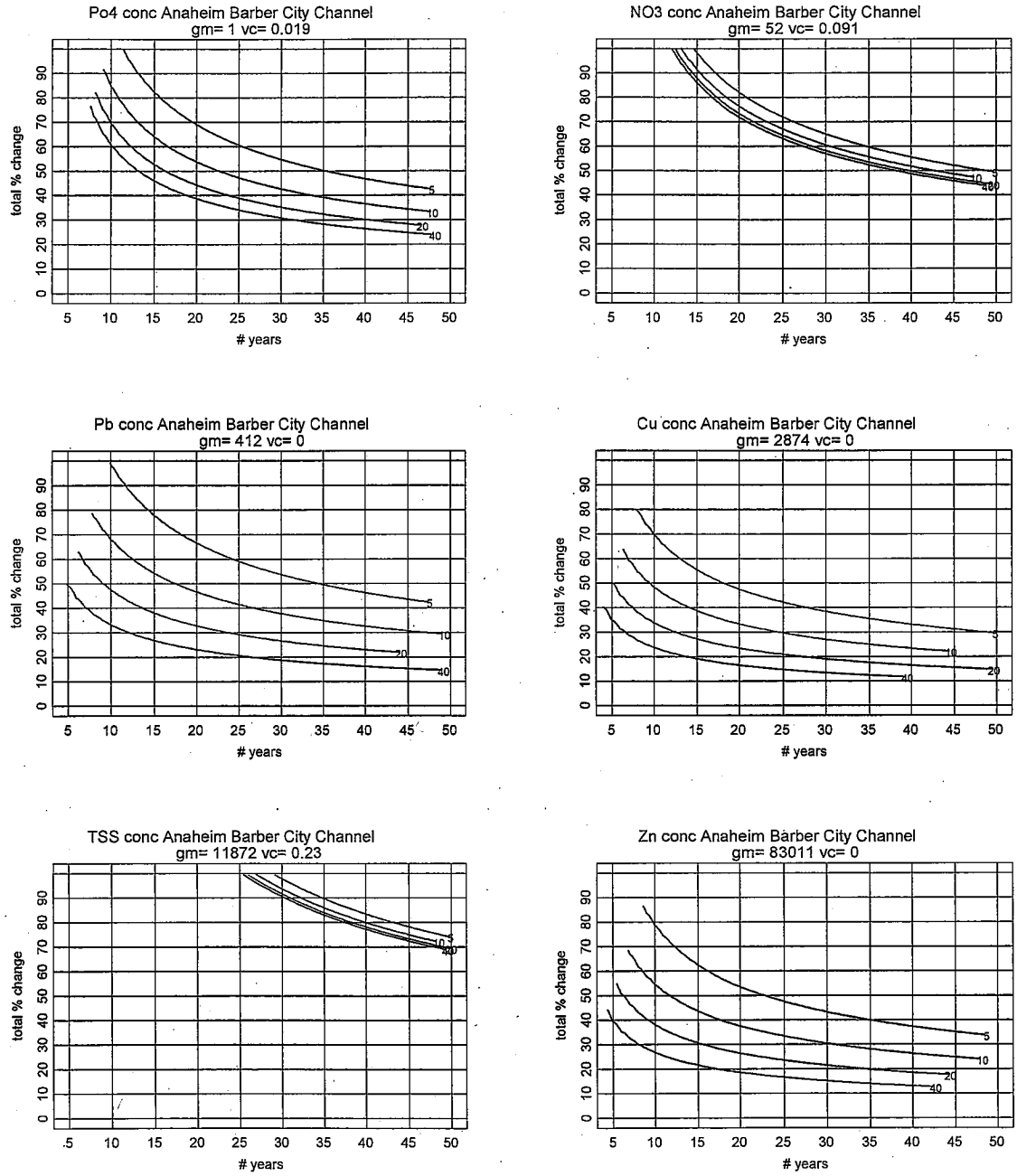


Figure A2.5. Statistical power analysis results for a trend monitoring design for event mean concentrations (EMC) of several parameters at Anaheim Barber City Channel, a long-term mass emissions station in Orange County.

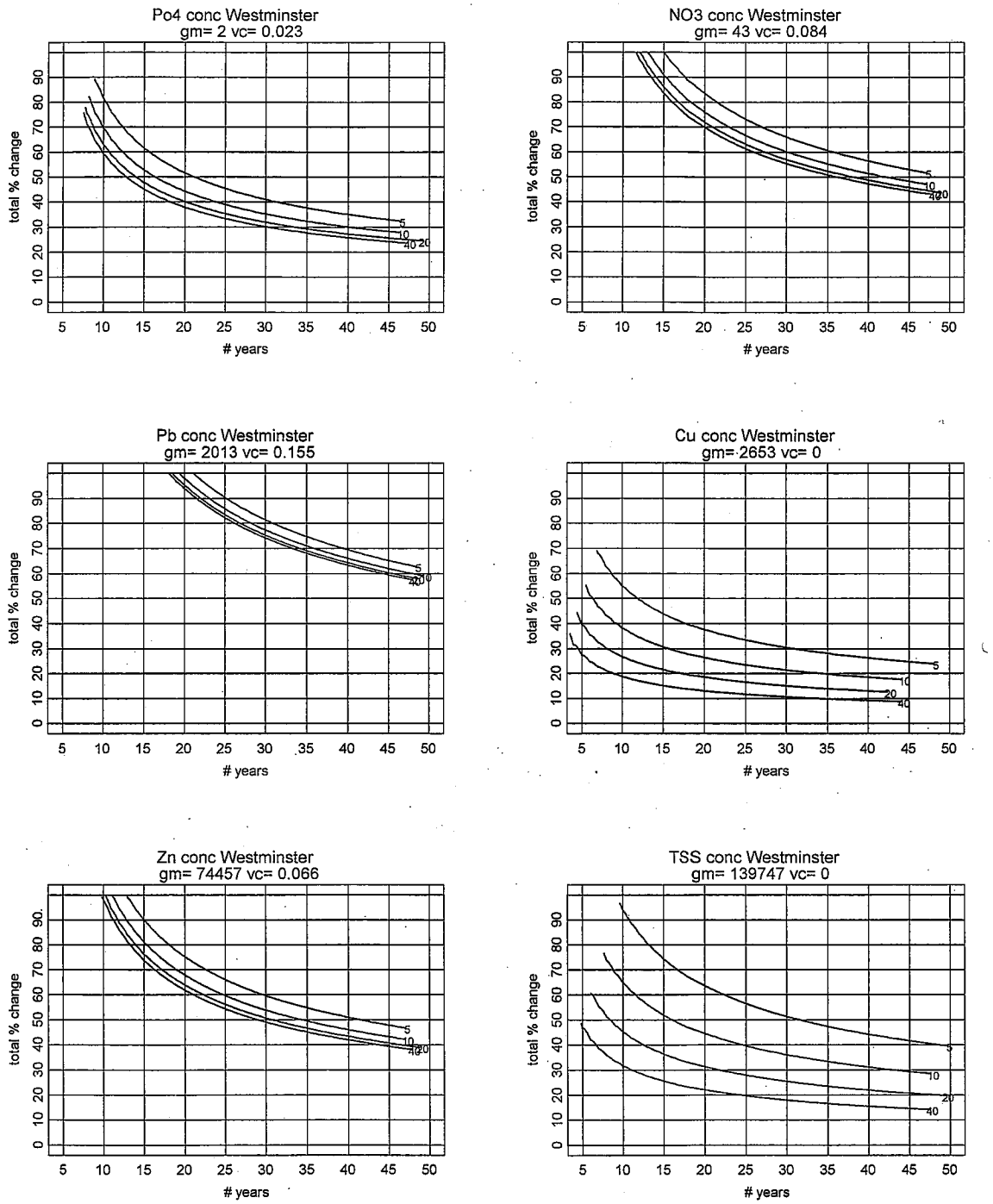


Figure A2.6. Statistical power analysis results for a trend monitoring design for event mean concentrations (EMC) of several parameters at Westminster Channel, a long-term mass emissions station in Orange County.

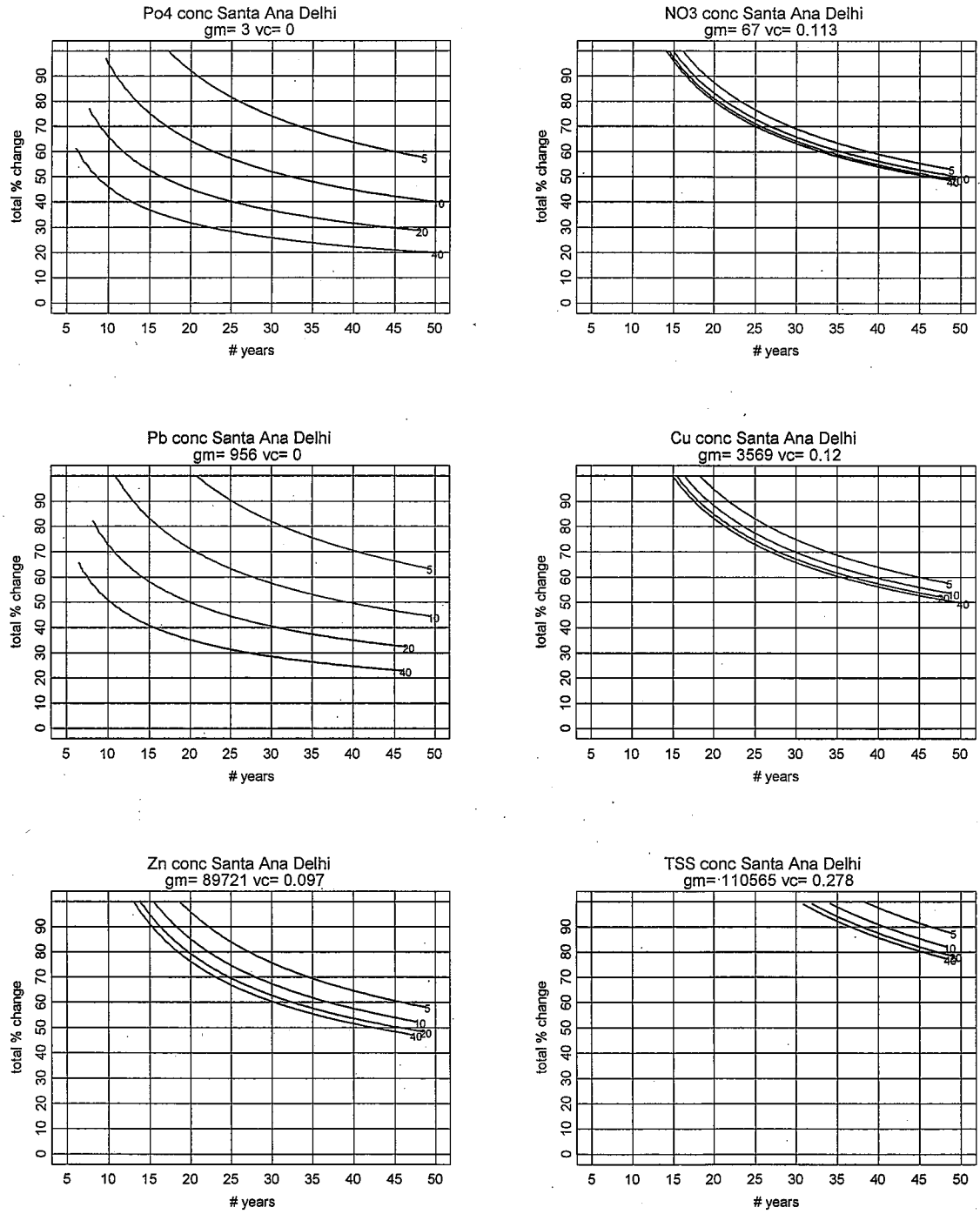


Figure A2.7. Statistical power analysis results for a trend monitoring design for event mean concentrations (EMC) of several parameters at Santa Ana Delhi Channel, a long-term mass emissions station in Orange Couinty.

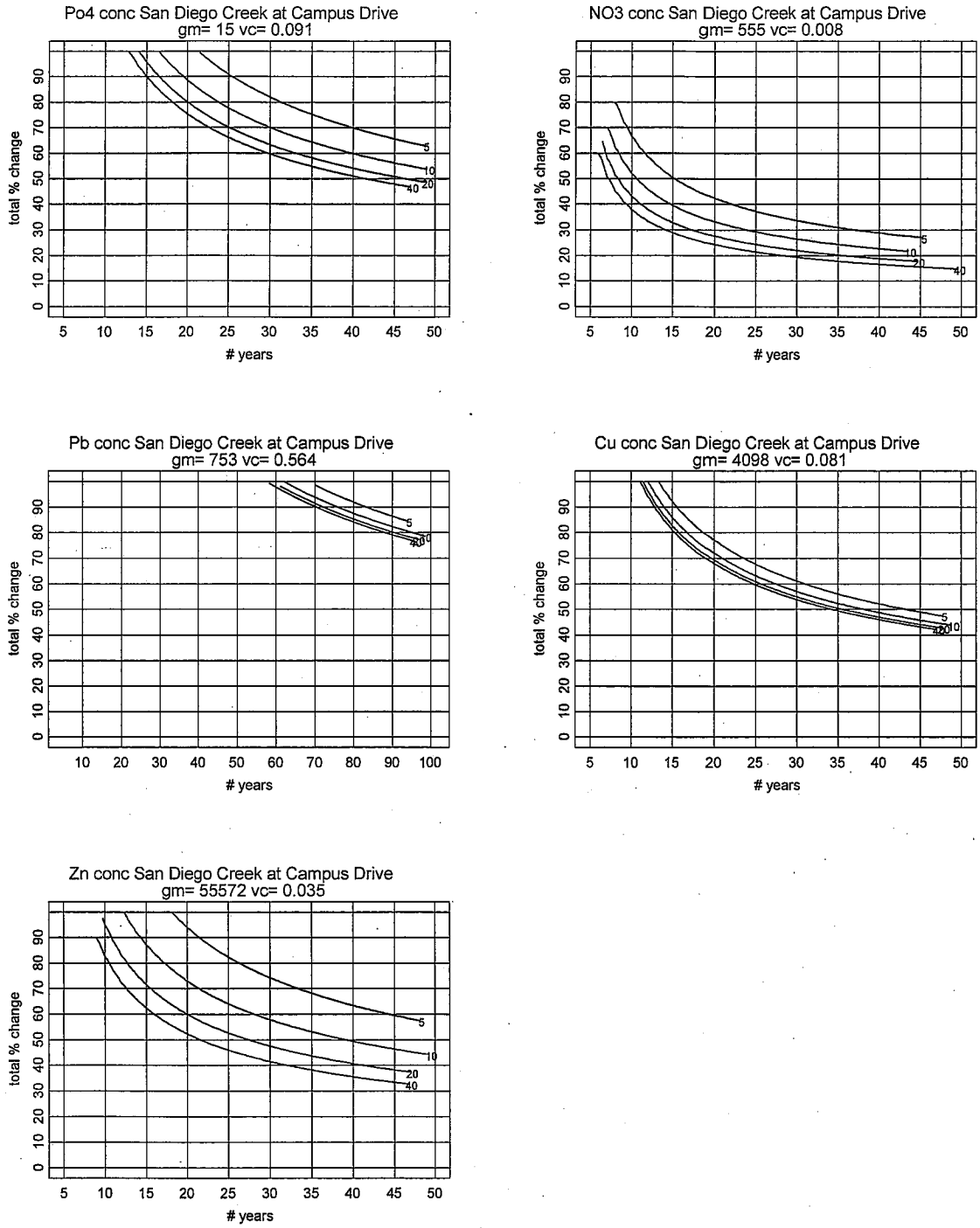


Figure A2.8. Statistical power analysis results for a trend monitoring design for event mean concentrations (EMC) of several parameters at San Diego Creek at Campus Drive, a long-term mass emissions station in Orange County.

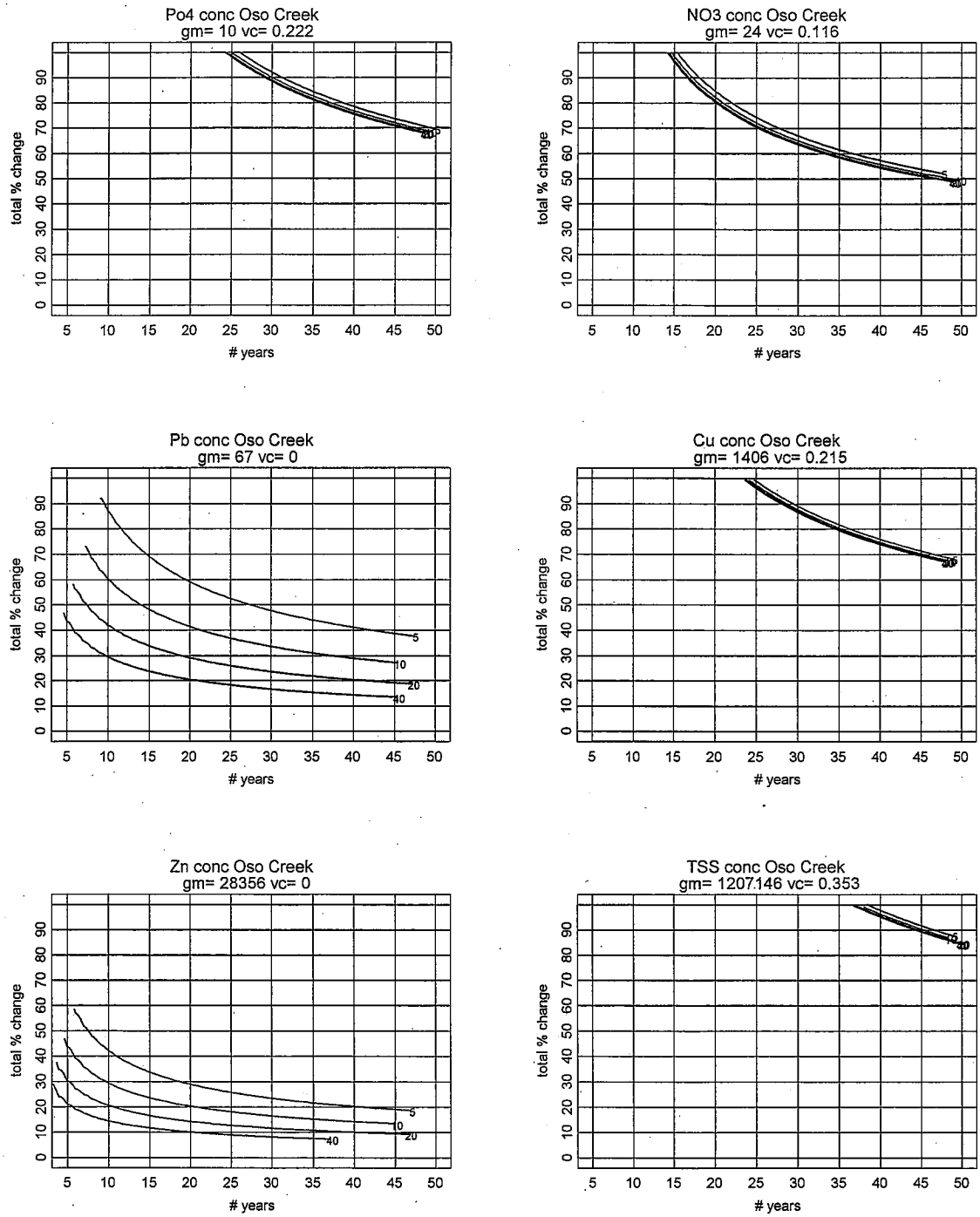


Figure A2.9. Statistical power analysis results for a trend monitoring design for event mean concentrations (EMC) of several parameters at Oso Creek, a long-term mass emissions station in Orange County.

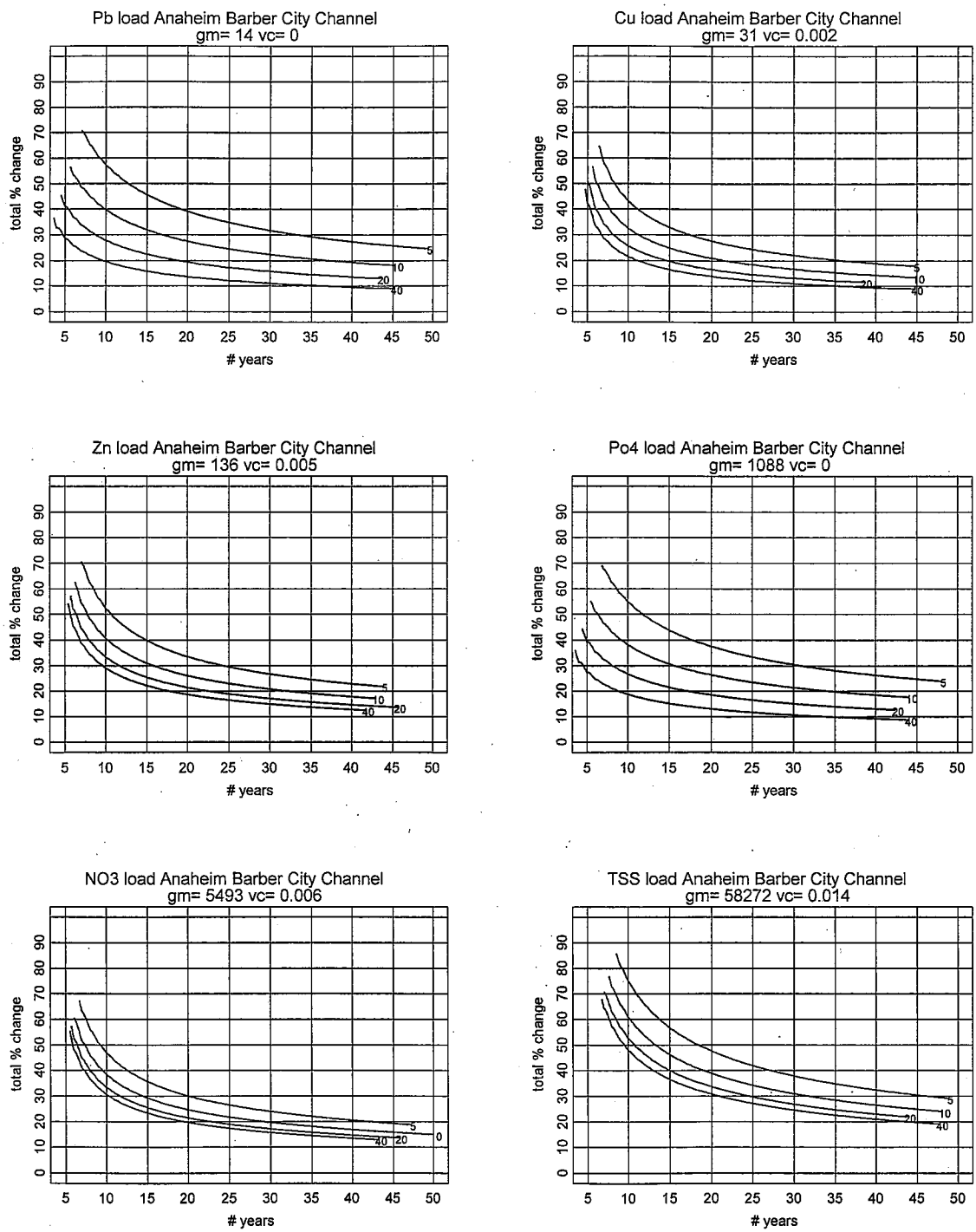


Figure A2.10. Statistical power analysis results for a trend monitoring design for loads of several parameters at Anaheim Barber Channel, a long-term mass emissions station in Orange County.

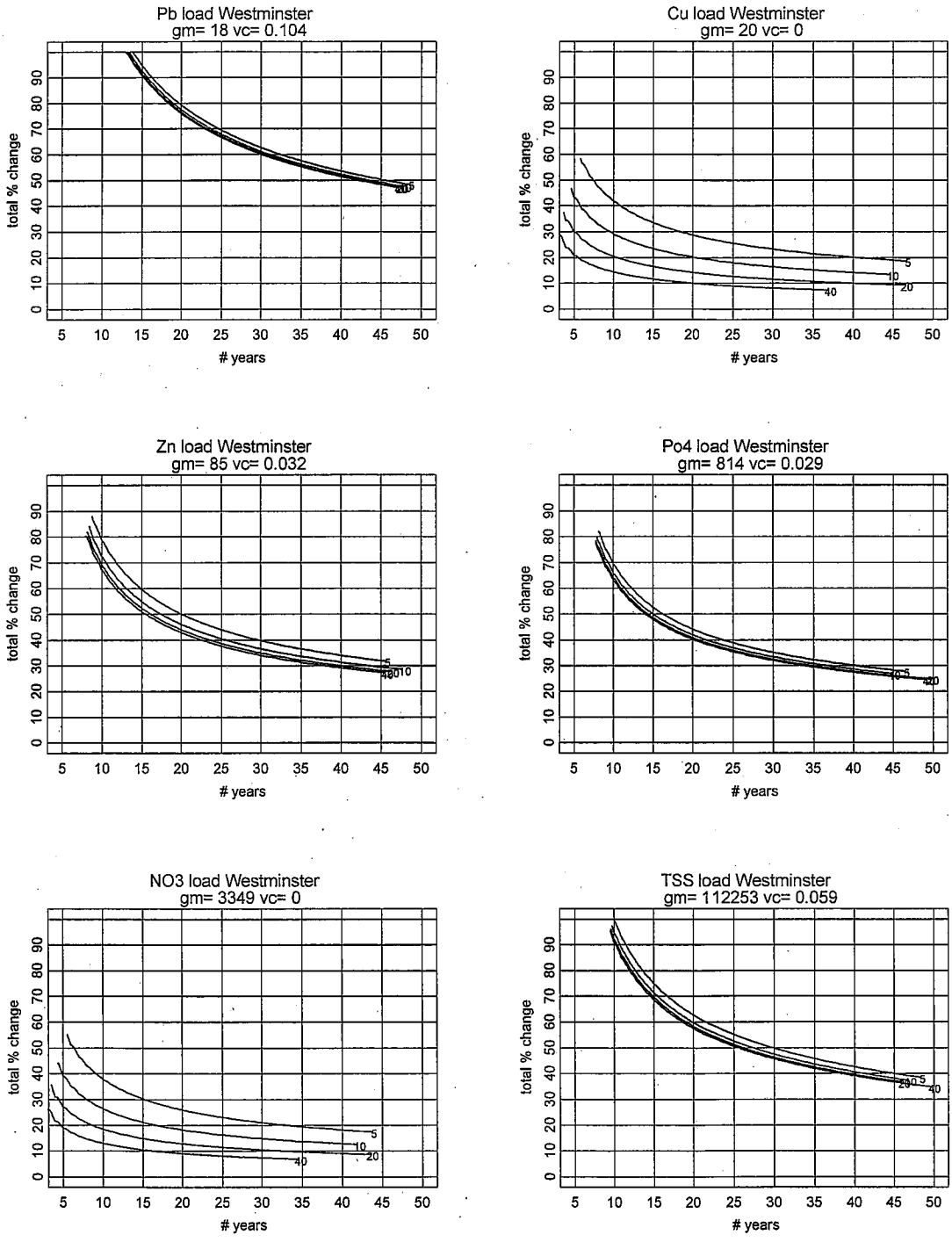


Figure A2.11. Statistical power analysis results for a trend monitoring design for loads of several parameters at Westminster Channel, a long-term mass emissions station in Orange County.

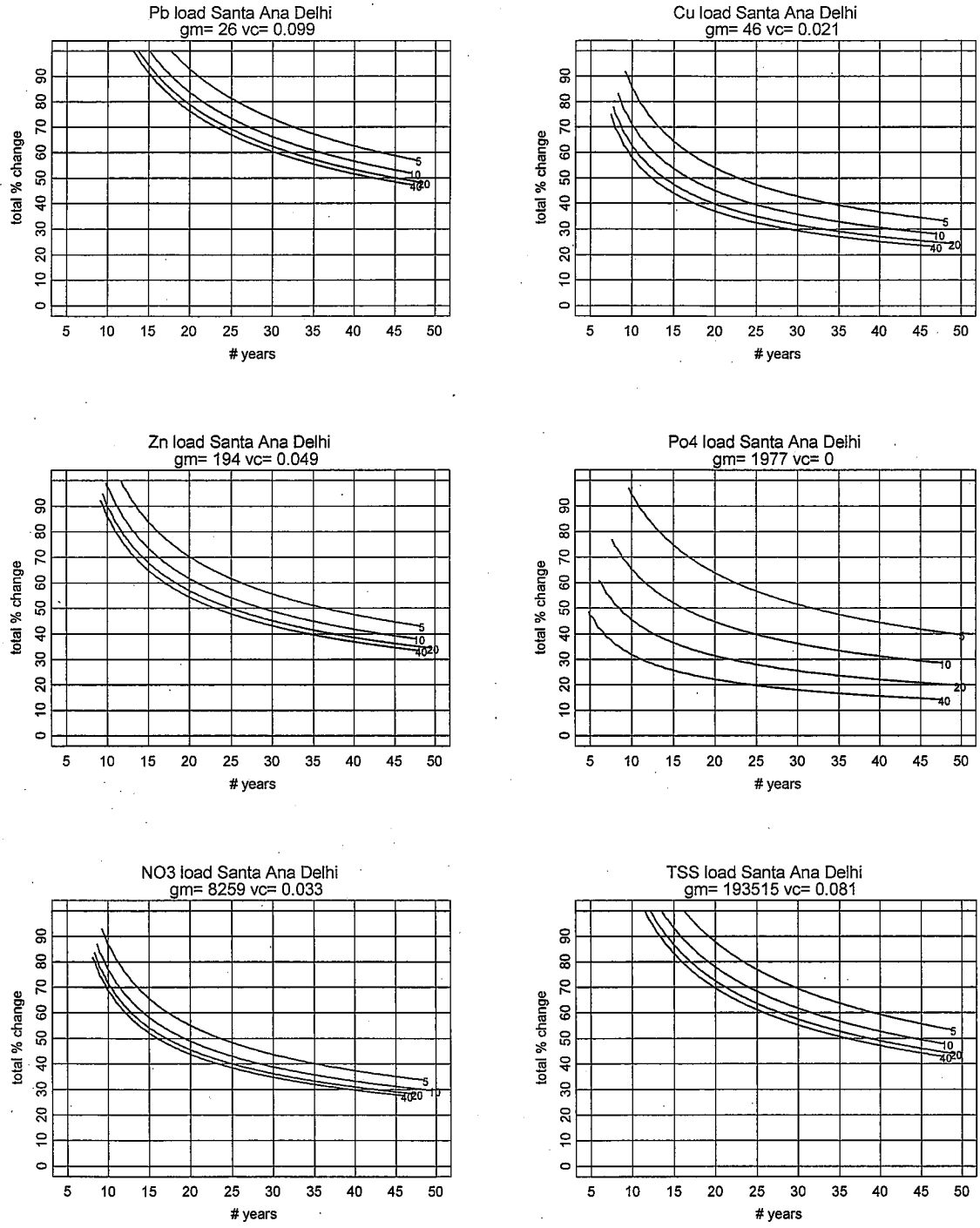


Figure A2.12. Statistical power analysis results for a trend monitoring design for loads of several parameters at Santa Ana Delhi Channel, a long-term mass emissions station in Orange County.

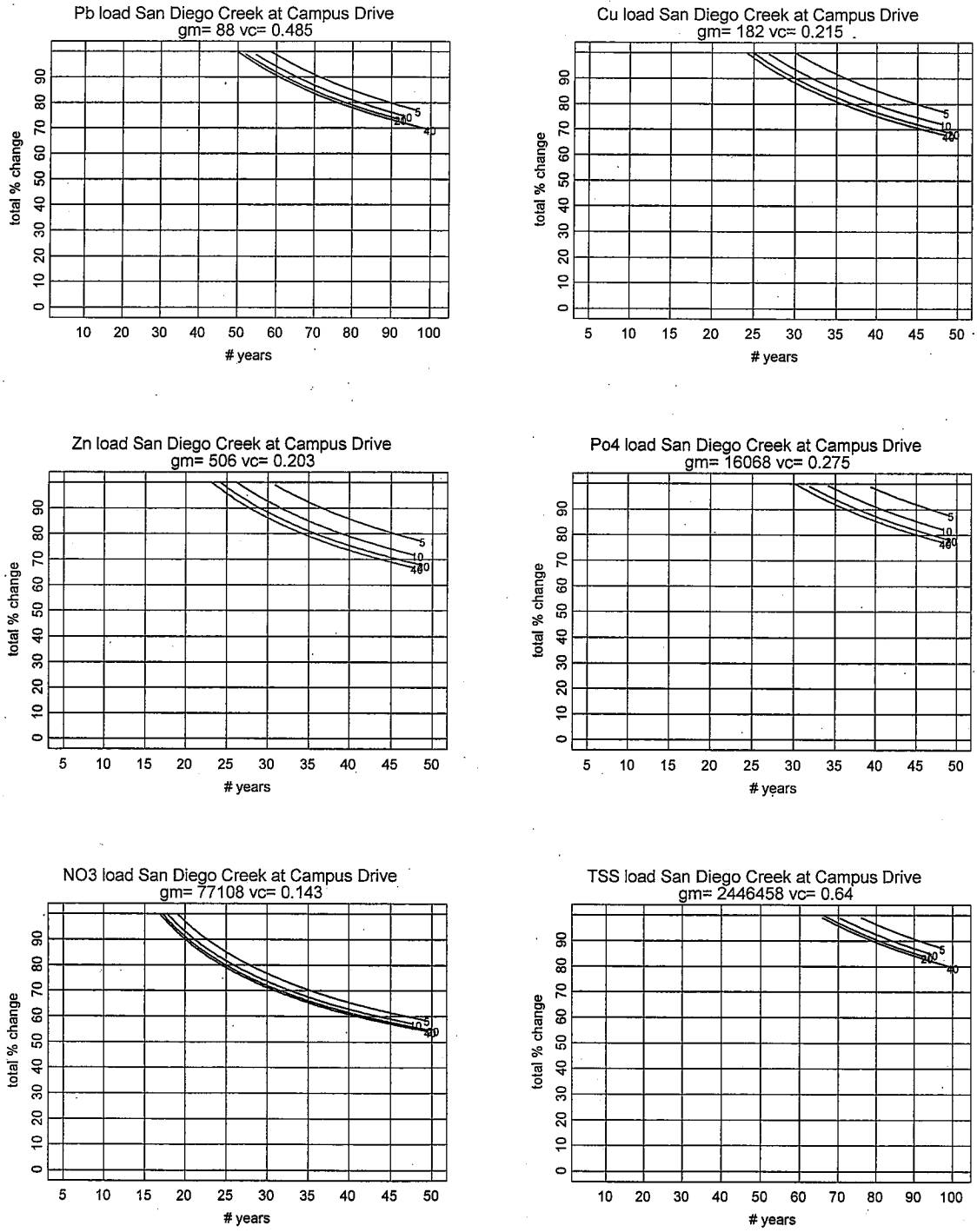


Figure A2.13. Statistical power analysis results for a trend monitoring design for loads of several parameters at San Diego Creek at Campus Drive, a long-term mass emissions station in Orange County.

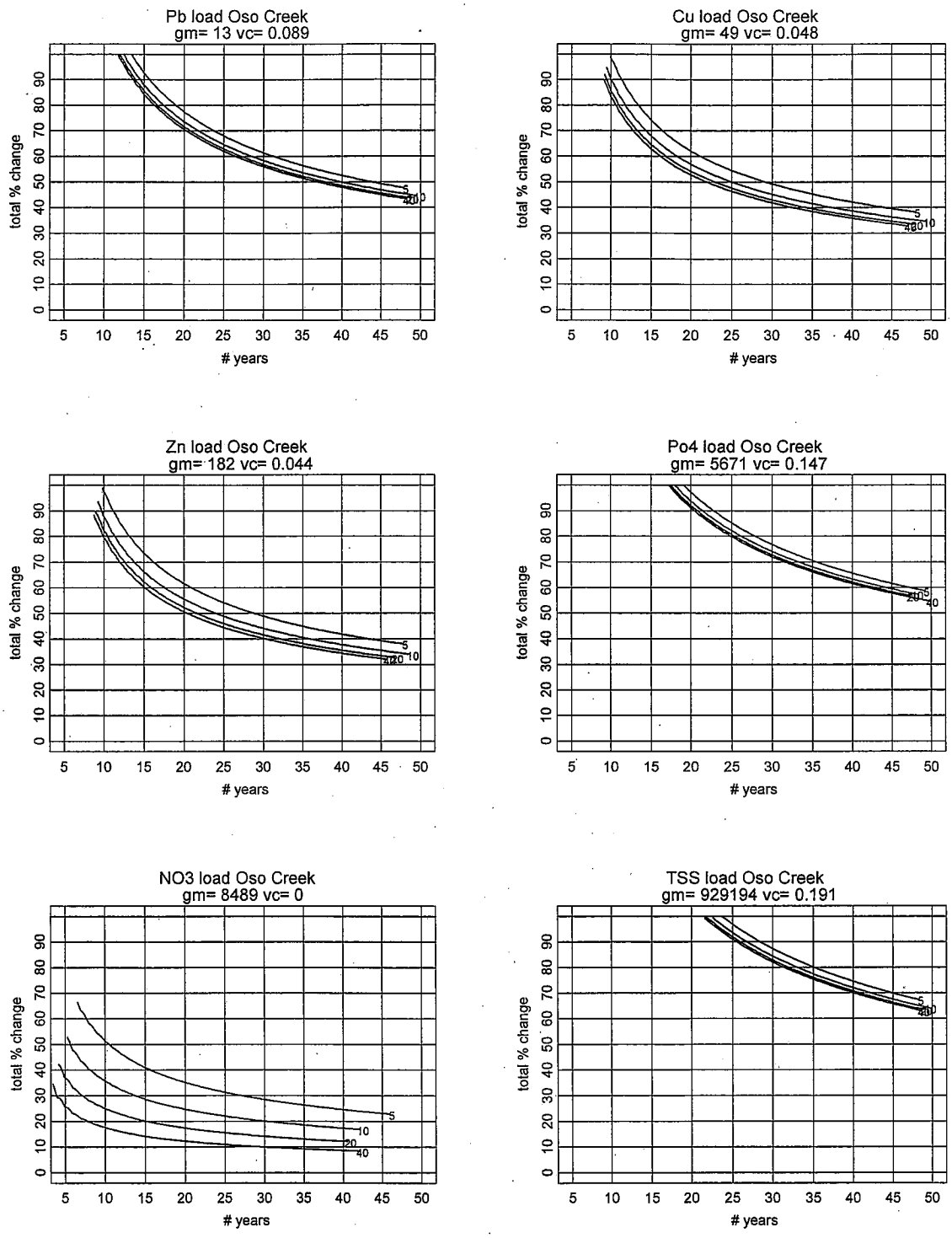


Figure A2.14. Statistical power analysis results for a trend monitoring design for loads of several parameters at Oso Creek, a long-term mass emissions station in Orange County.

Appendix 3: Source Identification Case Studies

The following case studies present examples of source identification efforts conducted to determine the rough proportion of input from urban runoff sources. They include several different kinds of problems and study approaches and exemplify the variety of methods that might be employed to address Question 3. The majority of the case studies were conducted in dry weather. This reflects difficulties of performing source identification studies in wet weather, given the large flow volumes and the typically increased number of possible sources. The cases also exhibit a range of level of effort, from evaluation of routine monitoring data and interviews to a series of iterative special field studies.

A3.1 Contaminated sediment taskforce (LA Harbor)

Sediments in ports, harbors, and marinas are subject to numerous pollutant inputs including sediments, trace metals, and organic contaminants. These sediments eventually need to be dredged to maintain navigable waterways, but the level of sediment contamination has a tremendous effect on the eventual disposal of these dredged materials. Clean sediments can be used for beach replenishment or even disposed at sea, but contaminated sediments need to be sent to a landfill or some other confined disposal area so they will not harm the environment. As the Port Districts, RWQCB and Coastal Commission (collectively known as the Contaminated Sediment Task Force) design a long-term dredged material management program, they are carefully considering ways to reduce the inputs of pollutants to the areas that need periodic maintenance dredging. One way to accomplish this is to identify and reduce or eliminate the sources of pollutants to these locations.

In order to begin reducing pollutant loads, the Contaminated Sediments Task Force asked SCCWRP to estimate the relative magnitude of pollutant loading from several potential sources to Los Angeles and Long Beach Harbors and Marina del Rey. A particular emphasis of the study was estimating loads from the Los Angeles River and Dominguez Channel to San Pedro Bay and from Ballona Creek to Marina del Rey. Primary questions addressed included:

- What are the predominant sources of contaminants?
- What are the long-term (i.e. decadal) trends in annual loading?
- What is the typical range of annual loading that should be expected?
- Which watersheds typically contribute the greatest annual loading?
- What land use types are the largest contributors to annual loading?

These questions were evaluated with an assessment study that involved existing data and limited modeling to estimate watershed loading patterns. Because historic data were somewhat limited, SCCWRP estimated loads with a ratio estimation technique. This involves establishing a relationship between flow and loads using available data and then applying this relationship to an entire storm season. Flow was estimated by applying rainfall data and standard runoff coefficients to different land use types. This combination of methods allowed loads to be approximated for a variety of land uses for entire years in the periods 1971-72, 1979-80, 1986-87, and 1987-88.

The analysis confirmed that the largest source of contamination to San Pedro Bay is watershed-derived loading from the Los Angeles River and Dominguez Channel watersheds. The Los Angeles River watershed contributed the greatest overall mass loading, but the Dominguez Channel watershed contributed the largest proportional loading (i.e. loading normalized for

watershed size). In general, industrial and residential land uses are the largest contributors of contaminants. Data from the 1990s also revealed that dry season (i.e. non-storm) loading may make up a significant portion of total annual loading, and in dry years, can be the predominant source of contaminants to the harbor. Analysis of temporal trends in the data showed that metals loading has not substantially changed since the 1970s, but loading of DDT and PCBs has declined.

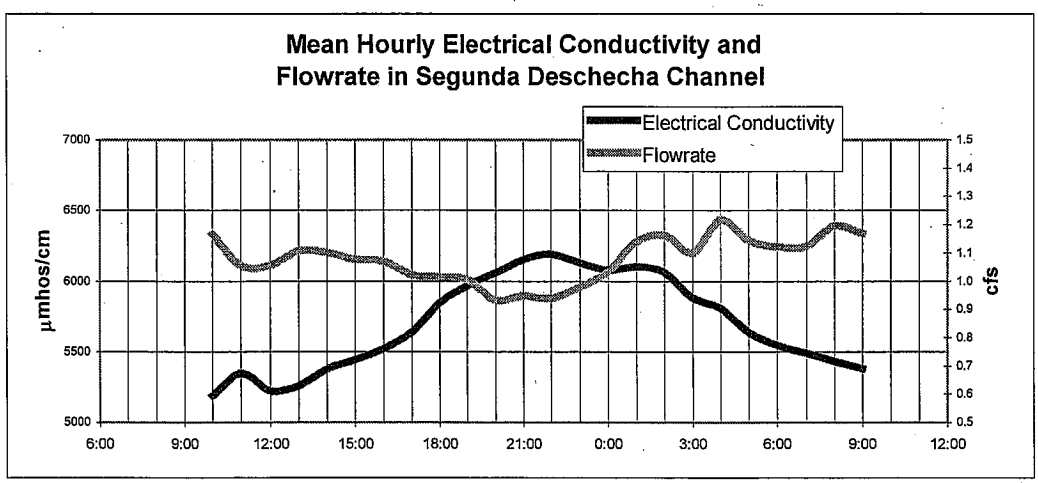
Annual loadings of metals varied between 10^3 and 10^5 kg/year, with zinc and copper loading typically exceeding loads of other metals. Variations in annual loading appear to correspond with changes in rainfall and runoff; however, direct analysis of the relationship between rainfall intensity and duration and loading produced only weak correlation coefficients. This correlation would likely be improved by analyzing a larger data set on more homogenous land use types.

Because the study depended on available data, not all possible sources of loading could be evaluated and key data gaps remained. These included the lack of data on loading of PAH and pesticides, lack of long-term data on dry season loading, lack of information on inputs from the Dominguez Channel Watershed, and the need for more temporally resolved loading data from specific land use types. Information on the transport and fate of runoff-derived contaminants within the study area would also be needed to improve estimates of the impact of loadings on sediment contamination.

Summary based on SCCWRP Technical Report #143. Watershed-based sources of contaminants to San Pedro Bay and Marina del Rey: Patterns and trends. October 13, 2003. ftp://ftp.sccwrp.org/pub/download/PDFs/413_cstf_watershed.pdf

A3.2 Elevated total dissolved solids in Prima and Segunda Deshecha channels (Orange County)

Routine monitoring during the 2001-2002 monitoring year documented elevated levels of total dissolved solids (TDS) at monitoring stations in Prima and Segunda Deshecha channels in Orange County. Special studies involving hourly measurements of conductivity and flow rate, conducted in both channels, showed that the peak TDS concentration was not a function of tide (i.e., did not reflect a higher concentration of saltwater) and that the TDS concentration was inversely proportional to flow rate in the channel (see the figure below).



This strongly suggested that urban runoff diluted a naturally high level of dissolved solids in the channels. Subsequent to this finding, an upstream reconnaissance survey identified the presence of hundreds of weepholes in the concrete sidewalls of the channel. These weepholes appeared to be allowing subsurface drainage to leach salts from soils and carry them into the channel. Preliminary sampling of three weepholes during the reconnaissance survey showed them to have extremely high levels of electrical conductivity, an indicator of TDS. In addition, the crystalline residue on the channel walls near the routine monitoring location was found to have high concentrations of sodium and soluble sulfate.

These findings provided the basis for a more substantial upstream source identification study in the Prima Deshecha channel in March 2002 that included monitoring at several weepholes, as well as upstream and downstream of the weepholes. The resulting data (see following table) indicate that the seepage from the channel seams and weepholes increases the concentrations of dissolved solids in the channel downstream of the seepage.

Reach of Channel	Time	Monitoring Point	EC (mhos)	TDS (mg/L)
At Diamante	13:00	Prima Deshecha Channel (M01)	Dry	
At Calle Nuevo	13:40	M01	5,150	
u/s Avenida Vacquero	14:48	M01 50' u/s weeping seam	5,510	4,880
	14:45	weeping seam	19,870	18,900
	14:42	M01 50' d/s weeping seam	7,510	7,330
	14:50	36" pipe discharging to M01	3,800	
d/s I-5	15:20	M01 in Shorecliff Golf Course	7,550	
u/s Calle Grande Vista	15:37	M01 50' u/s bubbling weepholes	7,750	7,490
	15:34	Bubbling weepholes	14,480	12,800
	15:40	48"pipe discharging to M01, 20' d/s weepholes	5,850	
At Calle Grande Vista	15:30	M01	8,480	

The conclusion was corroborated by soil samples collected from the levee of the Prima Deshecha channel above a weeping seam and from the levee of San Juan Creek, a channel with no history of elevated TDS levels (see following table).

Location	Chloride	Soluble Sulfate	Calcium	Magnesium	Potassium	Sodium
	mg/kg	%	mg/kg	mg/kg	mg/kg	mg/kg
Prima Deshecha Ch. Levee	61.3	0.447	861	275	37.2	582
San Juan Creek levee	13/7	0.048	34.1	11.1	15.4	202

Information obtained from the 2001-2002 annual report of the Orange County Stormwater Program

A3.3 SCCWRP's study of sources of loads to the LA River

The Los Angeles River drains most of Los Angeles County and extends 56 miles, starting from its headwaters in the San Fernando Valley, flowing past downtown Los Angeles, and eventually draining to San Pedro Bay near Long Beach. The highly developed watershed is 834 mi^2 and is comprised of residential (35%), commercial (5%), industrial (8%), and open land (51%) uses. The river's mainstem and tributaries are listed as impaired waterbodies for many constituents including nutrients (N), bacteria (fecal coliform), and trace metals (copper, lead, and zinc). The three primary sources of these pollutants included water reclamation plants (WRPs), major

tributaries, and storm drain outfalls. As part of efforts to establish TMDLs for the river, SCCWRP conducted a short-term study to characterize the water quality in the Los Angeles River and the various loads to the system.

This study was comprised of two parts. The first identified and sampled the inputs to the Los Angeles River and major tributaries. The second sampled the mainstem of the river to assess spatial distributions of water quality. The input monitoring was conducted using citizen volunteers while the spatial distribution monitoring was conducting using professionals. Visual observations were made of the outfall size and location, flow, and general characteristics (such as water discoloration; the presence of foam or oily sheens, trash or algae; and water quality). Flow was measured using either timed-volumetric or depth-velocity methods.

Water quality parameters included flow, total suspended solids (TSS), total organic carbon (TOC), biological oxygen demand (BOD5), nutrients (nitrate, nitrite, ammonia, TKN, and total phosphorous), and trace metals (cadmium, chromium, copper, iron, lead, nickel, mercury, and zinc). Sampling was accomplished on September 11, 2000 and included eight locations along the mainstem of the Los Angeles River and at the head of all seven tributaries Existing flow gages maintained by the Los Angeles County Department of Public Works provided flow information.

Table A3-3 shows the relative magnitude of the various inputs to the LA River. The majority of the dry weather flow in the river arose from the three inland POTW discharges in this watershed. In accordance, POTWs were the largest source of nutrients and some trace metals. In contrast, storm drains were the major source of bacteria and the remaining trace metals during dry weather. This preliminary sampling effort provided data sufficient to characterize the relative contributions of the major sources of pollutant loads to the system and a basis for more detailed source identification and loadings studies in the future.

Table A3-3. Total pollutant loads and the relative contributions among major sources to theLos Angeles River on September 10-11, 2000.

Constituent		Total Mass Emissions	Units	% Contribution POTWs	Tributaries	Storm Drains
Bacteria						
	E. coli	12,022	(10^9)/day	0	11	89
	Enterococcus	2,948	(10^9)/day	0	33	67
	Total Coliforms	113,854	(10^9)/day	1	65	35
Metals						
	Copper	3.7	kg/day	73	22	6
	Iron	39	kg/day	4	23	73
	Lead	0.53	kg/day	0	54	46
	Nickel	0.19	kg/day	0	0	100
	Zinc	11	kg/day	79	17	4
Nutrients						
	Ammonia-N3, 357		85	14	0	34
	Nitrate-N		kg/day	32	35	2
	TKN		kg/day	82	17	2
	Total Phosphate-P		kg/day	82	15	3

Ackerman, D., K. Schiff, H. Trim, and M. Mullin. 2003. Characterization of water quality in the Los Angeles River. *Bulletin of the Southern California Academy of Sciences* 102:17-25 or at ftp://ftp.sccwrp.org/pub/download/PDFs/2001_02ANNUALREPORT/08_ar08-drew.pdf.

A3.4 Elevated levels of diazinon in Bouquet Canyon Creek (Los Angeles County)

Toxicity tests conducted in late 2001 on water from Bouquet Canyon Creek documented elevated toxicity (4 – 5 toxic units). Subsequent TIEs showed the toxicity to be due primarily to diazinon, and water samples collected through late 2002 from inputs to the Creek (tributaries and storm drains) showed extremely high levels of diazinon (as high as 4000 ng/l). Following these findings, the Regional Board instructed Los Angeles County and the City of Santa Clarita to investigate the potential sources of diazinon and to eliminate any illicit discharges found.

By late November 2002, preliminary reconnaissance efforts, which included qualitative land use characterization) had identified several potential sources, including homeowner associations, exterminator companies, landscaping companies, and discharge outfalls. These efforts, including review of sales reports from hardware stores, suggested that there was no dominant single source of diazinon but, rather, that the diazinon contamination stemmed from widespread use by residents in the area. This conclusion led to the implementation of an aggressive pollution prevention approach in the area.

Monitoring continued at several key sites in parallel with the ongoing pollution prevention efforts. Monitoring data showed that, through March 2003, diazinon levels had dropped substantially (see following table), although some levels remained above the California Department of Fish and Game acute (0.08 ug/l) and chronic (0.05 ug/l) water quality criteria for diazinon.

Sample date	NR1	NR5	S2	S3	S7
08/28/02	5.698[1]	No data	4.214	No data	No data
10/16/02	0.95	3.76	1.19	0.46	0.53
11/20/02	0.20	0.02	0.17	No sample	No sample
01/14/03	0.34	No sample	0.16	0.41	0.31
02/03/03	0.05	No sample	0.04	0.08	0.08
03/05/03	0.15	No sample	0.10	0.22	0.08

[1] All data values reported as ug/l

The City of Santa Clarita is continuing with their educational outreach program, as part of ongoing pollution prevention efforts, to reduce diazinon levels to below State standards.

Information obtained from correspondence between the Los Angeles Regional Water Quality Control Board and the City of Santa Clarita.

A3.5 Elevated ammonia in Calleguas Creek stormwater flow (Ventura County)

Routine monitoring during the November 2001 detected an extremely high value of ammonia (52 mg/l) in Calleguas Creek. After the value was confirmed by reanalysis at the chemistry laboratory, Program staff conducted reconnaissance in the Calleguas watershed to attempt to identify the source of the ammonia. The reconnaissance was carried through in-person and telephone interviews to assess uses of ammonia in the watershed, which has a large percentage of agricultural land use. These interviews revealed that celery farmers typically inject ammonia into celery during wet weather to prevent the celery from becoming pithy.

Based on this information, the Program established five additional sites at the confluence of tributaries and at the inputs of major drains entering the creek from agricultural lands. This

sampling, conducted in dry weather, found no additional "hits" of ammonia. Nor did routine wet weather monitoring detect any further instances of elevated ammonia. Based on the information obtained about the use of ammonia by celery farmers, the presence of several celery farms upstream of the monitoring point, and the absence of any additional findings of elevated levels in either wet or dry weather samples, Program staff concluded that the elevated ammonia was most likely due to an unreported spill that occurred during the injection process. Because agriculture is exempt from the municipal NPDES permit, this was not pursued further.

Information obtained from personal communication with Ventura County Public Works Agency staff.

Appendix 4: Bacterial Die-off Rates in Freshwater Streams

This appendix reviews data on the inactivation of indicator microorganisms in freshwater as a basis for prioritizing sources of fecal contamination in southern California for further source identification work.

Fecal indicator bacteria, and the pathogenic organisms that they are meant to be the proxies for, have a limited ability to survive in most aquatic environments. Factors such as pH, temperature, solar (both UV and visible) irradiation, predation, osmotic stress, nutrient deficiencies, particulate levels, turbidity, oxygen concentrations, and microbial community composition affect bacteria inactivation once they reach receiving waters (Berry and Noton 1976, Mancini 1978, Kapuscinski and Mitchell 1980, Fujioka *et al.* 1981, Gerba and Bitton 1984, Auer and Niehaus 1992, Davies-Colley *et al.* 1994 , and Johnson *et al.* 1997). Indicator bacteria inactivation, that is, the rate at which the indicator bacteria die, is considered to be adequately represented by a first-order equation (see Thomann and Mueller, 1987). The first order decay rate (or inactivation) is usually referred to as k_D, and is usually reported as a per hour, or per day rate (e.g. 0.1 h^{-1}). In practice, people often use the term T_{90}, which describes the decline of bacteria in the time that it takes to obtain 90% mortality of the original number of bacteria, assuming a first-order loss. Throughout the rest of this document, the process will be referred to as inactivation, rather than decay, due to the fact that inactivation refers more specifically to the loss of the metabolic capabilities of the cell.

It is possible, with an adequate knowledge of environmental conditions, to create simple models of indicator bacteria inactivation using first order decay constants. In addition, several die-off equations can potentially be used in sequence with each other in order to estimate inputs from several stream inputs. There are existing models, like QUAL2E, that will permit the input of a particular coliform bacteria concentration, with temperature information, to estimate indicator bacteria levels downstream.

This document will outline a range of inactivation rates that could potentially be utilized to model bacterial inactivation in freshwater streams of southern California. However, it is particularly important to remember key points regarding bacterial inactivation rates, and the attempt to model such. First, even though most studies of rates of inactivation of indicator bacteria have been focused on single factors, we know that the process of inactivation is complex, and dependent upon multiple factors. For this reason, most of the studies that have been conducted in the laboratory to date should be reviewed suspiciously. Most of the studies have focused on analyzing the effects of one, two, or three factors independently (like temperature, pH, and TSS), and in doing so have ignored the biological complexities of the inactivation process. Second, solar irradiation/UV light is known to be one of the most important factors governing bacterial inactivation, and many studies have simply been conducted in the laboratory using UV lamps, ignoring the range of damage caused by visible, UV A, UV B, and UV C. Third, most studies have been conducted using laboratory strains of either *E. coli* or *enterococcus spp.* bacteria. These bacteria may not reflect the naturally found phylogenetic diversity of indicator bacteria inoculated into aquatic environments. Therefore the laboratory strains may be more susceptible to degradation than their outdoor counterparts. Finally, many different methods have been used to assess inactivation/decay/degradation. This is an issue that deserves much attention but in the interest of brevity, an example might be more useful. If two methods, membrane filtration, and chromogenic substrate kits (like Colilert-18®), were used to study rates of inactivation of *E. coli* in freshwater, the rate of inactivation determined with the use of the membrane filtration method would be much more rapid than that observed using the chromogenic substrate kits. The reason

for this discrepancy is that bacterial cells find it much more difficult, energetically speaking, to form a colony on a plate (the criteria for growth by membrane filtration), than to breakdown a growth substrate enzymatically (the criteria for growth by chromogenic substrate kits).

Given the cautions outlined above in interpreting data on bacterial inactivation, there are many useful studies that have been conducted that can be used to provide some general estimates of bacterial inactivation rates. Most studies have been conducted at a variety of temperatures, but presented here are the studies that have been conducted at temperate water temperatures that are applicable to southern California waters (ranging from 8-22° C).

One of the first things to do to understand the inactivation process is to determine whether or not sunlight will be considered in your estimates of bacterial inactivation rates. The detrimental effect of sunlight on survival of enteric bacteria in aquatic systems has been recognized for decades (Fujioka *et al.* 1981). Sunlight is capable of increasing inactivation rates by at least a factor of five compared to dark inactivation rates. Barcina *et al.* (1990) reported that EC was more resistant to damage by sunlight than *E. coli,* but Noble et al. (2003) demonstrated greater inactivation rates for EC than for *E. coli* even under low solar irradiation levels. The effect of sunlight is important to note, especially in sub-temperate latitudes such as southern California, where fluctuations in solar irradiation need to be considered. Published kD values for *E. coli* in freshwater can range from 0.03 to 0.06 h^{-1} (e.g., Barcina *et al.* 1986, Auer and Niehaus 1992, Menon 1993, and Mezrioui *et al.* 1995). However, other reports with kD as low as 0.001 h^{-1} and as high as 0.29 h^{-1} have been reported by Davies and Evison (1991), and Sinton *et al.* (2002), respectively. Obviously, different studies have revealed different rates of inactivation.

Given the concentration of the indicator bacteria of interest (C, in cfu or MPN/100 ml), an average decay coefficient (k_D, h^{-1}), a distance (D, meters or kilometers), and a stream or river velocity (U, translate into meters or kilometers per hour). An expected concentration of indicator bacteria can be calculated by:

$$C = C_0 * \exp(-k_D * D/U)$$

A general recommendation might be to assess the given conditions (high sunlight, low sunlight, etc.) and use a low rate of inactivation (conservative estimate), and a high rate of inactivation (liberal estimate) to provide a range of values of indicator bacteria that you will achieve downstream. The following table provides estimates of rates of inactivation, from several well-conducted and rigorously designed studies. Most rates of inactivation fall within the ranges observed here. In addition, the studies conducted by Noble et al. (2003) were specifically conducted in southern California waters, and so are a system specific representation of inactivation.

Table A4-1: Rates of inactivation for a range of studies.

Indicator	k_D (hr^{-1})	Notes	Reference
Total coliform	0.041-0.23	Ranges, freshwater, measured for 20° C	Thomann and Mueller, 1987
Total coliform	0.02	Riverine freshwater	Baudisova, 1997
Fecal coliforms	0.0162 0.007	Waste stabilization pond Raw sewage (conducted in the dark)	Sinton et al. 2002

Fecal coliforms	0.086 0.275	Waste stabilization pond Raw sewage (conducted in sunlight)	Sinton et al. 2002
E. coli	0.008	Natural surface water	Medema et al. 1997
E. coli	0.134	High solar radiation	Noble et al. 2003
E. coli	0.054	Low solar radiation	Noble et al. 2003
E. coli	0.001	Freshwater, dark	Davies and Evison 1991
E. coli	0.0171 0.023	Waste stabilization pond Raw sewage (conducted in the dark)	Sinton et al. 2002
E. coli	0.078 0.287	Waste stabilization pond Raw sewage in freshwater (conducted in sunlight)	Sinton et al. 2002
E. coli	0.03-0.06	Freshwater	Barcina et al. 1986,
Enterococci	0.27	High solar radiation	Noble et al. 2003
Enterococci	0.24	Low solar radiation	Noble et al. 2003
Enterococci	0.0168 0.012	Waste stabilization pond Raw sewage (conducted in the dark)	Sinton et al. 2002
Enterococci	0.276 0.137	Waste stabilization pond Raw sewage (conducted in sunlight)	Sinton et al. 2002
Enterococcus faecalis	0.016-0.038	Freshwater at 20° C	Thomann and Mueller, 1987

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Appendix 5: TIE Prioritization Metric

This appendix describes the calculation of a metric for prioritizing TIEs (Toxicity Identification Evaluations) to better identify the potential source(s) of toxicity in receiving waters. As discussed in the main body of the report, the model monitoring design recommends that a full year of toxicity testing be conducted and then TIEs be performed in the subsequent year, based on the relative magnitude and persistence of toxicity at the monitoring stations. The metric described below results in a single number for each site for each year and is an approach for combining the magnitude of toxicity (measured as mortality relative to a control), the breadth of toxicity across multiple test species, and the persistence of toxicity over multiple monitoring events in a given year. The metric provides users the ability to weight each of these three components differently, depending on the nature of toxicity and the specific management concern(s). However, all sites being considered for TIEs must be evaluated with the same metric weighting in order to ensure a consistent comparison among sites.

The experimental design is illustrated below:

	Time 1	Time 2	Time 3
Species 1			
Species 2			
Species 3			

At a specific site, three different species toxicity tests are performed at three different times over the course of the monitoring year. Each cell of the design contains a measure of the strength of water toxicity. A test with no measured toxic effects is represented by a value of zero.

The index is computed as the cell average toxicity value adjusted for consistency of toxic hits within species (rows) and/or time (columns). A toxic hit is defined as a toxicity value greater than zero. The consistency of toxicity within columns (across species) is measured by a cumulative score that depends on the numbers of toxic hits in the columns. For each column with three toxic hits, 1 is added to the total score (see the tables below), and for each column with two toxic hits, ½ is added to the total score. Nothing is added to the total score for 0 or 1 toxic hits in a column. A similar total score based in toxic hits in the rows is computed for consistency within rows.

Variables used to compute the index value are:

C_{col} = the column consistency score,

C_{row} = the row consistency score,

A_{col}=percent adjustment for column consistency,

A_{row}=percent adjustment for row consistency, and

M=the mean of all cells.

The index is computed as

$$I = M\left(1 + \frac{A_{col}}{100}\frac{C_{col}}{3} + \frac{A_{row}}{100}\frac{C_{row}}{3}\right). \qquad (1)$$

The value 3 in equation (1) is the maximum consistency score for rows (C_{row}) or columns (C_{col}). Thus, when the consistency score is maximal, the full percent adjustment (A) is added to the value in the parentheses, and lesser amounts are added for less than maximal scores. The values of 100 in equation (1) convert the adjustment percents to proportions.

It can be seen that equation (1) is the cell mean with upward adjustments for consistency within rows or columns. The user must decide what percent adjustment of the cell mean will be associated with the maximum score for both rows and columns. For example, if the user wants to emphasize consistency of toxicity across species at the same time, the user could set A_{col}=30 and A_{row}=0, which will adjust the cell mean upward by 30% for maximal within-column consistency, and ignore within-row consistency. Some example calculations with these A values are provided for below.

Example data with minimum within-column consistency might be as follows:

	Time 1	Time 2	Time 3	# hits
Species 1	30	40	20	3
Species 2	0	0	0	0
Species 3	0	0	0	0
# hits	1	1	1	

The calculations for these data with A_{col}=30 and A_{row}=0 are shown in equation (2).

$$I = M\left(1 + \frac{A_{col}}{100}\frac{C_{col}}{3} + \frac{A_{row}}{100}\frac{C_{row}}{3}\right) = 10\left(1 + \frac{30}{100}\frac{0}{3} + \frac{0}{100}\frac{1}{3}\right) = 10 \qquad (2)$$

Example data with some within-column consistency might be as follows:

	Time 1	Time 2	Time 3	# hits
Species 1	30	0	0	1
Species 2	40	0	0	1
Species 3	20	0	0	1
# hits	3+1	0	0	

The calculations for these data with A_{col}=30 and A_{row}=0 are shown in equation (3).

$$I = M\left(1 + \frac{A_{col}}{100}\frac{C_{col}}{3} + \frac{A_{row}}{100}\frac{C_{row}}{3}\right) = 10\left(1 + \frac{30}{100}\frac{1}{3} + \frac{0}{100}\frac{0}{3}\right) = 11 \qquad (3)$$

Note that the index value for the data used in equation (3) is higher than the index value for the data used in equation (2). This is because the equation (3) data have more within-column consistency and the A values were set to emphasize the within-column consistency. A more dramatic difference between the two index values would have resulted if a higher value for A_{col} was used.

It is important to stress that the intended use of the index (I) values is to help prioritize stations for follow-up TIEs. Thus, stations with higher index values would be a higher priority when allocating a fixed amount of resources for TIEs.

STORMWATER MONITORING COALITION LABORATORY GUIDANCE DOCUMENT

Richard Gossett
CRG Marine Laboratories, Inc.
2020 Del Amo Blvd. Suite 200
Torrance, CA 90501

David Renfrew
Enviromatrix Analytical, Inc.
4340 Viewridge Ave.
San Diego, CA 92123

Kenneth Schiff
Southern California Coastal Water Research Project
7171 Fenwick Lane
Westminster, CA 92683
www.sccwrp.org

August 2004

Technical Report #420

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1.0 INTRODUCTION

One goal of the southern California Stormwater Monitoring Coalition (SMC) is to compile monitoring data from separate programs to make region-wide assessments. This task has been difficult, thus far, because the various monitoring programs have differing project goals and objectives, differing mandates from regulatory agencies, differing sampling designs, and differing laboratory analytical methods. The goal of this document is to increase the comparability among stormwater monitoring agencies by minimizing the differences in results due to laboratory analysis. The comparability issues that revolve around goals, objectives, and study designs are being addressed through a related, but separate document.

There are at least three reasons that laboratory analytical data are not comparable including differences in target analytes, reporting levels (detection limits), and laboratory methods. Reviews of the current monitoring programs have identified some of the difficulties in laboratory analysis (Ackerman and Schiff, 2003). First, some monitoring programs measure as few as 32 analytes while others measure as many 128 analytes. Second, the range in reporting levels can vary by more than an order of magnitude among programs for some constituents. As an example, one program had a reporting limit for zinc of 5 µg/l while for another it was 50 µg/l. As a result, the frequency of non-reportable quantities was approximately 5% and 20% for each of the programs, respectively. To date, no one has attempted to compare the laboratory procedures among laboratories to assess any bias associated with this analytical component.

Many of these obstacles have been surmounted through the intercalibration studies of the SMC (Appendix A). This intercalibration study, which was conducted with most of the major laboratories currently analyzing stormwater samples throughout the region (Table 1), was aimed at developing a consensus based approach for achieving minimal levels of comparability among typically disparate laboratories. The success of this study was primarily due to three factors including communication and commitment among laboratory personnel, setting performance-based criteria for establishing standards of success, and round robin testing using locally derived reference materials. This was the same model that was used in developing consistency among laboratories that conduct marine monitoring in the southern California region (Noble *et al* 2002 and Gossett *et al* 2003).

1.1 Objectives and Goals of this Document

The objective of this guidance manual is to capture the performance-based guidelines established during the SMC intercalibration studies for ongoing analysis of stormwater samples for municipal agencies throughout southern California. The goal of this document is to set minimum standards of sensitivity, precision, and accuracy across laboratories so that individual data sets can be combined with estimated levels of confidence for making regional assessments of stormwater quality. The philosophy of performance-based guidelines is key to achieving this comparability. Although every laboratory involved in the stormwater intercalibration study was certified by the State of

California Environmental Laboratory Accreditation Program (ELAP), inventories of existing methods demonstrated that most analytes were not analyzed in exactly the same manner across all laboratories. This will continue as new laboratories, or new equipment at existing laboratories, continue to proliferate. Rather than mandate specific methods that are inflexible and discourages existing laboratories to achieve faster, more sensitive, and more inexpensive methods, this document merely sets minimum levels of comparability so that data sets can be combined no matter what technology currently exists. In a few cases, the workgroup experimentally demonstrated that greater specificity of sample preparation procedures maximized comparability. These procedures are also outlined herein.

Table 1. Collaboratorators for the SMC intercalibration study.

Contact Name	Company
Philip Carpenter	Toxscan Laboratories
Alan Ching	Weck Laboratories
Larry Chrystal	Edward S. Babcock and Sons
Andrew Eaton	MWH Laboratories
Rich Gossett	CRG Marine Laboratories
Norman Hester	Truesdail Laboratories
Wei Leung	Los Angeles County
Jim McCall	Associated Laboratories
Dave Renfrew	Enviromatrix Analytical
Kenneth Schiff	SCCWRP
Bob Stearns	CalScience Environmental Laboratories
David Terz	FGL Environmental

This guidance manual is a living document. It should be revisited each time an intercalibration exercise is conducted and can be expanded to include additional constituents, additional laboratories, or to refine the recommended performance-based sensitivity, accuracy, and precision requirements as new information becomes available.

This document and laboratory intercalibration study is not a certification program. The guidelines set by this document merely express the desired needs of the stormwater agencies throughout the southern California region. Therefore, these stormwater agencies may wish to use these guidelines in establishing specifications for work assignments or requests for proposals to conduct stormwater analyses. Alternatively, or in combination, stormwater regulatory agencies may use these specifications in the development of regulatory expectations for laboratory performance by monitoring agencies.

2.0 GUIDANCE INFORMATION

This document consists of four elements. First is a list of target analytes and minimum levels of sensitivity (reporting levels). Second are minimum levels of accuracy and precision. Third are recommended protocols for method specific comparability. Fourth are participation requirements for intercalibration studies.

2.1 Analytes and Reporting Levels

2.1.1 Target Analytes

A core group of target analytes was specified for comparability (Table 2). This list includes total suspended solids (TSS), nutrients, and trace metals. This list was based on three criteria. First, these analytes are consistently measured by the existing monitoring programs throughout the region. Second, these constituents are routinely detected in stormwater samples. Third, although standard methods exist, there is sufficient disparity in protocols among laboratories that consistency guidance is warranted.

The list of target analytes is not meant to be an exhaustive list of all constituents that could or should be measured in individual programs. For example, there are no pesticides, herbicides, or polynuclear hydrocarbons on the list of target analytes. Any or all of these target analytes may be the focus of individual monitoring programs. At this point in time, however, there has not been an intercalibration study conducted for these constituents to make performance-based recommendations for stormwater laboratories.

While the list of target analytes focuses on total trace metals, they can be applied to dissolved trace metals. Since the analytical methodology is similar among both total and dissolved metals, the performance-based guidelines may be applied to both.

2.1.2 Reporting levels

Targeted reporting levels (RLs) are provided in Table 2. This guidance was based on the philosophy that analyses should be sufficient to assess if samples are below water quality thresholds of concern. In this instance, the water quality thresholds of concern are established in the California Toxics Rule (CTR). Further, reporting levels should be technologically achievable, but far enough below water quality thresholds that exceedences cannot be attributable to methodological uncertainty. Therefore, the philosophical approach for selecting RLs was to select guidelines that were one-half of the lowest water quality threshold. In the case of the CTR, there are thresholds for both marine and fresh waters. For a participating laboratory to achieve these reporting levels, it should include a calibration standard at or below this level (e.g. the reporting level is a quantitation level and not an MDL).

Table 2. Target analytes and Reporting Levels for the Stormwater Monitoring Coalition Monitoring Program.

Analyte	SMC Target Reporting Level	California Toxics Rule Limit (Freshwater)	California Toxics Rule Limit (Seawater)	Units
General Constituents				
TSS	5	-	-	mg/L
Nitrate+Nitrite as N	0.2	-	-	mg/L
Ammonia as N	0.1	-	-	mg/L
Total Phosphorus as P	0.1	-	-	mg/L
Total Kjeldahl Nitrogen	0.2	-	-	mg/L
Total Organic Carbon	1	-	-	mg/L
		-	-	
Total Metals				
Arsenic	2	150	36	µg/L
Cadmium	1	2.2	9.3	µg/L
Chromium (total)	5	11	50	µg/L
Copper	2	9	3.1	µg/L
Nickel	4	52	8.2	µg/L
Lead	1	2.5	8.1	µg/L
Selenium	2	5	71	µg/L
Silver	1	3.4	1.9	µg/L
Zinc	10	120	81	µg/L

2.2 Accuracy and Precision

2.2.1 Laboratory Intercalibration Studies

Intercalibration studies evaluate the accuracy and precision of analysis among laboratories. For this document, interlaboratory precision guidelines were developed by analyzing one set of three replicate samples for each of three matrices by eleven different laboratories throughout southern California (Appendix A). These matrices included a specially-prepared performance evaluation (PE) sample, an urban runoff sample, and a rural runoff sample (Table 3). Each of the laboratories demonstrated competence with the easiest of matrices, the PE sample. Interlaboratory variability increased with the more difficult stormwater matrices, but iterative intercalibrations focused on understanding and minimizing the source of variability. This improved laboratory comparability until it approached the variability associated with the PE sample (i.e. 25% CV among labs).

Unlike the PE sample where the concentrations are known, the runoff samples contained unknown levels of constituents so assessing accuracy and precision becomes more

difficult. A population-based estimator was used to assess meaningful differences for interlaboratory variability similar to the approach used by Gossett et al (2003). Acceptable performance for the simulated rainfall samples was achieved if the results were within ± 2 standard deviations of the mean for the pooled results. Population-based estimators provide an unbiased method for assessing extreme variability; at least 90% of all laboratories will pass this guideline if the variability is normally distributed. The actual guidelines established from the intercalibration exercises during 2003 are shown as an example in Table 3. Each subsequent intercalibration exercise will result in specific numerical guidelines that will likely differ from those in 2003 since different runoff samples will be used. It should also be noted that some parameters were more precise than others. Data users should recognize that these ranges represent the current "state-of-the-art" for accuracy of routine analytical conditions in laboratories analyzing stormwater samples.

Table 3 Laboratory replicate analysis data quality objectives (DQOs) for Accuracy.

		ERA[a]			LU[b]			LR[c]		
Target Analyte	Units	Mean	+2 SD	-2 SD	Mean	+2 SD	-2 SD	Mean	+2 SD	-2 SD
General Constituents										
TSS		-			73.8	84.8	62.7	3201	3649	2753
Ammonia-N	mg/L	0.72	1.01	0.43	0.78	1.78	0.25	5.35	6.77	3.93
Nitrate-N+Nitrite-N	mg/L	0.74	0.88	0.60	1.31	2.29	0.85	5.31	11.20	rl
Total Kjeldahl Nitrogen	mg/L	0.87	1.24	0.50	0.63	1.42	RI	1.48	2.92	0.04
Total Phosphorus as P	mg/L	0.85	0.93	0.77	0.11	0.21	0.02	0.57	1.41	rl
Trace Metals										
Arsenic	µg/L	4.1	7.2	1.1	1.7	10.6	rl	5.3	11.3	rl
Cadmium	µg/L	7.7	9.2	6.1	1.0	4.1	rl	2.1	2.9	1.4
Chromium	µg/L	34.4	44.4	24.4	5.7	9.8	1.5	41.3	76.4	6.2
Copper	µg/L	6.0	7.5	4.5	10.3	16.3	4.4	23.0	39.0	7.1
Lead	µg/L	2.4	4.7	0.0	8.7	11.4	6.0	12.7	23.2	2.1
Nickel	µg/L	24.5	36.4	12.5	5.5	9.7	1.3	45.6	74.1	17.2
Selenium	µg/L	4.2	6.2	2.1	1.6	2.6	0.6	1.4	2.9	rl
Silver	µg/L	5.1	6.3	3.8	<1	-	-	1.1	4.2	rl
Zinc	µg/L	74.5	126.0	22.8	117.0	274.0	rl	96.3	163.0	29.4

[a] certified reference material
[b] simulated rainfall runoff from an urban catchment
[c] simulated rainfall runoff from a rural catchment
- no limit
rl below reporting level in table 2

2.2.2 *Ongoing Analyses*

Reproducibility among replicate sample analyses provides a determination of within laboratory precision. Analysis of spiked samples or reference materials provides a mechanism for assessing within laboratory accuracy. General guidance provided by the ELAP and the US EPA (40 CFR 136) are recommended for assessing within laboratory precision and accuracy on an ongoing basis. Laboratories should assess ongoing precision by analyzing two replicate sample matrix spikes per sample batch of 20 samples or less. It is also recommended that a set of laboratory replicate samples are analyzed with each batch of samples to indicate precision using actual sample matrices as compared to spikes. The relative percent difference (RPD) between these replicate analyses and replicate spikes should meet the guidelines specified in Table 4 for results that are at least 10 times the RL, unless the samples are grossly contaminated. If samples contain such large quantities of contaminants that the laboratory feels the MS/MSD results can not be reasonably met, then a detailed case narrative should accompany the analytical results. RPD criteria are not specified for concentrations less than 10x the RL because the variability increases significantly as you approach the RL.

Table 4. Laboratory precision guidelines for ongoing analysis of stormwater samples

Analyte	RPD Limits for Results >10x the RL
General Constituents	
TSS	0-20%
Nitrate-N+Nitrite-N	0-20%
Ammonia-N	0-20%
Total Phosphorus as P	0-20%
Total Kjeldahl Nitrogen	0-20%
Total Organic Carbon	0-20%
Metals, Total	
Arsenic	0-20%
Cadmium	0-20%
Chromium (total)	0-20%
Copper	0-20%
Nickel	0-20%
Lead	0-20%
Selenium	0-20%
Silver	0-20%
Zinc	0-20%

Accuracy of the method used is defined as the degree of difference between observed values and true values from the analysis of certified or standard reference materials, matrix spikes, or blank spikes. The extent to which it will be a good measure of accuracy depends on the complexity of the selected matrix; stormwater matrix is typically a very complex mixture of

unknown constituents and potential interferences. Therefore, these guidelines recommend ongoing analysis of a Matrix Spike (MS) and Matrix Spike Duplicate (MSD) for all parameters except Total Suspended Solids (TSS) on a once per batch of 20 or less samples. For TSS, a Certified Reference Material is recommended for testing accuracy since spike samples for this parameter are infeasible. Accuracy limits for MS and MSD are provided in Table 5. These accuracy limits mimic ELAP and US EPA guidelines. Accuracy limits for Certified Reference Materials are provided by the supplier.

Table 5. Laboratory accuracy guidelines for ongoing analysis of stormwater samples.

Analyte	Percent Recovery Limits for MS/MSD results with concentrations > 10x the RL
General Constituents	
TSS	-[a]
Nitrate-N+Nitrite-N	80-120%
Ammonia-N	80-120%
Total Phosphorus as P	70-130%
Total Kjeldahl Nitrogen	70-130%
Total Organic Carbon	80-120%
Metals, Total	
Arsenic	80-120%
Cadmium	80-120%
Chromium (total)	80-120%
Copper	80-120%
Nickel	80-120%
Lead	80-120%
Selenium	80-120%
Silver	80-120%
Zinc	80-120%

[a] defined by supplier of CRM

Additional QAQC requirements listed in the methods referenced by each laboratory should conform to the requirements listed within that method by Standard Methods or the US EPA (i.e. Blank Spikes). Since spiked samples can be complicated by matrix interferences and this can confound assessments of accuracy, the analysis of a Certified Reference Material is also a recommended option for the monitoring agency.

3.0 STANDARDIZATION

Although this document is founded on performance-based guidelines enabling flexibility within each laboratory to achieve consistency, the laboratory intercalibration studies have identified four protocols whereby recommended standardization can dramatically increase comparability. This standardization includes sub-sampling, Total Phosphorus digestion, TKN digestion, and trace metal digestion techniques.

3.1 Sub-sampling Techniques

Sub-sampling techniques are an important component of both within and among lab variability. This was especially true for particle-laden samples, such as those from more rural catchments with unlined channels. Particle-bound constituents have the potential to be dramatically biased if sub-sampling techniques selectively target or avoid particles within samples. To this end, standardized laboratory techniques for sub-sampling were developed for splitting large volume stormwater samples collected in the field into smaller bottles for distribution to the laboratory and for subsequent sampling of smaller aliquots in the laboratory at the time of analysis.

3.1.1 Sub-sampling of large volume field samples (courtesy of Kinnetic Laboratories)

In order to ensure that sample containers destined for an analytical laboratory all contain water that is similar and representative of the original composite sample, it is important to maintain a well-mixed composite sample during sub-sampling and to prevent stratification and the settling out of heavier particles. This is accomplished by the use of a large-capacity stirrer and a 2 to 3-inch, pre-cleaned, Teflon-coated stir bar; larger stir bars can be used for larger volume containers. Adjustment of stirring speed is important. Speeds that are too fast will create a large vortex within the composite bottle that can actually concentrate heavier particles and should be avoided. Speed should be based on a visual assessment of the most even mixing throughout the composite bottle.

Sub-sampling from the homogenized composite bottle is accomplished using a peristaltic pump and pre-cleaned (inside and outside) sub-sampling hose. Filling sample containers by pumping from the composite bottle is best performed by two people. One person is responsible for filling individual sample containers and one person is responsible for constantly moving the intake tubing up and down in the water column of the composite sample. Based on experimental evidence, this up and down movement of the intake is a procedure that helps obtain a more representative sub-sample. This is because there can still be some stratification of heavier particles in the composite sample despite the mixing created by the stirrer. The up and down movement of the intake tubing should be limited to approximately 80-90 percent of the depth of the water column and should never touch the bottom of the composite bottle.

3.1.2 Sub-sampling of sample containers for analysis

The goal of sub-sampling bottles in the laboratory for analysis is similar to field sampling techniques, to maintain a homogeneous particle distribution. Analysis of particle-associated constituents will be biased if non-representative particle suspensions are used for analysis. In order to maintain homogeneous particle distributions, we recommend the use of sub-sampling techniques described by the US Geological Survey (Charles J. Patton, USGS National Water Quality Laboratory, Denver, CO). Appropriately, a similar technique to large container sub-sampling is used (Section 3.1.1). Briefly, a "+" shaped magnetic stirring bar is placed into the sample container and the sample is stirred while a sub-sample is aspirated and dispensed into the processing container.

3.2 Total Kjeldahl Nitrogen

Total Kjeldahl Nitrogen analysis was affected by the digestion technique during the laboratory intercalibration exercise. This was due to the influence caused by particle content and size distribution. Therefore, minimum standardization of the digestion procedure is recommended. Either micro or macro Kjeldahl digestions are acceptable. However, the length of time of digestion should be set at a minimum of 1 hour at 380°C, until copious fumes are generated and the digestion solution turns yellow, and then for an additional 30 minutes (to ensure adequate recovery) prior to analysis.

3.3 Total Phosphorus

Total Phosphorus analysis was also affected by the digestion technique during the laboratory intercalibration exercise. Therefore, minimum standardization of this digestion procedure is also recommended. The US Geological Survey National Water Quality Laboratory compared several digestion techniques using our simulated stormwater sample. These results suggest that the Kjeldahl digestion and acid persulfate digestions were higher than the alkaline persulfate digestions. However, most of the laboratories in both rounds of the intercalibration exercise used the acid persulfate digestion and the results were still quite variable. Kjeldahl digestion may reduce this variation because it is more rigorous, but this technique has not been tested by the SMC laboratories and is not presently an approved technique. Therefore, we are recommending that an acid persulfate digestion be used until this issue can be resolved during the next intercalibration exercise.

3.4 Trace Metals

Trace metal analysis was also affected by the digestion technique during the laboratory intercalibration exercise. This is because trace metal concentration may be influenced by particle content and size distribution. Therefore, minimum standardization of trace metal digestion is recommended. Trace metals should be digested using a nitric/hydrochloric acid digestion at 95°C for 2-4 hours until the sample has evaporated from 50mL down to 10mL.

Dissolved metals analysis should be performed on filtered samples and do not require digestion if the Turbidity is < 1. Sample spiking for the Matrix Spikes should be done prior to filtering. All other criteria for trace metals in this guidance document are applicable to both total and dissolved metals.

4.0 PARTICIPATION REQUIREMENTS

4.1 Proficiency Testing

The SMC recommends laboratories performing analyses for Stormwater Monitoring Programs pass a SMC performance evaluation (PE) sample and participate successfully in SMC intercalibration exercises. The PE and intercalibration exercises are strongly recommended to be performed on an annual basis. This frequency is recommended because: 1) new laboratories may wish to participate; 2) existing laboratories need to evaluate new personnel; and 3) new and existing laboratories with new equipment or altered laboratory techniques need to be evaluated. Intercalibrations must occur within the first six months of the calendar year to ensure evaluation prior to the following wet season that typically begins on October 15th .

SMC PE samples are to be spiked between 1 and 10 times the established reporting limit (Table 2) for the analytes of concern for minimum proficiency. All sample results must meet the criteria provided by the commercial supplier of the sample to evaluate accuracy. PE samples are to be coordinated through the SMC, or their representives on a Chemistry subcommittee, and can be purchased from private company such as Environmental Resources Associates, Inc (ERA), Wibby Environmental, APG, or other proficiency test sample providers.

Intercalibration studies require laboratories to analyze three replicates of two runoff samples, one from an urban and one from a rural catchment. Each intercalibration study should be performed with two iterations to evaluate consistency and allow for laboratory corrective actions if deficient analysis resulted from the first iteration. Sample results must fall within ± 2 standard deviations of the mean of the pooled results as determined by the Chemistry Committee (see Section 2.2.1).

4.2 New Laboratories

New labs that have not participated in previous intercalibration exercises may still be able to analyze stormwater samples during the present wet season. These labs, however, will need to provide resources to purchase a PE sample with the same requirements used in the intercalibration study in Section 4.1 (i.e. samples will be spiked at 1 to 10 times the established reporting limit in Table 2). These samples must be delivered to the new laboratory blind and as whole volume samples. All new laboratories are required to participate in the next intercalibration exercise to remain qualified for the SMC program.

5.0. DEFINITIONS

Batch – An analytical batch consists of 20 or fewer client samples.

Method Blank (MB) – Analyte free water that is carried through the entire analytical process. The method blank is used to evaluate contamination contributed from the method. Analyte detections in the method blank must be less than 10x the analyte result for a client sample to be considered usable without flagging.

Duplicate – A client sample analyzed in duplicate. Duplicate RPD should be summarized in the report.

Blank Spike/Blank Spike Duplicate – A blank spike (laboratory control sample) is a certified standard reference material that is spiked into a reagent blank. It is carried through all steps of sample preparation to demonstrate method performance inclusive of sample preparation steps. The blank spike should be spiked near the mid point of the calibration curve.

Matrix Spike/Matrix Spike Duplicate – A matrix spike is a regular sample that is split into three sub-samples. Two of the replicates are spiked with analyte solution at the same concentration and are defined as the matrix spike and matrix spike duplicate (MS/MSD). The MS/MSD samples are carried through the sample preparation and analysis procedure with each batch of 20 or less samples. The MS/MSD results provide information regarding laboratory precision, sample matrix effects, and method efficiency.

RPD- Relative Percent Difference is calculated using the following formula:

$$RPD = (Result1 - Result2)/((Result1 + Result2)/2)*100$$

6.0 REFERENCES

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APPENDIX A

Intercalibration of Stormwater Analytical Laboratories Throughout the Southern California Region

Richard Gossett[1] and Kenneth Schiff[2]

[1]CRG Marine Laboratories
Torrance, CA

[2]Southern California Coastal; Water Research Project
Westminster, CA

January 30, 2003

ABSTRACT

Despite spending large resources on monitoring in southern California, stormwater agencies lack the ability to assess cumulative runoff inputs, make comparisons among watersheds in different counties, or efficiently track trends in stormwater concentrations or loads at regional spatial scales. Data compilation among the various monitoring programs is due to a lack of comparability in their lists of target analytes, methods used for measuring these constituents, varying levels of sensitivity (i.e. detection and reporting limits), and unequal levels of quality assurance and quality control. The goal of this study was to quantify the comparability of chemical analysis among multiple analytical laboratories for a standard list of constituents to be measured in stormwater runoff. In order to accomplish this goal, all of the laboratories that conduct analysis of stormwater samples for large municipalities throughout the region were given three types of samples (in triplicate) including a reference sample, a runoff sample from an urban catchment, and a runoff sample from a rural catchment. Most laboratories performed well on the reference material, typically reporting results less than 20% different from the true value. The more challenging urban runoff sample generated greater interlaboratory variability. The coefficient of variation (CV) averaged 20% for nutrients (NH3-N, NO2+NO3, TKN, Total P) and 38% for trace metals (arsenic, cadmium, chromium, copper, lead, nickel, selenium, silver and zinc). The greatest interlaboratory variability was for the rural runoff sample, which averaged 56% CV for both nutrients and trace metals. The greater variability in the runoff samples was attributable to increasing particulates; mean TSS ranged from <0.1 mg/L in the reference material to 3,200 mg/L in the rural runoff sample. After standardizing methods for potential subsampling bias and digestion efficiencies, interlaboratory precision improved with the re-analysis of the rural runoff sample.

INTRODUCTION

Municipal stormwater agencies expend remarkable effort monitoring the water quality of wet and dry weather discharges and their impacts in Southern California aquatic ecosystems. Schiff et al. (2002) estimated that the four coastal stormwater management agencies spend nearly $2M annually on monitoring circa 1997. At that time, the majority of monitoring costs arose from sampling storm events and measuring an array of chemical constituents. These results were then used to estimate mass emissions of these constituents from urbanized watersheds, compare concentrations to water quality thresholds, and identify potential pollutant-related impacts to receiving waters.

Despite this relatively large effort in Southern California, integrating stormwater monitoring data among these programs is difficult. Schiff (1997) attempted to use these monitoring data to make multi-county, regionwide assessments of stormwater loading. Several factors limited the ability to compile data including both sampling and laboratory parameters. Specifically for laboratory parameters, programs lacked comparability in their lists of target analytes, methods used for measuring these constituents, varying levels of sensitivity (i.e. detection and reporting limits), and unequal levels of quality assurance and quality control.

These differences are likely the result of a monitoring focus on site specific needs, rather than an attempt to integrate individual programs to assess cumulative runoff inputs, make comparisons among watersheds, efficiently track trends in stormwater concentrations or loads at regional spatial scales or find ways to reduce monitoring redundancy thereby increasing efficiency. Most runoff monitoring is mandated by the National Pollutant Discharge Elimination System (NPDES) administered by the state and/or federal government. In the case of southern California, there are at least 11 different municipal stormwater NPDES permits distributed across seven counties and three state regulatory agencies. The regulatory jigsaw puzzle, which typically follows jurisdictional boundaries and not watershed boundaries, has (at least in part) led to the widely varying monitoring programs we see today.

The goal of this study was to assess the comparability of laboratory analysis among multiple analytical laboratories for a standard list of constituents to be measured in stormwater runoff. In order to accomplish this goal, we gathered all of the laboratories that conduct analysis of stormwater samples as part of the NPDES monitoring programs for large municipalities in southern California. The objective was to determine if comparability among these laboratories was sufficient, or could be reasonably altered, to enable the compilation of data sets among individual monitoring programs to make regionwide assessments.

METHODS

The intercalibration for stormwater samples was conducted with the 11 most active laboratories in southern California that perform analyses in this matrix (Table 1). All but one of these laboratories are privately-held firms. The laboratories proceeded through a three-step process following a similar study by Gossett et al (2003): 1) create an inventory of existing methods and protocols; 2) conduct an intercalibration study to assess comparability; and 3) conduct iterative studies to improve comparability.

The core set of target analytes selected for intercalibration included total suspended solids (TSS), nutrients, and trace metals. This list was based on three criteria derived from the inventory. First, these analytes were consistently measured by the existing monitoring programs throughout the region. Second, these constituents were routinely detected in stormwater samples. Third, although all of the laboratories used standard methods (Table 2 and 3), there was sufficient disparity in protocols among laboratories that comparability examination was warranted.

Three types of samples were distributed to each of the participating laboratories for step 2 in the intercalibration study. The first sample was a reference material with known concentrations (Environmental Resources Associates, CO). This sample, prepared in the easiest of matrices, was selected because it provided a minimal base of comparison without confounding factors inherent in a stormwater runoff matrix. The second sample was runoff from an urban catchment, which was comprised of a parking lot (approximately 36 m^2) that received primarily weekday use. The third sample was runoff from a rural catchment comprised of undeveloped Mediterranean scrub/chaparral landscape (approximately 42 m^2) in the Santa Monica Mountains National Forest. These two runoff samples were selected because they represent the range of sample matrices that might be encountered by any of the laboratories as part of their stormwater monitoring programs. There was no attempt to standardize methods among labs prior to analysis of these three samples for step 2.

Simulated rainfall was used to generate runoff from the urban and rural sites for this study. Rainfall was simulated following Tiefenthaler and Schiff (2003) using distilled water and applied using a low flow spray nozzle attached to a battery operated pump. Rainfall continued until sufficient runoff was collected (approximately 50L) in a large volume container. Subsamples from this large composite sample were placed in smaller containers for shipment to the individual laboratories. A sufficient number of subsamples were collected so that three replicate bottles, selected at random, were sent to each lab on ice within 24 hours. In order to ensure that subsample containers destined for analytical laboratories all contained samples that were similar and representative of the original composite sample, a 3-inch "+" stirring bar was used during subsampling to prevent stratification and the settling of heavier particles. Stirring speed was adjusted so that no vortex was created and subsamples were collected using a peristaltic pump with an intake that was raised and lowered through the water column of the composite sample bottle during pumping. At no time did the pump intake touch the bottom of the composite bottle.

Step 3 of the intercalibration was an iterative analysis for those constituents that appeared to be problematic in step 2. The iterative analysis focused on particulate associated constituents (TKN, Total P, and trace metals). Two specific changes were made between steps 2 and 3. First, each laboratory was supplied with the exact volumes needed for digestion and analysis so no subsampling of the laboratory containers was needed. Since different laboratories used differing volumes, particles were collected dry from the same urban and rural sampling sites and weighed to create samples with identical TSS concentrations. The collected particles were dried (60C for 24 hrs) and thoroughly mixed to ensure representative distribution, then brought to the appropriate volume with distilled water. The premeasured sample containers were randomly selected and shipped in triplicate to each of the analytical laboratories on ice within 24 hours. The second specific change was standardization of digestion methods for trace metals. All labs digested their trace metal samples using nitric/hydrochloric acid at 95°C for 2-4 hours until the sample evaporated from 50mL down to 10mL.

Data analysis for this study required three steps. The first step was to identify the characteristics of the three different samples used in the study. To accomplish this evaluation we examined the mean concentration of each constituent for all laboratories combined. The second step was to identify the interlaboratory variability for each sample independently. To accomplish this comparison, we examined the coefficient of variation (CV) cumulatively for all constituents, by class of constituents (general constituents and trace metals), and by individual constituent. For the reference material, we also examined accuracy since we knew the true value *a priori*. The third step was to identify if interlaboratory variability improved in subsequent iterations as a result of standardization in subsampling and digestion techniques. To accomplish this evaluation, we compared the CV in the first and second iteration for the rural runoff sample on a constituent -by- constituent basis.

RESULTS

The reference material, the urban runoff, and the rural runoff samples had varying levels of target analytes (Table 4). For example, the reference material consisted of distilled water spiked with dissolved components (i.e. no TSS), while the TSS from the urban sample averaged 74 mg/L and the TSS from the rural sample averaged 3200 mg/L. The mean concentrations of NO2+NO3-N, TKN, TOC, copper, lead and zinc were lowest in the reference material. In contrast, the rural runoff sample had the highest mean concentrations of NO2+NO3-N, TKN, Total P, arsenic, cadmium, chromium, copper, nickel and lead. Finally, the urban runoff sample had mixed results compared to the reference material or rural runoff samples. The urban runoff sample had the highest concentrations of NH3-N, TOC, and zinc, but had the lowest concentrations of Total P, arsenic, cadmium, chromium, nickel, and selenium.

Analysis of the reference material demonstrated that, on average, the laboratories performed quite well with this type of sample (Table 5). The mean concentration for all

laboratories was less than 5% different from the true value for NH3-N, NO3+NO2-N, Total-P, arsenic, cadmium, chromium, nickel, selenium, silver, and zinc. TKN, copper, and lead were greater than 5%, but still less than 25% different from the True Value. In addition, the interlaboratory variability was reasonably precise. The CV among labs ranged from 9 to 42% (depending upon the constituent), averaging 12% for all constituents combined. The precision was better for general constituents (average CV of 7%) compared to trace metals (average CV of 22%).

The laboratories had mixed success with the comparability of nutrient and trace metal results in the urban runoff sample (Table 6a). The range of precision for the 10 laboratories varied between eight and 79% CV, averaging 32% CV for all constituents combined. Laboratory precision was slightly better for the general constituents (average of 20% CV) compared to trace metals (average of 38% CV). The greatest interlaboratory variability for general constituents was observed for NH3-N and Total P. This variation could be reduced with the removal of one or two data points (i.e. lab C for NH3-N or lab E for Total P). Where trace metal concentrations were routinely detectable, interlaboratory comparability was reduced. The range of precision for the 10 laboratories varied from 13% CV for copper to 55% for lead. Once again, much of the variability was attributable to one or two labs (i.e. lab H for lead).

The interlaboratory precision decreased in the rural runoff sample (Table 6b). The range of precision for the 10 laboratories varied between eight and 169% CV, averaging 56% CV for all constituents combined. Laboratory precision was similar for the general constituents (average of 57% CV) compared to trace metals (average of 56% CV). The greatest interlaboratory variability for general constituents was observed for NH3-N and TKN. Once again, some laboratories stood out as distinctly different (i.e. lab C for NH3-N). Even with the removal of this potential outlier, the resulting precision was poor (45% CV) most likely due to its relatively low levels (two labs were non-detectable). The Total P concentrations, in contrast, ranged from 1.1 to 3.5 mg/L with a relatively even distribution among labs. Where trace metal concentrations were routinely detectable, interlaboratory comparability still averaged 56% CV. The greatest interlaboratory variability was arsenic (80% CV) and the least was for cadmium (24% CV). In this instance, we found that three labs were routinely lower for many metals (i.e. labs F, H, and J), while another four were routinely higher than the grand mean (labs A, C, G, and I).

Improvements in sub-sampling and adjustments to digestion techniques resulted in improved comparability among laboratories for analysis of the simulated rural runoff sample (Figure 1). Despite decreases in concentration of 50 to 75% for each of the constituents (Table 4), precision increased in seven of the 10 constituents evaluated in the rural runoff sample. For two of the constituents where precision decreased, the differences between the two iterations were marginal (cadmium, and lead). These two constituents had the greatest precision of all the trace metals in the first iteration. The most noticeable lack of increased precision was for Total P where concentrations ranged from less than 0.1 to 0.57 mg/L among laboratories.

DISCUSSION

This study demonstrated that, in general, a performance-based approach could be an effective mechanism for ensuring comparability among multiple labs conducting stormwater analysis. All of the laboratories used in this study were using standard methods, but none of the laboratories were using exactly the same protocols. This is because standard methods allows for flexibility in methods and analytical techniques. Despite these differences, we could reach a level of precision among labs that approached the precision expected within a lab.

The largest hurdles to accomplishing regional scale comparability was performing intercalibration studies on the typical matrices that might be encountered, which for stormwater runoff is often very complex. All of the laboratories performed well on samples conducted in the easiest of matrices; the reference material was comprised of a very clean matrix. Other than the fact that we used relatively low levels of spiked compounds, these samples are no different than the ongoing Performance Evaluation standards they receive from the US EPA or are used by the State of California for laboratory accreditation. The key to success for this study was the use of runoff samples for intercalibration. Runoff samples are inherently turbid with many potential interferences. We found that the presence of particulates was amongst the most confounding factors in the comparability among laboratories. In fact, the greater the particulate concentration, the more dissimilar the intercalibration results became. The presence of particulates, though, is the nature of stormwater runoff samples. TSS concentrations throughout southern California range from 1 mg/L to 8,700 mg/L and averages near 300 mg/L (Ackerman and Schiff 2003).

We do not think that the increased interlaboratory variability we observed for the runoff samples with particulates was a result for poor subsampling and distribution among the participating laboratories. Conservative tracers such as TSS, TOC, pH, and conductivity indicated that the samples were relatively well-mixed prior to sample distribution. For example, TSS results from the urban runoff sample varied, on average, by 4% CV for all laboratories combined; four laboratories were within 1 percent of the overall mean. Similarly, laboratories were within 5% CV for the highly turbid rural runoff sample.

The presence of particulates confounds laboratory comparability, especially for particle-bound constituents such as trace metals and some nutrients. There were two sources of variability examined in this study. The first was inconsistent sub-sampling techniques in the laboratory that will lead to differences in particulate concentrations and ultimately to different overall concentrations. The second source of particulate-associated variability was differences in digestion technique. More rigorous digestions will liberate more constituent and ultimately lead to differences in total concentrations. Although these two factors weren't altered independently, this study found that they cumulatively contribute to overall variability in highly turbid samples like rural runoff. Based on these findings, recommendations for standardizing subsampling and digestion techniques for trace metals and TKN are warranted. Increased comparability for Total P, however, was not

achieved through improved sub-sampling and further work on standardizing laboratory procedures for this constituent will likely be needed for this constituent.

There were several intangible effects from the intercalibration study that will enhance comparability region-wide. Studies such as these foster communication among laboratories. While not a hard and fast rule, many laboratories savored the communication with their peers, which often does not happen in a competitive market like laboratory analysis. The communication about methodological details helped elucidate differences among methods, even though all were certified by the State and were following standard methods. Much of this communication was fostered in the early stages of the study as they helped develop the design of the study and the process by which they were going to be evaluated. Involvement in the process led to vestment on the part of the laboratories in not just passing a performance evaluation sample, but improving their overall laboratory performance on an ongoing basis. The final factor that helped ensure success was recognizing the end use of the data they were generating. Traditionally, laboratories in southern California have not attempted to become regionally consistent because no one has suggested that regional application of the data was needed. While most laboratories can treat samples as commodity-based work, laboratory personnel are typically well-trained scientists and recognize the effort attributable to data end users.

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Table 1. Collaborators for the SMC intercalibration study.

Contact Name	Laboratory
Philip Carpenter	Toxscan Laboratories
Alan Ching	Weck Laboratories
Larry Chrystal	Edward S. Babcock and Sons
Andrew Eaton	MWH Laboratories
Rich Gossett	CRG Marine Laboratories
Norman Hester	Truesdail Laboratories
Wei Leung	Los Angeles County
Jim McCall	Associated Laboratories
Dave Renfrew	Enviromatrix Analytical
Kenneth Schiff	SCCWRP
Bob Stearns	CalScience Environmental Laboratories
David Terz	FGL Environmental

Table 2. Methods Inventory for the Trace Metals.

LAB ID	PREPARATORY METHOD (Except Mercury)	PREPARATORY METHOD (Mercury Only)	ANALYTICAL METHOD	INSTRUMENT
A	EPA3010: Hot Block Digestion w/HNO3	EPA245.1	EPA200.7	ICPAES (Hg by Cold Vapor AA)
B	EPA200.8: Hot Block Digestion @ 95°C for 3 Hours Using HNO3/HCl	EPA245.1	EPA200.8	ICPMS (Hg by Cold Vapor AA)
C	No Data	No Data	No Data	No Data
D	EPA200.2:	No Data	EPA200.8	ICPMS
E	EPA200.8: Hot Block Digestion @ 95°C for 4 Hours Using HNO3/HCl	No Data	EPA200.8	ICPMS
F	EPA200.8: Digest Using HNO3	EPA245.7	EPA200.8	ICPMS (Hg by Cold Vapor Atomic Fluorescence)
G	EPA3020: Modblock Digestion @ 85°C to 25mL Using HNO3/HCl	EPA3020: Modblock Digestion @ 85°C to 25mL Using HNO3/HCl	EPA6020	ICPMS
H	EPA200.8: Digestion @ 65°C Using HNO3/HCl	EPA200.8: Digestion @ 65°C Using HNO3/HCl	EPA200.8	ICPMS
I	No Data	No Data	No Data	No Data
J	No Data	No Data	No Data	No Data
K	No Data	No Data	EPA200.8	ICPMS

Table3. Methods inventory for the General Chemistry parameters.

LAB ID	TOTAL SUSPENDED SOLIDS	AMMONIA-N	NITRATE-N + NITRITE-N	TOTAL KJELDAHL NITROGEN	TOTAL PHOSPHORUS	pH	SPECIFIC CONDUCTANCE	TOTAL ORGANIC CARBON
A	EPA160.2	EPA350.1	EPA300.0	EPA351.2	EPA365.4	EPA150.1	EPA120.1	EPA415.1
B	EPA160.1	EPA350.1	EPA300.0	EPA351.1	SM4500P E	EPA150.1	SM2510B	SM5310C
C	No Data	No Data	No Data	No Data	No Data	No Data	No Data	No Data
D	EPA160.2	SM4500NH3 F	EPA300.0	SM4500N B	EPA365.3	SM4500H+ B	SM2510B	SM5310C
E	No Data	No Data	No Data	No Data	No Data	No Data	No Data	No Data
F	EPA160.2	EPA350.2	EPA300.0	EPA351.1	EPA365.2	EPA150.1	EPA120.1	EPA415.1
G	SM2540D	SM4500NH3 B,C	SM4500NO3 E	SM4500N C	SM4500P B,E	EPA150.1	SM2510B	SM5310B
H	SM2540D	SM4500NH3 F	SM4500NO3 E	Not Analyzed	SM4500P C	EPA150.1	SM2510B	Not Analyzed
I	SM2540D	SM4500NH3 H	EPA300.0	EPA351.2	SM4500P B,E	SM4500H+ B	SM2510B	SM5310B
J	SM2540D	SM4500NH3 B,F/G	EPA300.0	SM4500NH3 B, F/G	EPA365.3	SM4500H+ B	SM2510B	EPA415.1
K	EPA160.2	EPA350.2	EPA300.0	EPA351.3	EPA365.3	EPA1500.1	EPA120.1	EPA415.1

Table 4. Comparison of grand mean concentrations for intercalibration samples.

PARAMETER	Mean ± 1SD				
	Reference Material	Round 1 Urban	Round 2 Urban	Round 1 Rural	Round 2 Rural
Total Suspended Solids (mg/L)	-	74±6	-	3200±244	-
NH3-N (mg/L)	0.722±0.124	0.78±0.26	-	0.35±0.59	-
NO2+NO3 (mg/L)	0.74±0.07	1.31±0.22	-	5.35±0.69	-
TKN (mg/L)	0.87±0.18	1.87±0.24	0.48±0.18	5.31±2.87	1.17±0.25
Total Phosphorus (mg/L)	0.85±0.06	0.16±0.05	0.16±0.07	2.09±0.89	0.61±0.44
Total Organic Carbon (mg/L)	2.58±0.93	15.8±1.9	-	12.3±2.8	-
Arsenic (µg/L)	4.28±1.34	2.0±0.4	ND	18.7±14.9	7.6±3.4
Cadmium (µg/L)	7.65±0.77	0.53±0.19	ND	9.61±2.34	2.13±0.62
Chromium (µg/L)	34.4±4.5	7.49±5.88	7.23±4.05	119±87	39±21
Copper (µg/L)	6.01±0.67	32.7±4.1	11±4.5	97±53	24±11
Nickel (µg/L)	24.5±5.1	9.55±2.89	6.88±3.48	159±85	43±16
Lead (µg/L)	2.35±0.99	15.6±8.6	9.0±2.3	32.5±15.3	14±7.3
Selenium (µg/L)	4.36±1.02	1.83±1.07	ND	3.47±1.98	1.76±0.63
Silver (µg/L)	5.07±0.58	ND	ND	ND	ND
Zinc (µg/L)	74.5±22.9	329±66	121±25	331±188	100±43

[a] 5 out of 10 reported ND for this result and were not included in the calculation.

Table 5. Results for the Certified Reference Material from Environmental Resource Associates.

PARAMETER	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F	Lab G	Lab H	Lab I	Lab J	True Value	All Labs	CV (%)
GENERAL CHEMISTRY													
NH3-N (mg/L)	0.60	0.724	1.03	0.720	0.878	0.737	0.540	0.753	0.660	0.578	0.71	0.72	17
NO3+NO2-N (mg/L)	0.70	0.81	0.80	0.80	0.874	0.77	0.720	0.677	0.71	0.61	0.775	0.74	9
TKN (mg/L)	0.80	0.84	1.00	0.98	1.04	0.68	1.20	n/a	0.69	0.70	0.71	0.87	21
Total –P (mg/L)	0.86	0.93	0.88	0.77	0.82	0.89	0.88	0.83	0.90	0.84	0.88	0.85	7
TRACE METALS													
Arsenic (µg/L)	4.00	4.20	8.40	3.25	<2	3.1	3.60	4.47	4.20	4.03	4.05	4.28	31
Cadmium (µg/L)	7.40	7.90	5.70	8.05	6.78	8	7.42	8.45	7.25	7.95	8.0	7.65	10
Chromium (µg/L)	30.0	32.0	49.0	34.0	34.0	34	32.4	34.6	33.0	32.5	32.9	34.4	13
Copper (µg/L)	5.2	5.4	7.5	6.4	6.5	6.4	6.7	5.9	5.4	5.3	5.01	6.01	11
Nickel (µg/L)	22.6	24.0	38.0	26.0	24.1	13	24.4	25.1	22.5	24.0	24.5	24.5	21
Lead (µg/L)	2.1	2.0	5.7	2.2	2.1	2.5	1.7	2.3	1.8	2.0	2.05	2.35	42
Selenium (µg/L)	4.4	<2	n/a	3.05	3.27	3.6	5.2	5.0	5.4	4.0	4.2	4.4	23
Silver (µg/L)	4.9	4.9	6.9	5.05	4.72	5.5	4.83	4.99	4.65	4.8	4.94	5.07	20
Zinc (µg/L)	65.2	76.0	145	51.5	63.9	72	64.1	84.4	67.5	65.4	70.8	74.5	31

Table 6A. Summary of intercalibration results for Urban Runoff.

	Lab A		Lab B		Lab C		Lab D		Lab E		Lab F		Lab G		Lab H		Lab I		Lab J		All Labs	
PARAMETER	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	CV(%)
GENERAL CHEMISTRY																						
TSS (mg/L)	71.7	0.6	74.3	1.2	72.3	1	73.3	1	72.8	2.3	85.7	0.6	77.1	2.9	73.7	0.6	63	4	74.7	0.6	74.0	8
NH3-N (mg/L)	0.97	0.21	0.62	0.01	1.35	0.08	0.84	0.01	0.46	0.12	0.82	0.02	0.72	0.02	0.43	0.06	0.76	0.01	0.8	0.01	0.78	33
NO3+NO2-N (mg/L)	1.3	0	1.5	0	1.35	0.01	1.5	0	1.38	0.06	1.7	0	0.99	0.01	0.95	0.02	1.27	0.06	1.2	0	1.31	17
TKN (mg/L)	1.63	0.06	1.97	0.06	1.69	0.19	2.13	0.06	1.98	0.21	1.57	0.12	1.93	0	-[a]	-	1.83	0.15	2.12	0.06	1.87	13
Total P (mg/L)	0.12	0.01	0.13	0.01	0.18	0.02	0.16	0.01	0.27	0.01	0.14	0	0.13	0.01	<0.1	-	0.13	0.01	0.12	0	0.16	31
TRACE METALS																						
Arsenic (µg/L)	<2	-	2.57	0.38	<5.6	-	1.9	0.17	<2	-	<2	-	1.42	0.25	2.14	0.04	<2	-	1.91	0.06	2.0	20
Cadmium (µg/L)	<1	-	<0.5	-	<5.6	-	<0.5	-	0.69	0.41	<1	-	0.55	0.05	0.51	0.01	0.38	0.03	<1	-	0.53	36
Chromium (µg/L)	6.8	0.53	6	0.4	23	1.7	<1	-	7.09	0.26	<5	-	4.94	0.09	1.88	0.06	8.07	0.59	5.61	0.28	7.49	79
Copper (µg/L)	34.2	1	36.3	5.8	36.3	1.2	36.7	0.6	34.7	0.9	28.3	0.6	33.1	0.2	29.7	0.6	32.7	0.6	25.1	0.2	32.7	13
Nickel (µg/L)	9.57	0.55	10.3	0.6	21	-	8.27	0.29	9.73	0.18	<4	-	10.7	0.7	6.27	0.16	10.7	0.6	7.07	0.49	9.55	30
Lead (µg/L)	13	0.3	13.3	0.6	20	3.6	12.3	0.6	13.1	0.1	11.3	0.6	11.5	0.3	39.6	1	12	0	9.61	0.06	15.6	55
Selenium (µg/L)	<2	-	<2	-	-	-	<2	-	1.28	0.38	<2	-	<2	-	1.35	0.12	3.43	1	1.25	0	1.83	58
Silver (µg/L)	<1	-	<0.5	-	<5.6	-	<0.5	-	<1	-	<1	-	<1	-	<0.5	-	0.13	-	<1	-	ND	-
Zinc (µg/L)	329	7	320	10	478	127	347	6	284	3	287	6	297	1	363	2	303	6	287	1	329	20

[a] Data is either not applicable or not reported.

Table 6B. Summary of intercalibration results for Rural Runoff.

	Lab A		Lab B		Lab C		Lab D		Lab E		Lab F		Lab G		Lab H		Lab I		Lab J		All Labs	
PARAMETER	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	CV(%)
GENERAL CHEMISTRY																						
TSS (mg/L)	3240	1	3320	25	3110	65	3070	115	3050	70	3670	75	3090	72	3440	183	2900	346	3120	3	3200	8
NH3-N (mg/L)	<0.1	-[a]	<0.05	-	1.87	0.16	0.19	0.0	0.10	0.05	0.17	0.01	0.05	0.02	0.09	0.02	0.13	0.01	0.19	0.01	0.35	169
NO3+NO2-N (mg/L)	5.2	0.1	5.6	0.0	5.52	0.04	5.97	0.06	5.49	0.10	6.57	0.06	4.10	0.05	4.39	0.17	5.2	0.0	5.49	0.0	5.35	13
TKN (mg/L)	4.77	0.12	9.23	0.71	7.31	0.67	5.33	0.06	1.87	0.07	6.03	0.97	1.4	0.0	-	-	2.6	0.2	9.22	0.37	5.31	54
Total P (mg/L)	3.53	0.01	1.14	0.19	1.18	0.11	1.67	0.06	1.22	0.02	2.53	0.06	1.80	0.04	1.57	0.10	3.47	0.21	2.78	0.02	2.09	43
TRACE METALS																						
Arsenic (µg/L)	22.6	1.9	9.7	0.5	42	7	6.53	0.67	32.7	0.8	2.9	0.8	29.0	0.3	4.42	0.07	35.7	1.2	1.68	0.17	18.7	80
Cadmium (µg/L)	11.4	0.2	10	0	12.7	1.5	10.7	0.6	11.0	0.3	6.6	0.1	11.7	0.1	5.97	0.17	9.5	0.3	6.68	0.04	9.61	24
Chromium (µg/L)	170	7	150	0	52.7	3.2	130	0	193	7	24.7	7.2	199	1	6.01	0.13	260	0	7.78	0.01	119	73
Copper (µg/L)	139	3	110	0	157	31	107	6	122	4	25.7	2.1	133	3	21.2	1.4	137	6	15.2	0.3	97.0	55
Nickel (µg/L)	231	4	190	0	243	58	190	0	194	6	39.7	2.1	213	3	31.2	0.8	217	6	37.1	1.0	159	53
Lead (µg/L)	34.9	0.4	35.3	3.1	50.3	4.5	38.3	0.6	44.1	0.8	8.7	2.9	43.1	0.5	25.5	1.6	42.3	0.6	2.60	0.05	32.5	47
Selenium (µg/L)	2.9	0.2	<2	-	-	-	<2	-	3.58	0.67	<2	-	3.65	0.23	1.83	0.10	7.0	1.0	1.02	0.03	3.47	57
Silver (µg/L)	<1	-	<0.5	-	<5.6	-	<0.5	-	<1	-	<1	-	7.99	12.5	0.06	-	0.45	0.02	<1	-	ND	-
Zinc (µg/L)	462	7	390	10	551	43	360	10	442	14	80.7	9.9	424	2	56.7	2.2	497	6	41.7	0.2	331	57

[a] Data is either not applicable or not reported.

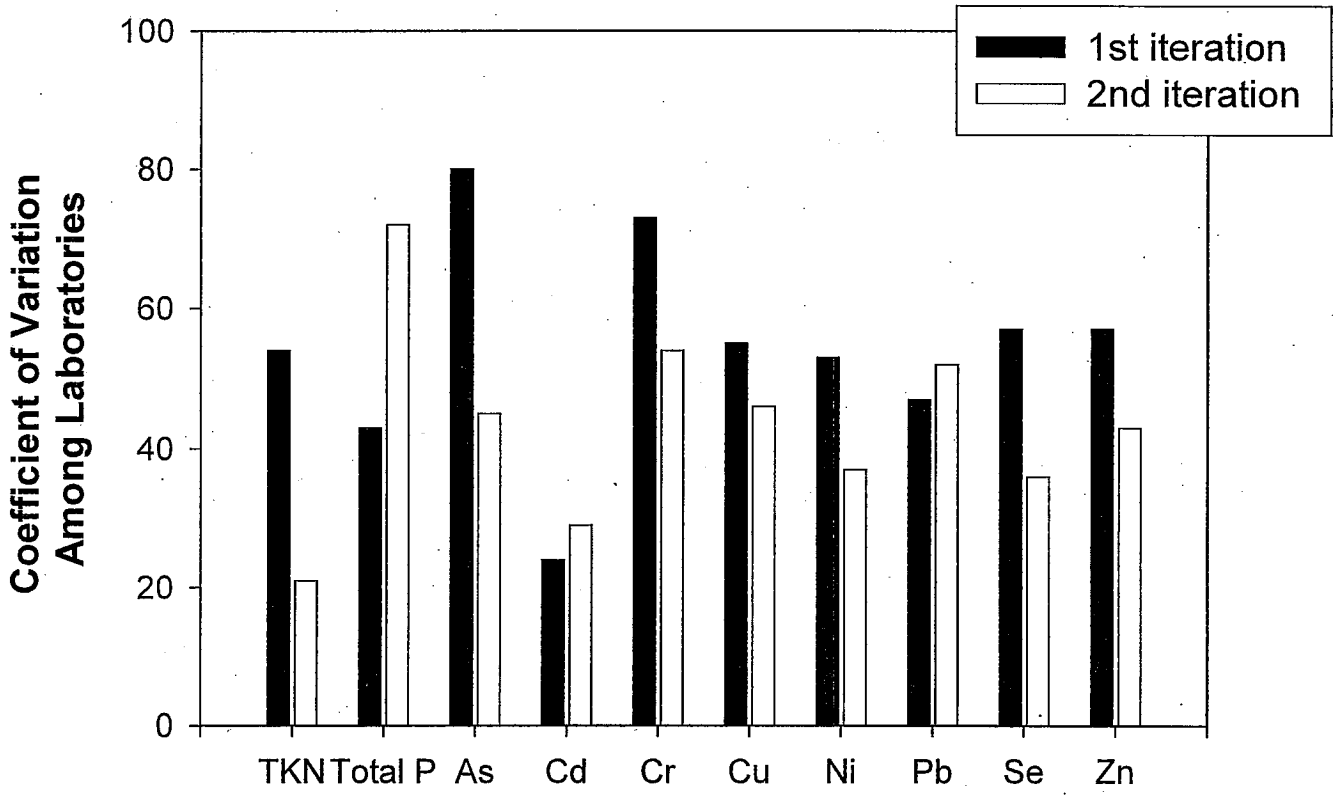

Figure 1. Comparison of coefficients of variation among laboratories between subsequent iterations of intercalibration exercises for the rural runoff sample.

STORMWATER MONITORING COALITION LABORATORY GUIDANCE DOCUMENT

Second Edition

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and

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Southern California Coastal Water Research Project

Technical Report 521 - September 2007

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Richard Gossett
CRG Marine Laboratories, Inc.

and

Kenneth Schiff
Southern California Coastal Water Research Project

Southern California Coastal Water Research Project
3535 Harbor Blvd., Suite 110
Costa Mesa, CA 92626

www.sccwrp.org

September 2007

Technical Report 521

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INTRODUCTION

A continuing goal of the southern California Stormwater Monitoring Coalition (SMC) is to compile monitoring data from separate programs to make region-wide assessments. This task has been difficult, thus far, because the various monitoring programs have differing project goals and objectives, differing mandates from regulatory agencies, differing sampling designs, and differing laboratory analytical methods. The goal of this document is to provide the basis for ensuring comparability among stormwater chemical analytical laboratories. The comparability issues that revolve around goals, objectives, and study designs were addressed through a related, but separate document (Bernstein and Schiff, 2003).

There are at least three reasons why laboratory analytical data are not comparable including differences in target analytes, reporting levels (detection limits), and laboratory methods. In 2003, a laboratory intercalibration study sponsored by the SMC established common reporting levels, target analytes, and iterative round robin exercises that surmounted many of these obstacles (Gossett, Renfrew, and Schiff, 2003). However, there are new laboratories, or new staff within existing laboratories, which have not been evaluated and periodic intercalibrations are a necessity. In an effort to improve and update laboratory performance, a new intercalibration study was conducted with 14 laboratories that conduct stormwater analysis in California (Table 1). Like the previous exercise, this study was aimed at developing a consensus-based approach for achieving minimal levels of comparability among typically disparate laboratories. The success of the 2003 exercise and this subsequent study was primarily due to three factors including communication and commitment among laboratory personnel, setting performance-based criteria for establishing standards of success, and round robin testing using locally derived reference materials.

Objectives and Goals of this Document

The objective of this guidance manual is to update and present the performance-based guidelines established during the SMC interlaboratory studies of 2003 and 2007. This document sets the minimum standards of sensitivity, precision, and accuracy across laboratories so that individual data sets can be combined with estimated levels of confidence for making regional assessments of stormwater quality. The philosophy of performance-based guidelines is key to achieving this comparability. Although every laboratory involved in the stormwater intercalibration study was certified by the State of California Environmental Laboratory Accreditation Program (ELAP), inventories of existing methods demonstrated that most analytes are not analyzed in exactly the same manner. This will continue as new laboratories, or new equipment at existing laboratories continues to proliferate. Rather than mandate specific methods that are inflexible and discourages existing laboratories from achieving faster, more sensitive, and more cost-effective methods, this document merely sets minimum levels of comparability so that data sets can be combined no matter what technology currently exists. These procedures are also outlined herein.

This guidance manual is a living document. It should be revisited each time an intercalibration exercise is conducted and can be expanded to include additional constituents, additional laboratories, or to refine the recommended performance-based sensitivity, accuracy, and precision requirements as new information becomes available.

This document and laboratory intercalibration study is not a certification program. The guidelines set by this document merely express the desired needs of the stormwater agencies throughout the southern California region. Therefore, these stormwater agencies can use these guidelines in establishing specifications for work assignments or requests for proposals to conduct stormwater analyses. Alternatively, or in combination, stormwater regulatory agencies may use these specifications in the development of regulatory expectations for laboratory performance by monitoring agencies.

Table 1. Participating Laboratories in the 2007 SMC Intercalibration Study (listed alphabetically).

Laboratory Name	Contact
Advanced Technology Labs	Bing Roura
Associated Labs	Jim McCall
California Department of Fish and Game	Patricia Bucknell
CalScience Environmental Labs	Larry Lem
Capco Analytical Services	Dan Farah
City of Los Angeles- EMD	Mahesh Pujari
CRG Marine Labs	Rich Gossett
FGL Environmental	David Terz
Los Angeles County- ACWM	Wai Leung
MWH Labs	Andrew Eaton
Soil Control Labs	Mike Galloway
Truesdail Labs	Norm Hester
UC Santa Barbara MSI	George Paradis
Weck Labs	Alan Ching

GUIDANCE INFORMATION

This document consists of four elements. First is a list of target analytes and minimum levels of sensitivity (reporting levels). Second are minimum levels of accuracy and precision. Third are recommended protocols for method specific comparability. Fourth are participation requirements for intercalibration studies and the laboratory evaluation criteria and results.

Analytes and Reporting Levels

Target Analytes

A core group of target analytes was specified for comparability (Table 2). This list includes total suspended solids (TSS), total organic carbon (TOC), nutrients, and trace metals. This list was based on three criteria. First, these analytes are consistently measured by existing monitoring programs throughout the region. Second, these constituents are routinely detected in stormwater samples. Third, although standard methods exist, there is sufficient disparity in protocols among laboratories that consistency guidance is warranted.

The list of target analytes is not meant to be an exhaustive list of all constituents that could or should be measured in individual programs. For example, there are no pesticides, herbicides, or polynuclear hydrocarbons on the list of target analytes. Any or all of these target analytes may be the focus of individual monitoring programs. At this point in time, however, there has not been an intercalibration study conducted for these constituents to make performance-based recommendations for stormwater laboratories.

While the list of target analytes focuses on total trace metals, they can be applied to dissolved trace metals. Since the analytical methodology is similar among both total and dissolved metals, the performance-based guidelines may be applied to both. Bear in mind that for dissolved trace metals, the preparation step of filtration is a major source of potential contamination and was not evaluated in this study.

Reporting Levels

Targeted reporting levels (RLs) are provided in Table 2. This guidance was based on the philosophy that analyses should be sufficient to assess if samples are below water quality thresholds of concern. In this instance, the water quality thresholds of concern are established in the California Toxics Rule (CTR). Further, reporting levels should be technologically achievable, but far enough below water quality thresholds that exceedences cannot be attributable to methodological uncertainty. Therefore, the philosophical approach for setting RLs was to select guidelines that were one-half of the lowest water quality threshold concentration. In the case of the CTR, there are thresholds for both marine and fresh waters. For a participating laboratory to achieve these reporting levels, it should include a calibration standard at or below this level (e.g. the reporting level is a quantitation level and not an MDL).

Table 2. Target analytes and Reporting Levels for the Stormwater Monitoring Coalition Monitoring Program.

Analyte	Units	SMC Target Reporting Level	California Toxics Rule Limit (Freshwater)	California Toxics Rule Limit (Seawater)
General Constituents				
TSS	mg/L	5	-	-
Nitrate+Nitrite as N	mg/L	0.2	-	-
Ammonia as N	mg/L	0.1	-	-
Total Phosphorus as P	mg/L	0.1	-	-
Total Kjeldahl Nitrogen	mg/L	0.2	-	-
Total Organic Carbon	mg/L	1	-	-
			-	-
Total Metals				
Arsenic	µg/L	2	150	36
Cadmium	µg/L	1	2.2	9.3
Chromium (total)	µg/L	5	11	50
Copper	µg/L	2	9	3.1
Nickel	µg/L	4	52	8.2
Lead	µg/L	1	2.5	8.1
Selenium	µg/L	2	5	71
Silver	µg/L	1	3.4	1.9
Zinc	µg/L	10	120	81

Accuracy and Precision

Analysis of spiked samples or reference materials provides a mechanism for assessing within laboratory accuracy. Reproducibility among replicate sample analyses provides a determination of within laboratory precision. General guidance provided by ELAP and the US EPA (40 CFR Part 136) are recommended for assessing within laboratory accuracy and precision by analyzing two replicate sample matrix spikes per batch of 20 or less samples. Matrix spike concentrations of approximately 10 times the lab reporting level are recommended for most meaningful spike recovery measurements. As general guidance, matrix spike concentrations should be in the same general range as relevant regulatory limits to truly represent accuracy and precision at these concentrations. It is also recommended that a set of laboratory replicate samples are analyzed with each batch of samples to indicate precision using actual sample matrices, which is typically larger due to natural variation of sample homogeneity. The relative percent difference (RPD) between replicate spikes for the parameters listed in Table 2 should be less than 20%. Accuracy limits for MS and MSD are provided in Table 3. These accuracy limits mimic ELAP and US EPA guidelines. Accuracy limits for Certified Reference Materials are provided by the supplier.

Additional QAQC requirements in the methods referenced by each laboratory should conform to the requirements listed within that method by Standard Methods or the US EPA

(i.e. Blank Spikes). Project specific QAQC requirements may also be listed in the Quality Assurance Project Plan (QAPP). Since spiked samples can be complicated by matrix interferences, this can confound assessments of accuracy. Therefore, the analysis of Certified Materials, when available, is also a recommended (but not required) option for the monitoring agency.

Intercalibration studies evaluate the accuracy and precision of analysis among laboratories. For this document, interlaboratory precision guidelines were developed by analyzing each of three matrices by fourteen different laboratories (Table 1) throughout southern California. These matrices included a specially prepared performance evaluation (PE) sample, an urban runoff sample, and a rural runoff sample.

Table 3. Laboratory accuracy and precision guidelines for concentrations greater than 10x the RL for ongoing analysis of stormwater samples.

Target Analyte	Precision (RPD of Duplicate Samples)	Accuracy (Percent Recovery of MS or MSD [a])
General Constituents		
TSS	0 - 20	-[b]
Ammonia-N	0 – 20	80 – 120
Nitrate-N+Nitrite-N	0 – 20	80 - 120
Total Kjeldahl Nitrogen	0 – 20	70 – 130
Total Phosphorus as P	0 – 20	70 – 130
Total Organic Carbon	0 – 20	80 – 120
Trace Metals		
Arsenic	0 – 20	80 – 120
Cadmium	0 – 20	80 – 120
Chromium	0 – 20	80 – 120
Copper	0 – 20	80 – 120
Lead	0 – 20	80 – 120
Nickel	0 – 20	80 – 120
Selenium	0 – 20	80 – 120
Silver	0 – 20	80 – 120
Zinc	0 – 20	80 – 120

[a]- For certified reference materials, use supplier recommendations
[b]- defined by supplier

For each of the 3 matrices, the grand mean and standard deviation were calculated using the pooled results from all fourteen laboratories (Table 4). A Grubb's test was used to identify outliers that were removed along with the "not detected" values from the data set prior to calculating the grand means and standard deviations. It should be noted that not all laboratories analyzed every target analyte so that the number of results varied by parameter. Upper and lower "Warning" limits were based on two standard deviations within the grand mean and upper and lower "Control" limits were based on three standard deviations within the grand mean.

Table 4. Grand mean, warning limits, and control limits of each matrix from the combined results of all fourteen laboratories participating in the intercalibration. Warning limits and control limits are indicated as maximum difference from the mean (e.g., TSS for LU control limits are 106 to 138 ppm).

		ERA[a]			LU[b]			LR[c]		
Target Analyte	Units	Mean	Warning Limit (±2 SD)	Control Limit (±3 SD)	Mean	Warning Limit (±2 SD)	Control Limit (±3 SD)	Mean	Warning Limit (±2 SD)	Control Limit (±3 SD)
General Constituents										
TSS	mg/L	-	-	-	122	11	16	337	83	124
Ammonia-N	mg/L	0.551	0.51	0.76	1.33	0.45	0.66	0.341	0.166	0.249
Nitrate-N+Nitrite-N	mg/L	1.88	0.19	0.29	0.675	0.109	0.162	0.755	0.144	0.216
Total Kjeldahl Nitrogen	mg/L	0.796	0.554	0.834	2.95	0.89	1.33	3.64	2.13	3.19
Total Phosphorus as P	mg/L	0.746	0.171	0.258	0.288	0.220	0.330	0.388	0.338	0.507
Total Organic Carbon	mg/L	-	-	-	26.5	6.4	9.5	6.63	3.77	5.64
Trace Metals										
Arsenic	µg/L	7.05	1.59	2.38	3.13	0.81	1.22	2.58	1.14	1.70
Cadmium	µg/L	2.96	0.33	0.49	0.306	0.074	0.111	0.466	0.112	0.168
Chromium	µg/L	30.2	3.4	5.2	5.51	2.08	3.12	13.2	4.2	6.4
Copper	µg/L	12.7	2.3	3.5	116	25	37	19.3	8.8	13.2
Lead	µg/L	7.10	0.71	1.06	8.74	1.30	1.96	8.87	2.53	3.77
Nickel	µg/L	32.1	4.0	6.03	13.1	2.0	2.94	5.08	1.15	1.72
Selenium	µg/L	17.7	4.3	6.6	1.88	0.89	1.33	1.44	0.81	1.21
Silver	µg/L	6.74	1.18	1.77	0.106	0.041	0.063	0.069	0.060	0.09
Zinc	µg/L	79.1	21.7	32.6	153	28	42	468	106	159

[a] certified reference material
[b] simulated rainfall runoff from an urban catchment
[c] simulated rainfall runoff from a rural catchment
- no limit

Using the same data set as the grand means and standard deviations, the overall relative standard deviation (RSD) for each matrix was calculated and is presented in Table 5. Precision among laboratories is expected to be less than 30% for those analytes that are present at concentrations at least 10 times the MDL. The RSD for Ammonia and TKN was greater than 30% for the ERA sample but within 30% for the LU and LR samples. Total P RSD was greater than 30% for the LU and LR samples, but the concentration was below 10 times the MDL. The same was true for Silver in the LR sample.

Table 5. Relative standard deviation results for each matrix for the combined results from all fourteen laboratories.

		Relative Standard Deviation		
Target Analyte	Units	ERA[a]	LU[b]	LR[c]
General Constituents				
TSS	%	-	5	12
Ammonia-N	%	46	17	24
Nitrate-N+Nitrite-N	%	5	8	10
Total Kjeldahl Nitrogen	%	35	15	29
Total Phosphorus as P	%	11	38	43
Total Organic Carbon	%	-	12	28
Trace Metals				
Arsenic	%	11	13	22
Cadmium	%	6	12	12
Chromium	%	6	19	16
Copper	%	9	11	23
Lead	%	5	7	14
Nickel	%	6	7	11
Selenium	%	12	24	28
Silver	%	9	20	44
Zinc	%	14	9	11

[a] certified reference material
[b] simulated rainfall runoff from an urban catchment
[c] simulated rainfall runoff from a rural catchment
- no limit

Standardization

Although this document is founded on performance-based guidelines enabling flexibility within each laboratory to achieve consistency, the laboratory intercalibration studies have identified four protocols whereby recommended standardization can dramatically increase comparability. This standardization includes sub-sampling, Total Phosphorus digestion, TKN digestion, and trace metal digestion techniques.

Sub-Sampling Techniques

Sub-sampling techniques are an important component of both within and among laboratory variability. This was especially true for particle-laden samples, such as those from more rural catchments with unlined channels. Particle-bound constituents have the potential to be dramatically biased if sub-sampling techniques selectively target or avoid particles within samples. To this end, standardized laboratory techniques for sub-sampling were developed for splitting large volume stormwater samples collected in the field into smaller bottles for distribution to the laboratory and for subsequent sampling of smaller aliquots in the laboratory at the time of analysis.

Sub-sampling of large-volume composite containers

In order to ensure that sample containers destined for an analytical laboratory all contain water that is similar and representative of the original composite sample, it is important to maintain a well-mixed composite sample during sub-sampling and to prevent stratification and the settling out of heavier particles. This is accomplished by the use of a large-capacity stirrer and a 2 to 3-inch, pre-cleaned, Teflon-coated stir bar; larger stir bars can be used for larger volume containers. Adjustment of stirring speed is important. Speeds that are too fast will create a large vortex within the composite bottle that can actually concentrate heavier particles and should be avoided. Speed should be based on a visual assessment of the most even mixing throughout the composite bottle.

Sub-sampling from the homogenized composite bottle is accomplished using a peristaltic pump and pre-cleaned (inside and outside) sub-sampling hose. Filling sample containers by pumping from the composite bottle is best performed by two people. One person is responsible for filling individual sample containers and one person is responsible for constantly moving the intake tubing up and down in the water column of the composite sample. Based on experimental evidence, this up and down movement of the intake is a procedure that helps obtain a more representative sub-sample. This is because there can still be some stratification of heavier particles in the composite sample despite the mixing created by the stirrer. The up and down movement of the intake tubing should be limited to approximately 80-90 percent of the depth of the water column and should never touch the bottom of the composite bottle.

Sub-sampling of sample containers for analysis

The goal of sub-sampling bottles in the laboratory for analysis is similar to field sampling techniques, to maintain a homogeneous particle distribution. Analysis of particle-

associated constituents will be biased if non-representative particle suspensions are used for analysis. In order to maintain homogeneous particle distributions, we recommend the use of sub-sampling techniques described by the US Geological Survey (Charles J. Patton, USGS National Water Quality Laboratory, Denver, CO). Appropriately, a similar technique to large composite container sub-sampling is used. Briefly, a "+" shaped magnetic stirring bar is placed into the sample container and the sample is stirred while a sub-sample is aspirated and dispensed into the processing container.

Total Kjeldahl Nitrogen

Total Kjeldahl Nitrogen (TKN) analysis was affected by the digestion technique during the laboratory intercalibration exercise. This was due to the influence caused by particle content and size distribution. Therefore, minimum standardization of the digestion procedure for stormwater samples is recommended. Either micro or macro Kjeldahl digestions are acceptable. However, the length of time of digestion should be set at a minimum of 1 hour at 380ºC, until copious fumes are generated and the digestion solution turns yellow, and then for an additional 30 minutes (to ensure adequate recovery) prior to analysis.

Total Phosphorus

Total Phosphorus analysis was also affected by the digestion technique during the laboratory intercalibration exercise. Therefore, we are recommending standardization of the digestion procedure to the use of an acid persulfate digestion.

Trace Metals

Trace metal analysis was also affected by the digestion technique during the laboratory intercalibration exercise. This is because trace metal concentration may be influenced by particle content and size distribution. Therefore, minimum standardization of trace metal digestion is recommended for stormwater samples. Trace metals should be digested using a nitric/hydrochloric acid digestion at 95ºC for 2-4 hours until the sample has evaporated from 50mL down to 10mL.

Dissolved metals analysis should be performed on filtered samples and does not require digestion if the turbidity is <1. Sample spiking for the Matrix Spike should be done after filtering. Filtration is a common source of contamination and when measuring dissolved metals. All other criteria for trace metals in this guidance document are applicable to both total and dissolved metals.

Participation Requirements

Proficiency Testing

Laboratories performing analyses for SMC Stormwater Monitoring Programs should pass a SMC performance evaluation (PE) sample and participate successfully in SMC intercalibration exercises. The PE and intercalibration exercises are strongly recommended to be performed on an annual basis. This frequency is recommended

because: 1) new laboratories may wish to participate; 2) existing laboratories need to evaluate new personnel; and 3) new and existing laboratories with new equipment or altered laboratory techniques need to be evaluated. Intercalibrations should occur within the first six months of the calendar year to ensure evaluations prior to the following wet season that typically begins on October 15th.

SMC PE samples should be spiked between 1 and 10 times the established reporting limit (Table 2) for the analytes of concern for minimum proficiency. All sample results should meet the criteria provided by the commercial supplier of the sample to evaluate accuracy. PE samples are to be coordinated through the SMC, or their representatives on a Chemistry subcommittee, and can be purchased from private companies such as Environmental Resource Associates, Inc. (ERA), Wibby Environmental, APG, or other NELAC approved proficiency testing sample providers.

Intercalibration studies require laboratories to analyze one PE sample and three replicates of two runoff samples, one from an urban area and one from a rural catchment. Each intercalibration study should be performed with one or more iterations to evaluate consistency and allow for laboratory corrective actions if deficient analysis resulted from the first iteration.

New Laboratories

New laboratories that have not participated in previous intercalibration exercises may still be able to analyze stormwater samples during the present wet season. These labs, however, will need to provide resources to purchase a PE sample with the same requirements used in the intercalibration study in Section 3.1 (i.e. samples will be spiked at 1 to 10 times the established reporting limits in Table 2). These samples should be delivered to the new laboratory blind and as whole volume samples. All new laboratories are required to participate in the next intercalibration exercise to remain qualified for the SMC program.

Laboratory Intercalibration Exercise Evaluation Criteria

Laboratories participating in the intercalibration exercise will be evaluated to determine if their results are within acceptable accuracy and precision insuring comparability of data between the different SMC stormwater monitoring programs. For the present study, a scoring system was used to assign a numeric value and letter grade to each laboratory indicating the quality of their performance in the exercise. A grade of “C” or better is recommended for a laboratory to be eligible to perform analyses for SMC programs.

Scoring System and Results

Based on the combined results from all fourteen laboratories participating in the exercise, a Grubb’s test was performed to identify outlier data points. After removal of the outlier data, the “not detected” results were removed and the grand mean and standard deviation were calculated for each analyte. Upper and lower warning and control limits were based on 2 and 3 times the standard deviation, respectively (see table 4). The results, upper and

lower warning, and upper and lower control limits are presented in graph form in Appendix I.

A scoring system was established to rate each laboratory's performance. Each replicate analysis by a laboratory was given 3 points if the result for that sample-analyte combination was within the warning limit and 2 points if the result was within the control limit (Figure 1). Zero points were given if the result was outside the control limit. For example, if a laboratory got all three replicate results within 2 SD, then they received 9 points for that analyte. If two results were within 2SD and one was within 3SD, then the laboratory was given 3+3+2 or 8 points out of 9. The Relative Standard Deviation (RSD) was also calculated for each parameter and 1 point was given for each analyte/matrix with an RSD of < 20%. The total points awarded to each laboratory were combined for the General Constituents (TSS, Ammonia-N, Nitrate-N + Nitrite-N, TKN, TOC, and Total P) and then divided by the total possible points, then multiplied by 100 for the final score. Trace metals were all combined as a separate score using the same criteria.

Laboratories that did not analyze a particular analyte were not given a score for that parameter so that they were not penalized. The omitted analyte is listed in the scoring results summary so that SMC programs managers have the option of using another laboratory for that specific analysis. A letter grade was assigned based on 90-100% being an "A", 80-89% being a "B", 70-79% being a "C", and below 70% being an "F".

The results from the 2007 laboratory intercalibration exercise are presented in Table 6a and b. Since this is a public document and participation in this exercise is intended to be kept anonymous, the laboratories are listed by randomly assigned number. SMC member agencies will be given the key to the laboratory names upon request.

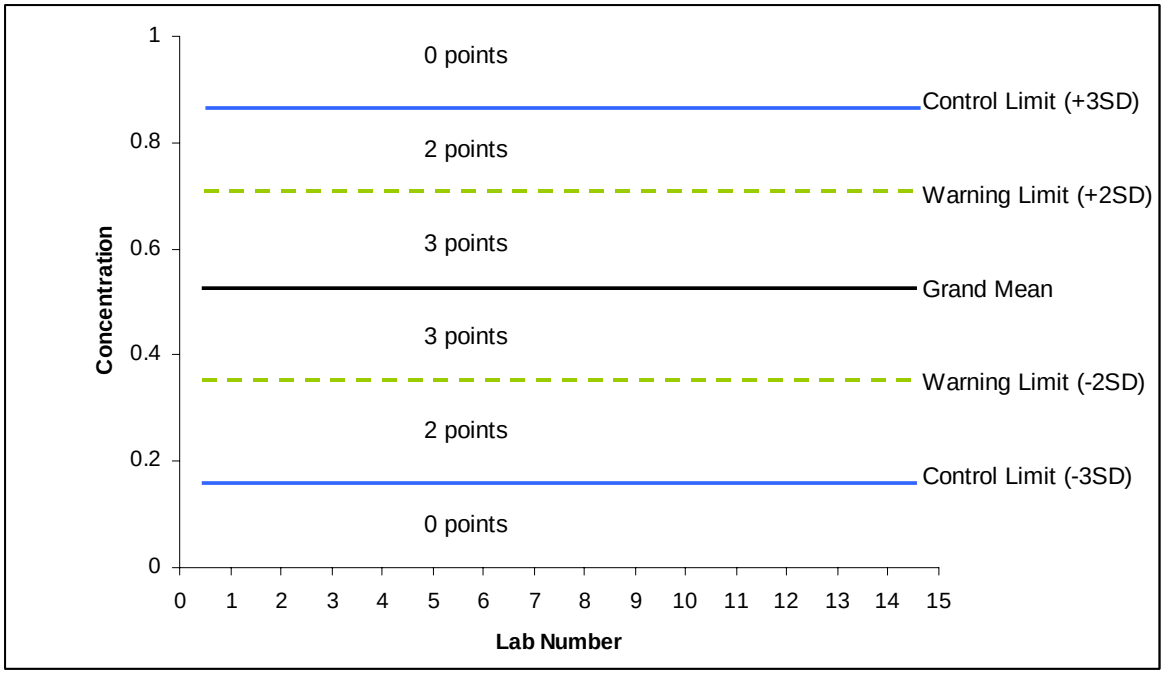


Figure 1. Example of scoring system for SMC intercalibration studies without any specific data shown.

Table 6a. Scoring results for General Constituents in the 2007 Laboratory Intercalibration Exercise.

Laboratory Number	Analytical Result Score	RSD Score	Percent Success	Letter Grade
1	93 out of 120	11 out of 12	79 %	C
2	120 out of 120	12 out of 12	100%	A
3 (did not analyze TKN or TOC)	79 out of 81	8 out of 8	98 %	A
4	116 out of 120	12 out of 12	97%	A
5	118 out of 120	12 out of 12	98%	A
6	98 out of 120	11 out of 12	83%	B
7	120 out of 120	12 out of 12	100%	A
8 (did not analyze TOC)	110 out of 120	11 out of 12	92%	A
9	112 out of 120	11 out of 12	93%	A
10 (did not analyze Nitrate-N + Nitrite-N or TOC)	11 out of 11	0 out 8	58%	F
11 (did not analyze TOC)	91 out of 102	9 out of 10	89%	B
12	117 out of 120	12 out of 12	98%	A
13 (did not analyze TSS, TKN, TOC, or Total P)	42 out of 42	4 out of 4	100%	A
14 (did not analyze TKN or TOC)	79 out of 81	8 out of 8	98%	A

Table 6b. Scoring results for Trace Metals in the 2007 Laboratory Intercalibration Exercise.

Laboratory Number	Analytical Result Score	RSD Score	Percent Success	Letter Grade
1	186 out of 189	18 out of 18	99%	A
2	180 out of 189	18 out of 18	96%	A
3	185 out of 189	18 out of 18	98%	A
4	186 out of 189	18 out of 18	99%	A
5	179 out of 189	18 out of 18	95%	A
6	189 out of 189	18 out of 18	100%	A
7	187 out of 189	16 out of 18	98%	A
8	not analyzed	-	-	-
9	189 out of 189	17 out of 18	100%	A
10	26 out of 189	16 out of 18	20%	F
11	187 out of 189	78 out of 18	99%	A
12	189 out of 189	18 out of 18	100%	A
13	not analyzed	-	-	-
14	178 out of 189	18 out of 18	95%	A

DEFINITIONS

Batch – An analytical batch consists of 20 or fewer client samples.

Method Blank (MB) – Analyte free water that is carried through the entire analytical process. The method blank is used to evaluate contamination contributed from the method. Analyte detections in the method blank must be less than 10x the analyte result for a client sample to be considered usable without flagging.

Duplicate – A client sample analyzed in duplicate. Duplicate RPD should be summarized in the report.

Blank Spike/Blank Spike Duplicate – A blank spike (laboratory control sample) is a certified standard reference material that is spiked into a reagent blank. It is carried through all steps of sample preparation to demonstrate method performance inclusive of sample preparation steps. The blank spike should be spiked near the mid point of the calibration curve.

Matrix Spike/Matrix Spike Duplicate – A matrix spike is a regular sample that is split into three sub-samples. Two of the replicates are spiked with analyte solution at the same concentration and are defined as the matrix spike and matrix spike duplicate (MS/MSD). The MS/MSD samples are carried through the sample preparation and analysis procedure with each batch of 20 or less samples. The MS/MSD results provide information regarding laboratory precision, sample matrix effects, and method efficiency.

RPD- Relative Percent Differnce is calculated using the following formula:

RPD=(Results1-Result2)/(Result1+Result2)/2)*100

RSD- Relative Standard Deviation is calculated using the following formula:

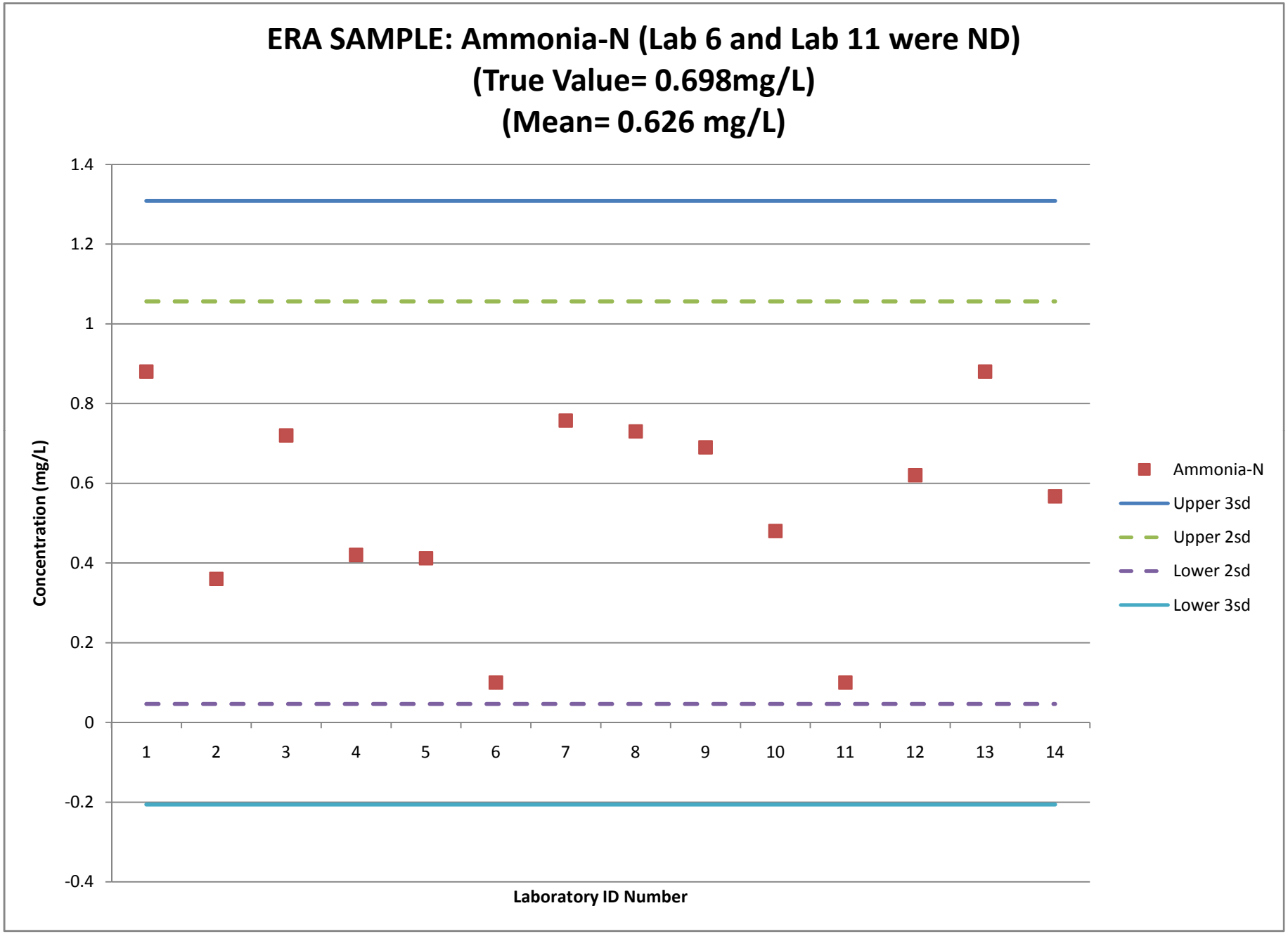
RSD=(Standard Deviation)/(Mean)*100

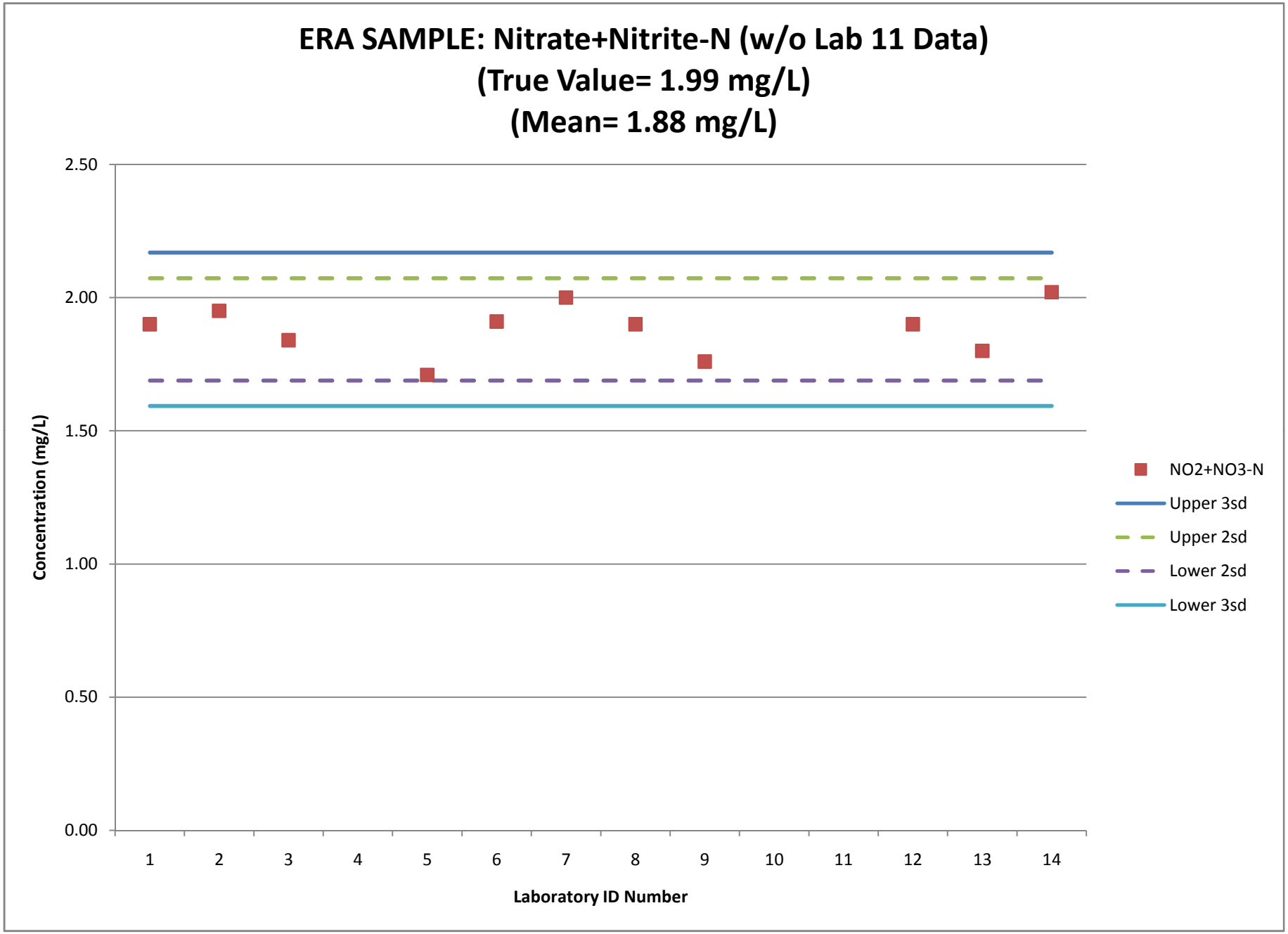
REFERENCES

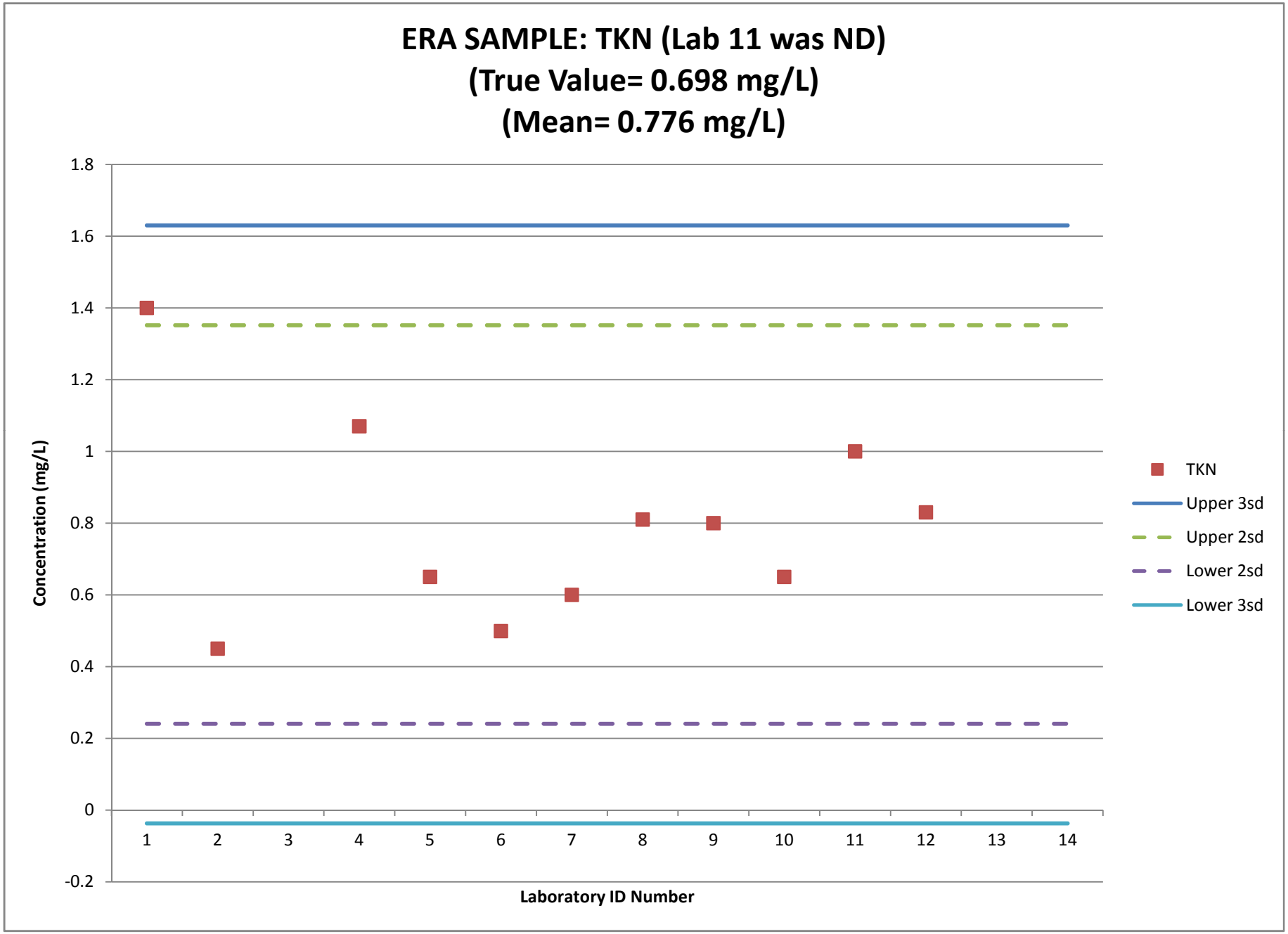
Bernstein, B. and K. Schiff. 2003. Model Stormwater Monitoring Program for Urban Runoff Programs.

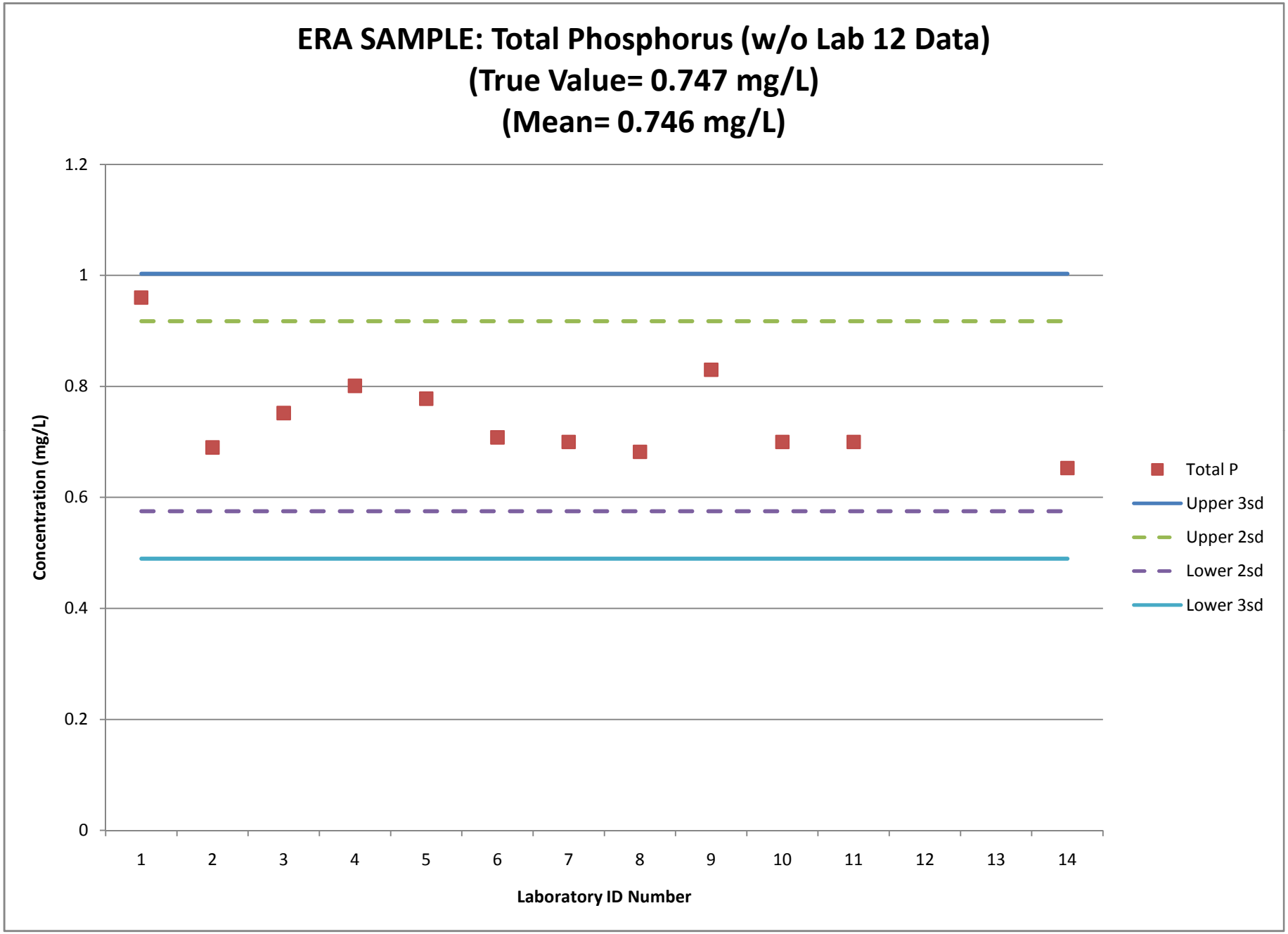
Gossett, R., Renfrew, D., and K. Schiff. 2003. Stormwater Monitoring Coalition Laboratory Guidance Document.

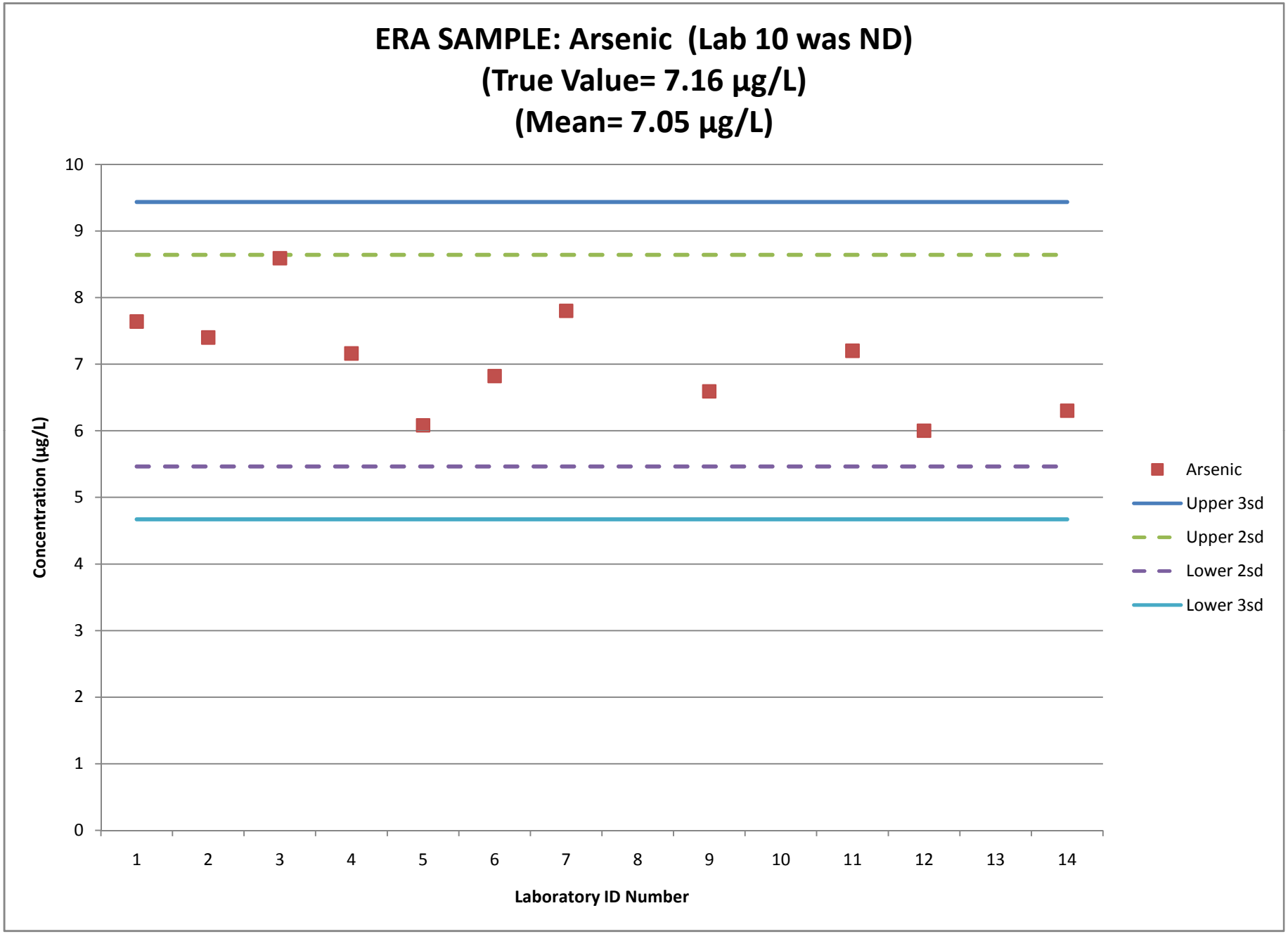
APPENDIX A

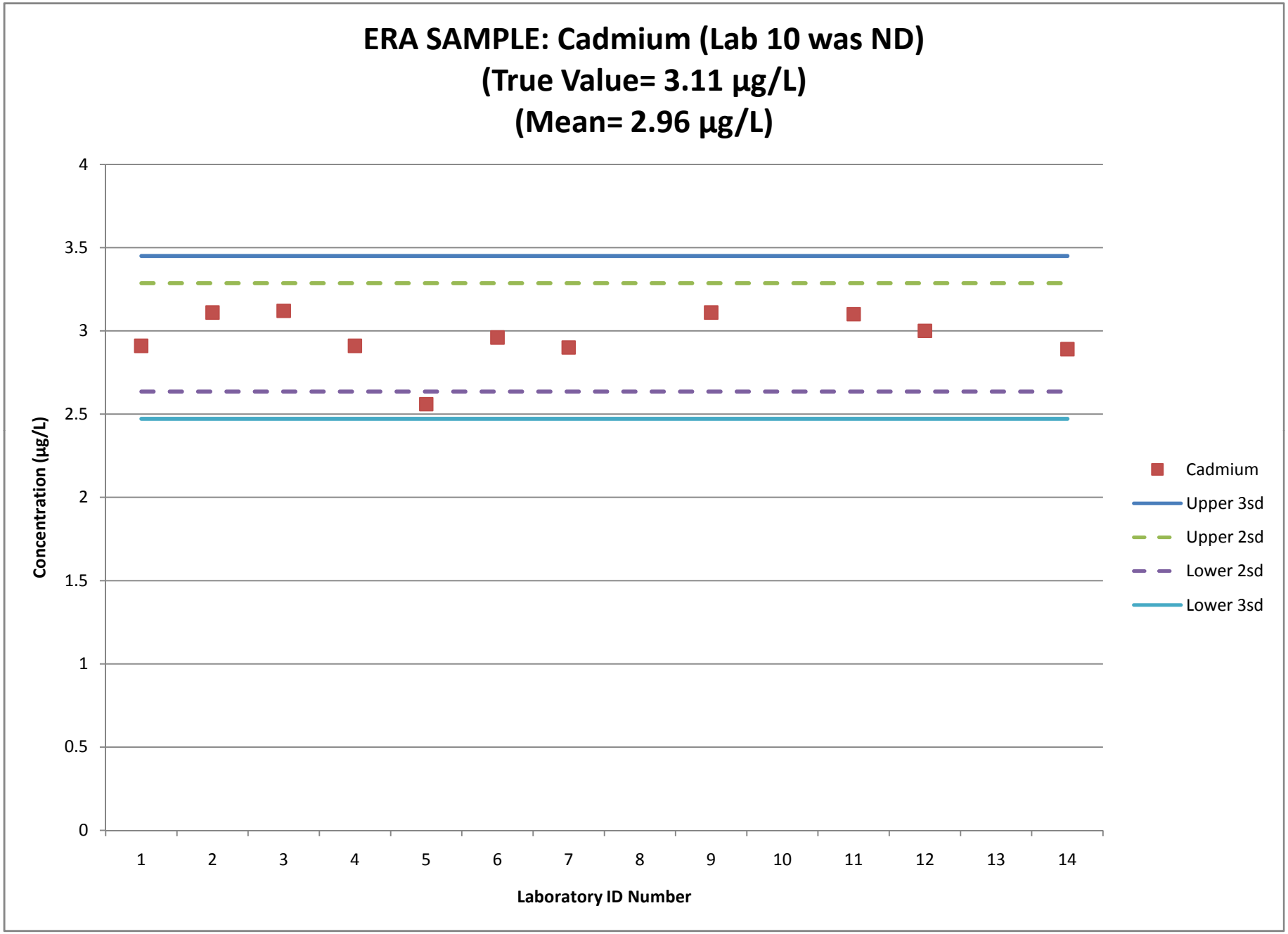

ERA SAMPLE: Ammonia-N (Lab 6 and Lab 11 were ND)
(True Value= 0.698mg/L)
(Mean= 0.626 mg/L)
Concentration (mg/L)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
Ammonia-N
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

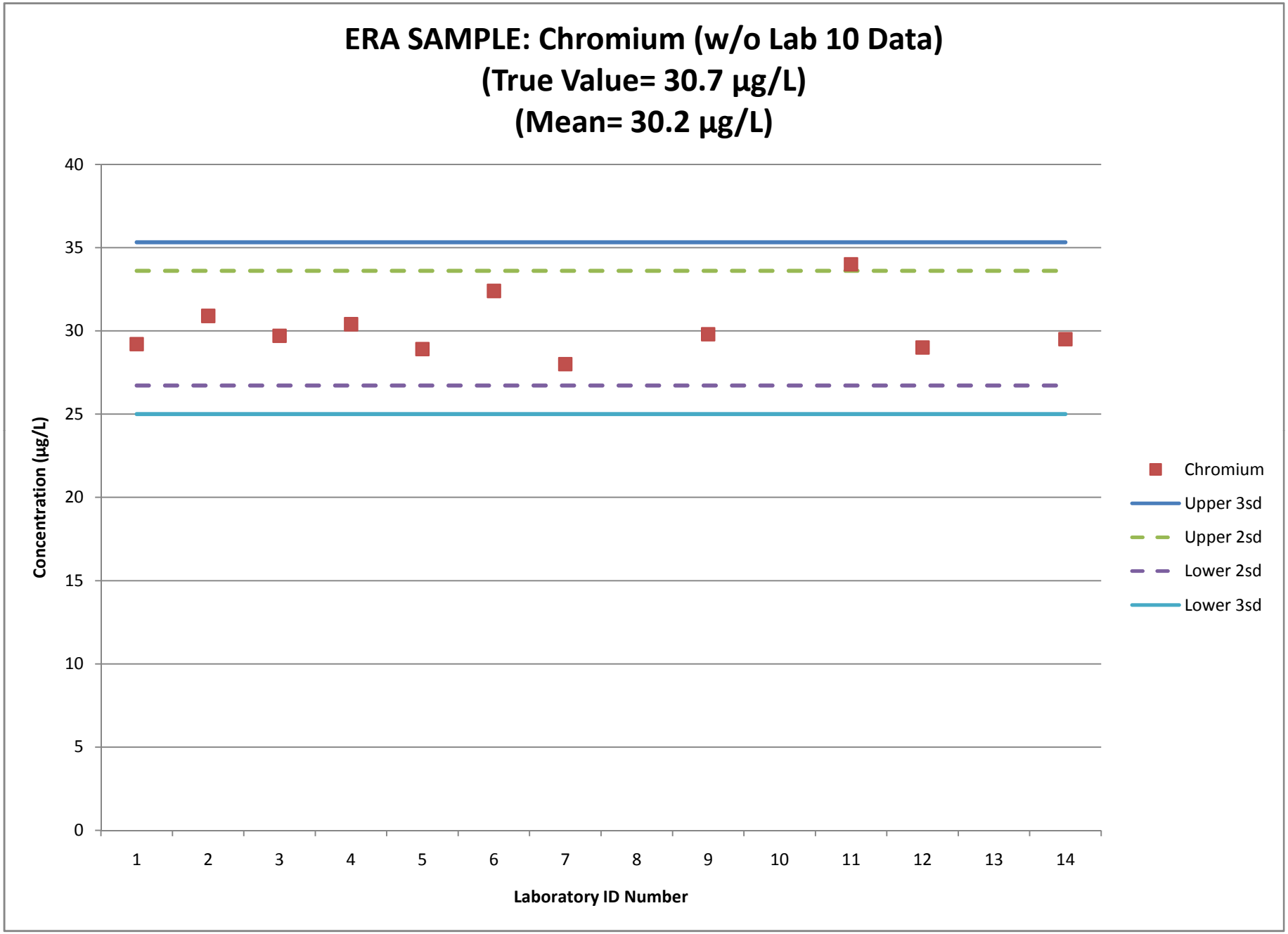

ERA SAMPLE: Nitrate+Nitrite-N (w/o Lab 11 Data)
(True Value= 1.99 mg/L)
(Mean= 1.88 mg/L)
Concentration (mg/L)
2.50
2.00
1.50
1.00
0.50
0.00
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
NO2+NO3-N
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

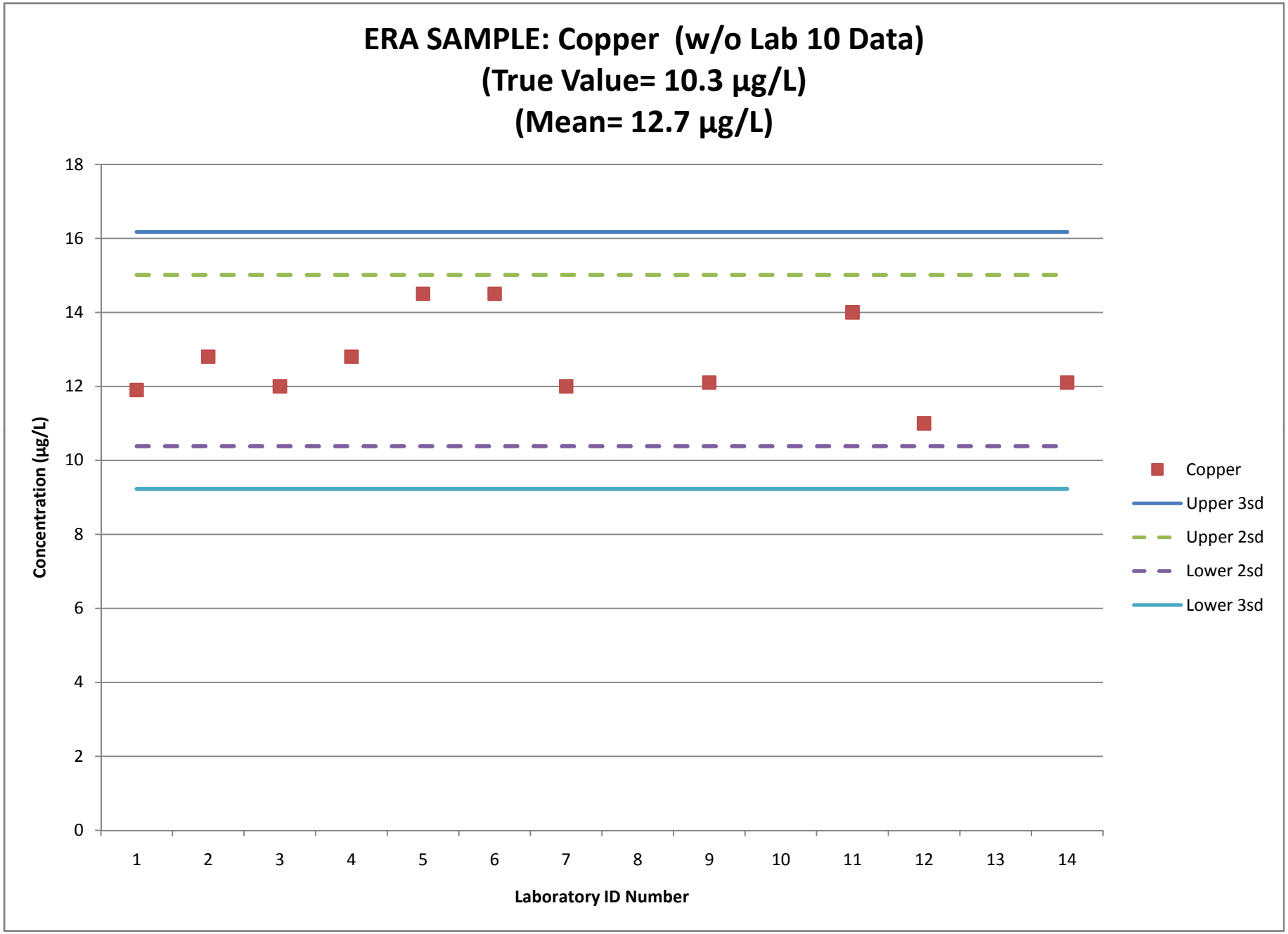

ERA SAMPLE: TKN (Lab 11 was ND)
(True Value= 0.698 mg/L)
(Mean= 0.776 mg/L)
Concentration (mg/L)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
TKN
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

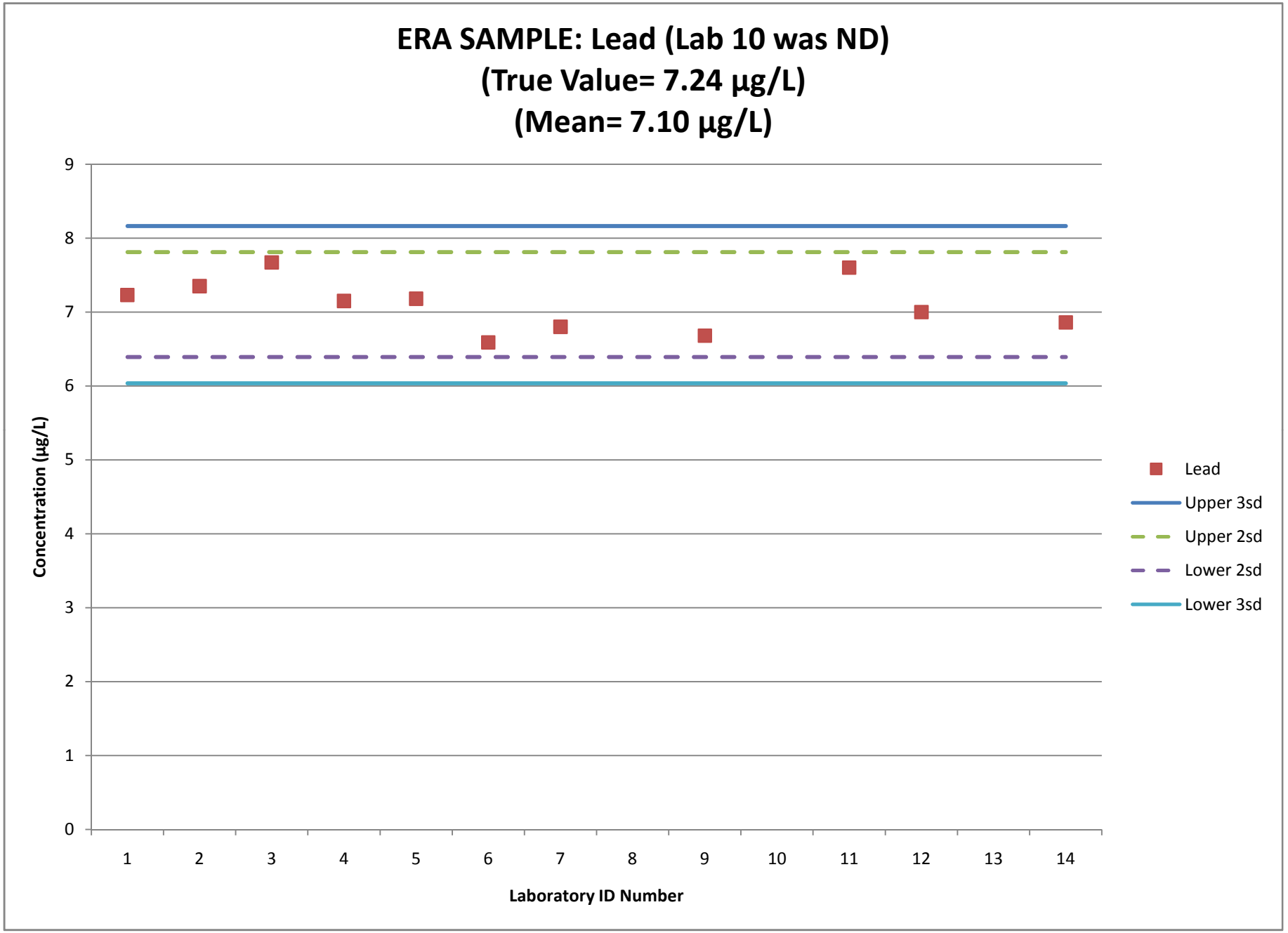

ERA SAMPLE: Total Phosphorus (w/o Lab 12 Data)
(True Value= 0.747 mg/L)
(Mean= 0.746 mg/L)
Concentration (mg/L)
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
Total P
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

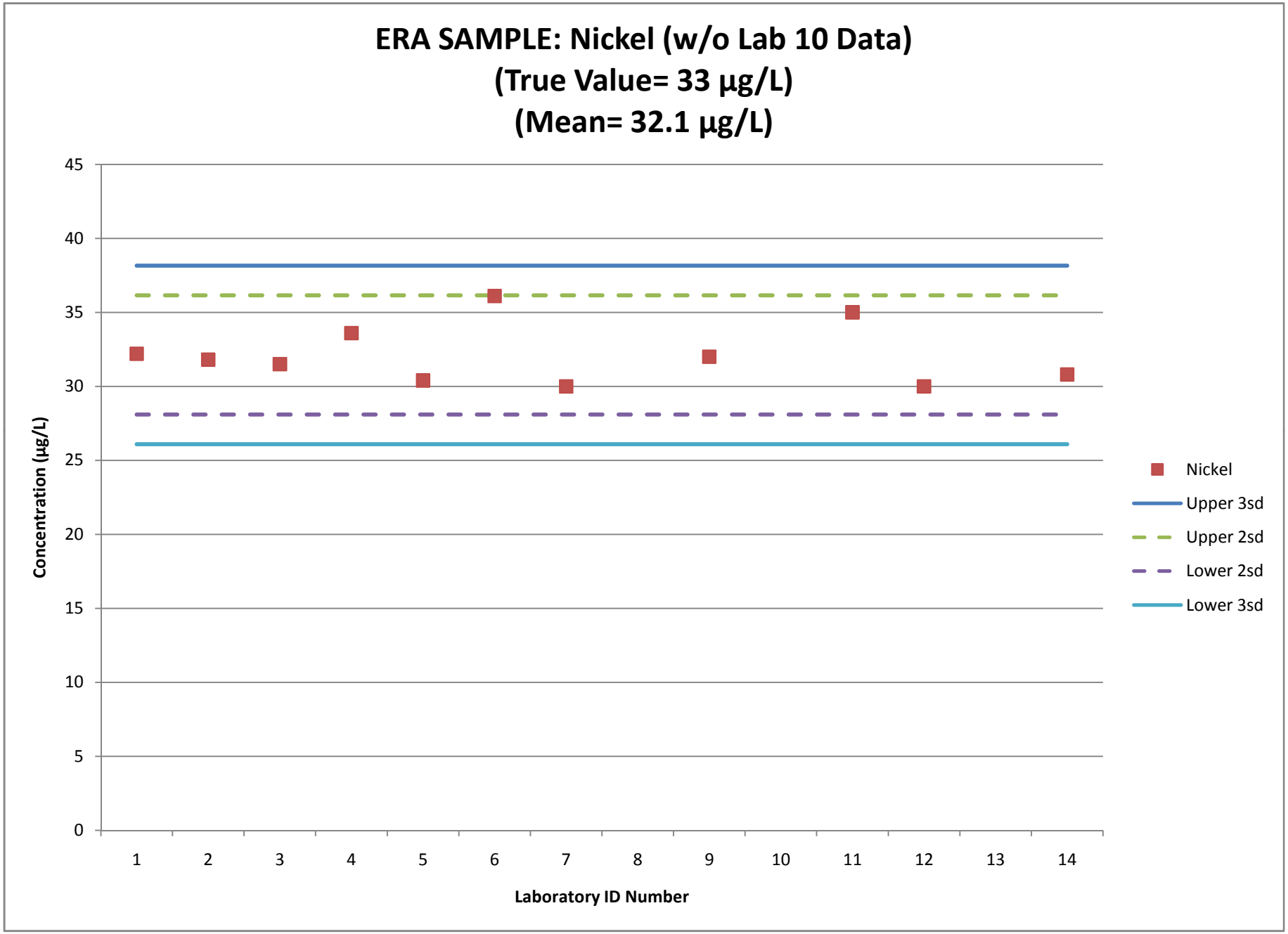

ERA SAMPLE: Arsenic (Lab 10 was ND)
(True Value= 7.16 µg/L)
(Mean= 7.05 µg/L)
Concentration (µg/L)
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
Arsenic
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

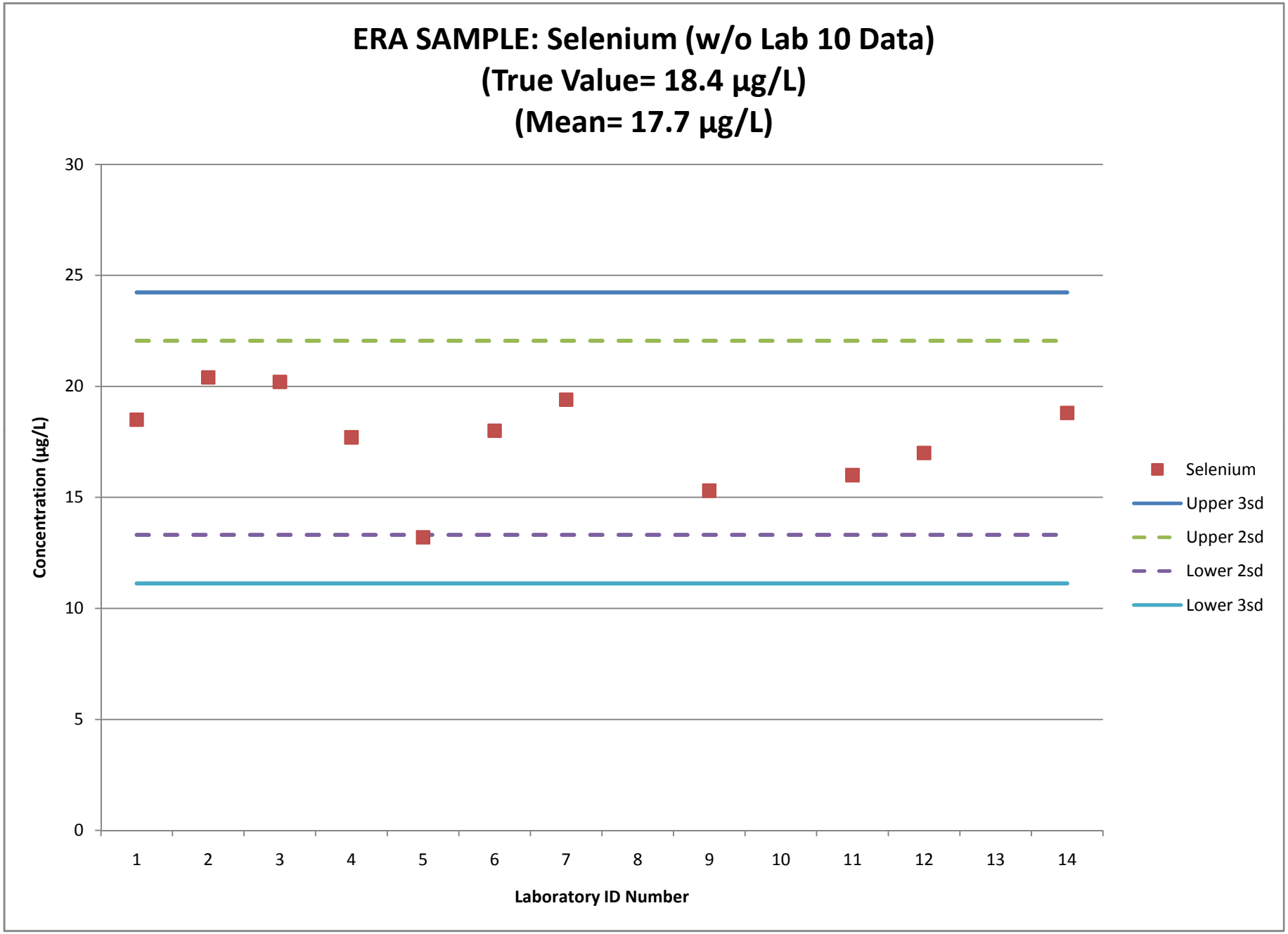

ERA SAMPLE: Cadmium (Lab 10 was ND)
(True Value= 3.11 µg/L)
(Mean= 2.96 µg/L)
Concentration (µg/L)
4
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
Cadmium
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

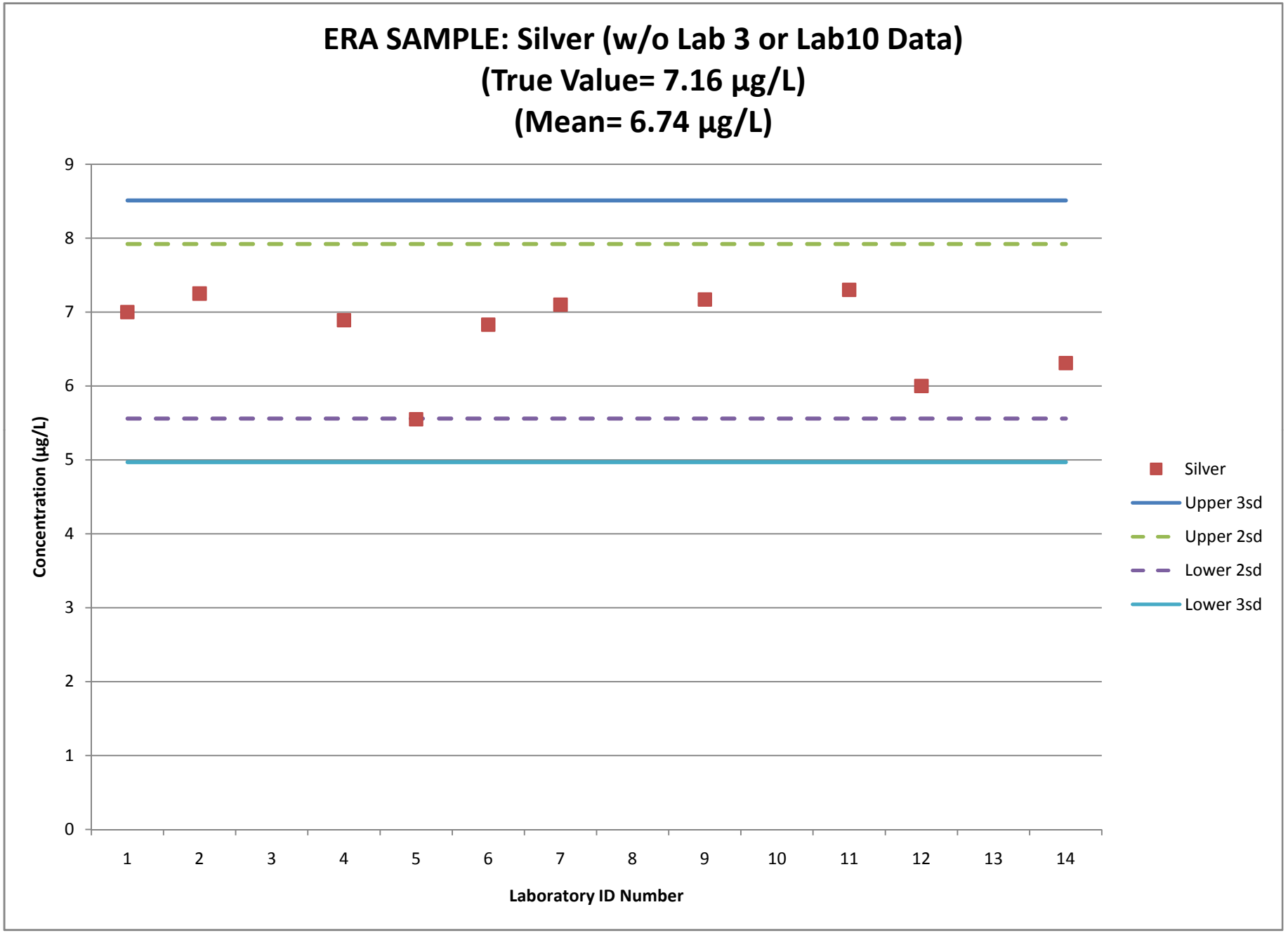

ERA SAMPLE: Chromium (w/o Lab 10 Data)
(True Value= 30.7 µg/L)
(Mean= 30.2 µg/L)
Concentration (µg/L)
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
Chromium
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

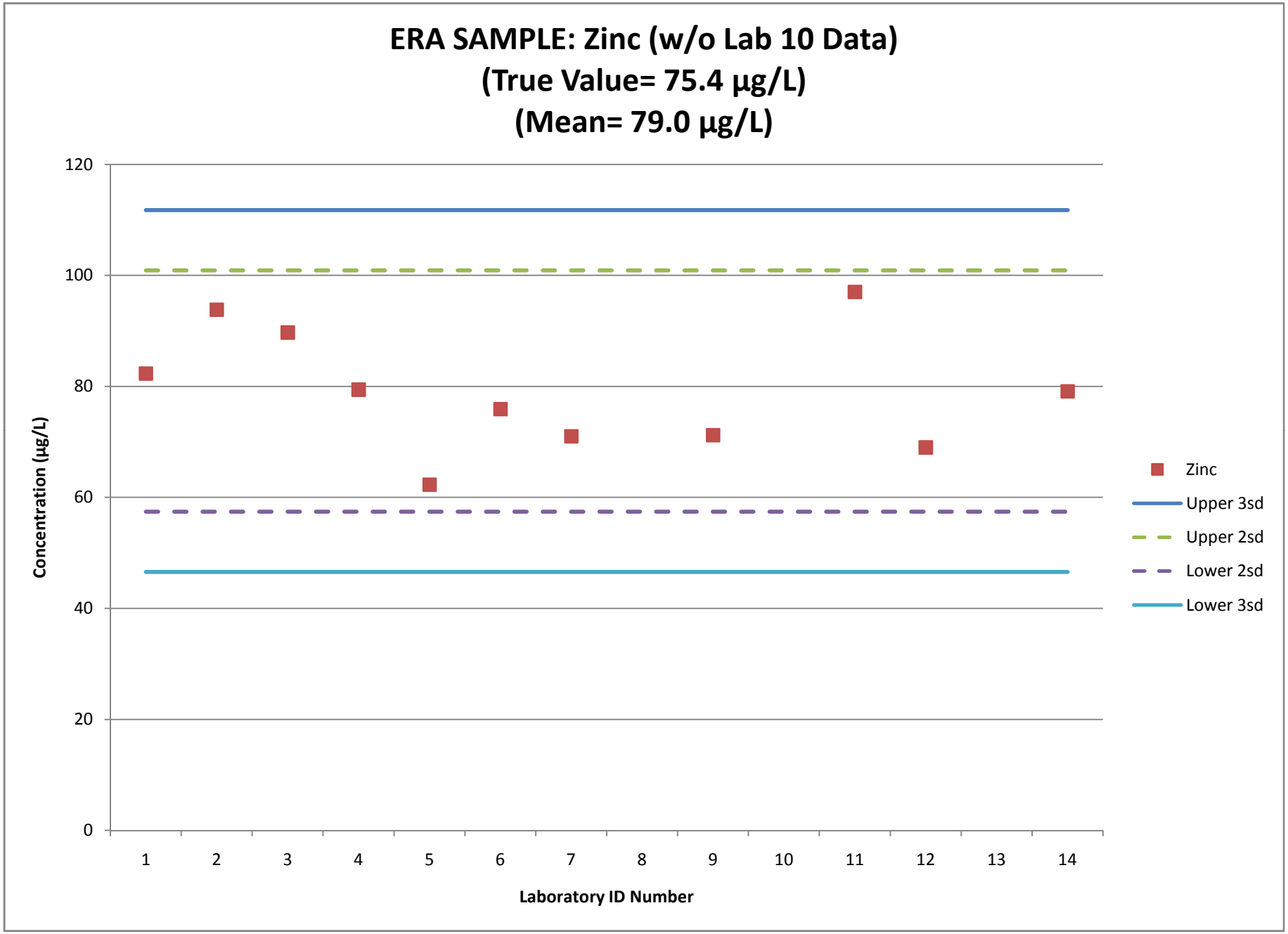

ERA SAMPLE: Copper (w/o Lab 10 Data)
(True Value= 10.3 µg/L)
(Mean= 12.7 µg/L)
Concentration (µg/L)
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
Copper
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

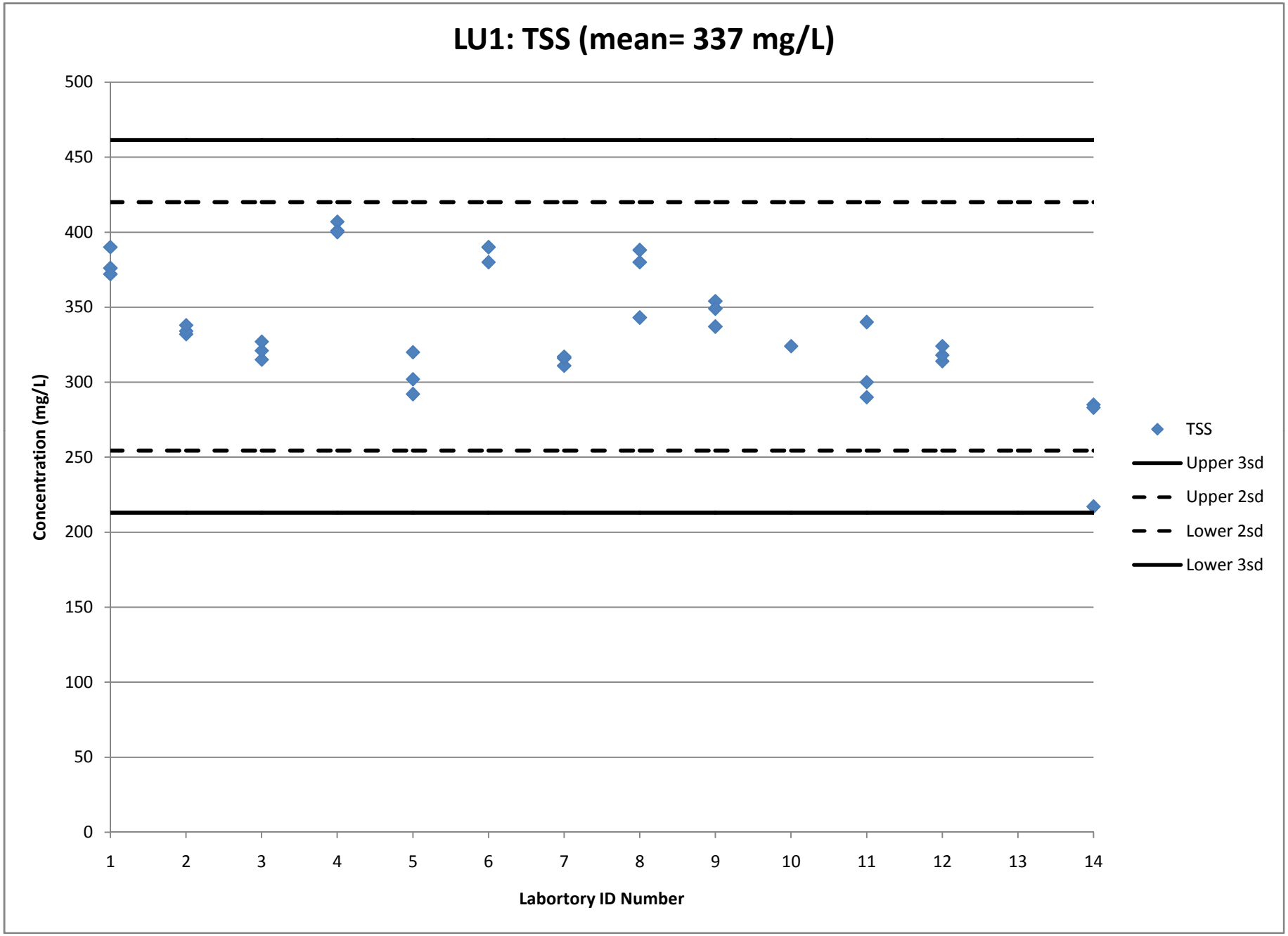

ERA SAMPLE: Lead (Lab 10 was ND)
(True Value= 7.24 µg/L)
(Mean= 7.10 µg/L)
Concentration (µg/L)
0
1
2
3
4
5
6
7
8
9
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
Lead
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

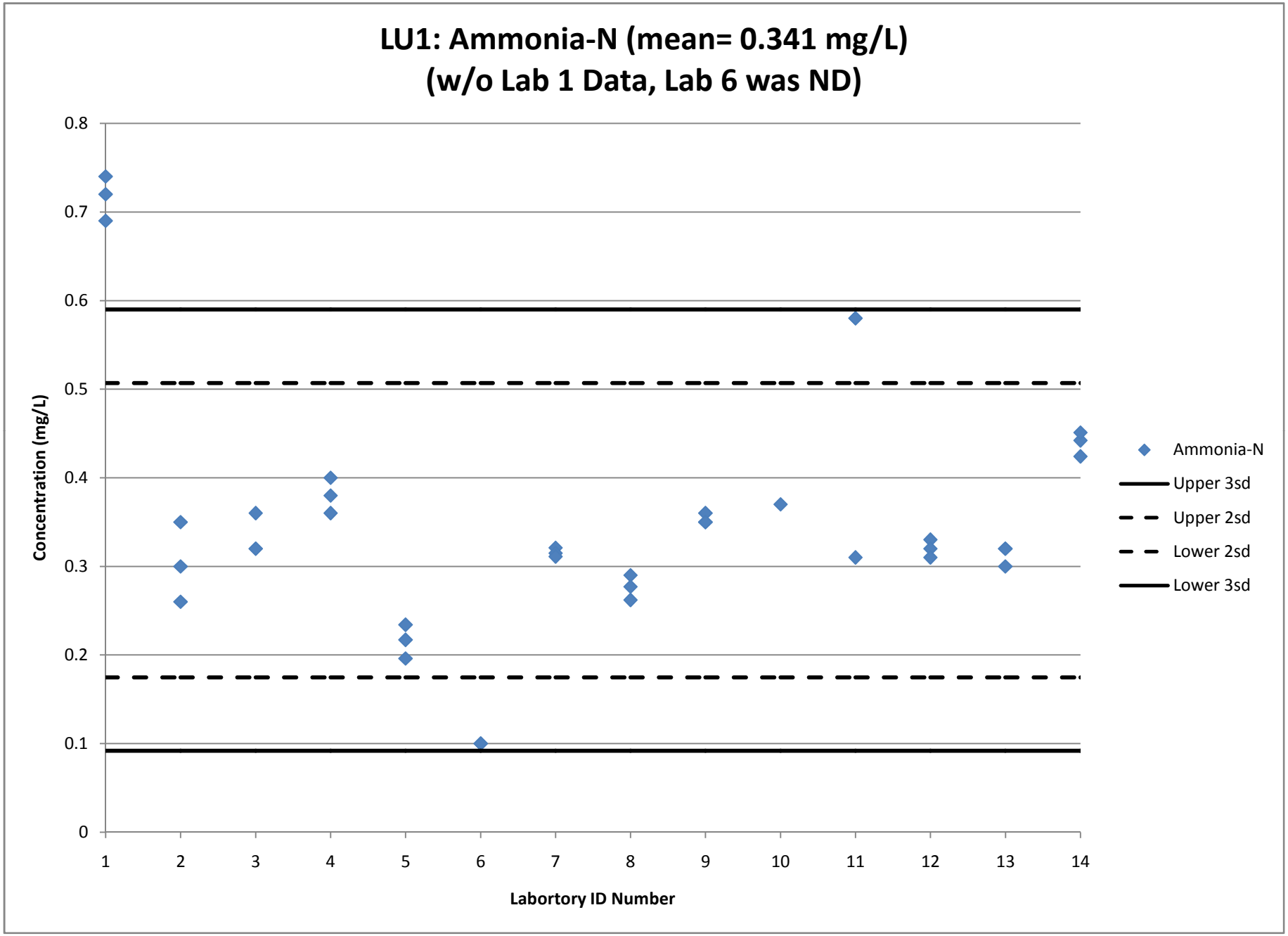

ERA SAMPLE: Nickel (w/o Lab 10 Data)
(True Value= 33 µg/L)
(Mean= 32.1 µg/L)
Concentration (µg/L)
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
Nickel
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

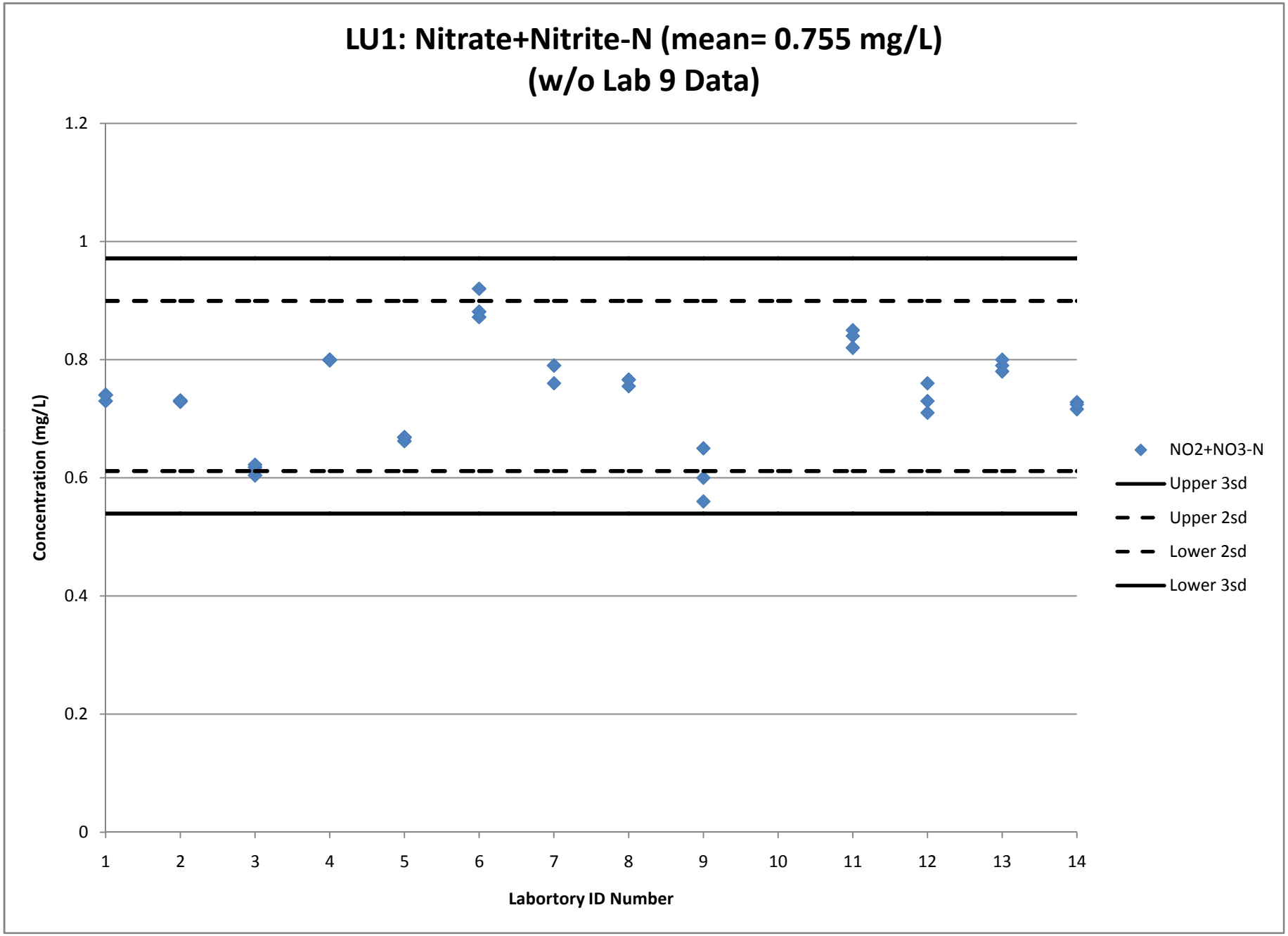

ERA SAMPLE: Selenium (w/o Lab 10 Data)
(True Value= 18.4 µg/L)
(Mean= 17.7 µg/L)
Concentration (µg/L)
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
Selenium
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

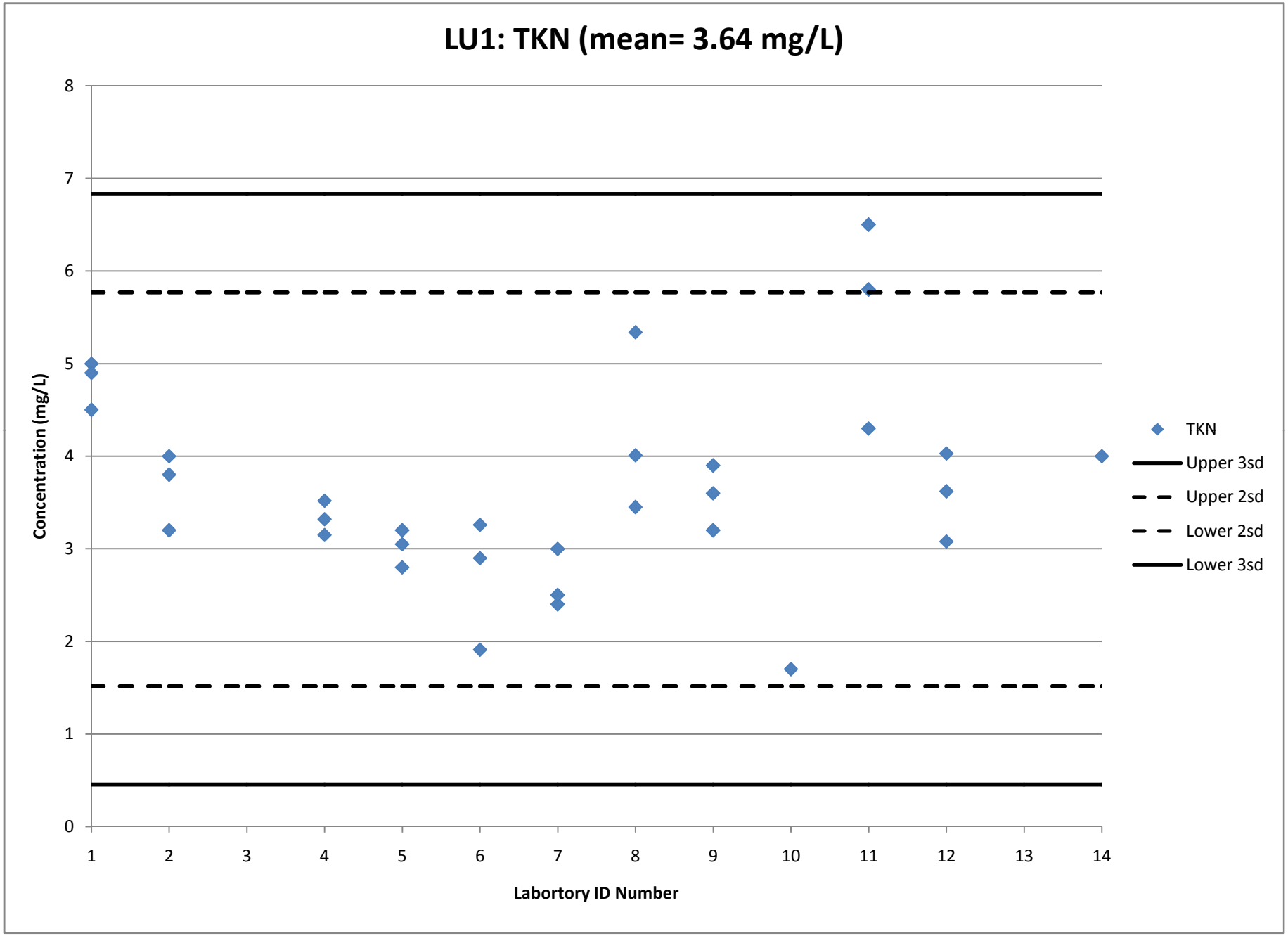

ERA SAMPLE: Silver (w/o Lab 3 or Lab10 Data)
(True Value= 7.16 µg/L)
(Mean= 6.74 µg/L)
Concentration (µg/L)
0
1
2
3
4
5
6
7
8
9
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
Silver
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

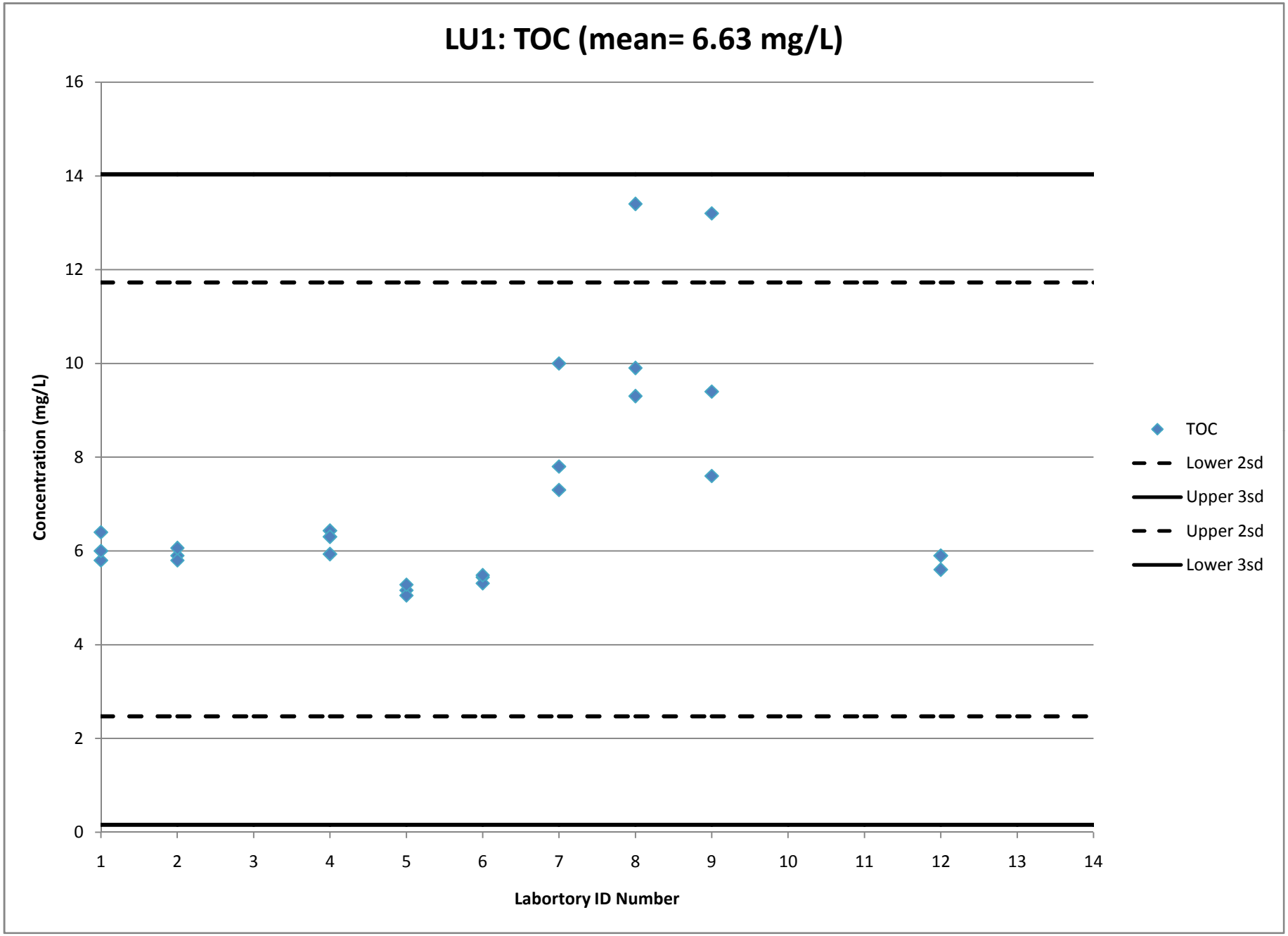

ERA SAMPLE: Zinc (w/o Lab 10 Data)
(True Value= 75.4 µg/L)
(Mean= 79.0 µg/L)
Concentration (µg/L)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Laboratory ID Number
Zinc
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

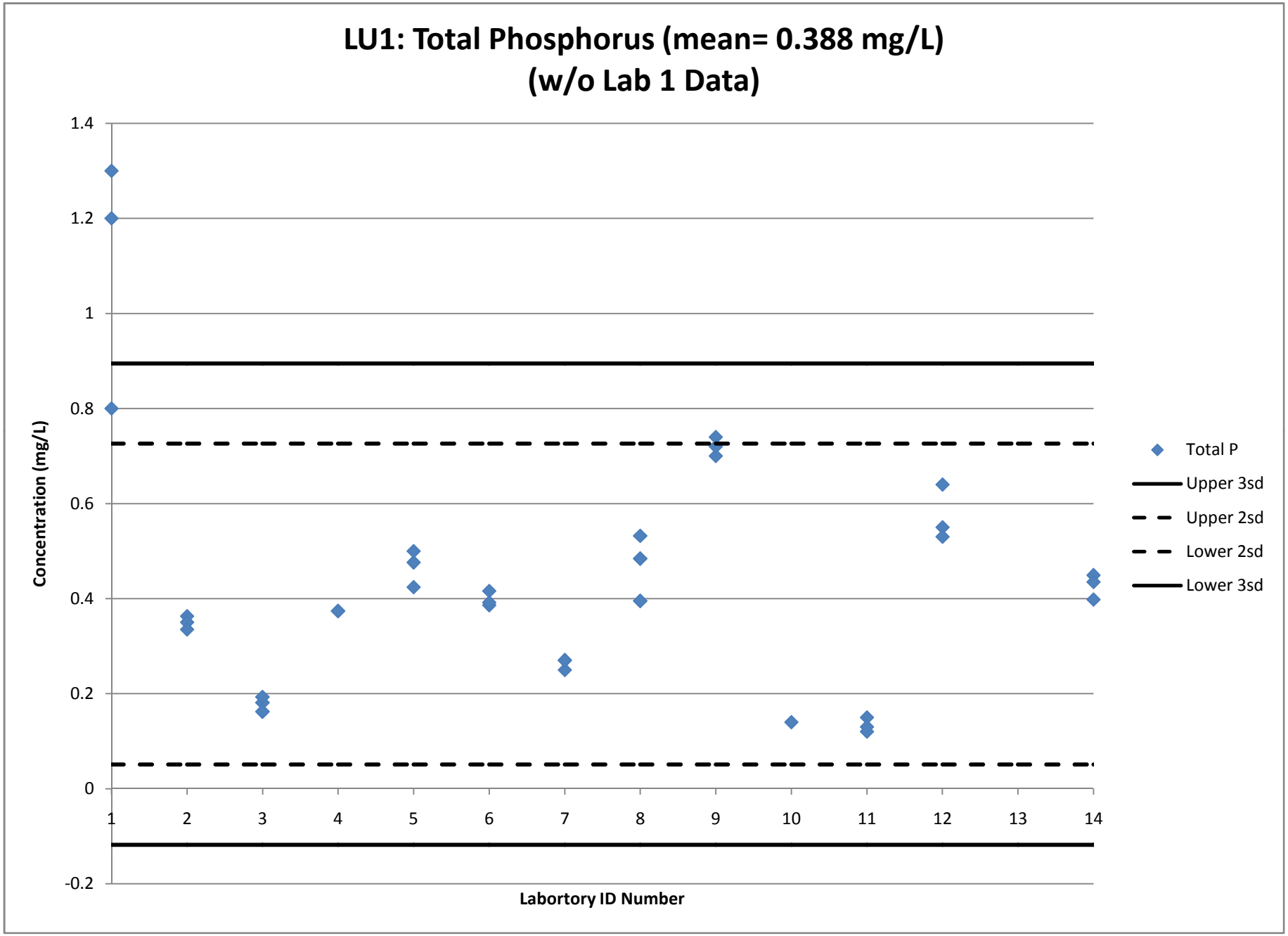

LU1: TSS (mean= 337 mg/L)
Concentration (mg/L)
500
450
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
TSS
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

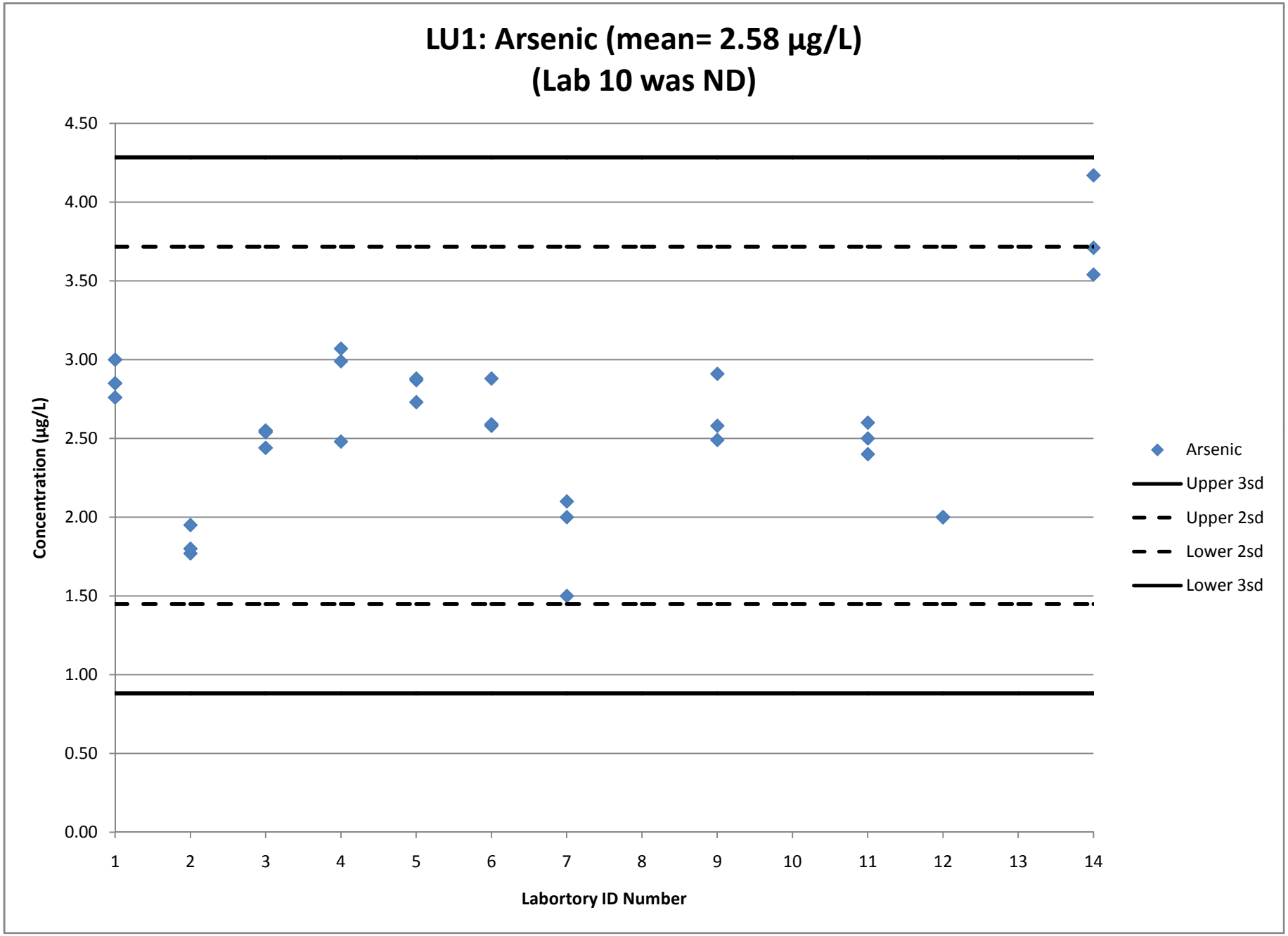

LU1: Ammonia-N (mean= 0.341 mg/L)
(w/o Lab 1 Data, Lab 6 was ND)
Concentration (mg/L)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Ammonia-N
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

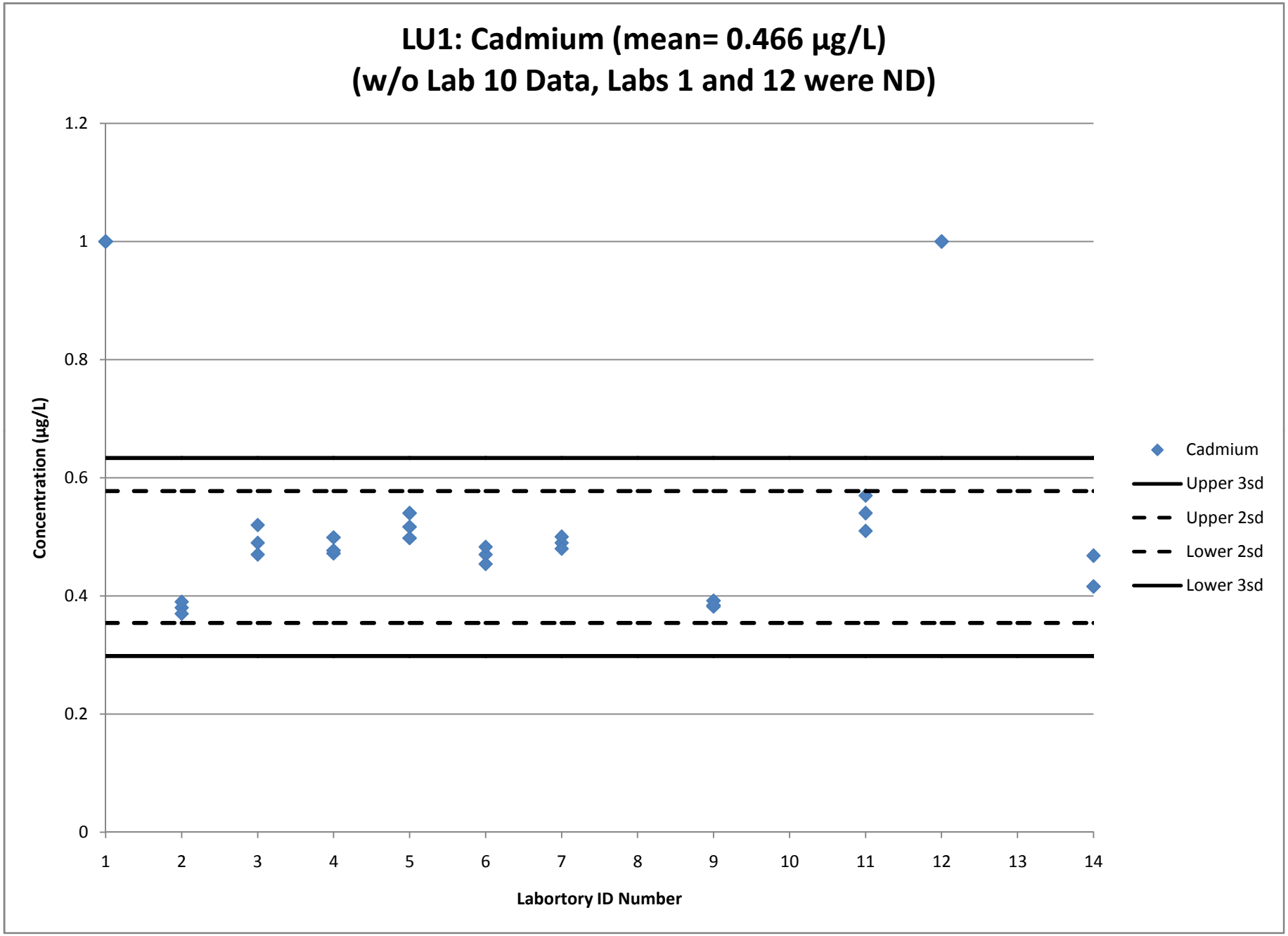

LU1: Nitrate+Nitrite-N (mean= 0.755 mg/L)
(w/o Lab 9 Data)
Concentration (mg/L)
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
NO2+NO3-N
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

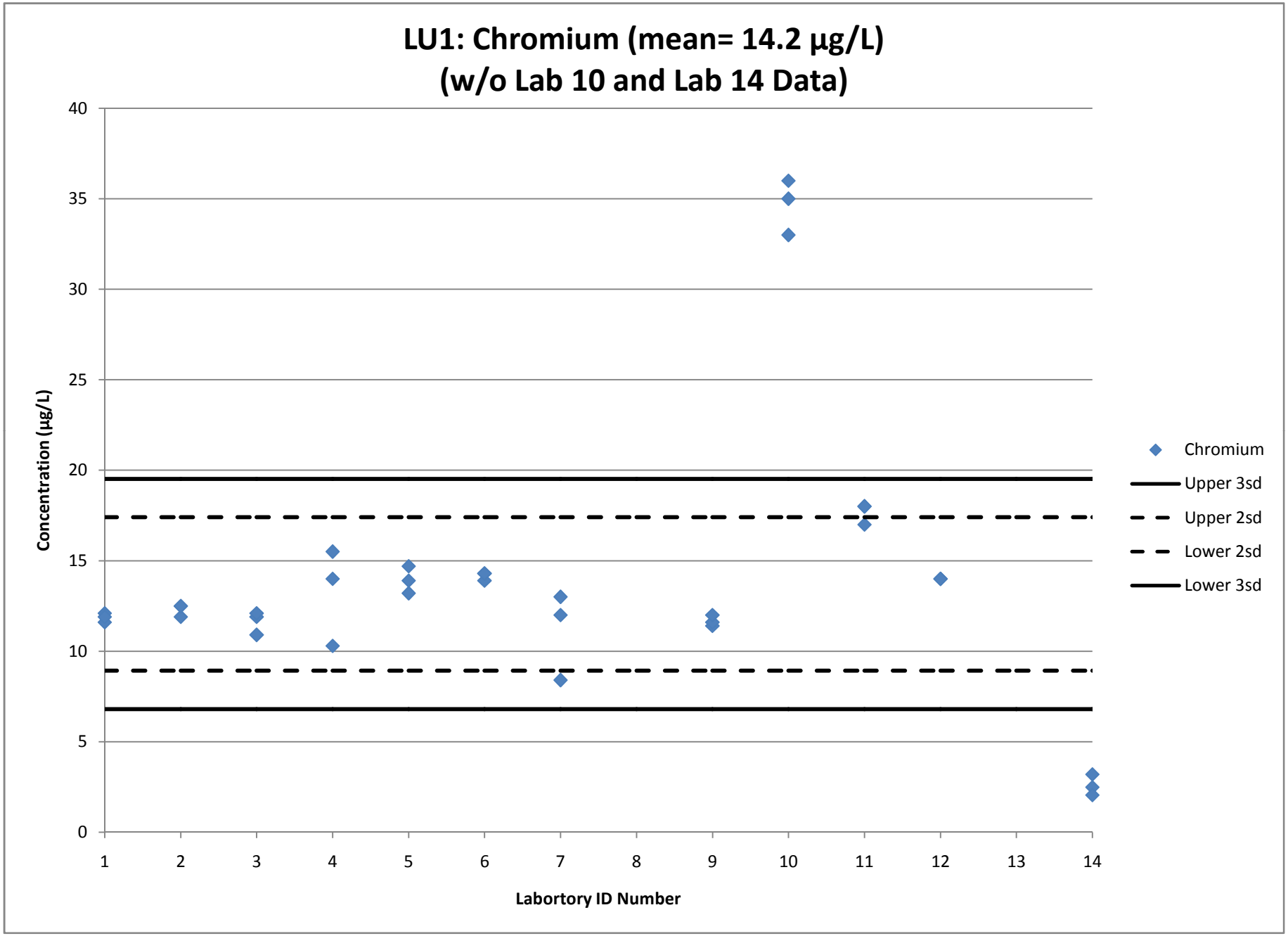

LU1: TKN (mean= 3.64 mg/L)
Concentration (mg/L)
Labortory ID Number
TKN
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd
0
1
2
3
4
5
6
7
8
1
2
3
4
5
6
7
8
9
10
11
12
13
14

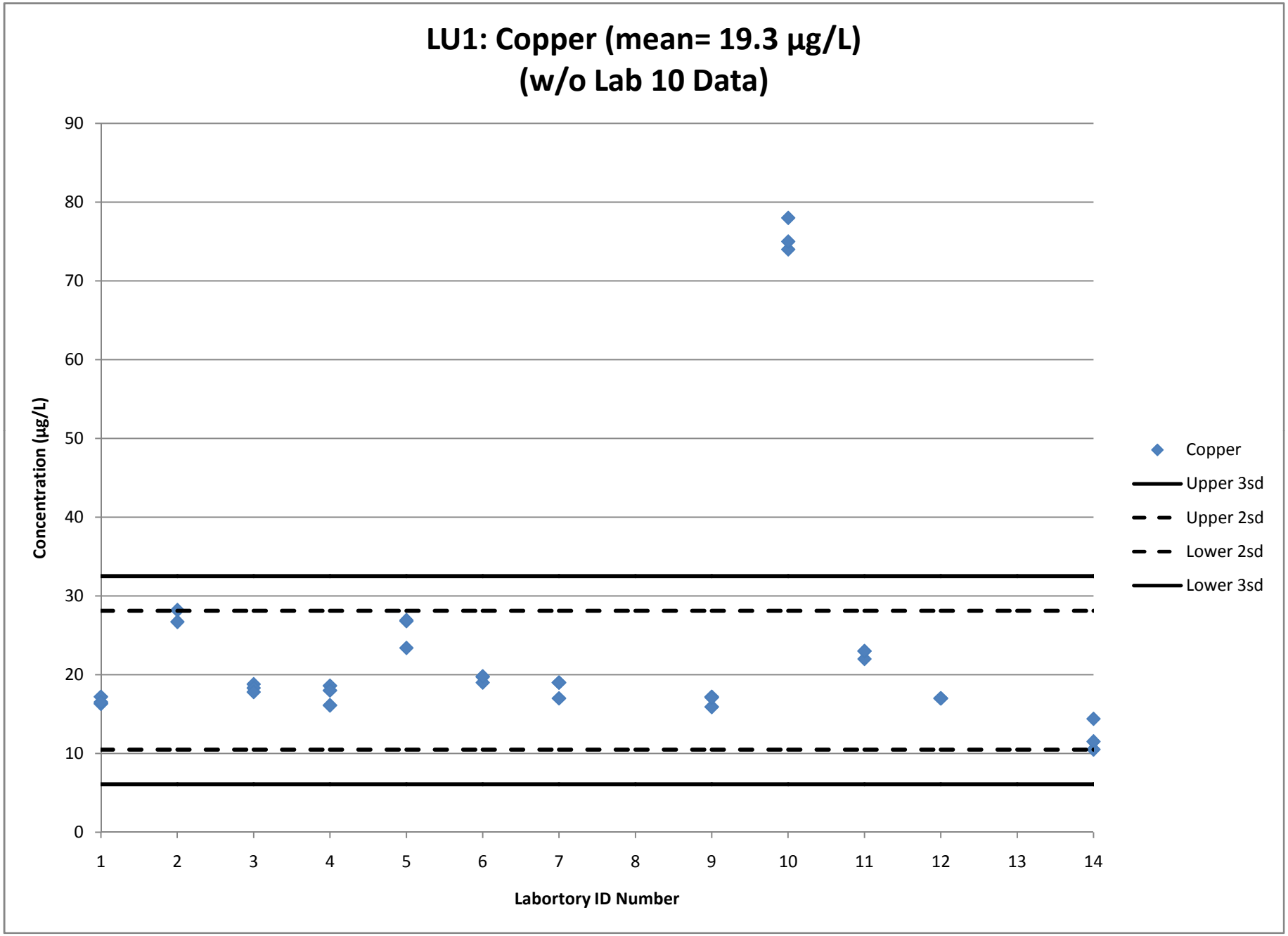

LU1: TOC (mean= 6.63 mg/L)
Concentration (mg/L)
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
TOC
Lower 2sd
Upper 3sd
Upper 2sd
Lower 3sd

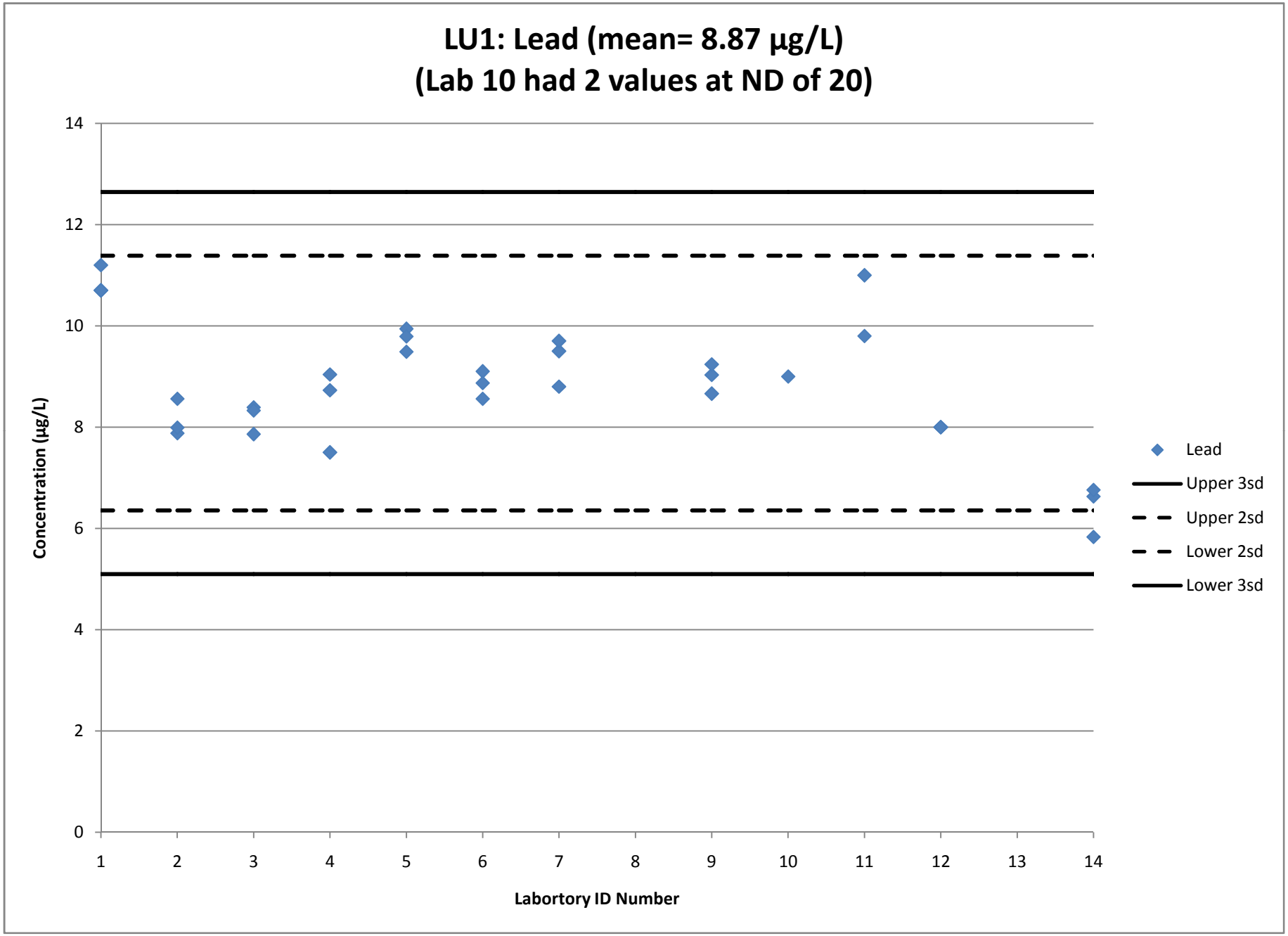

LU1: Total Phosphorus (mean= 0.388 mg/L)
(w/o Lab 1 Data)
Concentration (mg/L)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Total P
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

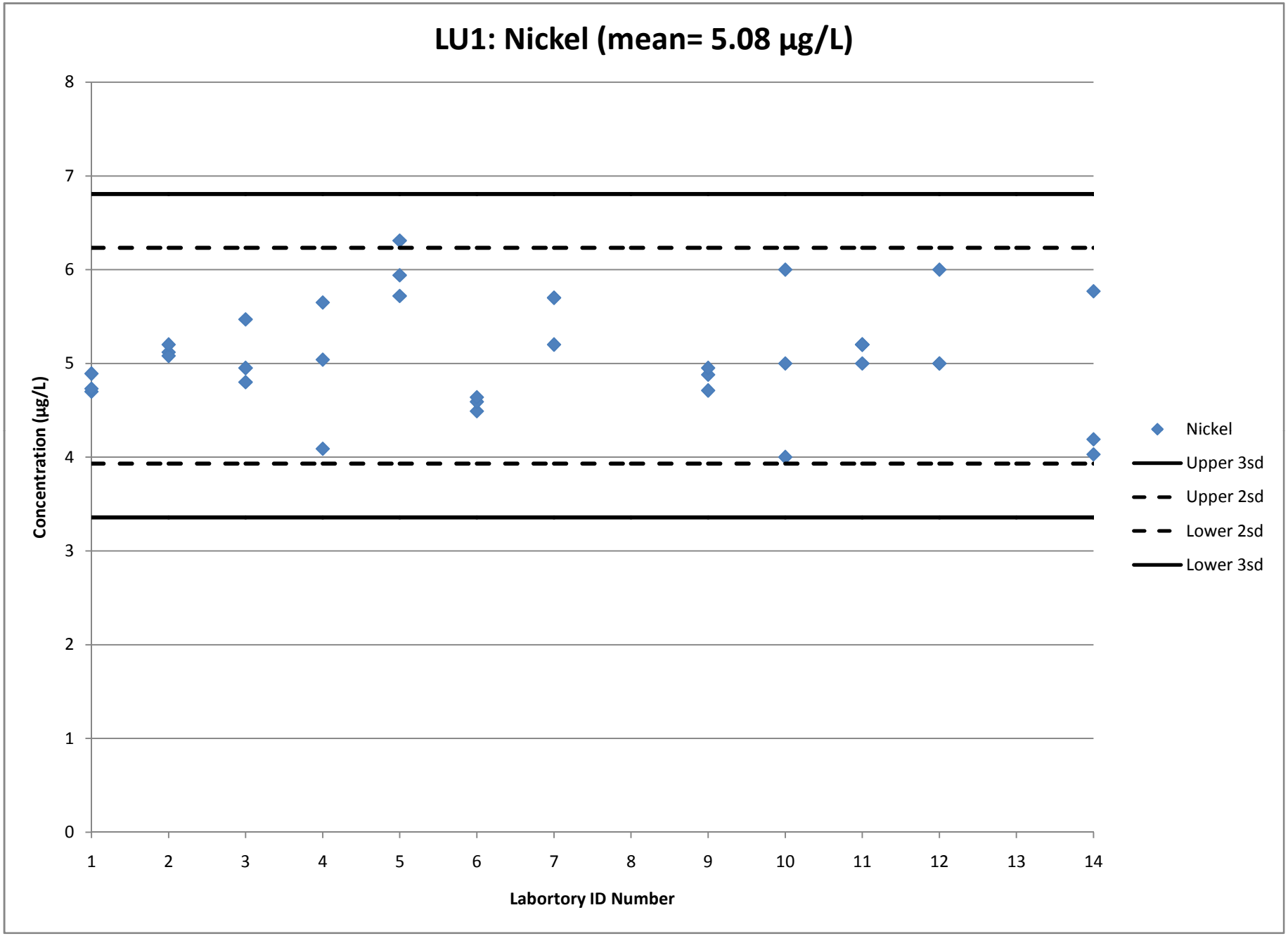

LU1: Arsenic (mean= 2.58 µg/L)
(Lab 10 was ND)
Concentration (µg/L)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Arsenic
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

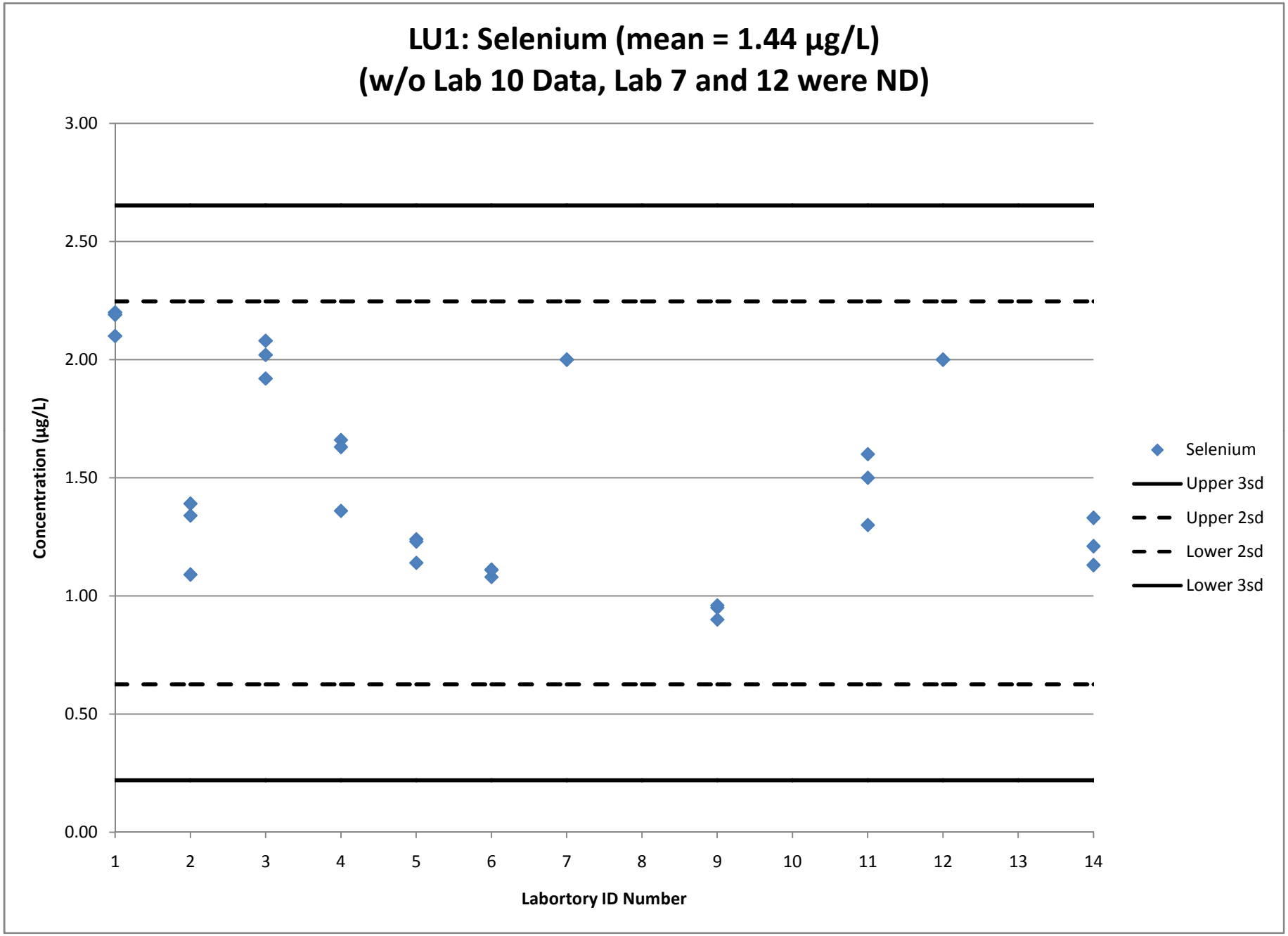

LU1: Cadmium (mean= 0.466 µg/L)
(w/o Lab 10 Data, Labs 1 and 12 were ND)
Concentration (µg/L)
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Cadmium
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

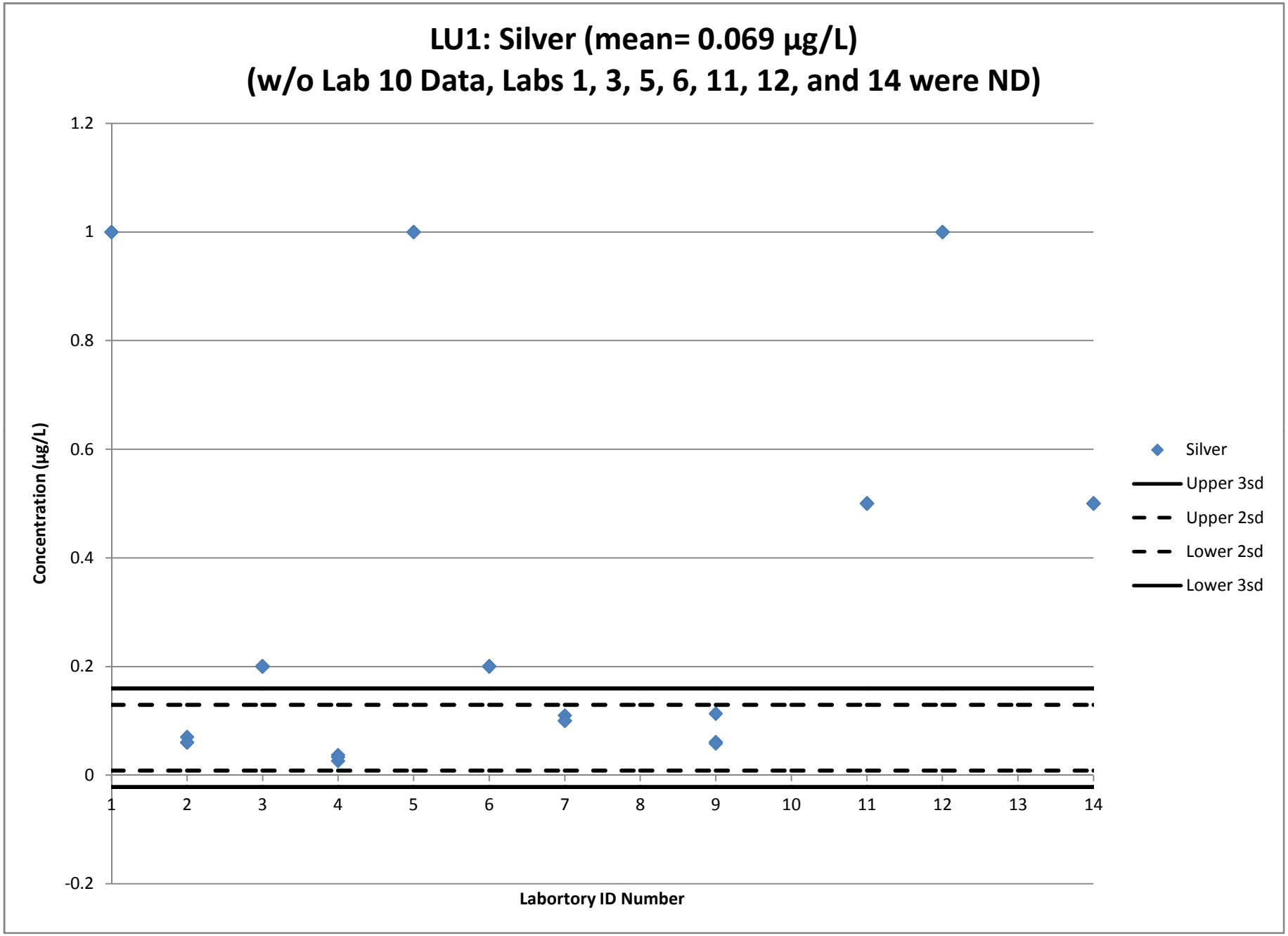

LU1: Chromium (mean= 14.2 µg/L)
(w/o Lab 10 and Lab 14 Data)
Concentration (µg/L)
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Chromium
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

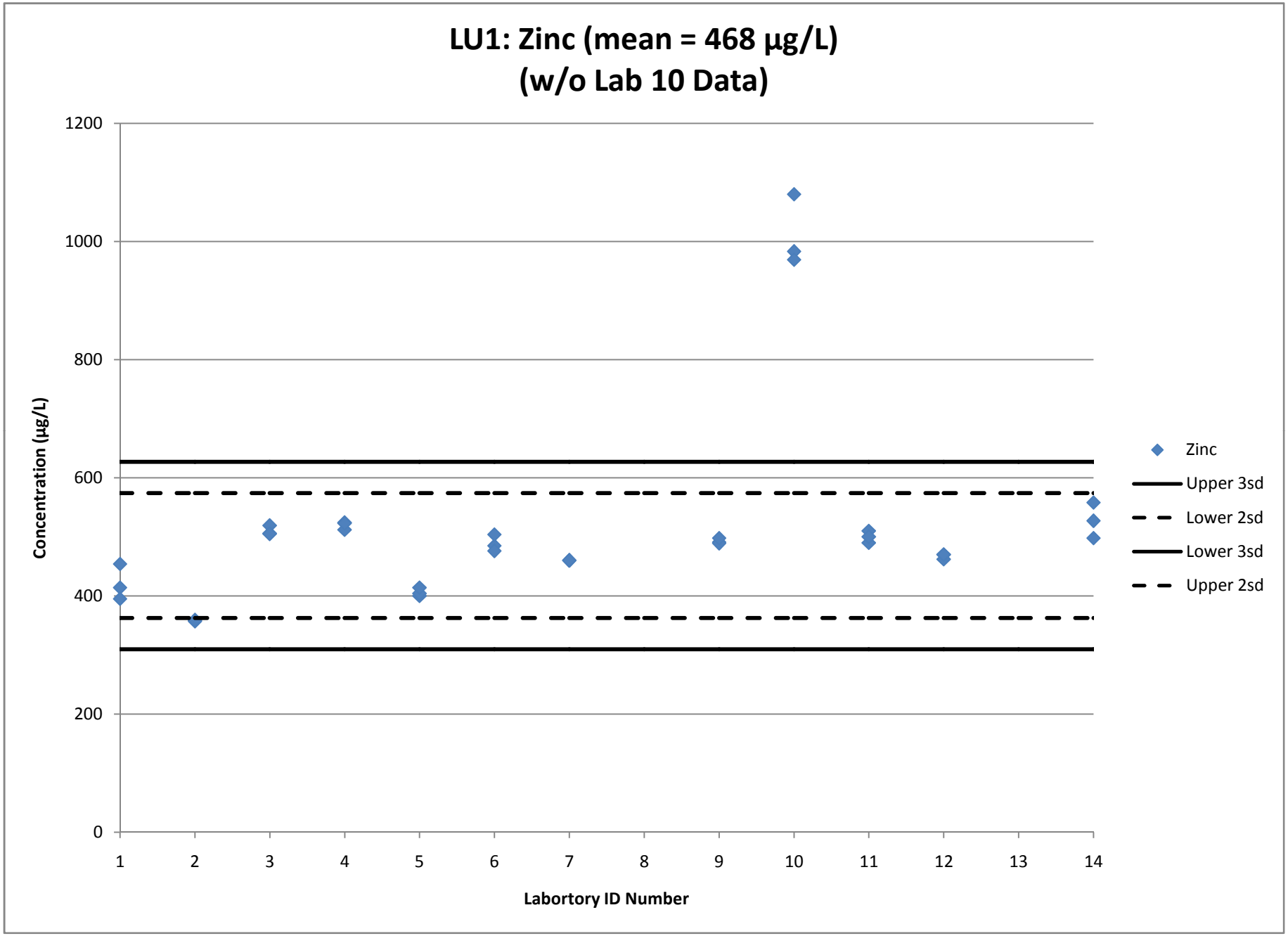

LU1: Copper (mean= 19.3 µg/L)
(w/o Lab 10 Data)
Concentration (µg/L)
0
10
20
30
40
50
60
70
80
90
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Copper
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

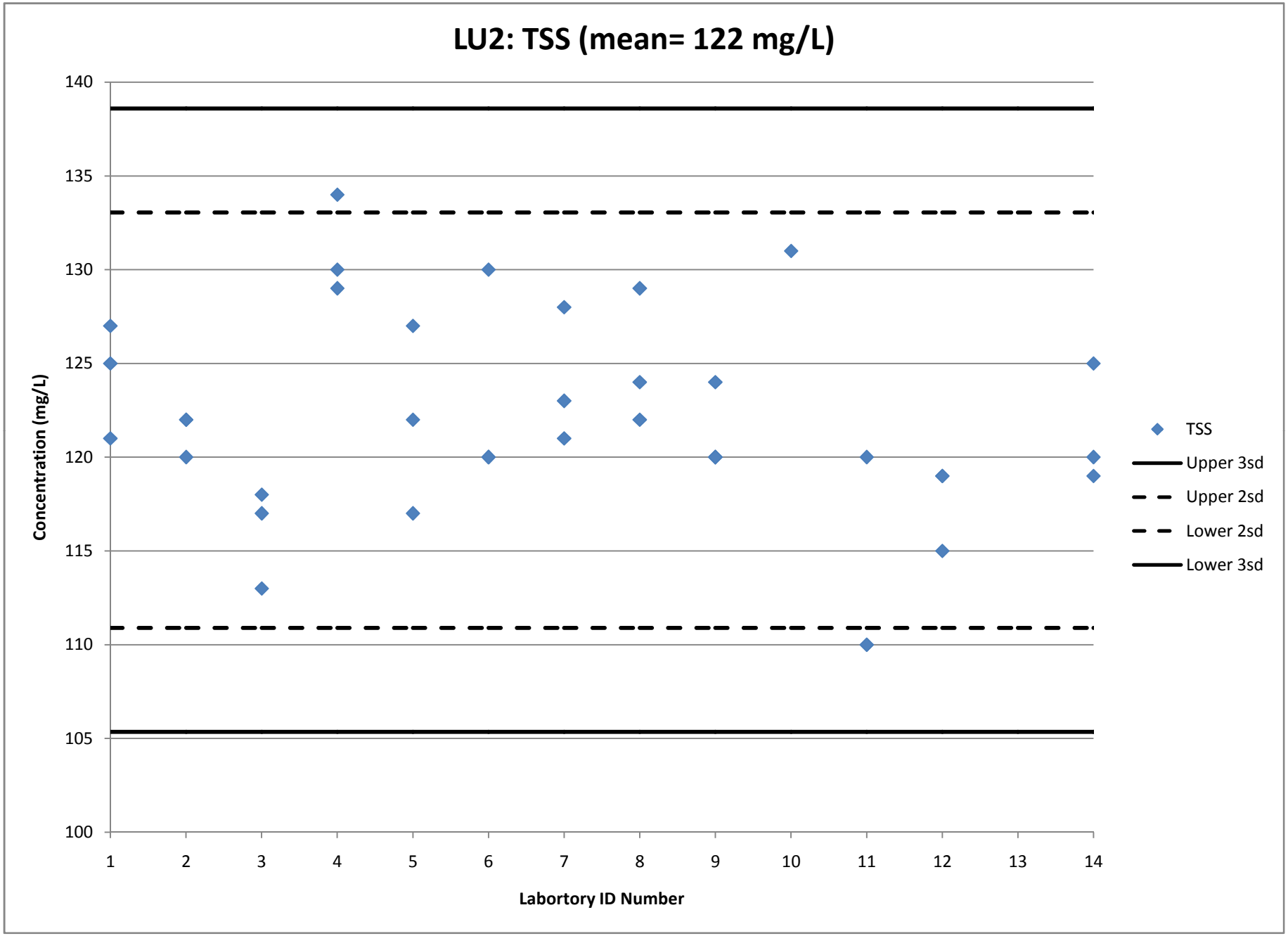

LU1: Lead (mean= 8.87 µg/L)
(Lab 10 had 2 values at ND of 20)
Concentration (µg/L)
0
2
4
6
8
10
12
14
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Lead
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

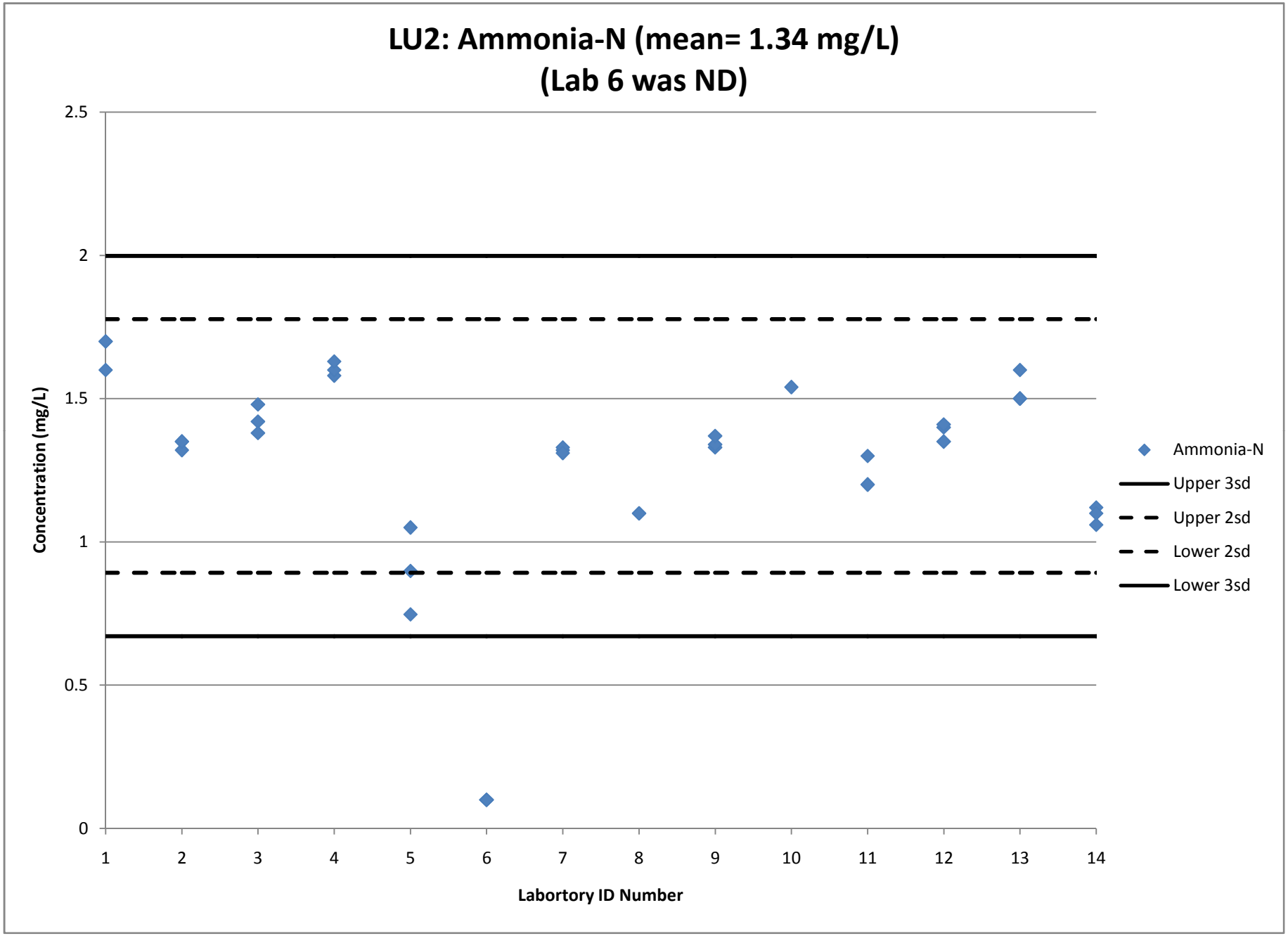

LU1: Nickel (mean= 5.08 µg/L)
Concentration (µg/L)
Labortory ID Number
0
1
2
3
4
5
6
7
8
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Nickel
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

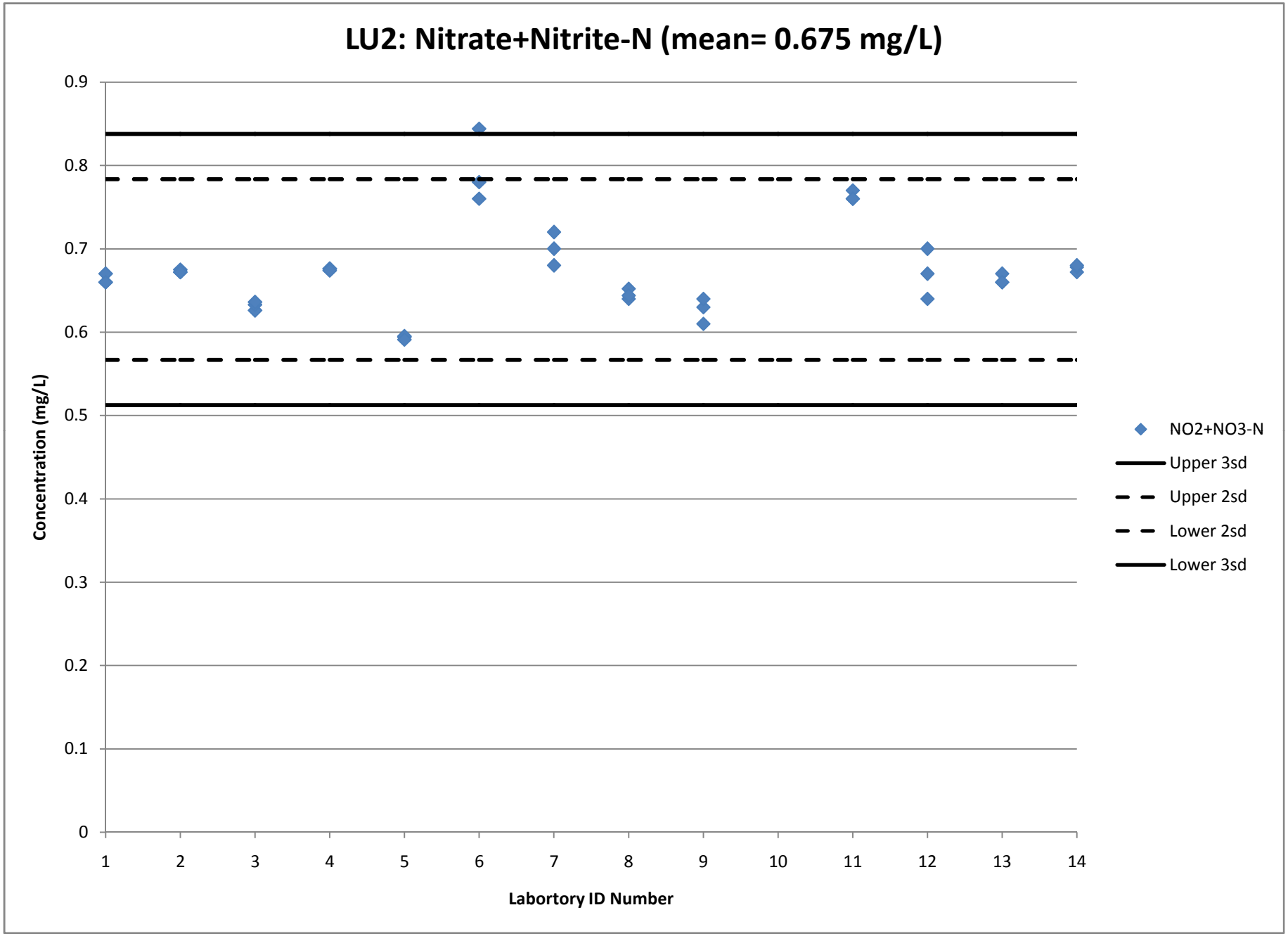

LU1: Selenium (mean = 1.44 µg/L)
(w/o Lab 10 Data, Lab 7 and 12 were ND)
Concentration (µg/L)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Selenium
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

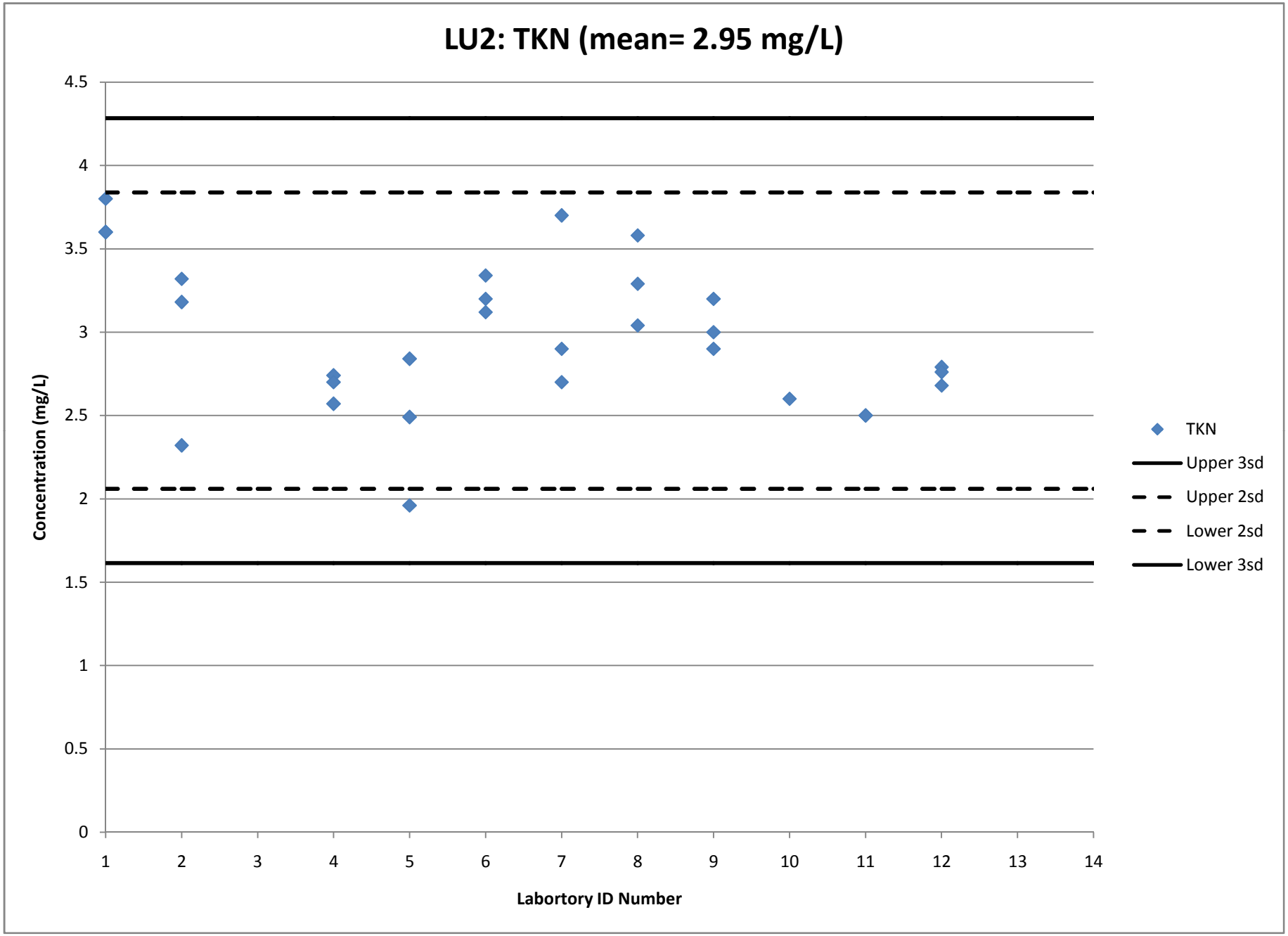

LU1: Silver (mean= 0.069 µg/L)
(w/o Lab 10 Data, Labs 1, 3, 5, 6, 11, 12, and 14 were ND)
Concentration (µg/L)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Silver
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

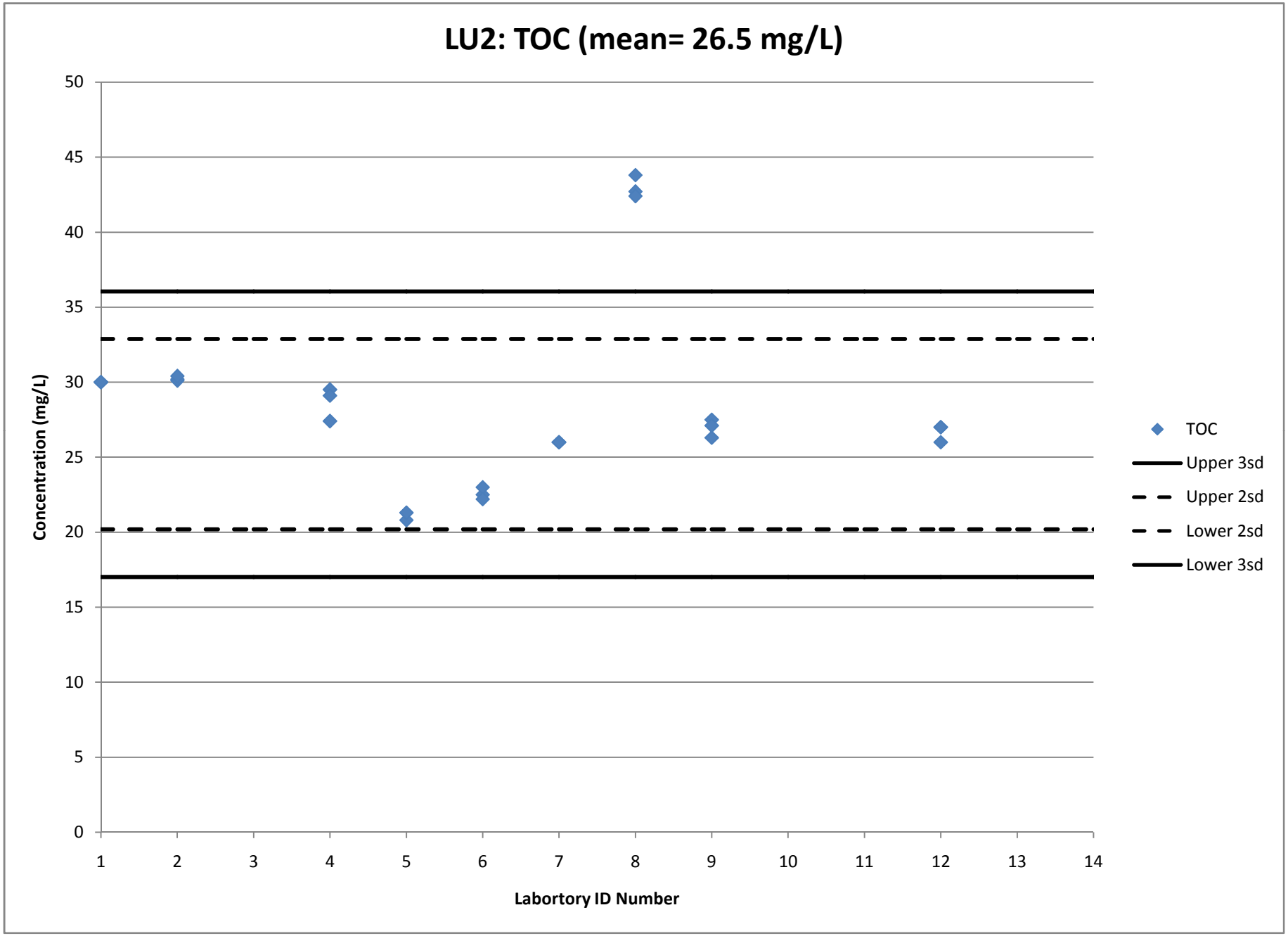

LU1: Zinc (mean = 468 µg/L)
(w/o Lab 10 Data)
Concentration (µg/L)
1200
1000
800
600
400
200
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Zinc
Upper 3sd
Lower 2sd
Lower 3sd
Upper 2sd

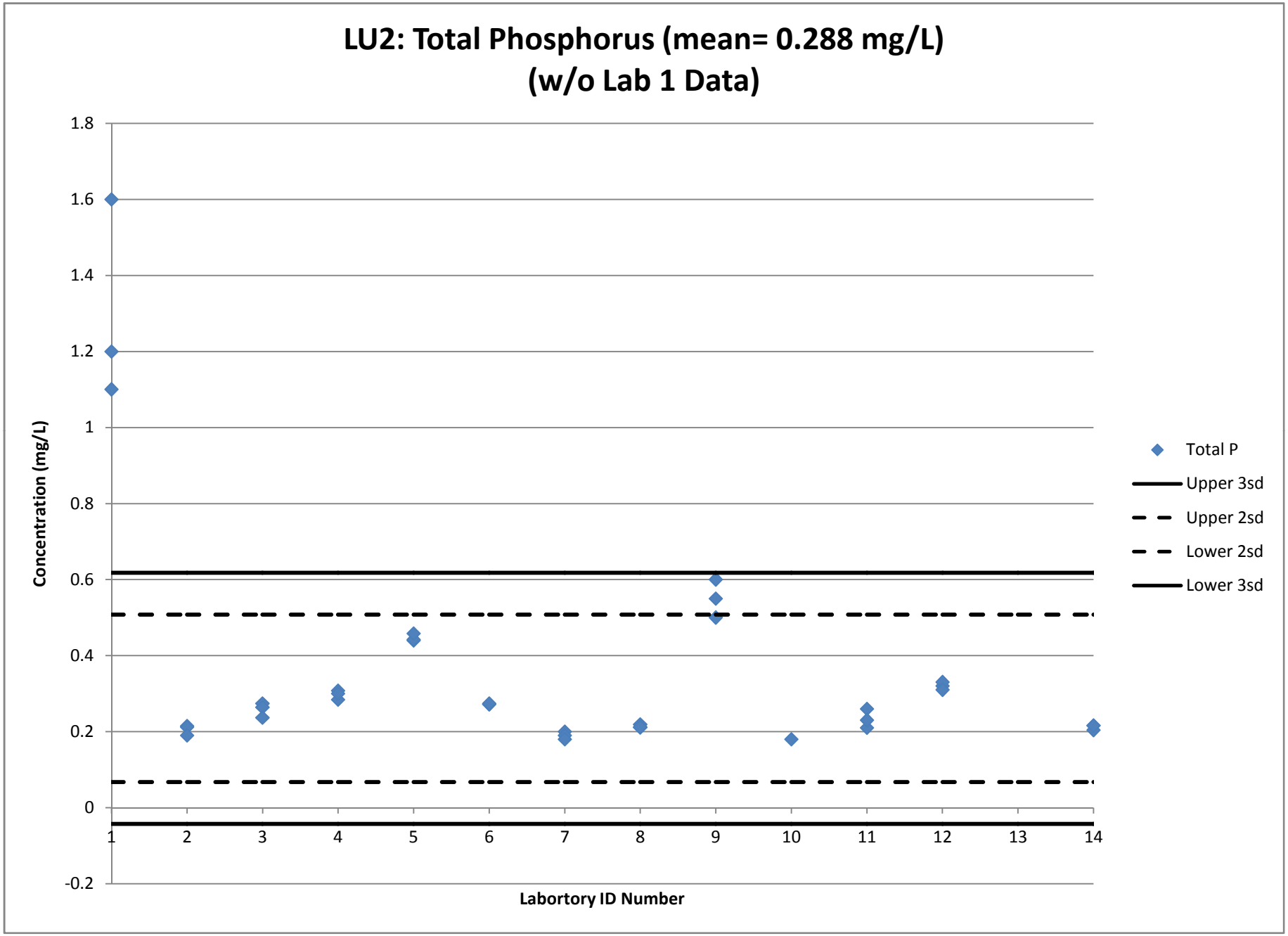

LU2: TSS (mean= 122 mg/L)
Concentration (mg/L)
140
135
130
125
120
115
110
105
100
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
TSS
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

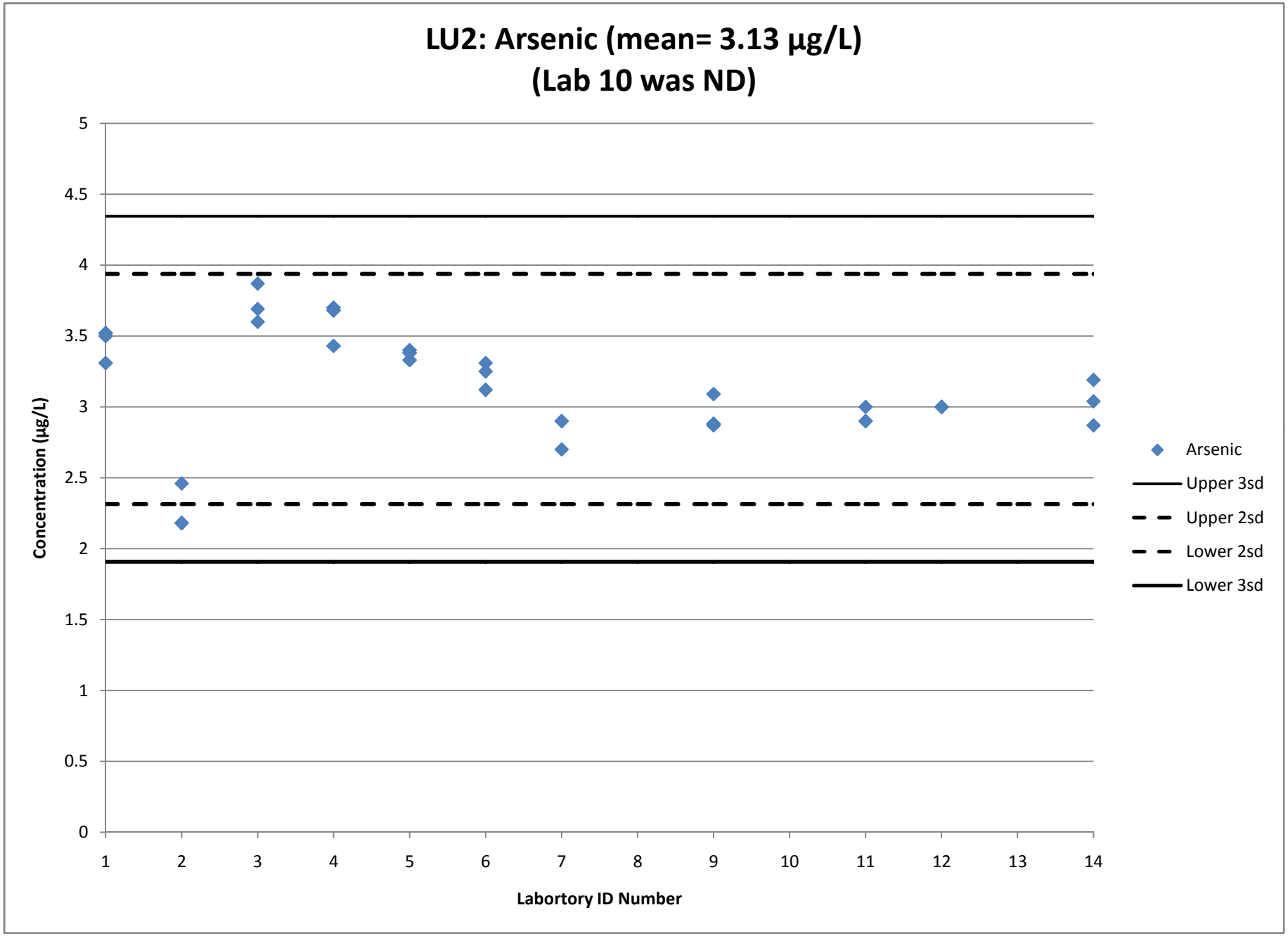

LU2: Ammonia-N (mean= 1.34 mg/L)
(Lab 6 was ND)
Concentration (mg/L)
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Ammonia-N
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

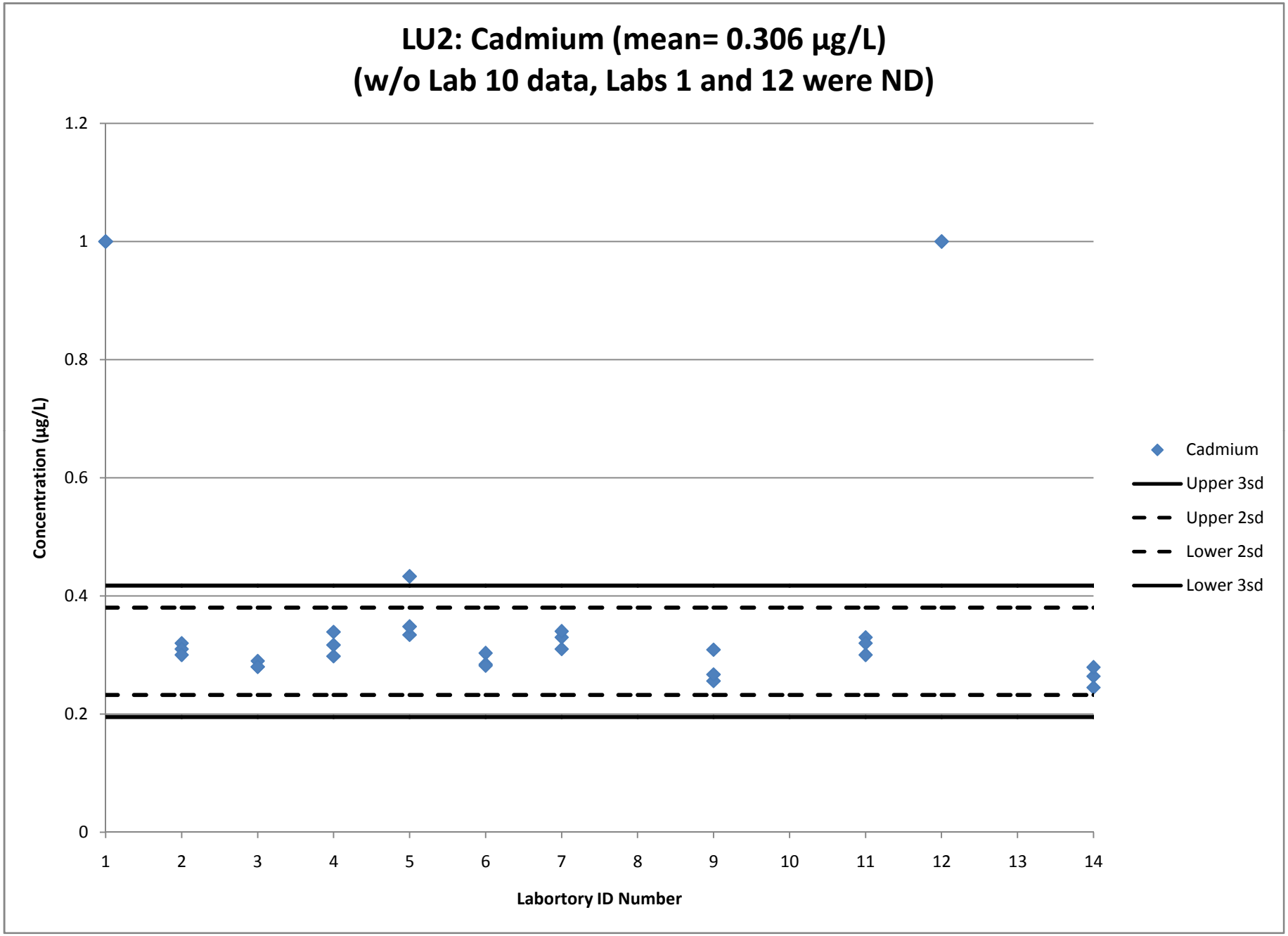

LU2: Nitrate+Nitrite-N (mean= 0.675 mg/L)
Concentration (mg/L)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
NO2+NO3-N
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

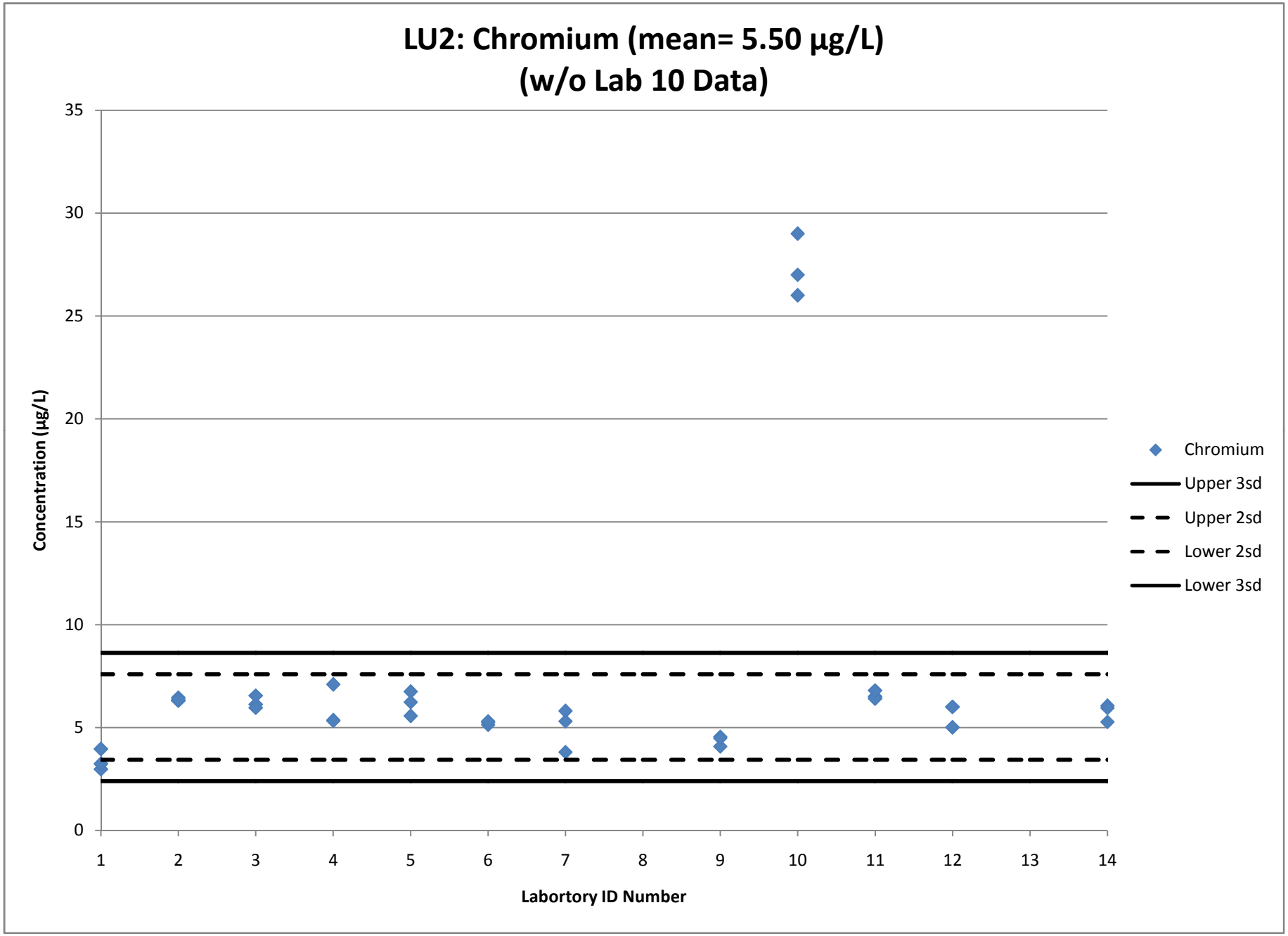

LU2: TKN (mean= 2.95 mg/L)
Concentration (mg/L)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
TKN
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

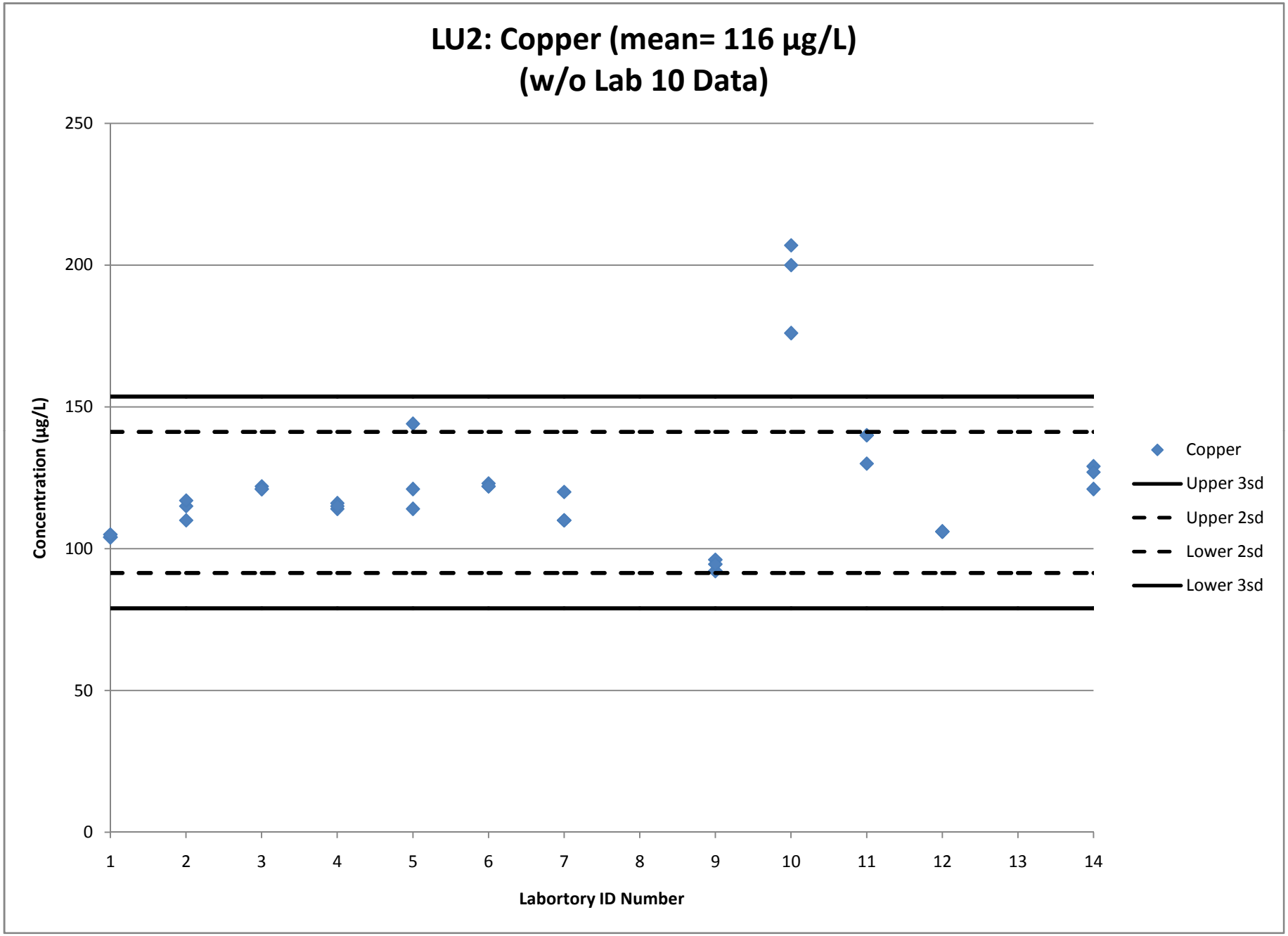

LU2: TOC (mean= 26.5 mg/L)
Concentration (mg/L)
50
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
TOC
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

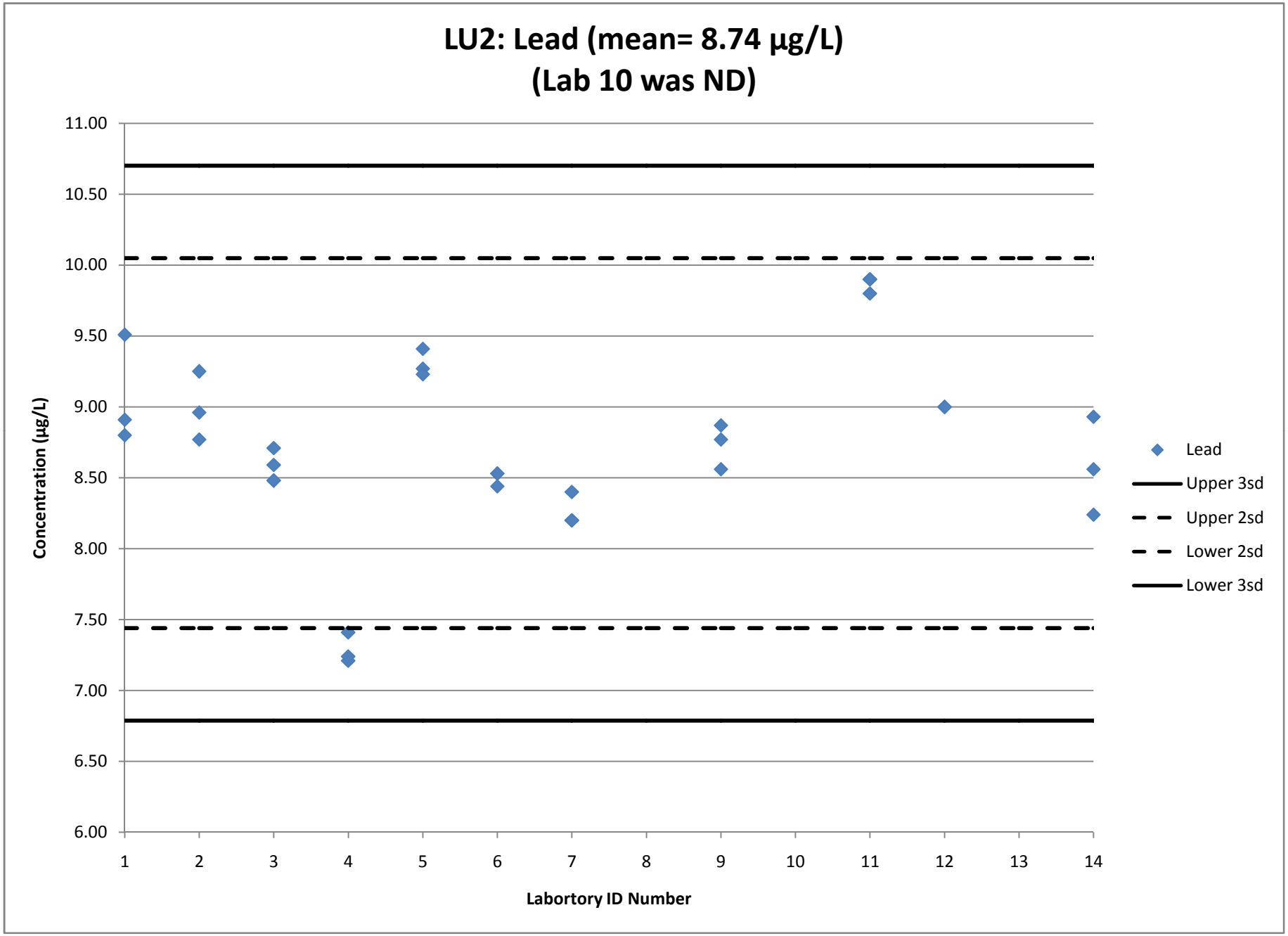

LU2: Total Phosphorus (mean= 0.288 mg/L)
(w/o Lab 1 Data)
Concentration (mg/L)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Total P
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

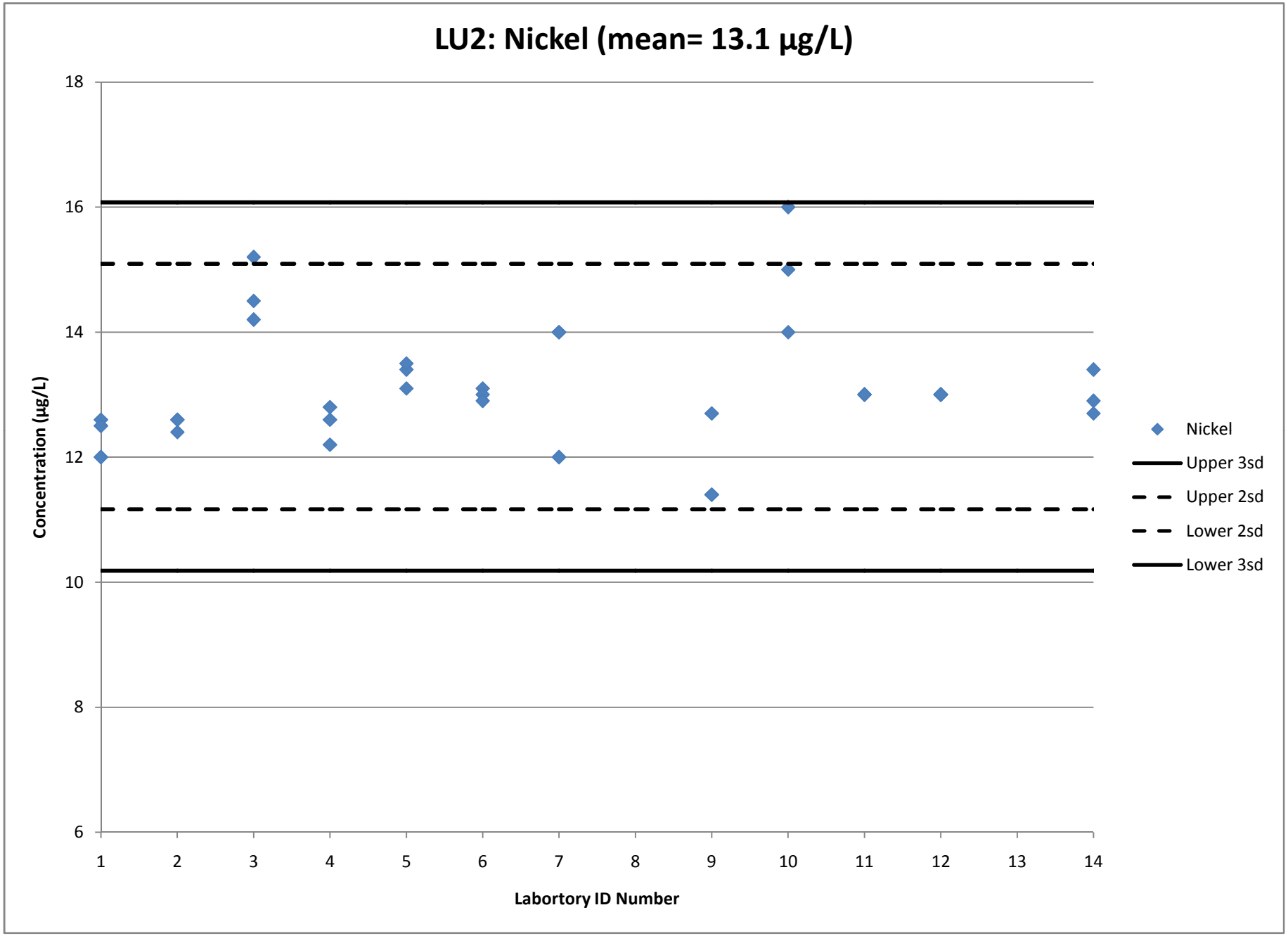

LU2: Arsenic (mean= 3.13 µg/L)
(Lab 10 was ND)
Concentration (µg/L)
Labortory ID Number
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Arsenic
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

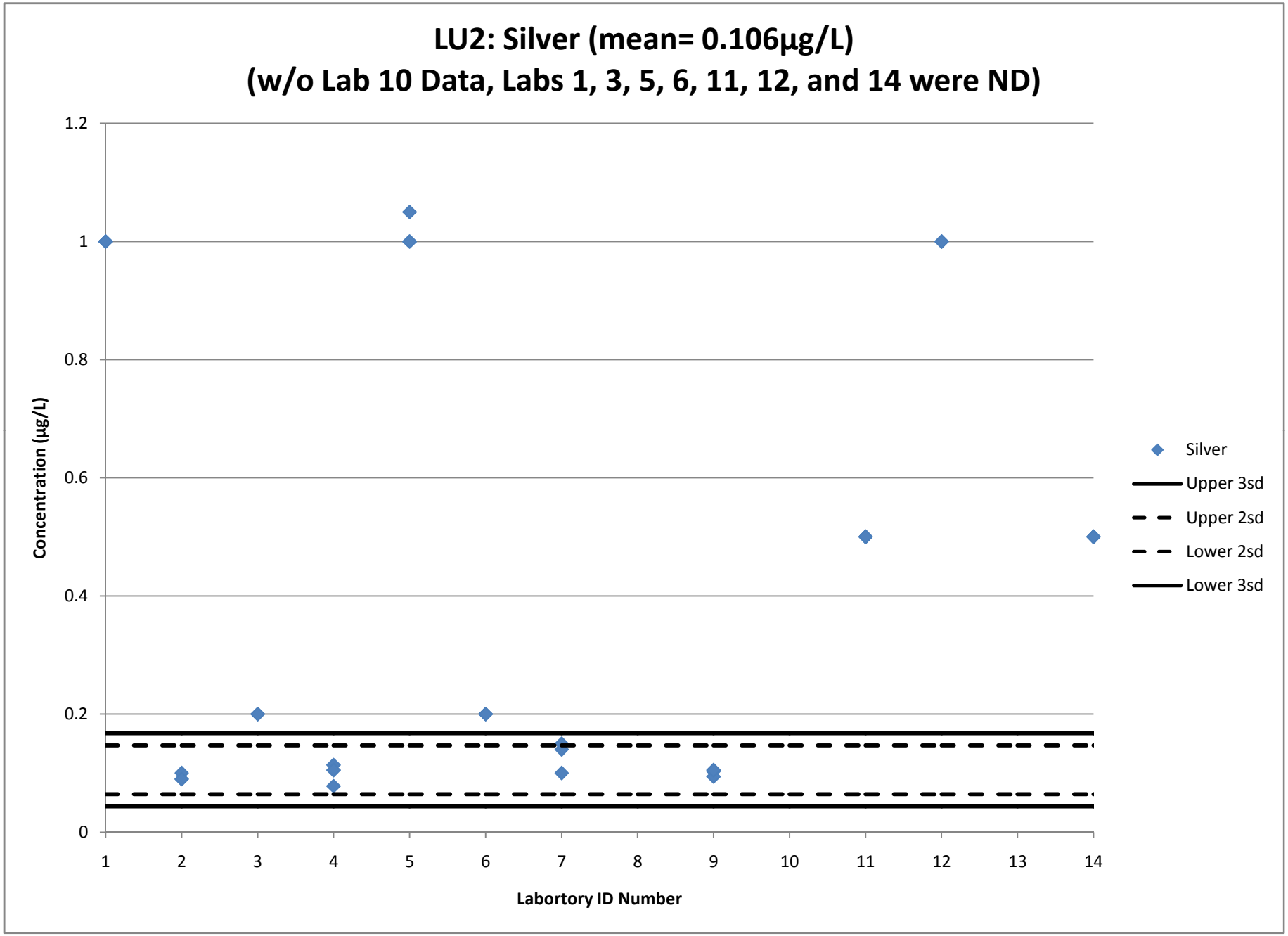

LU2: Cadmium (mean= 0.306 µg/L)
(w/o Lab 10 data, Labs 1 and 12 were ND)
Concentration (µg/L)
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Cadmium
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

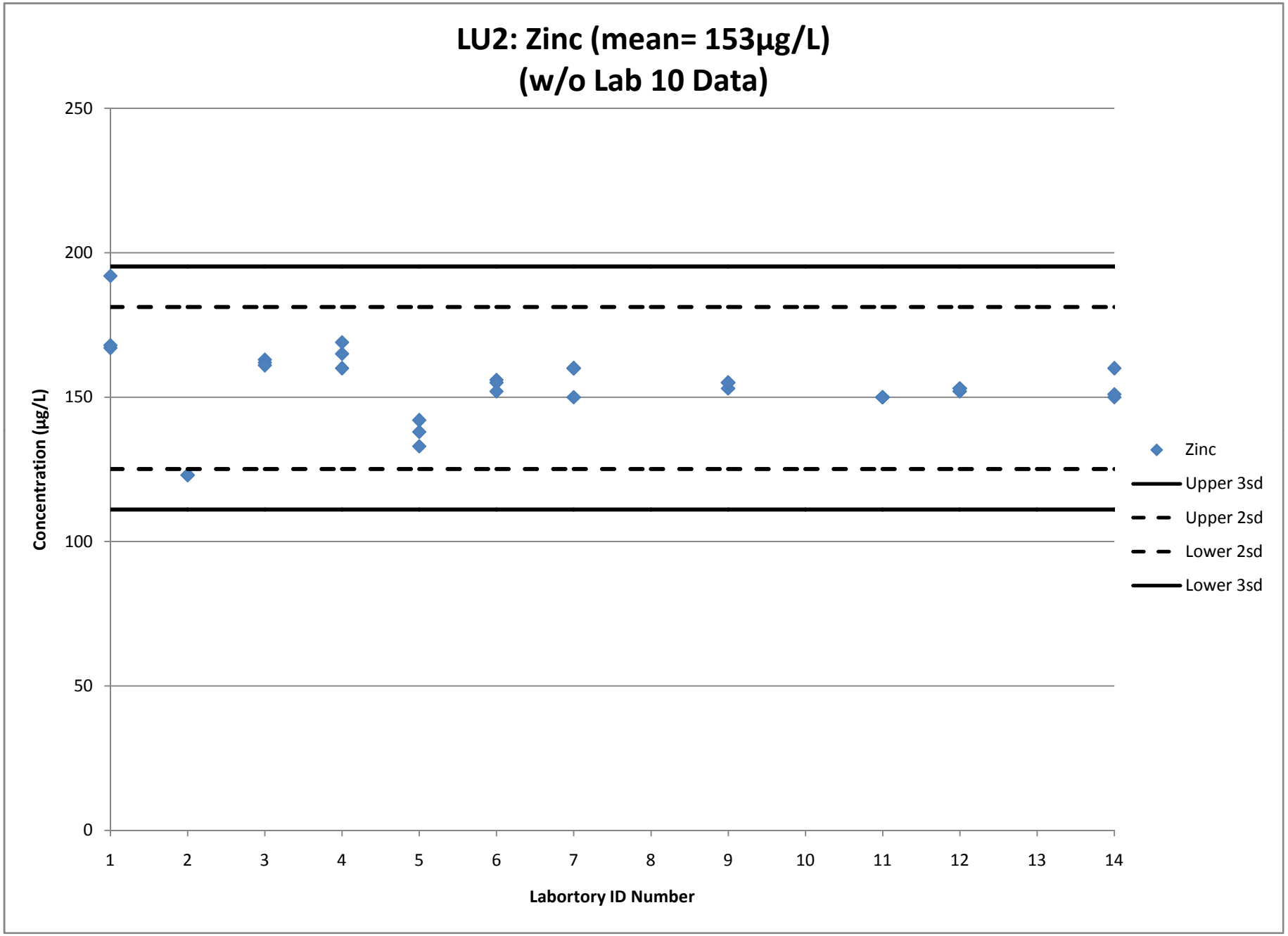

LU2: Chromium (mean= 5.50 µg/L)
(w/o Lab 10 Data)
Concentration (µg/L)
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Chromium
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd


LU2: Copper (mean= 116 µg/L)
(w/o Lab 10 Data)
Concentration (µg/L)
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Copper
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd


LU2: Lead (mean= 8.74 µg/L)
(Lab 10 was ND)
Concentration (µg/L)
11.00
10.50
10.00
9.50
9.00
8.50
8.00
7.50
7.00
6.50
6.00
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Lead
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd


LU2: Nickel (mean= 13.1 µg/L)
Concentration (µg/L)
18
16
14
12
10
8
6
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Nickel
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd


LU2: Silver (mean= 0.106µg/L)
(w/o Lab 10 Data, Labs 1, 3, 5, 6, 11, 12, and 14 were ND)
Concentration (µg/L)
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Silver
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd


LU2: Zinc (mean= 153µg/L)
(w/o Lab 10 Data)
Concentration (µg/L)
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Labortory ID Number
Zinc
Upper 3sd
Upper 2sd
Lower 2sd
Lower 3sd

SOURCES, PATTERNS AND MECHANISMS OF STORM WATER POLLUTANT LOADING FROM WATERSHEDS AND LAND USES OF THE GREATER LOS ANGELES AREA, CALIFORNIA, USA

Technical Report 510
March 2007

Eric D. Stein
Liesl L. Tiefenthaler
Kenneth C. Schiff

Southern California Coastal Water Research Project

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Southern California Coastal Water Research Project
3535 Harbor Blvd., Suite 110
Costa Mesa, California 92626, USA
www.sccwrp.org

March 20, 2007

Technical Report 510

EXECUTIVE SUMMARY

Storm water runoff and the associated contaminants from urban areas is one of the leading sources of water quality degradation in surface waters (US EPA 2000). Runoff from pervious and impervious areas (i.e., streets, parking lots, lawns, golf courses and agricultural land) carries accumulated contaminants (i.e., atmospheric dust, trace metals, street dirt, hydrocarbons, fertilizers and pesticides) directly into receiving waters (Novotny and Olem 1994). Because of the environmental effects of these contaminants, effective storm water monitoring and management requires identification and characterization of the sources, patterns, and mechanisms that influence pollutant concentrations and loads. Concentrations and loads of pollutants in urban storm water have been documented in some portions of the country (Hoffman et al. 1984; Buffleben et al. 2002; Simpson et al. 2002); however, little is known about the mechanisms and processes that influence spatial and temporal factors that affect the magnitude and patterns of constituent loading from specific land uses. Specifically, storm water managers need to understand how sources vary by land use type, how patterns of loading vary over the course of a single storm, how loading varies over the course of a storm season, and how applicable national or regional estimates of land use-based loading are to southern California.

To investigate these issues, the Southern California Coastal Water Research Project (SCCWRP) conducted a storm water sampling program over five seasons (2000-01 through 2004-2005). Constituent concentrations were measured over the entire storm duration from eight different land use types over 11 storm events in five watersheds in the greater Los Angeles, CA region (Figure ES-1). In addition, runoff samples were also collected from twelve mass emission sites (in-river) during 15 different storm events. A total of 71 site-events were sampled, comprised of 33 land use site-events and 38 mass-emission site events. These data were collected to better characterize contributions of specific land use types to loading of bacteria, trace metals, and organic compounds and to provide data for watershed model calibration. The specific goals of this study were (1) to examine constituent event mean concentrations (EMC), fluxes, and mass loadings associated with storm water runoff from representative land uses; (2) to investigate within storm and within season factors that affect constituent concentrations and fluxes; (3) to evaluate how constituent loadings compare to loadings from point sources, and (4) to assess how the concentrations of constituents in runoff compare to published data and water-quality criteria.

To understand the complex spatial and temporal patterns that affect storm water runoff in the greater Los Angeles region, runoff and constituent concentrations from a variety of land uses and mass emission sites were sampled over a range of different storm sizes and antecedent conditions. Between 10 and 15 discrete grab samples were collected for each site-event and the samples analyzed individually to provide time vs. concentration plots (i.e., pollutographs) for each site-event. Samples were analyzed for a broad range of constituents including trace metals, organic compounds and bacteria. Storms were targeted to capture early vs. late season conditions and large vs. small rainfall events. Understanding both intra-storm and inter-storm variability provides a more complete assessment of factors that influence constituent loading, and will allow us to develop dynamic watershed models that are able to predict pollutant runoff from specific land use types and watersheds under a variety of conditions.

General Conclusions

1. Storm water runoff from watershed and land use based sources is a significant contributor of pollutant loading and often exceeds water quality standards

Results of this study indicate that urban storm water is a substantial source of a variety of constituents to downstream receiving waters. Substantially high constituent concentrations were observed throughout the study at both mass emission (ME) and land use (LU) sites. Constituent concentrations frequently exceeded water quality criteria. Storm water concentrations of trace metals exceeded California Toxic Rule (USEPA 2000) water quality criteria in more than 80% of the wet weather samples collected at ME sites. This was partly due to industrial land use sites where 100% and 87% of runoff samples exceeded water quality criteria for zinc and copper, respectively. Furthermore, fecal indicator bacteria (FIB) at both ME and LU sites consistently exceeded California single-sample water quality standards. In fact levels of FIB at the recreational (horse) and agricultural LU sites were as high as those found in primary wastewater effluent in the U.S., with densities of 10^{6}-10^{7} MPN/100mL.

2. All constituents were strongly correlated with total suspended solids

Land use had a strong influence on constituent concentrations. Total suspended solids (TSS) was strongly correlated with constituent EMCs at most land use sites, although not all correlations were statistically significant. This correlation was primarly influenced by highly urbanized land uses and a single undeveloped open space land use. High TSS loads in rivers contribute to water quality impairments, habitat loss and to excessive turbidity resulting in impairments in recreational, fish/wildlife, and water supply designated uses of the rivers. These results suggest that controlling TSS at specific land uses may result in reducing other particle-bound constituents.

3. Storm water EMCs, fluxes and loads were substantially lower from undeveloped open space areas when compared to developed urbanized watersheds

Storms sampled from less developed watersheds (i.e., Santa Monica Canyon and Arroyo Sequit) produced constituent EMCs and fluxes that were one to two orders of magnitude lower than comparably sized storms in urbanized watersheds (i.e., Los Angeles River and Ballona Creek) (Figure ES-2). Furthermore, the higher fluxes from developed watersheds were generated by substantially less rainfall than the lower fluxes from the undeveloped watersheds, presumably due to increased impervious surface area in developed watersheds. Stein and Yoon (2007) reported similar wet weather runoff results from undeveloped land uses while investigating pollutant contributions from natural sources. The contrasts between the different watershed scale mass-emission sites were also apparent at the small, homogeneous land use sites.

4. Land use based sources of pollutant concentrations and fluxes varied by constituent

No single land use type was responsible for contributing the highest loading for all constituents measured. For example industrial land use sites, contributed higher storm EMCs and fluxes of all trace metals than other land use types. (Figure ES-3). Recreational (horse) land use sites contributed significantly higher storm fluxes for *E. coli* while agricultural land use sites contributed the highest TSS fluxes. Substantially higher TSS fluxes were also observed at the industrial sites. PAHs were not preferentially generated by any one land use type, rather

analyses of individual PAHs demonstrated a consistent predominance of high molecular weight (HMW) PAH compounds indicative of regional pyrogenic PAHs (i.e., atmospheric deposition) as a major source material of the PAHs found in urban storm water.

5. Storm water runoff contributed a similar range of constituent loading to regional point sources

Storm water runoff of trace metals from the urban watersheds in this study produced a similar range of annual loads as those from point sources; such as large publicly owned treatment plants (Table ES-1). Nevertheless, when combined with dry estimates of pollutant loading from Stein and Tiefenthaler (2005), the total non-point source contribution from all watersheds in the greater Los Angeles area far exceeds that of the point sources (Table ES-1).

6. The Los Angeles region contributed a similar range of storm water runoff pollutant loads as that of other regions of the United States

Comparison of constituent concentrations in storm water runoff from land use sites from this study reveal median EMCs that are comparable to current U.S. averages reported in the National Storm water Quality Database (NSQD; Pitt *et al*, 2003) (Figure ES-4). Comparison to the NSQD data set provides insight to spatial and temporal patterns in constituent concentrations in urban systems. Similarities between levels reported in the NSQD and this study suggest that land-based concentrations in southern California storm water are generally comparable to those in other parts of the country.

7. Storm water runoff concentrations improved over time when compared with the Nationwide Urban Runoff Program (NURP.

Results showed an improvement in water quality between constituent concentrations reported by NURP in 1983 and those observed in this study (Los Angeles River Watershed (LARW). Long-term overall trends of decreasing median constituent EMCs were observed at all land uses with the exception of total zinc, which showed an increase in median EMCs over the course of the studies (Figure ES-4). For example, lead concentrations have exhibited a 10-fold reduction over the last 20 years. Relatively low lead concentrations may reflect fate and transport characteristics of the particular systems sampled. However, a more likely explanation is that low concentrations of lead observed in these studies can be attributed to regulations banning the use of leaded gasoline.

8. Peak concentrations for all constituents were observed during the early part of the storm

Constituent concentrations varied with time over the course of storm events. For all storms sampled, the highest constituent concentrations occurred during the early phases of storm water runoff with peak concentrations usually preceding peak flow (Figure ES-5). In all cases, constituent concentrations increased rapidly, stayed high for relatively short periods and often decreased back to base levels within one to two hours. In contrast, the developed LU (recreational (horse) site; Figure ES 1-5c) had a peak concentration followed by intra-storm variable concentrations that mimicked flow. Although the pattern of an early peak in concentration was comparable in both large and small developed watersheds (Ballona Creek; Figure ES-5a, Los Angeles River Figure ES-5b), the peak concentration tended to occur later in the storm and persist for a longer duration in the smaller developed watersheds. Therefore

monitoring programs must capture the early portion of storms and account for intra-storm variability in concentration in order to generate accurate estimates of EMC and contaminant loading. Programs that do not initiate sampling until a flow threshold has been surpassed may severely underestimate storm EMCs.

9. The magnitude of a mass first flush effect at land use sites was a function of watershed size

Storm mass loading is a function of both concentration and magnitude of flow at various points during a storm. Cumulative mass loading of constituents from ME sites generally exhibited a weak "first flush" for trace metals and bacteria. For PAHs, a moderate first flush was observed where between 40% and 60% of the load was discharged during the first 25% of storm volume. In contrast to the ME sites, cumulative mass loading plots from small, homogenous land use sites exhibited moderate first flush for all constituents sampled. When all developed sites were analyzed together, the magnitude of the first flush effect decreased with increasing watershed size (Figure ES-6). The inverse relationship between first flush and catchment size has several potential mechanistic explanations including differences in relative pervious area, spatial and temporal patterns in rainfall, and pollutant transport through the catchment. Ultimately, the differences in first flush, whether due to imperviousness, travel time, or rainfall variability, suggest that management strategies aimed at capturing constituent loads should focus on more than just the initial portion of the storm at moderate to large catchments.

10. Highest constituent loading was observed early in the storm season with intra-annual variability driven more by antecedent dry period than amount of rainfall

Seasonal differences in constituent EMCs and loads were consistently observed at both ME and LU sites. In general, early season storms (October – December) produce significantly higher constituent EMCs and loads than late season storms (April-May), even when rainfall quantity was similar (Figure ES-7). This suggests that the magnitude of constituent load associated with storm water runoff depends, at least in part, on the amount of time available for pollutant build-up on land surfaces. The extended dry period that typically occurs in arid climates such as southern California maximizes the time for constituents to build-up on land surfaces, resulting in proportionally higher concentrations and loads during initial storms of the season. This seasonal pattern suggests that focusing management actions on early season storms may provide relatively greater efficiency than distributing lower intensity management actions throughout the season.

Further Research

This study establishes the relative contributions of land uses and watersheds to constituent loading in receiving water bodies. Having statistically significant data sets at regional, seasonal, and land use levels enables modelers to use the information for more sensitive calibration of models that may be used for contaminant load allocations. Similarly, these data sets can assist storm water engineers in the design of more effective monitoring programs and better performing treatment practices (i.e., BMPs) that address specific rainfall/runoff conditions.

Further research is needed to directly assess the relationship between constituent concentrations and particle-size distributions in storm water runoff from mass emission and land use sites to better understand the fate, transport and treatment of constituents in urban runoff.

Storm water borne metals, PAHs and (to a lesser extent) bacteria are typically associated with particulates to varying degrees depending on the constituent and the size distribution of suspended solids in the storm water runoff. Furthermore, the particle size distribution, and constituent partitioning can change over the course of a storm event (Furumai et al. 2002, Stein and Yoon 2007). Understanding the dynamic partitioning of constituents to various size particles is important to being able to estimate temporal and spatial patterns of constituent deposition in estuaries and harbors, and should be an area of future investigation.

Our understanding of the mechanisms of constituent loading from urban land uses could also be improved by estimating the percent of directly connected impervious area (DCIA) in each land use category (i.e., percent rooftop, sidewalks, paved driveways and streets) and its impacts on storm water runoff concentrations and loads. This could allow identification of critical source areas, which in turn could provide for more precise estimates of loading and more focused application of best management practices.

Table ES-1. Mean annual (± 95% confidence intervals) trace metal loading in the Los Angeles coastal region from different sources (mt = metric tons).

Source	Mean Annual Load / Year (mt ± 95% CI)		
	Total Copper	Total Lead	Total Zinc
Point Source Data[1,2] (2000-05)			
Large Publicly Owned Treatment Plants (POTWs)	10.9 ± 6.8	0.8 ± 0.8	13.9 ± 7.6
Low Volume Waste Power Generating Stations (PGS)	0.01	0.00	0.09
Wet Weather Runoff (2000-05)			
Los Angeles River	1.6 ± 1.2	1.4 ± 1.5	9.8 ± 9.4
Ballona Creek	0.7 ± 0.4	0.6 ± 0.3	4.3 ± 2.5
Dominguez Channel	0.4 ± 2.4	0.2 ± 1.1	2.1 ± 11.0
Total Annual Wet Weather Runoff	**2.7 ± 4.0**	**2.2 ± 2.9**	**16.2 ± 22.9**
2000-02 Dry Weather Urban Runoff [3,4]			
Los Angeles River	2.9 ± 19.9	0.1 ± 1.2	10.4 ± 80.6
Ballona Creek	0.2 ± 0.3	0.1 ± 0.4	0.7 ± 0.6
Total Annual Dry Weather Runoff	**3.1 ± 20.2**	**0.2 ± 1.6**	**11.1 ± 81.2**

[1]SCCWRP Biennial Report 2004-06 (Lyons G, Stein E).
[2]SCCWRP Biennial Report 2003-04 (Steinberger A, Stein E); PGS data represents year 2000 only.
[3]American Water Resources Association in Press (Stein E, Ackerman D).
[4]Water, Air and Soil Pollution, 2005. Vol. 164 (Stein E, Tiefenthaler L).

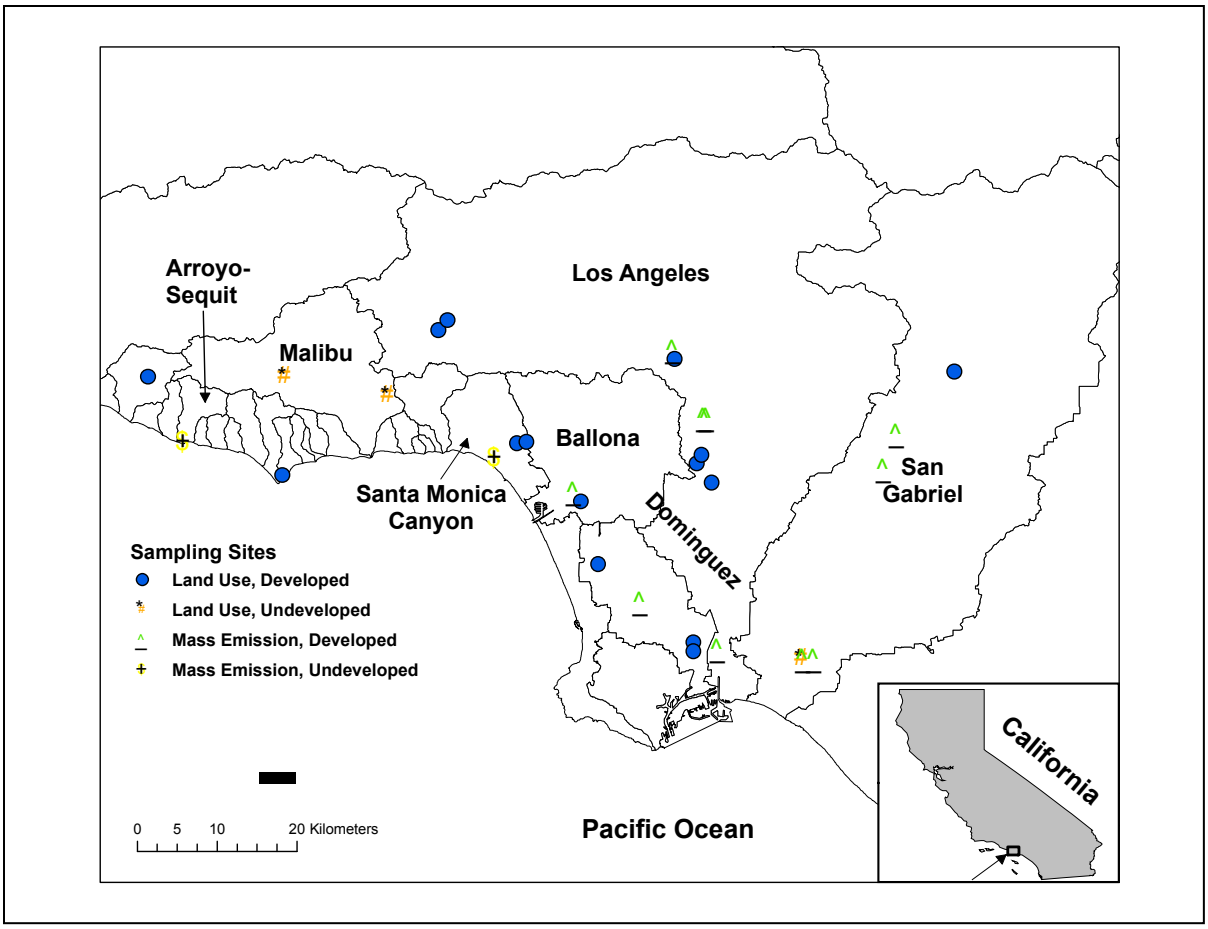

Figure ES-1. Map of in-river mass emission and land use sampling sites and watersheds within the greater Los Angeles region, California, USA.

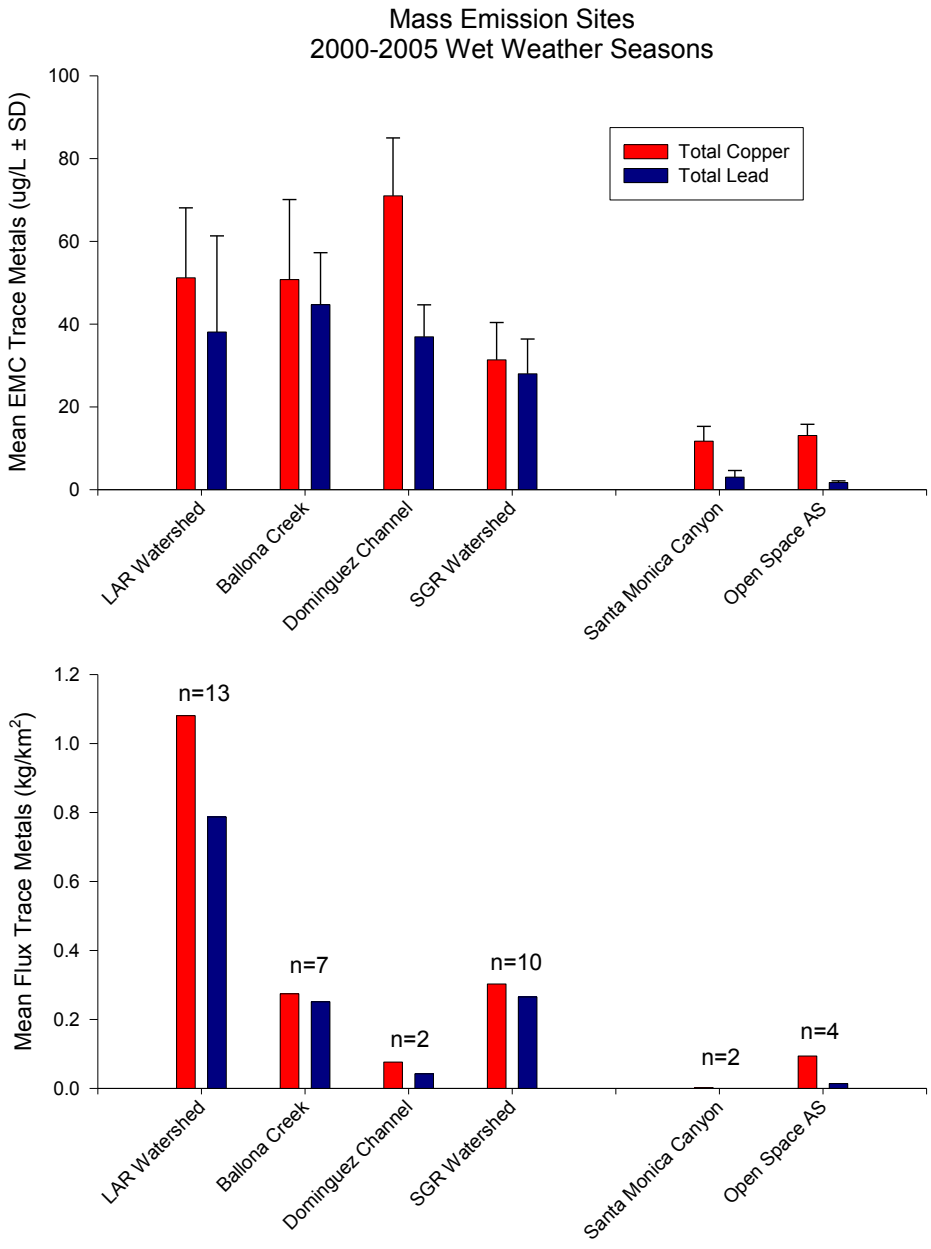

Figure ES-2. Average event mean concentrations (EMCs; a) and fluxes b) of total copper and lead loading from southern California watersheds during the 2000-2001 to –2004-2005 storm seasons. A similar pattern of higher loadings for the mass emission sites was observed for all other constituents measured in the study as well. Los Angeles River (LAR), San Gabriel River (SGR), and Arroyo Sequit (AS), number of storm events (n), and standard deviation (SD).

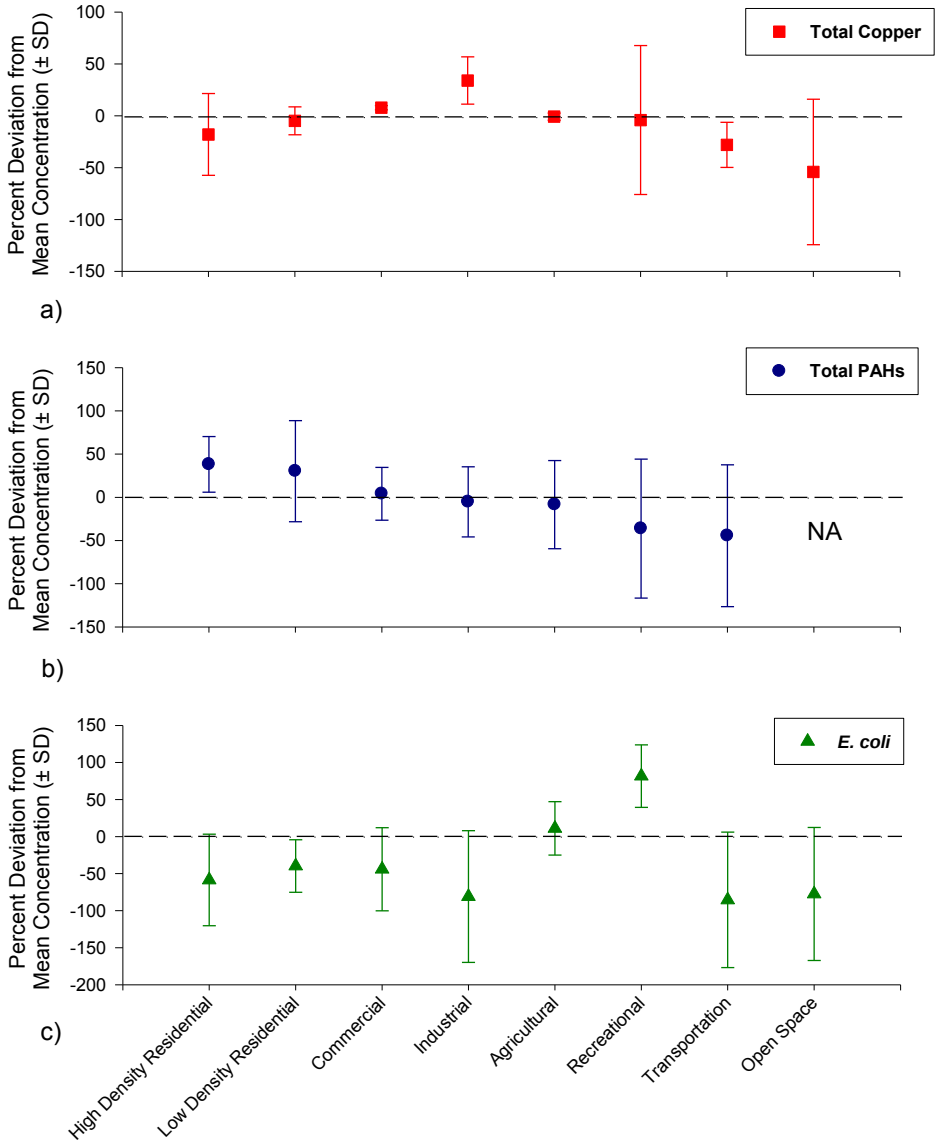

Figure ES-3. Percent deviation from mean concentration of total PAHs a), total copper b) and *E. coli* c) in storm water runoff from land use sites during the 2001-2005 storm seasons. The dashed line represents the overall mean concentration for each constituent. Standard deviation (SD). Not analyzed (NA).

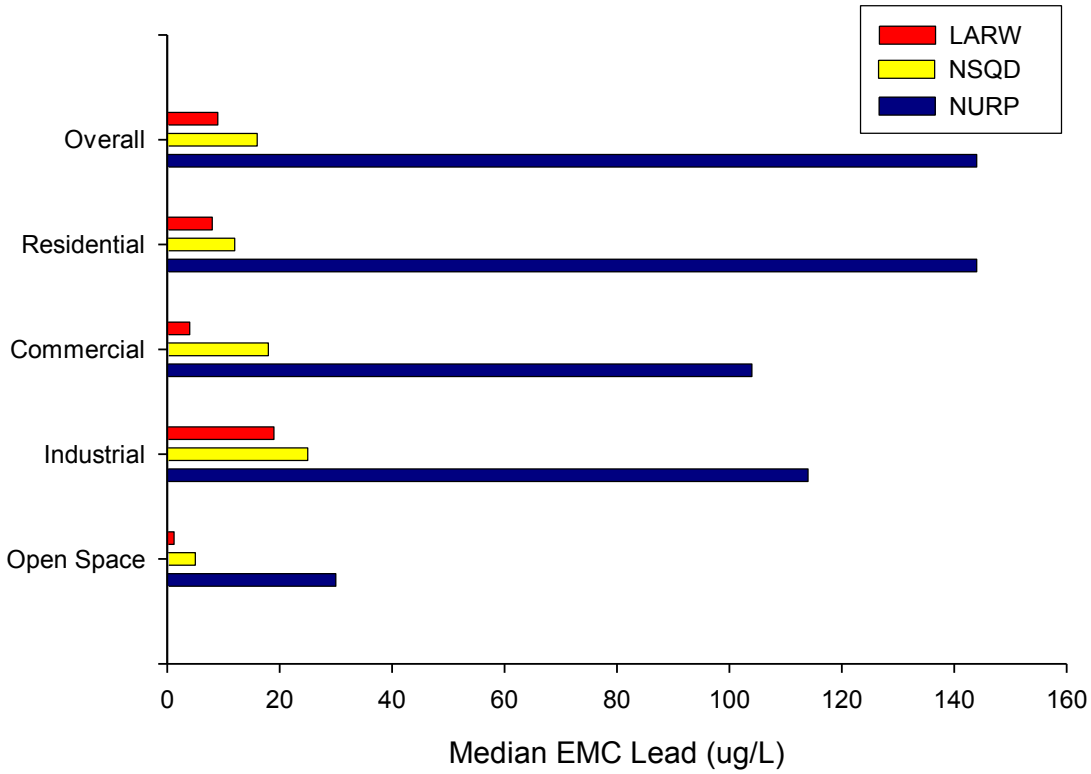

Figure ES-4. Comparison of median lead event mean concentration (EMCs) at specific land use sites during the 1983 Nationwide Urban Runoff Program (NURP, U.S. EPA 1983a), to the 1990 National Storm water Quality Database (NSDQ, Pitt et al. 2003) monitoring study and the 2001-2005 Los Angeles River Wet Weather (LARW) study. A similar pattern was observed for other constituents with the exception of zinc, which showed an increase in median EMCs over the course of the studies.

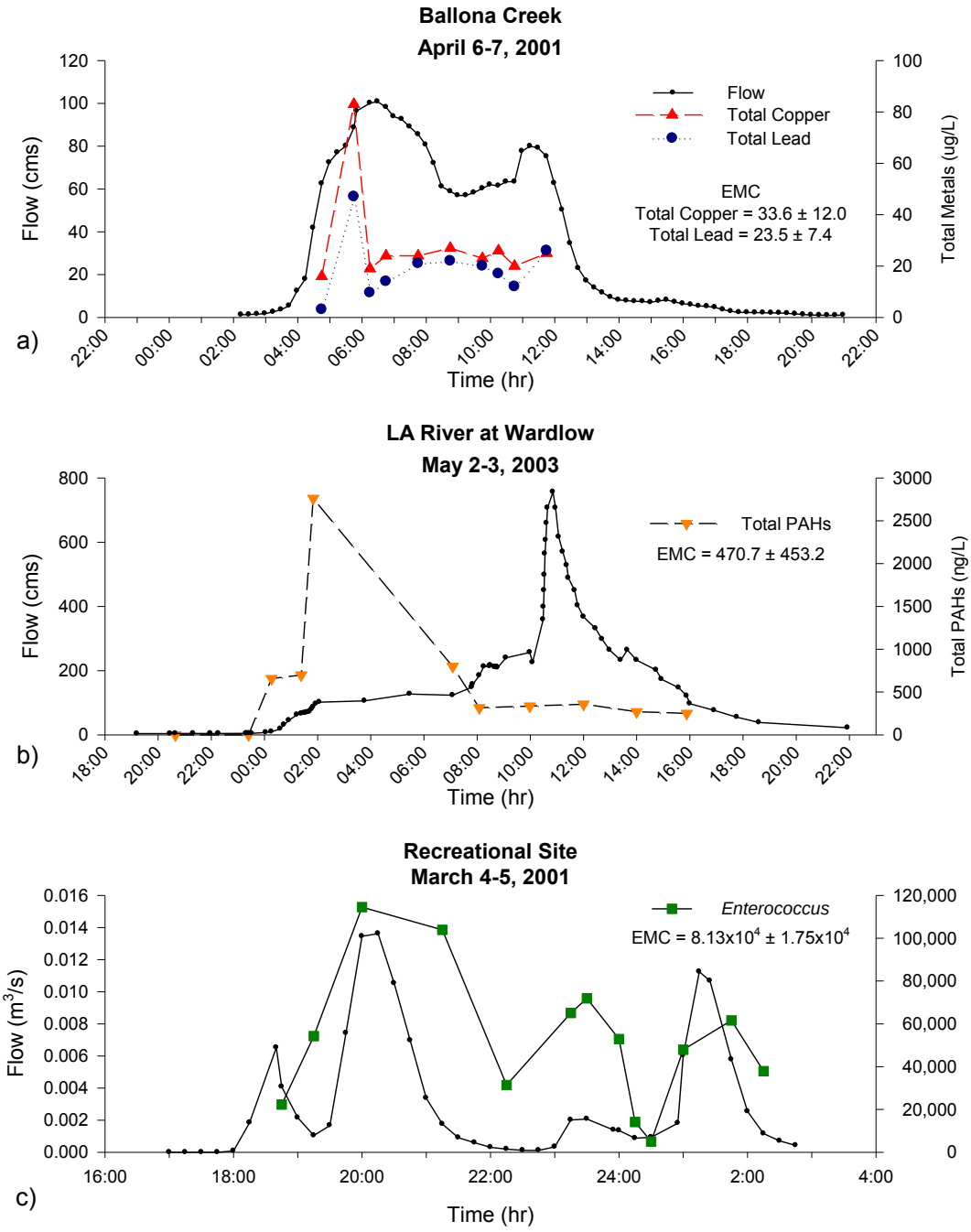

Figure ES-5. Variation in constituent concentrations with time for a storm event in the developed Ballona Creek a) and Los Angeles River watersheds b) and the developed recreational (horse) land use site c).

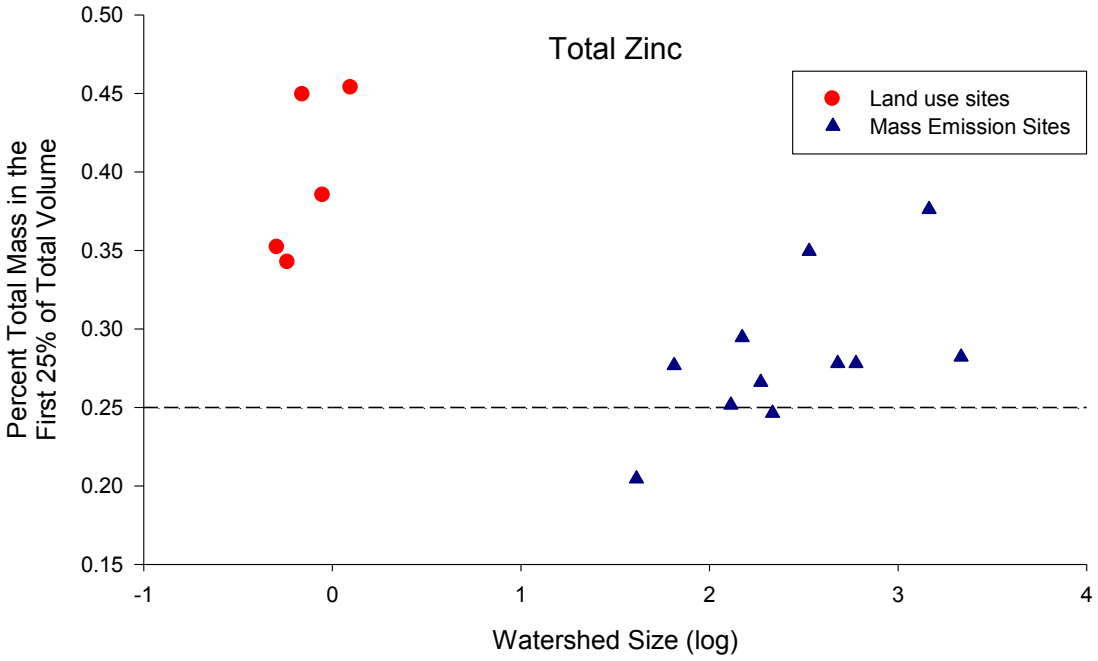

Figure ES-6. First flush patterns of total zinc (a) in relation to watershed size. Watershed size data is log transformed.

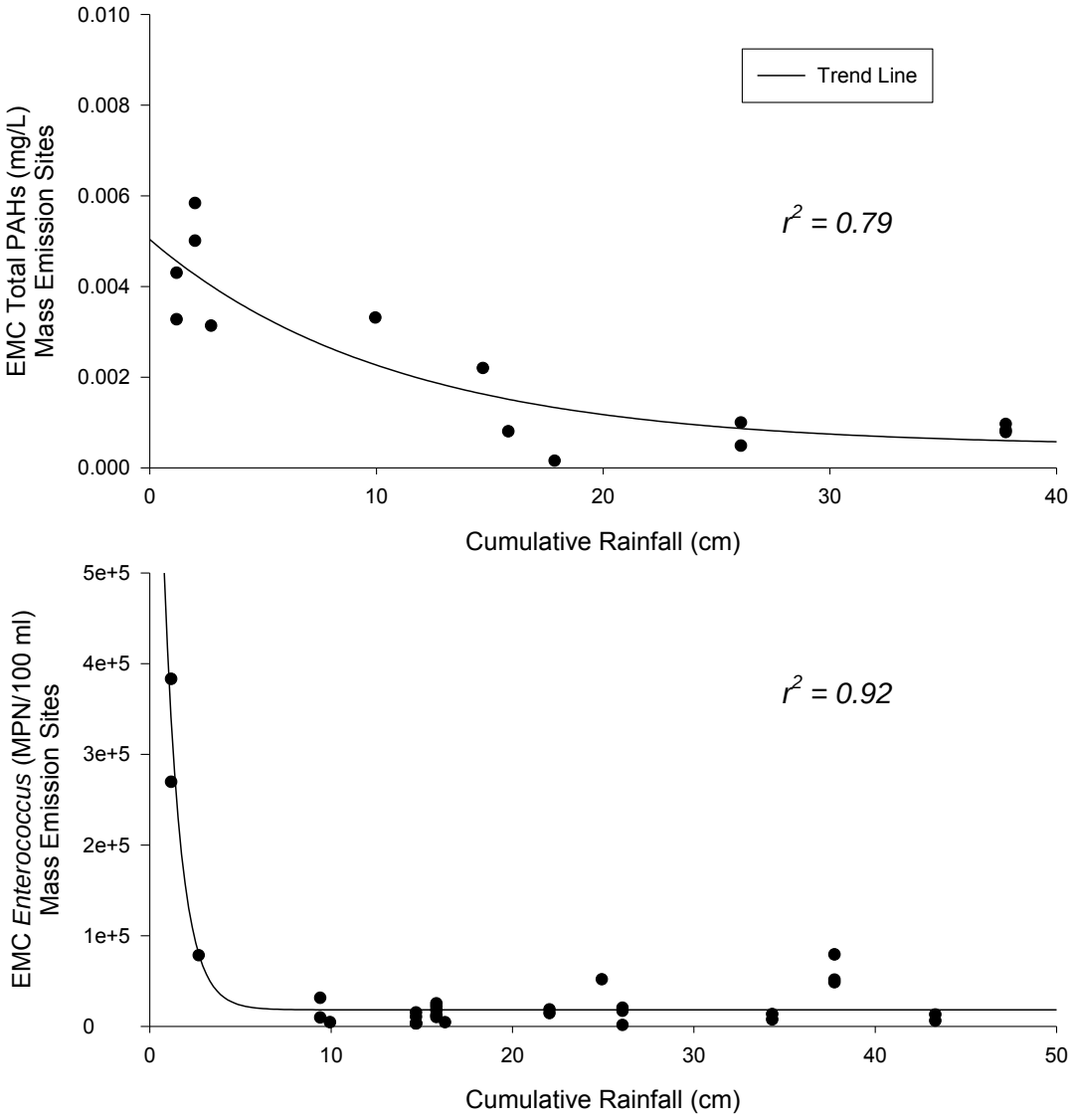

Figure ES-7. Cumulative annual rainfall versus event mean concentration (EMC) for a) polycyclic aromatic hydrocarbons and b) *E. coli*. Plots show data for mass emission sites only.

ACKNOWLEDGEMENTS

The authors would like to thank Jay Shrake, Sean Porter, Richard Rusnik, and the rest of the MacTec Engineering field crews for their assistance and dedication in collecting storm water samples. We also thank Shelly Moore of SCCWRP for her expertise with GIS software. Funding for this study was provided by the Los Angeles Regional Water Quality Control Board, Western States Petroleum Association, Los Angeles Contaminated Sediments Task Force, City of Los Angeles, Los Angeles Department of Water and Power, Los Angeles County Sanitation Districts, City of Carson, and by SCCWRP.

LIST OF ACRONYMS

AV	Acoustic Doppler Velocity
BMP	Best Management Practices
BST	Bacterial Source Tracking
CDF	Cumulative Density Frequency
CTR	California Toxics Rules
DCIA	Directly Connected Impervious Area
E. coli	*Escherichia coli*
EMC	Event Mean Concentration
FIB	Fecal Indicator Bacteria
FWM	Flow Weighted Mean
HMW	High Molecular Weight
ICPMS	Inductively Coupled Plasma-Mass Spectroscopy
LARW	Los Angeles River Wet-weather
LMW	Low Molecular Weight
ME	Mass Emission
NPDES	National Pollutant Discharge Elimination System
NSQD	National Storm water Quality Database
NURP	National Urban Runoff Program
PAHs	Polycyclic Aromatic Hydrocarbons
PCs	Principal Components
PCA	Principle Component Analysis
SCCWRP	Southern California Coastal Water Research Project
TSS	Total Suspended Solids
USEPA	United States Environmental Protection Agency

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SECTION 1. INTRODUCTION

Urban storm water runoff has been identified as a major cause of degradation of surface water quality (Characklis and Wiesner 1997, Davis et al. 2001, Buffleben et al. 2002). Studies in southern California have documented trace metals, polycyclic aromatic hydrocarbons (PAHs) and fecal indicator bacteria (FIB) as major constituents of concern in storm water runoff (Buffleben et al. 2002, McPherson et al. 2002; Gigliotti 2000; Menzie et al. 2002). As a result numerous stream reaches in the greater Los Angeles Basin are listed as impaired waterbodies under Section 303(d) of the Clean Water Act for a range of constituents (LARWQCB 1998a and 2002).

Past monitoring and assessment efforts have provided important insight into the general patterns of storm water loading. Previous studies have documented that the most prevalent metals in urban storm water are zinc, copper, lead, and to a lesser degree nickel and cadmium (Sansalone and Buchberger 1997, Davis et al. 2001). Recent FIB studies using *Escherchia coli* (*E. coli*), *Enterococcus* spp. and total coliforms (Noble et al. 2003 and Stein and Tiefenthaler 2005) have documented freshwater outlets such as storm drains to be especially high contributors of bacterial contamination. Routine storm water monitoring programs focus on quantification of average concentration or load at the terminal watershed discharge point. While important for overall status and trends assessment, such monitoring provides little insight into the mechanisms and processes that influence constituent levels in storm water.

To effectively manage storm water, managers need to gain a deeper understanding of factors that affect storm water quality. In particular, managers need to understand the sources, processes and mechanisms that affect runoff and associated constituent loading. Specifically, managers need to understand how sources vary by land use (LU) type, how patterns of loading vary over the course of a single storm, how loading varies over the course of a storm season, and how applicable national or regional estimates of LU based loading are to southern California. Such information is critical to designing and implementing effective management strategies and for calibrating watershed models that can be used to evaluate proposed strategies.

The goal of this study is to quantify the sources, patterns of concentrations, fluxes, and loads of trace metals, PAHs and fecal indicator bacteria from representative land use types in the greater Los Angeles, California region. In addition to quantifying differences between land use categories, our goal is to investigate within storm and within season patterns in order to identify mechanisms that influence patterns of constituent loadings. Finally, we compare the estimates of storm water metals, total suspended solids (TSS) and *E. coli* loading to data from point and non-point sources and to existing water quality standards to provide context for the magnitude of importance of storm water to overall metals, TSS and *E. coli* loading for the region.

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SECTION 2. METHODS

Study Areas

Storm water runoff was sampled from 19 different land use (LU) sites and 12 mass emission (ME) sites throughout the greater Los Angeles area (Figure 2-1). The 19 LU sites represented homogeneous distributions of eight land use types including high density residential, low-density residential, commercial, industrial, agricultural, recreational (horse), transportation or open space. The LU sites ranged in size from 0.002 to 2.89 km^2 (see Appendix A for more detailed land use information). In contrast to the smaller, homogeneous LU sites, ME sites had much larger catchments and consisted of heterogeneous land use distributions that commingle and ultimately discharge to recreational beaches and harbors along the Pacific Ocean. There were 10 urban ME sites and two nonurban ME sites sampled. Developed land use ranged from 49% to 94% of total watershed area in the 10 urban watersheds. Developed land use comprised less than 5% of the watershed area in the two non urban watersheds examined in this study. The 12 ME sites ranged in size from 31 to 2,161 km^2.

Rainfall

All of the LU and ME sites were sampled during the 2000–2001 through 2004–2005 storm seasons. Winter storms typically occur between October and May, providing 85% to 90% of the annual average rainfall (38.4 cm; Ackerman and Weisberg 2003). Annual precipitation in Los Angeles can be highly variable. For example, the 2004-2005 rainfall season brought 94.6 cm of precipitation to downtown Los Angeles making it the second wettest season in Los Angeles since records began in 1877 (National Weather Service; http://www.wrh.noaa.gov/lox/). In contrast, the 2001-2002 rainfall season totaled a mere 11.2 cm, 27 cm below the seasonal average. Consequently, the study period encompassed a wide range of precipitation conditions.

Sampling and Analysis

Twenty discrete storms were sampled, with each site sampled between one to seven individual storm events (Tables 2-1a and 2-1b). Rainfall amounts ranged from 0.12 to 9.68 cm and antecedent conditions ranged from 0 to 142 d without measurable rain. Rainfall at each site was measured using a standard tipping bucket that recorded in 0.025-cm increments. Antecedent dry conditions were determined as the number of days following the cessation of previous measurable rain. Water quality sampling was initiated when flows were greater than base flows by 20%, continued through peak flows, and ended when flows subsided to less than 20% of base flow. Because watersheds in southern California have highly variable flows that may increase orders of magnitude during storm events, these criteria are considered conservative. Flow at ME sites was estimated at 15-min intervals using existing, county-maintained flow gauges, or stage recorders in conjunction with historically derived and calibrated stage-discharge relationships. At ungauged ME sites and previously unmonitored LU sites, stream discharge was measured as the product of the wetted cross-sectional area and flow velocity. Velocity was measured using an acoustic Doppler velocity (AV) meter. The AV meter was mounted to the invert of the stream channel, and velocity, stage, and instantaneous flow data were transmitted to a data logger/controller on query commands found in the data logger software.

Between 10 and 15 discrete grab samples per storm were collected at approximately 30 to 60 min intervals for each site-event based on optimal sampling frequencies in southern California described by Leecaster et al. (2001). Samples were collected more frequently when flow rates were high or rapidly changing and less frequently during low-flow periods. All water samples were collected by one of three methods: 1) by peristaltic pumps with Teflon® tubing and stainless-steel intakes that were fixed at the bottom of the channel or pipe pointed in the upstream direction in an area of undisturbed flow, 2) by direct filling of the sample bottle either by hand or affixed to a pole, or 3) by indirect filling of intermediate bottles for securing large volumes. After collection, the samples were stored in precleaned glass bottles on ice with Teflon®-lined caps until they were shipped to the laboratory for analysis.

Chemical Analysis

Total Suspended Solids (TSS)

Total suspended solids (TSS) were analyzed by filtering a 10-mL to 100-mL aliquot of storm water through a tared 1.2-µm Whatman GF/C filter. The filters plus the solids were dried at 60°C for 24 h, cooled, and weighed.

Trace Metal Analysis

Whole samples (particulate plus dissolved) were prepared by nitric acid digestion followed by analysis using inductively coupled plasma-mass spectroscopy (ICPMS) according to USEPA Method 200.8 (US EPA 1991). Target analyses included aluminum, antimony, arsenic, beryllium, cadmium, chromium, copper, iron, lead, mercury, nickel, selenium, silver, and zinc. Quality assurance measurements indicated that all laboratory blanks were below method detection limits with duplicate samples within 10% reproducible difference.

Polycyclic Aromatic Hydrocarbon (PAHs) Analysis

Total PAH (∑PAH) was computed as the sum of the 26 individual PAH compounds quantified (Table 2-2). The individual PAHs were divided into low-molecular-weight (LMW) PAH compounds (<230, two to three rings) and high-molecular weight (HMW) PAH compounds (>230, four to six rings) for source analysis. The 26 specific PAHs were extracted, separated, and quantified by capillary gas chromatography coupled to mass spectrometry according to U.S. Environmental Protection Agency (US EPA) method 625 (US EPA 1991).

Fecal Indicator Bacteria Analysis

Concentrations of *E. coli* and *Enterococcus* spp.were measured by defined substrate technology using kits supplied by IDEXX Laboratories, Inc. (Westbrook, ME) according to the manufacturer's instructions. Briefly, 10-fold and 100-fold dilutions of the water samples were made with deionized water containing the appropriate media and sodium thiosulfate, mixed to dissolve, dispensed into trays (Quanti-Tray/2000), and heat sealed. *E. coli* was measured using the Colilert-18 reagents, while *Enterococcus* spp. were measured using Enterolert reagents. Samples were incubated overnight according to the manufacturer's instructions and inspected for positive wells. Conversion of positive wells from these tests to a most probable number (MPN) was done following Hurley and Roscoe (1983).

Data Analysis

Data analyses was broken into three sections; 1) comparison between LU sites; 2) comparison between developed and undeveloped watershed; and 3) assessment of within-season and within-storm variability. Comparison between LU sites focused on event mean concentrations (EMCs), load, flux, and principle components analysis (PCA). Prior to analysis constituent concentrations were log-transformed to improve normality. In all cases, non-detectable results were assigned a value of 1/2 the minimum detection limit, based on the inability to log transform a value of zero.

The EMC was calculated using Equation 1:

$$EMC = \frac{\sum_{i=1}^{n} C_i * F_i}{\sum_{i=1}^{n} F_i} \qquad \text{(Equation 1)}$$

where: EMC = flow-weighted mean for a particular storm; C_i = individual runoff sample concentration of ith sample; F_i = instantaneous flow at the time of ith sample; and n = number of samples per event. Constituent concentrations were log-transformed prior to calculations to improve normality. In all cases, non-detectable results were assigned a value of one-half the minimum detection limit, based on the inability to log transform a value of zero. Mass loading was calculated as the product of the EMC and the storm volume. Flux estimates facilitated loading comparisons among watersheds of varying sizes. Flux was calculated as the ratio of the mass load per storm and watershed area. Differences in concentration or flux between LU sites were tested using a one-way ANOVA, with a significance level (p) <0.05 , followed by Tukey-Kramer *post-hoc* test for multiple comparisons (Sokal and Rohlf 1969).

The PCA was used to identify the most important factors (i.e., groups of parameters, storm size and storm season) controlling data variability (Helene *et al*. 2000, SAS Inc. 2003, http://www.sas.com/textbook). As a multivariate data analysis technique, PCA reduces the number of dependent variables without sacrificing critical information (Qian et al. 1994). The number of principal components (PCs) extracted (to explain the underlying data structure) was defined by using the Kaiser criterion (Kaiser 1960) where only the PCs with eigenvalues greater than unity are retained. Scores derived from the PCA were plotted along the first two PC axes and examined visually for relationships that differentiate constituent concentrations among subclasses (e.g., land use types). PCA and ANOVA were used in a two-step process: The PCA was used to identify factors influencing variability and to group data into different sets based on the factors identified. ANOVA was then used to test for significant differences between the classes identified by the PCA.

The second analysis that compared developed and undeveloped ME sites followed similar approach as the LU sites focusing on EMCs, load, and flux. Differences between watershed types were determined using ANOVA.

The third analysis bifurcated into two approaches. The first compared seasonal patterns of total metal loading by plotting mass emissions against storm season (early = October to December, mid = January to March, and late = April to May) and cumulative annual rainfall. For this analysis, all ME sites were analyzed as a group to examine differences between early- and late-season storms across the sampling region.using ANOVA. The second approach compared flow and constituent concentration within-storm events. This comparison examined the time-concentration series relative to the hydrograph plots using a pollutograph. A first flush in concentration from individual ME storm events was defined as a circumstance when the peak in concentration preceded the peak in flow. This was quantified using cumulative loading plots in which cumulative mass emission was plotted against cumulative discharge volume during a single storm event (Bertrand-Krajewski et al. 1998). When these curves are close to unity, mass emission is a function of flow discharge. A strong first flush was defined as ≥75% of the mass was discharged in the first 25% of runoff volume. A moderate first flush was defined as ≥30% and ≤75% of the mass discharged in the first 25% of runoff volume. No first flush was assumed when ≤30% of the mass was discharged in the first 25% of runoff volume.

Further Analyses for Individual Constituents

Total Metal Comparison to The California Toxics Rules (CTR)

In order to investigate the percent of samples exceeding water quality standards total metals concentrations were compared to the California Toxics Rules (CTR) for inland surface waters (acute freshwater aquatic life protection standards, US EPA 2000). The standards for total copper, total lead and total zinc are 14.00, 81.65, and 119.82 respectively based on a hardness value of 100 mg/L.

The formula for calculating the acute objectives for copper, lead, and zinc in the CTR take the form of the following equation:

CMC = WER * ACF * exp[(ma)(ln(hardness)+ba]

Where: WER = Water Effects Ratio (assumed to be 1), ACF = Acute conversion factor (to convert from the total to the dissolved fraction), m_A = slope factor for acute criteria, and b_A = y intercept for acute criteria.

The CTR allows for the adjustment of criteria through the use of a water-effect ratio (WER) to assure that the metals criteria are appropriate for the site-specific chemical conditions under which they are applied. A WER represents the correlation between metals that are measured and metals that are biologically available and toxic. A WER is a measure of the toxicity of a material in site water divided by the toxicity of the same material in laboratory dilution water. No site-specific WER has been developed for any of the waterbodies in the Los Angeles River or San Gabriel River watersheds. Therefore, a WER default value of 1.0 was assumed. The coefficients needed for the calculation of objectives are provided in the CTR for most metals.

PAH Source Identification

PAHs were also analyzed to examine sources of PAHs. First, the FWM concentrations from the homogenous land use sites were compared. Differences between land use sites were investigated using a one-way analysis of variance (ANOVA) with a $p < 0.05$ significance level (Sokal and Rohlf 1969). Next, the relative proportion of individual PAH compounds and their ratios were evaluated to determine if the sources of PAHs suggested a pyrogenic (i.e., combustion by-products) or petrogenic (i.e., unburnt petroleum) signature. The ratio of fluoranthene (F) to pyrene (P; F/P) and the ratio of phenanthrene (P) to anthracene (A; P/A) were used to determine pyrogenic versus petrogenic sources of PAH. Pyrogenic sources predominate when F/P ratios approach 0.9 (Maher and Aislabe 1992). Pyrogenic sources predominate when P/A ratios ranged from 3 to 26 (Lake et al. 1979, Gschwend and Hites 1981).

Correlations between TSS, Flow and FIB

To explore the potential link between storm water runoff, TSS and FIB concentrations Spearman Rank correlation coefficients (a nonparametric measure of correlation) were computed between FIB, TSS and stream flow (Townsend 2002).

Cumulative Density Frequency Plots (CDFs) - Bacteria

Fecal indicator bacteria were used to assess whether storm water samples met State of California water quality thresholds by examining the relative frequency of exceedence for all storms combined at both ME and LU sites. Fecal indicator bacterial counts were plotted on logarithmic scales (a scale that minimizes differences and allows widely varying numbers to be shown) and compared to the CA single-sample criterion to estimate percent exceedances. The CA single-sample standard (assembly bill AB411) for ocean beaches has limits of 104 *Enterococcus* spp. bacteria in 100 ml of water, 400 *E. coli* colonies (400 MPN/100ml) and 10,000 total coliforms colonies (10,000 MPN/100mL). Cumulative density frequency plots (CDFs) were produced to compare observed bacterial concentrations to the CA quantitative standards and to calculate accumulated relative exceedance percentages.

An additional data analysis element examined the incidence of exceedences of California's AB411 water quality standards for fecal indicator bacteria compared to the size of the watershed. Watersheds were broken into small (<25 km^2), medium (20 km^2 - 99 km^2), and large (>100 km^2), with at least three watersheds falling into each category.

Constituent Comparison to Nationwide Results

Existing data sets provide insight into land use based loading, but do not provide the mechanistic understanding needed by storm water managers. Between 1977 and 1983 the U.S. EPA funded The Nationwide Urban Runoff Program (NURP), which compiled discharge data from separate storm sewers in different land uses to receiving waters. This project used 81 sites in 28 cities throughout the U.S. and included the monitoring of approximately 2,300 individual storm events (US EPA 1983a). The utility of the NURP data set is somewhat limited because it is 23 years old and only contains data from storm drains (vs. in-river measures). The National Storm water Quality Database (NSQD) was created in 2003 by the University of Alabama and the Center for Watershed Protection to examine more recent storm water data from a

representative number of National Pollutant Discharge Elimination System (NPDES) municipal separate storm sewer system (MS4) storm water permit holders (Pitt et al. 2003). The NSQD includes Phase I storm water monitoring data from 369 stations from 17 states and 9 rain zones and a total of 3,770 individual storm events between 1992 and 2003. Unfortunately, the NSQD does not contain any samples from the arid west. Furthermore, neither the NURP nor the NSQD provides time variable measurements that provide an understanding of the temporal processes that affect storm water loading.

Table 2-1a. Summary of storm events sampled at mass emission sites during 2001-2005 in Los Angeles, CA, USA.

Mass Emission Sites	Date of Storm Event	Size (km^2)	Rainfall (cm)	Antecedant Dry Days	Mean Flow (cm/s)	Peak Flow (cm/s)
Los Angeles River Developed Watersheds						
LA River above Arroyo Seco	1/26 - 1/27/2001	1460	1.80	1	27.3	114.0
	2/9 - 2/11/2001		1.42	1	22.4	165.2
	2/12 - 2/13/2001		9.68	0	62.6	262.5
LA River at Wardlow	1/26 - 1/27/2001	2161	1.80	1	15.0	50.9
	2/9 - 2/11/2001		1.42	1	1.4	6.0
	5/2 - 5/3/2003		3.56	4	209.9	756.7
	2/2 -2/3/2004		1.14	6	90.4	375.6
Verdugo Wash	1/26 - 1/27/2001	65	1.80	1	15.0	50.9
	2/9 - 2/11/2001		1.42	1	13.9	90.2
	11/12 - 11/13/2001		9.68	0	68.5	368.2
	10/31 - 11/1/2003		1.74	30	56.5	155.0
Arroyo Seco	2/9 - 2/11/2001	130	3.56	12	2.9	13.5
	4/7/2001		1.78	30	7.8	21.8
Ballona Creek	2/18 - 2/19/2001	338	1.50	3	38.1	107.0
	4/7/2001		1.24	31	32.6	100.9
	11/24 - 11/25/2001		1.52	11	53.1	396.2
	5/2 - 5/3/2003		2.03	4	52.8	134.4
	10/31 - 11/1/2003		2.03	30	62.0	148.1
	2/2 -2/3/2004		2.21	29	55.0	213.9
	2/21 -2/22/2004		3.41	18	44.8	95.6
Dominguez Channel	3/17 - 3/18/2002	187	0.28	10	4.8	14.0
	2/21 -2/22/2004		1.52	18	14.7	35.5
Undeveloped Watersheds						
Santa Monica Canyon	2/9 - 2/11/2001	41	3.74	1	0.1	1.1
	4/7/2001		3.05	50	0.6	3.0
Open Space Arroyo Sequit	5/2 - 5/3/2003	31	5.03	3	0.0	0.0
	2/25 -2/26/2004		4.12	1	3.4	21.9
	12/27 -12/28/2004		5.05	17	0.0	0.2
	1/7/05		5.54	2	0.3	0.9

Table 2-1b. Summary of storm events sampled at land use sites in Los Angeles, California USA during 2000/01-2004/05 storm seasons.

Land-use Type	Date of Storm Event	Size (km^2)	Rainfall (cm)	Antecedant Dry Days	Mean Flow (cm/s)	Peak Flow (cm/s)
High Density Residential (#1)	2/9 - 2/11/2001	0.52	1.93	2	0.082	0.563
	2/18 - 2/19/2001		0.61	4	0.060	0.233
High Density Residential (#2)	3/17 - 3/18/2002	0.02	0.20	10	0.000	0.003
	2/17/2002		0.89	19	0.001	0.006
	2/2 -2/3/2004		1.19	29	0.004	0.025
High Density Residential (#3)	12/28/2004	1.0	3.25	0	0.009	0.080
	2/11/2005		1.35	13	0.004	0.016
Low Density Residential (#1)	2/18 - 2/19/2001	0.98	0.61	4	0.068	0.097
	3/4 - 3/5/2001		1.42	6	0.017	0.071
	2/2 -2/3/2004		2.26	29	0.030	0.143
Low Density Residential (#2)	3/17 - 18/2002	0.18	2.13	19	0.008	0.116
Commercial (#1)	2/17/2002	2.45	0.74	19	0.337	1.340
Commercial (#2)	2/17/2002	NA	0.89	19	0.002	0.008
Commercial (#3)	2/18 - 2/19/2001	0.06	0.81	4	0.003	0.008
	4/7/2001		2.03	31	0.008	0.018
	3/17 - 18/2002		0.12	9	0.000	0.001
Industrial (#1)	2/9 - 2/11/2001	2.77	0.81	14	0.253	1.801
	2/18 - 2/19/2001		0.41	3	0.205	0.774
	3/17 - 18/2002		0.25	27	0.000	0.003
Industrial (#2)	2/17/2002	0.001	0.74	19	0.000	0.002
Industrial (#3)	4/7/2001	0.004	2.06	25	0.008	0.017
Industrial (#4)	3/15/2003	0.01	4.50	10	0.117	0.375
Agricultural (#1)	2/18 - 2/19/2001	0.98	0.81	5	0.014	0.042
	3/4 - 3/5/2001		8.13	3	0.021	0.053
	3/17 - 3/18/2002		0.23	9	0.012	0.031
	2/2 -2/3/2004		1.17	29	0.023	0.128
Agricultural (#2)	4/7/2001	0.8	2.06	25	1.723	3.801
Recreational (horse)	2/18 - 2/19/2001	0.03	0.61	4	0.015	0.044
	3/4 - 3/5/2001		1.42	6	0.003	0.014
Transportation (#1)	4/7/2001	0.01	3.05	25	0.022	0.057
Transportation (#2)	2/17/2002	0.002	0.74	19	0.001	0.006
Open Space (#1)	2/24-25/2003	9.49	3.00	11	0.160	0.360
Open Space (#2)	2/24-25/2003	2.89	2.57	11	0.180	0.680

Table 2-2. List of the 26 individual polycyclic aromatic hydrocarbon compounds measured during the study. Compounds were divided into low-molecular-weight (LMW) compounds (<230, two to three rings) and high-molecular-weight (HMW) compounds (>230, four to six rings) for source analysis.

LMW Compounds	Weight	No. Rings	HMW Compounds	Weight	No. Rings
1-Methylnaphthalene	156+170	2	Benz[a]anthracene	228	4
1-Methylphenanthrene	192+206	3	Benzo[a]pyrene	252	5
2,3,5-Trimethylnaphthalene	155+170	2	Benzo[b]fluoranthene	252	5
2,6-Dimethylnaphthalene	156+170	2	Benzo[e]pyrene	252	5
2-Methylnaphthalene	156+170	2	Benzo[g,h,i]perylene	276	6
2-Methylphenanthrene	192+206	3	Benzo[k]fluoranthene	252	5
Acenaphthene	154	2	Chrysene	228	5
Acenaphthylene	152	3	Dibenz[a,h]anthracene	278	5
Anthracene	178	3	Fluoranthene	202	4
Biphenyl	154	2	Indeno[1,2,3-c,d]pyrene	276	6
Fluorene	166	3	Methylanthracene	222	5
Naphthalene	128	2	Perylene	252	5
Phenanthrene	178	3	Pyrene	202	4

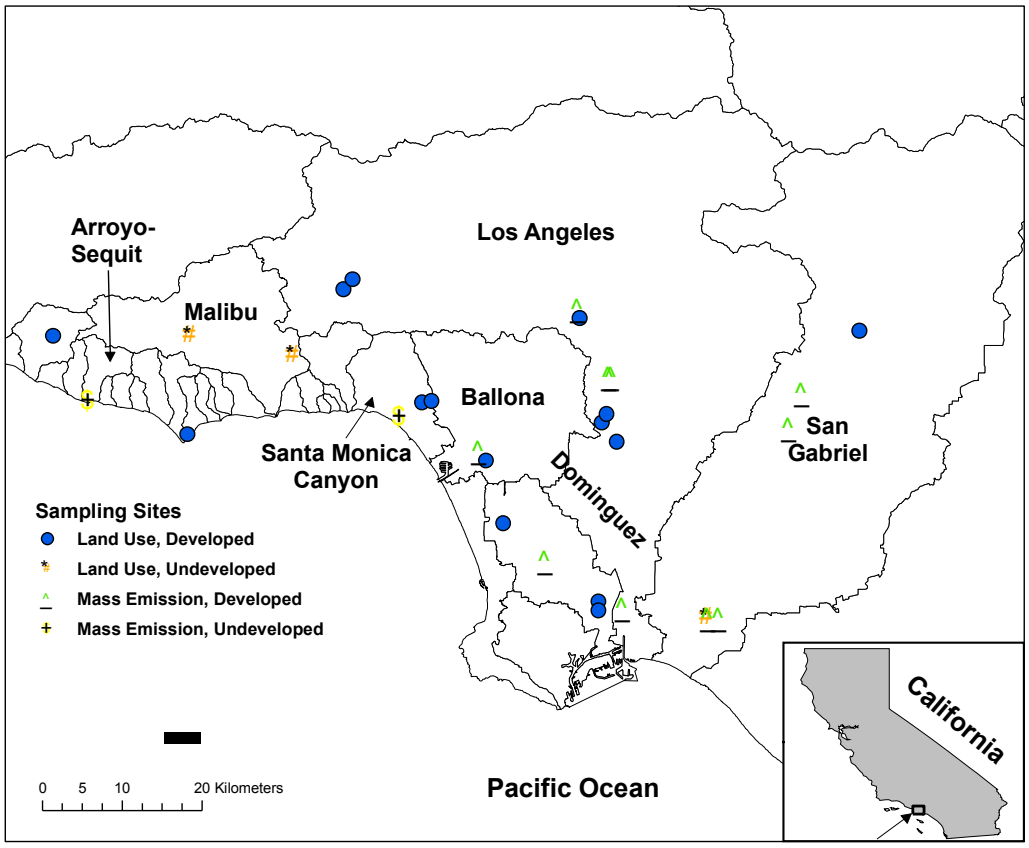

Figure 2-1. Map of watersheds with land use and mass emission sampling sites within the greater Los Angeles region, California USA. Undeveloped >90% open space.

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SECTION 3. TRACE METALS AND TOTAL SUSPENDED SOLIDS

Results

Comparison Between LU Sites

Industrial LU sites contributed a substantially higher flux of copper and zinc compared to the other LU sites evaluated (Figure 3-1). For example, mean total copper flux from the industrial LU was 1,238.0 g/km^2 while mean total copper flux from high density residential and recreational (horse) LU was 100.5 g/km^2 and 190.1 g/km^2, respectively. Trace metal flux from undeveloped LU sites was lower than those observed in developed LUs. For example, mean copper flux at open space LU sites was 23.6 g/km^2. In contrast to copper and zinc, the mean flux of total lead was greatest at agriculture, high density residential, and recreational (horse) LU sites (Figure 3-1). The mean flux of total lead at these three LU sites was at least an order of magnitude greater than any other LU sampled.

Industrial LU had the greatest mean EMC for copper and zinc relative to all other LU sites (Figure 3-2). For example, zinc EMCs at the industrial LU averaged 599.1 µg/l compared to 362.2 µg/l and 207.7 µg/l for commercial and high density residential LU sites, respectively. High density residential had the greatest EMC for lead relative to all other LU sites (Figure 3-2). For example, lead EMCs at high density residential LU averaged 28.4 µg/l compared to 24.1 µg/l and 7.8 µg/l for industrial and agricultural LU sites, respectively. Mean EMCs for all three metals from undeveloped LU sites were lower than those observed in developed LU sites. For example, mean copper, lead, and zinc EMCs from open space LU sites was 7.6 µg/l, 1.2 µg/l, and 23.2 µg/l, respectively.

Both industrial and agricultural LU sites contributed substantially higher fluxes of TSS compared to the other LU sites evaluated (Figure 3-1). For example, mean TSS flux from the industrial and agricultural LU sites were comparable around 3,150.3 kg/km^2 while mean TSS flux from recreational (horse) and high density residential LU was 2,211.1 kg/km^2 and 91.1 kg/km^2, respectively. Mean TSS flux from undeveloped LU sites were comparable to the remaining developed LU sites. For example mean TSS flux from open space LU sites was 513.8 kg/km^2 compared to 160.8 kg/km^2 and 94.0 kg/km^2 for low density residential and commercial LU sites, respectively.

Recreational (horse) LU had the greatest mean TSS EMC compared to all other LU sites. For example, TSS EMCs at the recreational (horse) LU averaged 530.7 mg/l compared to 111.1 mg/l and 92.0 mg/l for agricultural and industrial LU sites, respectively. Mean TSS EMCs from undeveloped LU sites were comparable to those observed in developed agricultural and industrial LU sites. TSS EMCs from open space LU sites averaged 134.8 mg/l.

Results of the PCA indicated that the land use was a predominate source of variability and that land use categories can be grouped based on differences in their intrinsic runoff and loading characteristics (Figure 3-3). Two Principal Components (PCs) had eigenvalues greater than one, with PC1 and PC2 accounting for 63% and 17% of the total variance, respectively. Factor loadings indicated that PC1 and PC2 described concentrations of copper, cadmium, lead, nickel, zinc, and TSS. The two dimensional plot of scores from PC1 and PC2 revealed that

industrial, recreational (horse), and open space LU types were distinct from other LU types based on the concentrations of these constituents. Comparison of the PC scores (or eigenvectors) using a one-way ANOVA indicated that both industrial (group D) and recreational (horse) (group F) sites were significantly different ($p < 0.001$) than open space (group H) sites. All other LU types were indistinguishable.

Comparison Between Developed and Undeveloped Watersheds

The contrasts between the different small, homogeneous LU sites were also apparent at the watershed scale (Figure 3-4). Total copper, total lead and total zinc EMCs and fluxes were significantly greater at ME sites from developed compared to undeveloped watersheds (ANOVA, $p = <0.001$). For the 15 storm events measured, the mean flux of total copper, total lead and total zinc from developed ME watersheds was 0.6, 0.5 and 3.0 kg/km^2 respectively. The mean flux of total copper, total lead and total zinc from undeveloped ME watersheds were 0.06 kg/km^2, 0.01 kg/km^2 and 0.1 kg/km^2 (Figure 3-1), respectively. Furthermore, the higher fluxes from developed ME watersheds were generated by substantially less rainfall than the lower fluxes from the undeveloped ME watersheds (2.8 ± 0.8 cm for storms in developed ME watersheds vs. 4.4 ± 0.8 cm for storms in undeveloped ME watersheds), presumably due to increased impervious surface area in developed watersheds. Similarly, total copper, total lead, and total zinc mean EMC concentrations from developed ME watersheds significantly exceeded those from undeveloped ME watersheds (46.1 ± 14.8 µg/L, 36.3 ± 15.3 µg/L, 251.9 ± 76.9 µg/L vs. 12.6 ± 3.0 µg/L, 2.2 ± 0.8 µg/L, and 27.0 ± 8.4 µg/L, respectively; ANOVA, $p = <0.001$).

The TSS concentrations from less developed ME watersheds were within the same order of magnitude as those from more developed ME watersheds. For example, annual TSS EMCs for developed ME watersheds averaged 246.3 mg/L for Los Angeles River compared to 217.0 mg/L for the undeveloped ME watersheds. However, TSS fluxes were substantially higher for developed ME watersheds. For the 15 storm events measured, mean TSS flux from the developed Los Angeles River and San Gabriel River watersheds were 3,116.8, and 398.8 kg/km^2 respectively, while mean TSS flux from undeveloped watersheds was 62.8 kg/km^2.

Within-Season and Within-Storm Variability

There were significant seasonal differences in total metal loading ($p < 0.001$). Early season storms had significantly higher total metal load than late season storms both within and between watersheds, even when rainfall quantity was similar (Figure 3-5). For example, the two early-season storms from Ballona Creek in water years 2001-2002 and 2003-2004 had total copper loadings that were approximately four times larger (ranging from 154.7 ± 16.0 to 160.8 ± 9.4 kg) than the two storms that occurred at the end of the rainy season (42.6 ± 3.8 to 64.2 ± 4.6 kg), despite the early- and late-season storms resulting from comparable rainfall. The results for total lead and total zinc showed a similar pattern.

Trace metal concentrations varied with time over the course of storm events (Figure 3-6). For all storms sampled, both the highest trace metal concentrations and the peak flow occurred in the early part of a storm event. In all cases, metal concentrations increased rapidly, often preceding peak flow. Concentrations stayed high for relatively short periods and often decreased

back to base levels within one to two hours. In contrast, the undeveloped watershed (Arroyo Sequit; Figure 3-6a) had appreciably lower peak concentrations than the developed watershed (Ballona Creek; Figure 3-6b). Although the pattern of an early peak in concentration was comparable in both undeveloped and developed watersheds, the peak concentration tended to occur later in the storm and persist for a longer duration in the undeveloped watersheds. Due to the small number of storms sampled in undeveloped watersheds, consistency of these patterns is inconclusive.

Cumulative mass loading of all trace metals from ME sites showed little variation over flow implying there was a weak "first flush" effect at these locations (Figure 3-7). In contrast, cumulative mass loading plots for total copper, lead and zinc from LU sites exhibited moderate first flush patterns in the residential, commercial, industrial, agricultural and open space LU categories. When all developed catchments were analyzed together, the magnitude of the first flush effect decreased with increasing watershed size (Figure 3-8). For the developed LU sites that had catchments generally less than 3 km^2 in size, between 30 and 50% of the total copper, total lead and total zinc load was discharged during the first 25% of storm volume. For the ME sites, where runoff was integrated across larger and more diverse landscapes, between 15 and 35% of the total mass of copper, lead, and zinc was discharged during the first 25% of storm volume.

Discussion

Concentrations, flux, and loading in storm water runoff exhibited some key patterns with important implications for managers tasked with controlling trace metals. First, the magnitude of trace metal concentrations and loads were higher at industrial land uses than other land use types. High pollutant loading from industrial sites observed in this study results, at least in part, from intrinsic properties of the industrial land use themselves. These intrinsic properties include high impervious cover (typically greater than 70%) and on-site source generation. Other authors have reported similar results. Sanger et al. (1999) reported that total metal concentrations in runoff from industrial catchments tended to be higher than those from residential and commercial catchments. Park and Stenstrom (2004) used Bayesian networks to estimate pollutant loading from various land uses in southern California and concluded that zinc showed higher EMC values at commercial and industrial land uses. Bannerman et al. 1993 identified industrial land uses as a critical source area in Wisconsin storm water producing significant zinc loads. Bannerman *et al.* 1993 further suggested that targeting best-management practices to 14% of the residential area and 40% of the industrial area could significantly reduce contaminant loads by up to 75%. In this study high density residential sites had considerably higher lead EMCs than low density residential sites. This difference likely results from greater impervious cover and higher source generation at high density residential sites. High density sites typically have greater road surface and more vehicular use, resulting in more lead. In addition, higher impervious cover more effectively conveys accumulated pollutants to streams and creeks. Substantially higher TSS fluxes were also observed at the industrial sites, which may explain the high trace metal concentrations often associated with fine particles. The City of Austin (City of Austin 1990) found lead and zinc EMCs were related to the TSS EMCs. Consequently, controlling TSS at industrial sites may also result in reducing other constituents with the same particle sizes.

A second key conclusion that may affect storm water management is that seasonal flushing was consistently observed at both land use and mass emission sites. This suggests that the magnitude of trace metal loads associated with storm water runoff depends, at least in part, on the amount of time available for build-up on land surfaces. The extended dry period that typically occurs in arid climates such as southern California maximizes the time for trace metals to build-up on land surfaces, resulting in proportionally higher concentrations and loads during initial storms of the season. Similar seasonal patterns were observed for polycyclic aromatic hydrocarbons (PAHs) in the Los Angeles region (Sabin and K. 2004, Stein et al. 2006). Han et al. (2006) also reported that antecedent dry period was the best predictor of the magnitude of pollutant runoff from highways. Other researchers (Anderson and Rounds 2003, Ngoye and Machiwa 2004) have reported corresponding temporal trends for other particle-bound contaminants. This seasonal pattern suggests that focusing management actions on early season storms may provide relatively greater efficiency than distributing lower intensity management actions throughout the season.

A third key conclusion is that trace metal concentrations varied throughout the duration of storm hydrographs. The greatest total metal concentrations occurred at or just before the peak in flow of the storm hydrograph for nearly every storm sampled. This hydrograph/pollutograph pattern was also observed for PAHs in the greater Los Angeles area (Stein et al. 2006). Tiefenthaler et al. (2001) observed similar pollutographs that showed peak suspended-sediment concentrations preceding the peak in discharge for the Santa Ana River. Similar time vs. concentration relationships were observed by Characklis and Wiesner (1997), who reported that the maximum concentrations of zinc, organic carbon and solids coincided with early peak storm water flows. The early occurrence of peak concentrations indicates that monitoring programs must capture the early portion of storms to generate accurate estimates of EMC and contaminant loading. Programs that do not initiate sampling until a flow threshold has been surpassed may severely underestimate storm EMCs.

Despite a strong and consistent pattern of high metal concentrations early in the storm hydrograph, cumulative mass loading plots exhibited only a moderate first flush of total copper, lead and zinc at the small land use sites and a weak first flush at the larger mass emission sites. Lee et al. (2002) also found that the magnitude of first flush varied by constituent, with metals generally showing the weakest first flush. Furthermore, first flush phenomena were strongest for small catchments and generally decreased with increasing catchment size. Han et al. (2006) reported that first flush characteristics increased with decreasing drainage area size. Characklis and Wiesner (1997) reported that storm water runoff of trace metals from the urban areas of Houston exhibited no discernable first flush effect; however, these measurement were from larger mass emission catchments.

The inverse relationship between first flush and catchment size has several potential mechanistic explanations including relative pervious area, spatial and temporal patterns in rainfall, and pollutant transport through the catchment. Smaller LU catchments have increased impervious area that allows contaminants to be easily washed off relative to larger ME watersheds with less impervious area that requires greater rainfall energy to washoff particles and associated contaminants. In our study, industrial, commercial and high-density residential LU sites were comprised of 72%, 72% and 33% imperviousness, respectively. In contrast, the

larger ME watersheds (>40 km^2) ranged from 32 to 59% impervious area. The undeveloped ME watersheds, which had the least within storm variability, were comprised of only 1% imperviousness. Pitt (1987) also found a first flush on relatively small paved areas that he associated with washoff of the most available material.

A corollary to the relationship between imperviousness and catchment size is travel time. Travel time becomes a factor because contaminants are rapidly delivered to the point of discharge within smaller, more impervious catchments relative to larger, less impervious catchments. In our study, the time of travel in the larger ME watersheds like Ballona Creek or Los Angeles River was estimated in hours while travel times in the small LU catchments was minutes. As a result, not all first flush in smaller catchments upstream arrive at a ME site at the same time, effectively diluting short peaks in concentration. Hence, the different times of concentration (i.e., travel times) from various portions of the watershed may obscure first flush patterns at larger mass emission sites.

Spatial and/or temporal differences in rainfall further complicate first flush in large watersheds. Adams and Papa (2000) and Deletic (1998) both concluded that the presence of a first flush depends on numerous site and rainfall characteristics. In smaller catchments, rainfall distribution is more uniform compared to larger watersheds. When rainfall is distributed uniformly, then particles and associated pollutants are potentially washed off at the same time. In larger catchments, rainfall lags between various parts of the watershed may take hours and rainfall quantity and/or duration may not be similar between subwatersheds. Ackerman and Weisberg (2003) quantified rainfall temporal and spatial variability and determined that these factors were an important consideration in hydrologic inputs to the coastal ocean of southern California. Ultimately, the differences in first flush, whether they were due to imperviousness, travel time, or rainfall variability, suggest that management strategies at most moderate to large catchments should focus on more than just the initial portion of the storm if the goal is to capture a majority of metals load.

Urban storm water runoff from this study appeared worthy of management concern because it represented a large mass emission source that frequently exceeded water quality criteria (Table 3-1). Cumulatively, the annual average loading of total copper, lead, and zinc from the Los Angeles River, Ballona Creek, and Dominguez Channel exceeded the mass emissions from industrial point sources such as power generating stations and oil refineries by orders of magnitude. Annual storm water loading from these three watersheds also rivaled, or exceeded, trace metal emissions from point sources such as publicly owned treatment works. One significant difference between these point sources and urban storm water is that southern California has a completely separate sanitary sewer collection system and urban storm water receives no treatment prior to discharge into estuaries or the coastal ocean. Assuming a hardness of 100 mg/L and that 15% of the total metals in storm water occur in the dissolved fraction (Young *et al.* 1980), storm water concentrations of copper and zinc exceeded California Toxic Rule (US EPA 2000) water quality criteria in more than 80% of the wet weather samples collected at mass emission sites. This was partly due to industrial LU sites where 100% and 87% of runoff samples exceeded water quality criteria for zinc and copper, respectively. Commercial LU sites exceeded water quality criteria in 79% and 72% of its runoff samples, respectively. Only 8% to 9% of the runoff samples exceeded the water quality criterion for lead at commercial

or industrial LU sites. Hall and Anderson (1988) concluded that industrial and commercial land use sites were the major source of trace metals most often considered toxic to aquatic invertebrates, with runoff from the commercial sites proving most frequently toxic to the test organism.

The focus on LU sites in this study enabled the comparison of median EMCs with data sets collected from other parts of the nation (Table 3-2). All of the median EMCs for total copper at LU sites from Los Angeles were greater than, or equal to, median EMCs at LU sites reported in the NSQD (Pitt et al. 2004). With the exception of the open LU, all of the median EMCs for zinc were greater at LU sites in Los Angeles than median EMCs at LU sites reported in the NSQD. In contrast, all of the median EMCs for lead were lower at LU sites in Los Angeles than median EMCs at LU sites reported in the NSQD. Of the 15 LU – EMC combinations, all but one of the median EMCs (industrial zinc) were lower in Los Angeles than median EMCs reported by NURP (US EPA 1983; Table 3-2). Unlike the NSQD that was focused on data from the 1990's, NURP data was collected during the 1970's. Therefore, the differences between median EMCs from NURP and median EMCs from Los Angeles were also a function of time. Certainly this factor affected median EMCs for lead, which was phased out of gasoline in the mid-1980s (Marsh and Siccama 1997, Hunt et al. 2005).

Further research is needed to directly assess the relationship between trace metal concentrations and particle-size distributions in storm water runoff from mass emission and land use sites to better understand the fate, transport and treatment of trace metals in urban runoff. Storm water borne trace metals are typically associated with particulates to varying degrees depending on the metal and the size distribution of suspended solids in the storm water runoff. Furthermore, the particle size distribution, and metal partitioning can change over the course of a storm event (Furumai et al. 2002). Understanding the dynamic partitioning of trace metals to various size particles is important to being able to estimate temporal and spatial patterns of trace metal deposition in estuaries and harbors, and should be an area of future investigation. Our understanding of the mechanisms of metal loading from urban land uses could also be improved by estimating the percent of directly connected impervious area (DCIA) in each land use category (i.e., percent rooftop, sidewalks, paved driveways and streets) and its impacts on storm water runoff concentrations and loads. This could allow identification of critical source areas, which in turn could allow for more focused application of best management practices.

Table 3-1. Mean annual (± 95% confidence intervals) trace metal loading from different sources (mt = metric tons).

	Mean Annual Load / Year (mt ± 95% CI)		
	Total Copper	Total Lead	Total Zinc
Point Source Data[1,2] (2000-2005)			
Large Publicly Owned Treatment Plants (POTWs)	10.9 ± 6.8	0.8 ± 0.8	13.9 ± 7.6
Low Volume Waste Power Generating Stations (PGS)	0.01	0.00	0.09
Wet Weather Runoff (2000-2005) [3]			
Los Angeles River	1.6 ± 1.2	1.4 ± 1.5	9.8 ± 9.4
Ballona Creek	0.7 ± 0.4	0.6 ± 0.3	4.3 ± 2.5
Dominguez Channel	0.4 ± 2.4	0.2 ± 1.1	2.1 ± 11.0
Total Annual Wet Weather Runoff	**2.7 ± 4.0**	**2.2 ± 2.9**	**16.2 ± 22.9**

[1]SCCWRP Biennial Report 2004-06 (Lyons G, Stein E).
[2]SCCWRP Biennial Report 2003-04 (Steinberger A, Stein E); PGS data represents year 2000 only.
[3]This study

Table 3-2. Comparison of Nationwide Urban Runoff Program (NURP) and National Storm water Quality Database to trace metals concentrations from specific land uses in the Los Angeles, California USA region. Median event mean concentration (EMCs) are in µg/L .

	Constituent Median EMC (µg/L)		
Land use Type	Total Copper	Total Lead	Total Zinc
Overall			
LARW[1]	20	9	151
NSQD[2]	16	16	116
NURP[3]	34	144	160
Residential			
LARW[1]	18	8	103
NSQD[2]	12	12	73
NURP[3]	33	144	135
Commercial			
LARW[1]	17	4	156
NSQD[2]	17	18	150
NURP[3]	29	104	226
Industrial			
LARW[1]	33	19	550
NSQD[2]	22	25	210
NURP[3]	27	114	154
Open Space			
LARW[1]	8	1	23
NSQD[2]	5	5	39
NURP[3]	NA[4]	30	195

[1]2001-2005 This Study
[2]The National Storm water Quality Database (NSDQ), Pitt et al. (2003)
[3]Nationwide Urban Runoff Program (USEPA 1983a)
[4]NA = Not analyzed

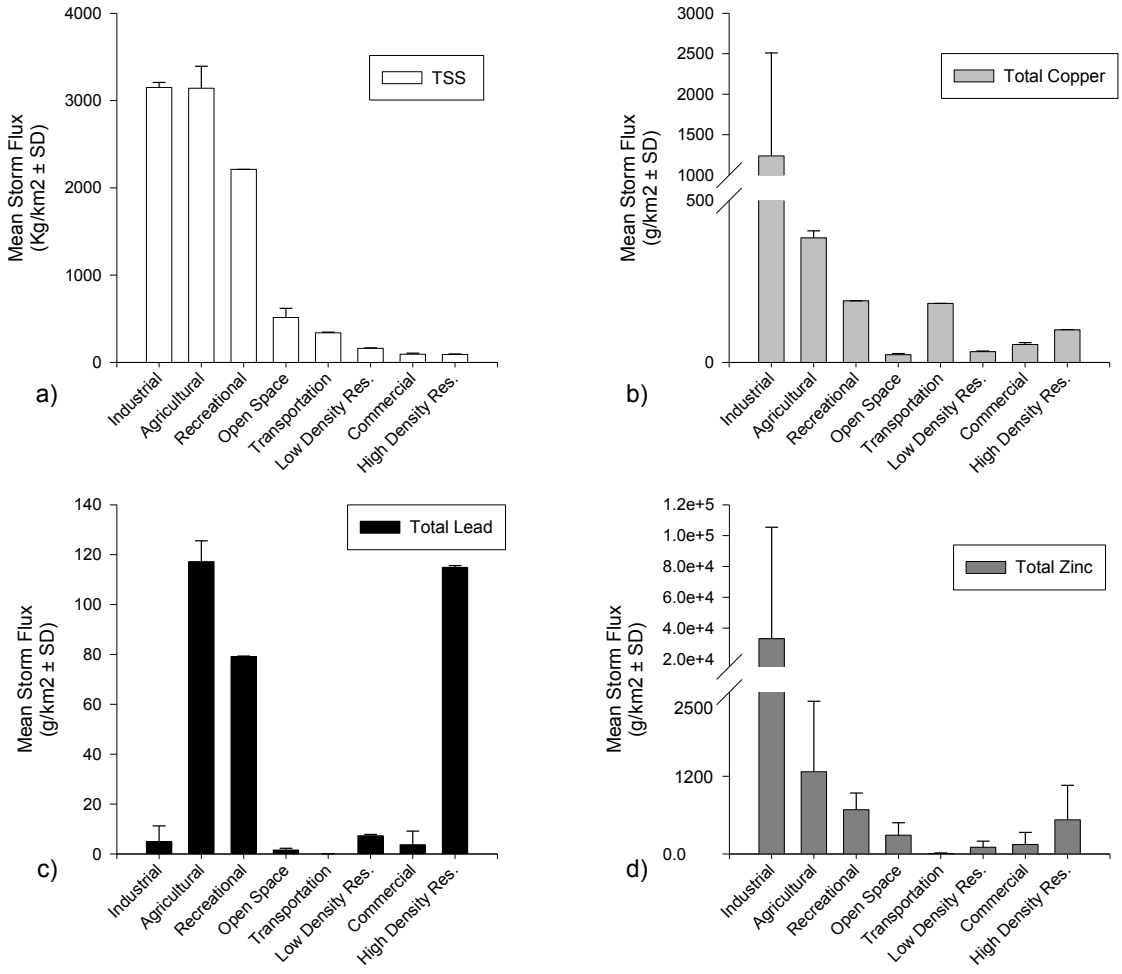

Figure 3-1. Mean storm flux of total suspended solids (TSS; a), total copper b), total lead (c), and total zinc (d) at land use sites during 2000/01-2004/05 storm seasons. Standard deviation (SD).

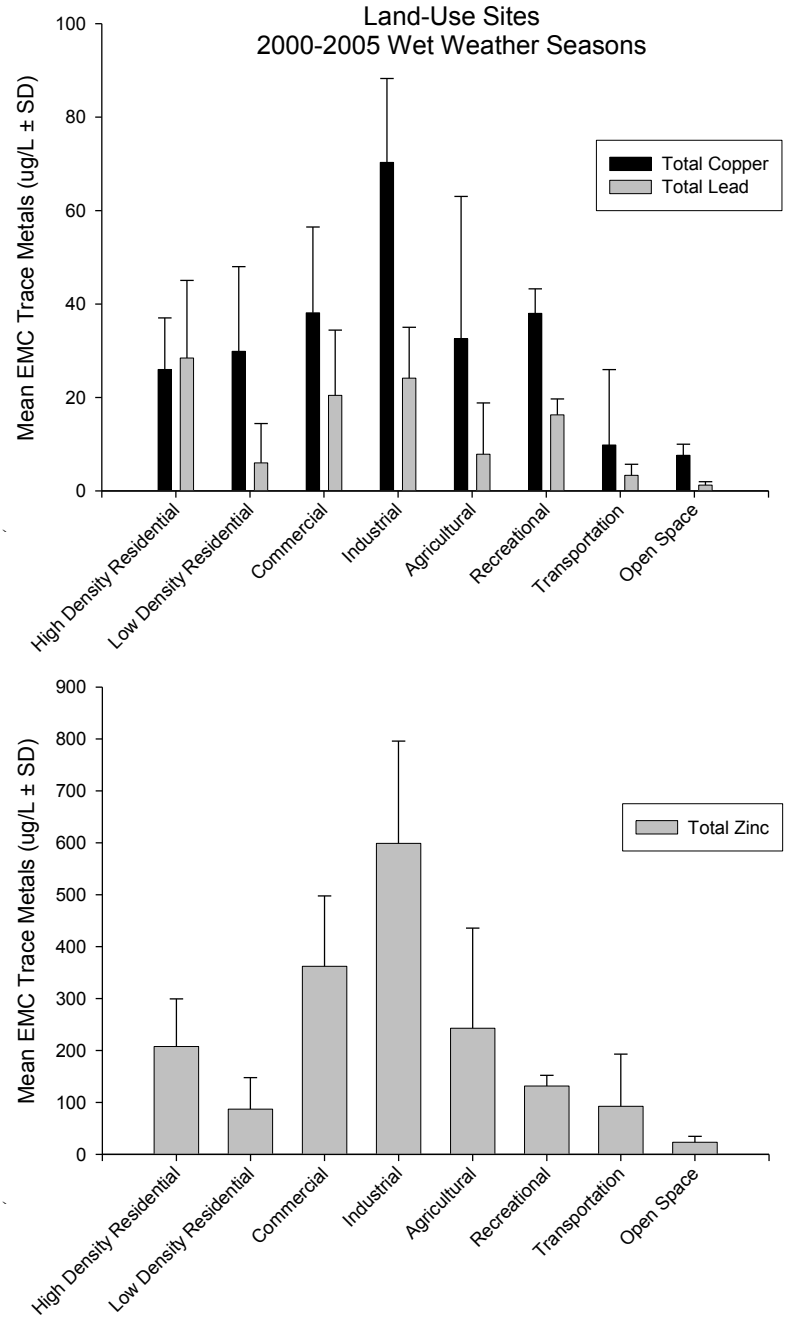

Figure 3-2. Mean storm EMCs of total copper and total lead (top) and total zinc (bottom) at specific land use sites during the 2000/01-2004/05 storm seasons. Standard deviation (SD).

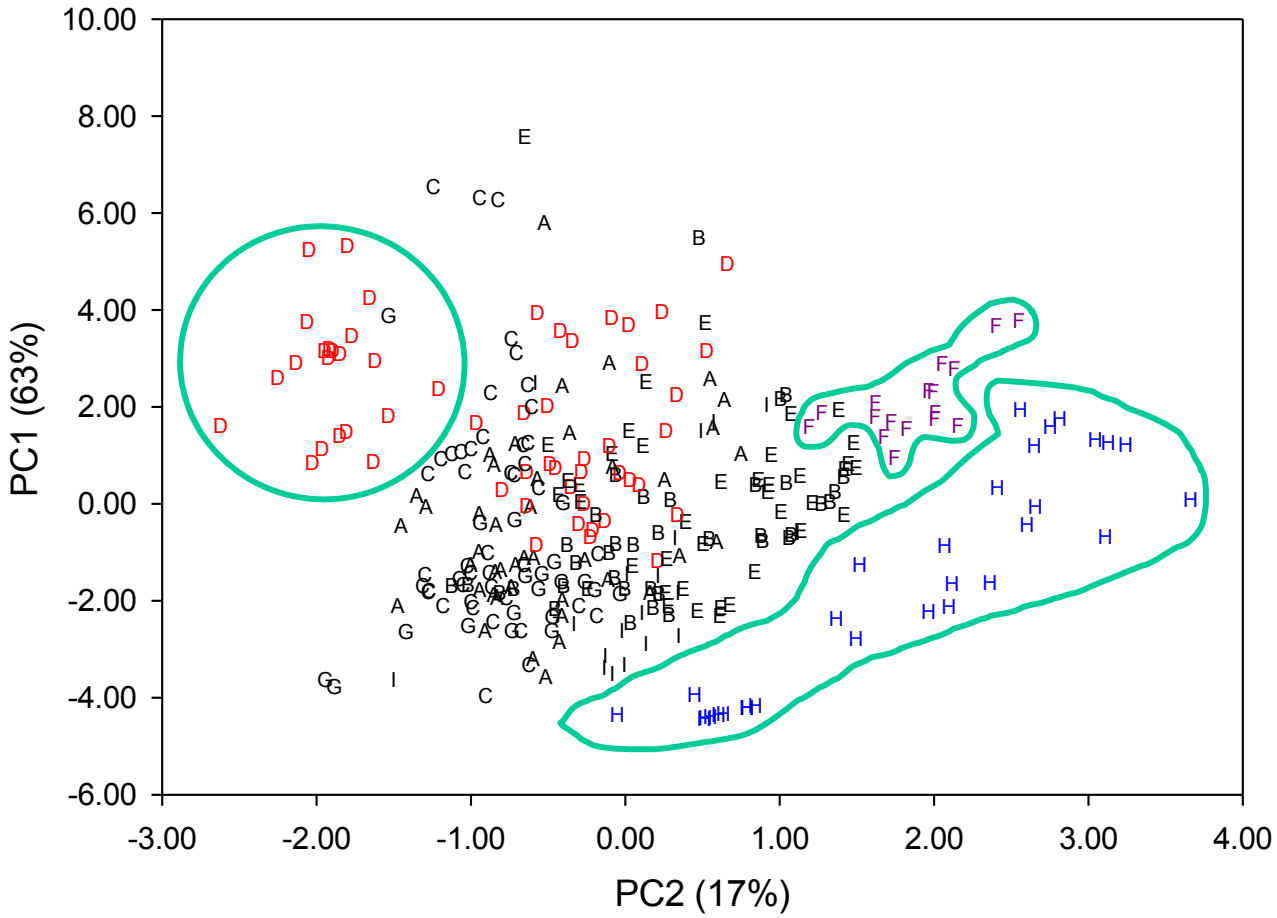

Figure 3-3. Plot of two principle components explaining 63% (y axis) and 17% (x axis) of the variation between trace metal concentrations at land use sites during 2000/01–2004/05 storm seasons. High density residential-Los Angeles River (A), Low density residential(B), C ommercial (C), Industrial(I) , Agricultural(E), Recreation (horse; F), Transportation(G), Open space(H), and High density residential-San Gabriel River (I).

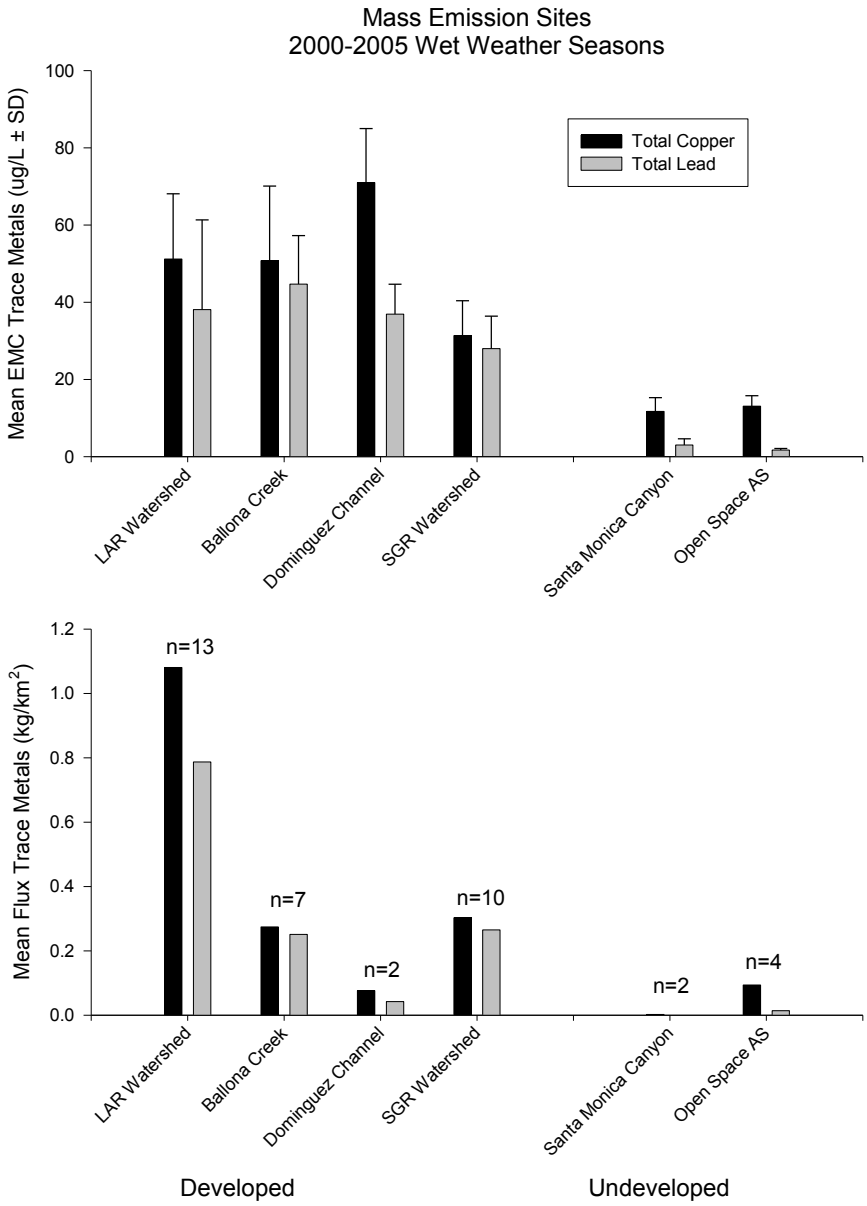

Figure 3-4. Average event mean concentrations (EMCs; top) and fluxes (bottom) of total copper and lead during the 2000/01 to 2004/05 storm seasons. Los Angeles River (LAR), San Gabriel River (SGR) and Arroyo Sequit (AS), number of storm events (n), and standard deviation (SD).

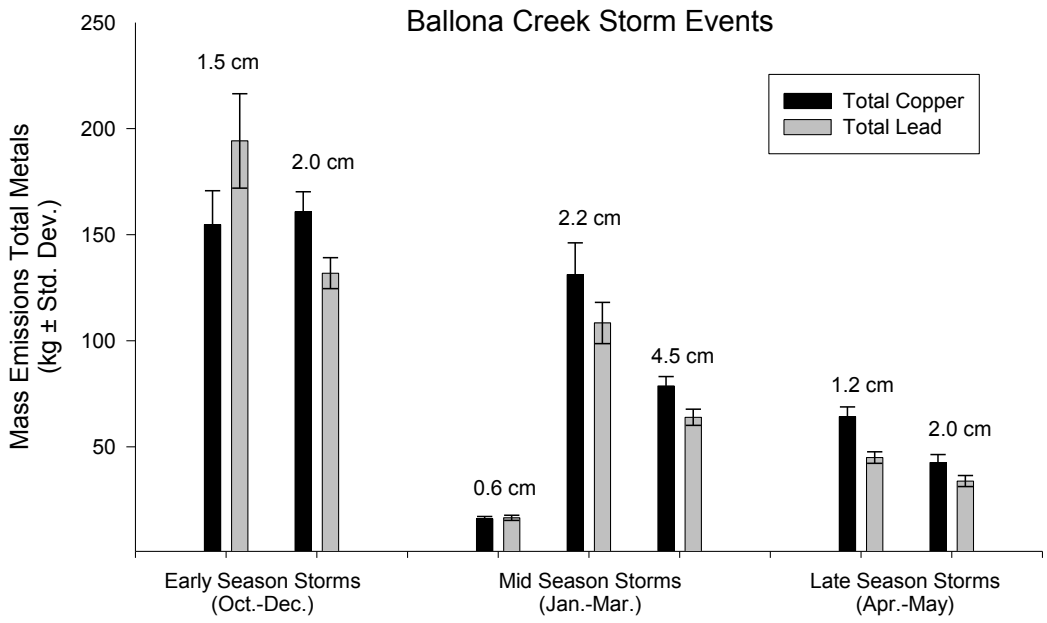

Figure 3-5. Metals loadings from early, mid, and late season storms in Ballona Creek during 2000/01 – 2004/05 storm seasons for total copper and total lead. The numbers above the bars in the graph indicate total event rainfall.

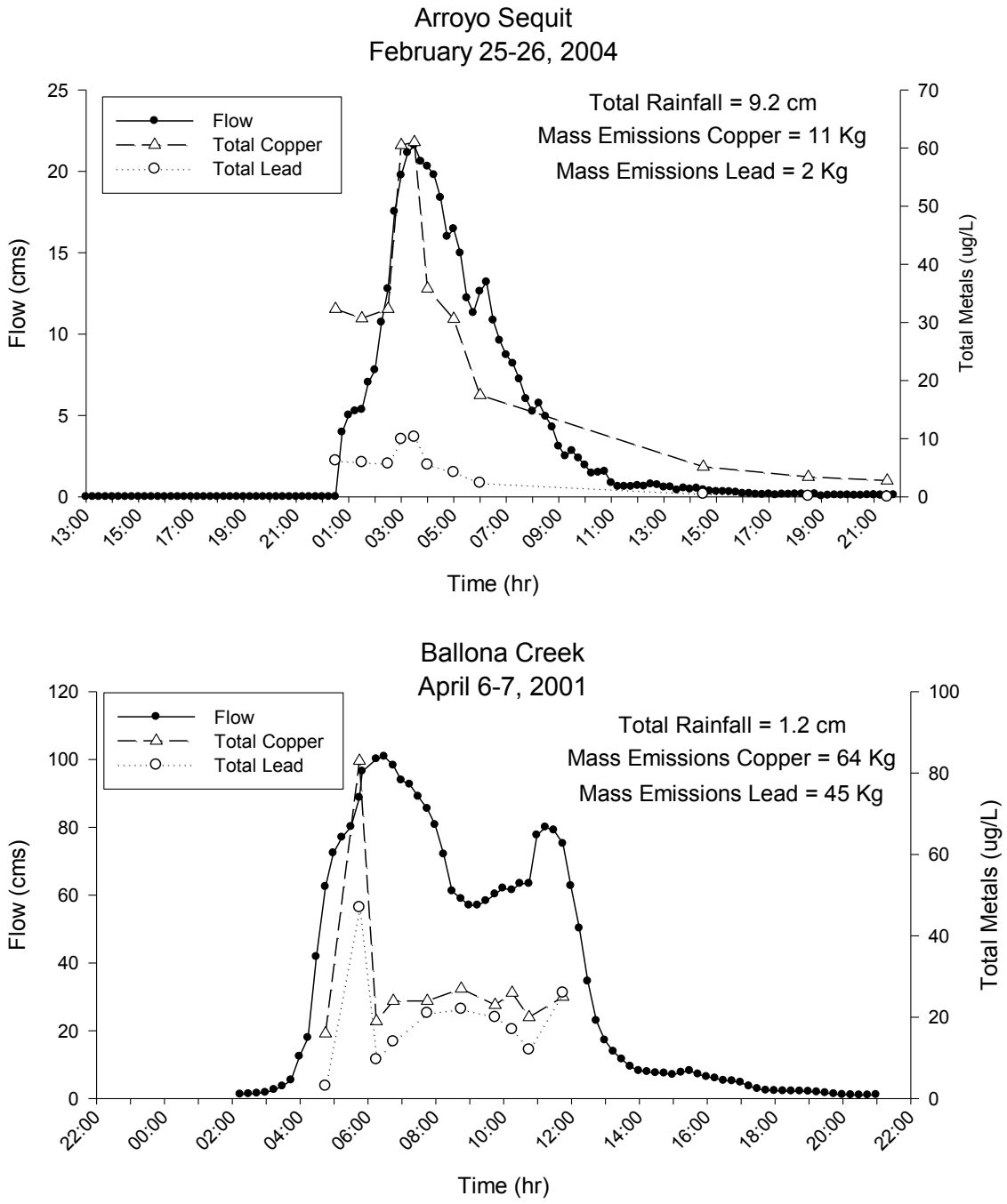

Figure 3-6. Variation in total copper and lead concentrations with time for a storm event in the undeveloped Arroyo Sequit watershed (top) and developed Ballona Creek watershed (bottom).

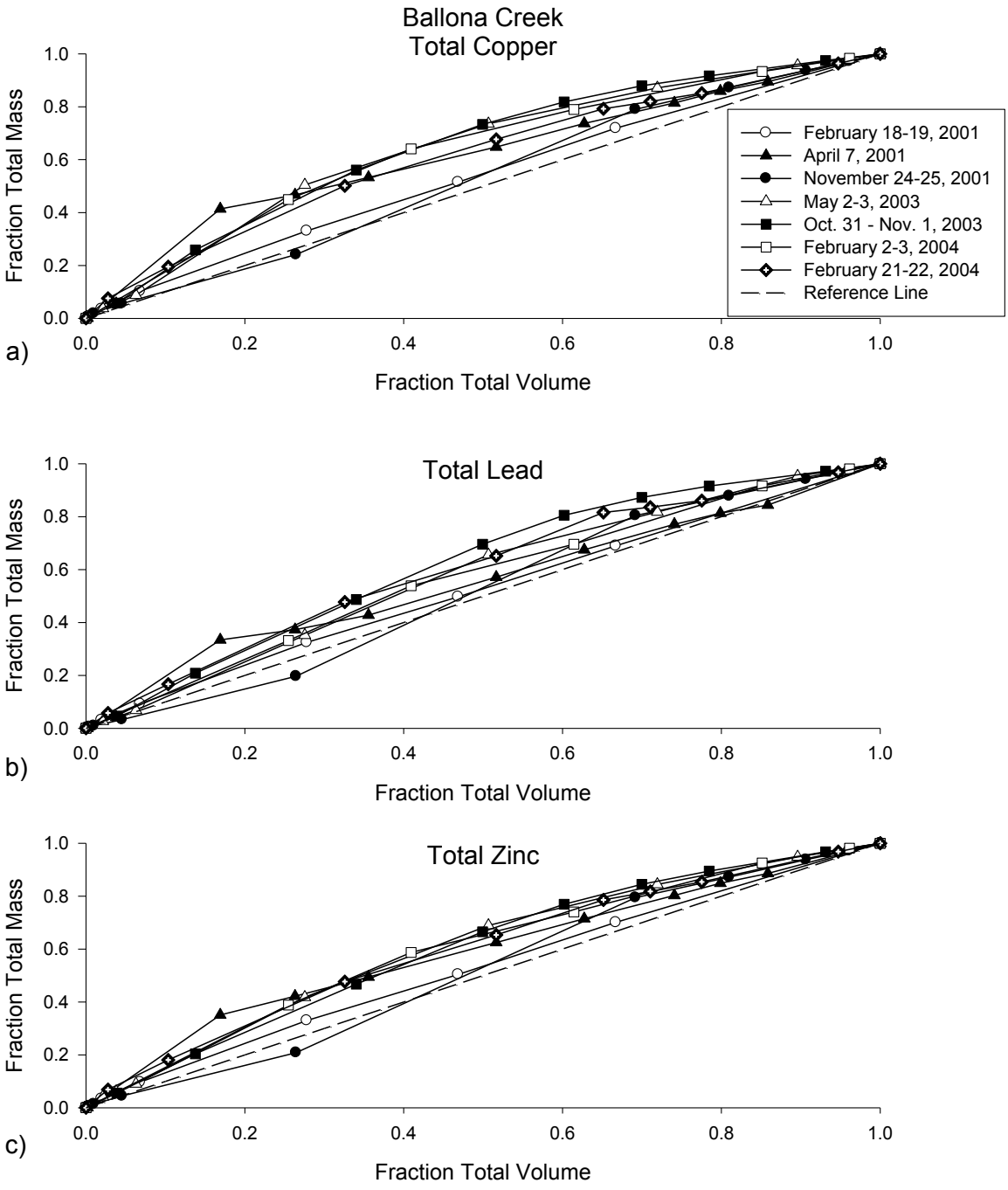

Figure 3-7. Cumulative load duration curves for total copper (a), total lead (b), and total zinc (c) for seven storms in the developed Ballona Creek watershed. Reference line indicates a 1:1 relationship between volume and mass loading. Portions of the curve above the line indicate proportionately higher mass loading per unit volume (i.e., first flush). Portions below the line indicate the reverse pattern.

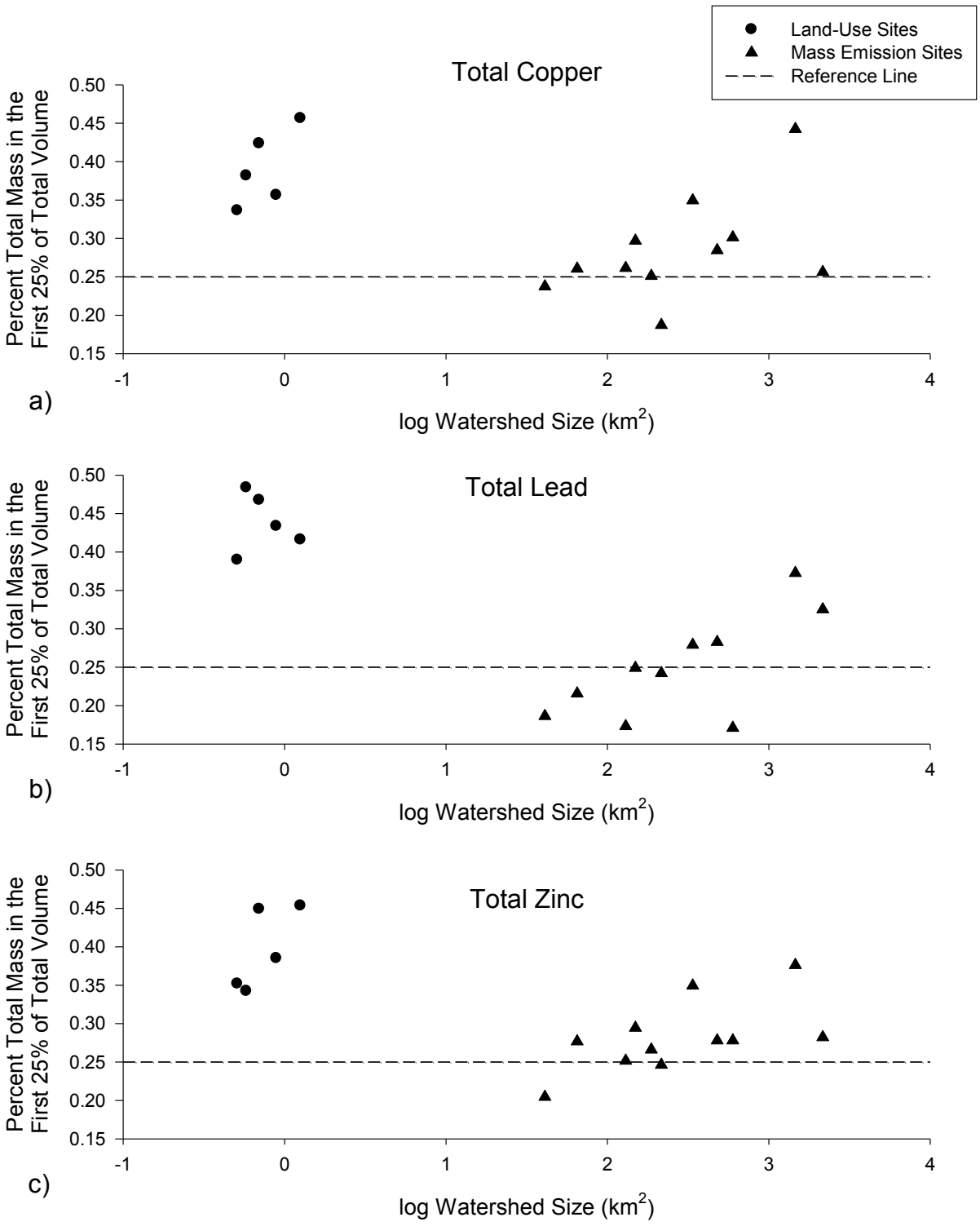

Figure 3-8. First flush patterns of total copper (a), total lead (b), and total zinc (c) in relation to watershed size. Dashed reference line indicates 25% of total mass loading in first 25% of total volume.

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SECTION 4. POLYCYCLIC AROMATIC HYDROCARBONS (PAHS)

Results

PAHs from Developed and Undeveloped Watersheds

In-river total PAH loads, concentrations, and fluxes were higher for developed versus undeveloped watersheds. For the 14 storm events measured, mean PAH load from developed watersheds was 5.6 ± 5.1 kg/storm, while mean load from undeveloped watersheds was 0.03 ± 0.02 kg/storm. Similarly, mean total PAH concentration from developed watersheds exceeded that from undeveloped watersheds (2,655.0 ± 1,768.1 ng/L vs 452.2 ± 444.9 ng/L; Tables 4-1 and 4-2). Flux of PAHs from developed watersheds was 46 times greater than that from undeveloped watersheds (Table 4-1). Mean PAH flux from the developed watersheds was 35.6 ± 69.8 g/km^2 compared to 0.75 ± 0.77 g/km^2 for the undeveloped watersheds. When the anomalously high fluxes from the Dominguez watershed are removed, flux from the developed watersheds was 7.8 ± 8.6 g/km^2, which is still greater than 10 times that of the undeveloped watersheds. Furthermore, the higher fluxes from developed watersheds were generated by substantially less rainfall than the lower fluxes from the undeveloped watersheds (1.85 ± 0.97 cm for storms in developed watersheds vs. 6.11 ± 4.32 cm for storms in undeveloped watersheds).

Annual PAH Loading in Storm water Runoff

The estimated annual output rate of total PAHs in the Los Angeles River watershed during the 2002–2003 water year was around 34.9 kg/year (Table 4-1). During this same period, Ballona Creek had an estimated annual output rate of approximately 20.0 kg/year into Santa Monica Bay. The following water year (2003–2004), the storm-water runoff discharge rate from Ballona Creek increased by a factor of four (72.9 kg/year). For comparative purposes, during the same time period, the Los Angeles River watershed discharged an estimated 150.6 kg/year of total PAHs into Santa Monica Bay. Annual output rates for undeveloped watersheds could not be estimated because those sites are not gauged, and consequently annual storm volumes are not available for estimation of annual PAH loads.

Effect of Rainfall Patterns

Antecedent dry period (expressed as cumulative rainfall) was strongly correlated with total PAH concentration, load, and flux in an exponentially nonlinear manner (r^2 = 0.54–0.81; Figure 4-1). Early-season storms have significantly higher PAH loads than late-season storms both within and between watersheds, even when rainfall quantity is similar. For example, the two early-season storms from Ballona Creek in water years 2002 and 2003 had total PAH loadings that were approximately four times larger (ranging from 7.9–8.3 kg) than the two storms that occurred at the end of the rainy season (1.1–1.8 kg), despite the early- and late-season storms resulting from comparable rainfall. When all watersheds are analyzed together, PAH concentration and load decrease with increasing cumulative rainfall until approximately 10 cm (average annual rainfall is 33 cm), beyond which the effect is markedly less dramatic (Figure 4-1).

PAH Variability Within Storms

The greatest total PAH concentrations occurred during the rising limb of the storm hydrograph for nearly every storm sampled. For example, peak concentrations (2,761 and 2,276 ng/L, respectively) occurred before the peak in flow (757 and 101 cms) in both the Los Angeles River and Ballona Creek (Figure 4-2). In the Los Angeles River example, peak total PAH concentrations occurred almost 8 h before the peak in storm flow. In the Ballona Creek example, a second peak in flow (75 cms) was also preceded by a second peak in total PAH concentration (1,015 ng/L).

Despite a strong and consistent pattern of first flush in concentration, cumulative mass loading plots exhibited only a moderate first flush of PAHs. Between 40% and 60% of the total PAH load was discharged in the first 25% of storm volume for the storms examined in this study. The mass loading plots for Ballona Creek (Figure 4-3) illustrate a consistent pattern of higher mass loading in the early portions of the storm, with a slightly stronger first flush in late-season storms. Land use sites showed a similar pattern of higher mass loading in the early portions of the storm however mid season storms (i.e., January-March) exhibited the strongest first flush.

Potential Sources of PAHs

Sources of PAHs were investigated by comparing concentrations and loads in runoff from homogeneous land uses sites. For all land use sites samples, mean PAH flux was between 0.33 and 140 g/km^2, while FWM concentration was between 4.6E ± 02 and 4.4E ± 03 ng/L (Table 4-3). Despite some apparent differences between land uses (e.g., high-density residential having higher concentrations and industrial having higher flux), no significant differences were observed in either concentration or flux among land use category (p = 0.94 and 0.60, analysis of covariance, with rainfall as a covariate).

The relative proportion of individual PAH compounds can be used to determine the source of PAHs in storm water. The HMW PAHs dominated LMW PAHs in runoff from all storms analyzed, suggesting a pyrogenic source. During the May 2–3, 2003, storm, HMW PAHs in runoff from the Los Angeles River and Ballona Creek accounted for 72% of the total PAH concentrations from these watersheds (Figure 4-4 and Table 4-2). Similarly, HMW PAHs in runoff from the Dominguez channel watershed in Los Angeles County, California, USA, accounted for 74% of the total PAH concentrations from its watershed. Even in the undeveloped Arroyo Sequit watershed, HMW PAHs accounted for 63% of the total PAH concentrations. In all storms and at all sites, the HMW compounds fluoranthene and pyrene were the dominant HMW PAHs. Analysis of the distribution of PAHs within each storm event shows that HMW PAHs are predominant uniformly throughout each storm regardless of land use (Figure 4-5). The exceptions were the industrial oil refinery and the agricultural sites, where the proportions of HMW and LMW PAHs were comparable throughout the storm. In all cases (except the oil refinery and agricultural sites), the relative contribution of LMW PAH compounds averaged 14 to 30% of the total PAH mass. Phenanthrene was the most dominant LMW PAH, comprising 7 to 21% of the total PAH contribution (Table 4-2).

The F/P ratio was between 0.9 and 1.2 for all storms in this study, indicating a strong predominance of pyrogenic PAH sources (Table 4-2). Furthermore, the P/A ratio was nearly always less than 21, once again indicating a strong predominance of pyrogenic PAH sources

(Table 4-2). Only one storm, March 17–18, 2002, at the Dominguez channel site, had a potential petrogenic source; the F/P ratio was 0.9, but the P/A ratio was .74. This result is consistent with the data from the land use sites, as the Dominguez watershed contains four major oil refineries. As with the distribution of HMW versusLMW PAHs, the F/P and P/A ratios indicate a consistent pyrogenic source for all lands use and mass emission sites regardless of the point within the storm (Figure 4-6). Again, the exception was at the industrial oil refinery, where the P/A ratio is low until the peak runoff occurs, at which time it rises to between 17 and 20. For both Ballona Creek and the Los Angeles River, a moderate, transient increase in the P/A ratio occurs coincident with the time of peak flow (Figure 4-4).

Discussion

Anthropogenic sources of total PAHS in storm-water runoff from urbanized coastal watersheds appears to be a significant source of PAHs to the southern California Bight. Estimates from this study based on FWM concentrations and gauged annual discharge volume indicate that during the study period approximately 92.8 and 32.7 kg/year of total PAH were discharged annually from the Los Angeles River and Ballona Creek watersheds, respectively. Over the same time period, the combined treated wastewater discharge from the city and county of Los Angeles (~2.8 x 10^6 m^3/d) discharged an estimated 740 kg of PAHs to the southern California Bight (Steinberger et al. 2003). The main difference between the two types of discharges is the delivery of the load to the coastal oceans; the treated wastewater discharge occurs in small, steady doses that occur daily, while storm-water loading occurs over the 10 to12 precipitation events that this region averages annually. The impact of the total PAHs in storm water discharged from urbanized watersheds is also reflected in receiving waterbody impacts. Regional monitoring of the southern California Bight revealed that the highest concentrations of PAHs were associated within bay and harbor areas that receive inputs from urbanized coastal watersheds (Noble et al. 2003). Bays and harbors only accounted for 5% of the total area of soft-bottom habitat but contained approximately 40% of the total PAH mass residing in southern California Bight surficial sediments. A second concern is the cost of remediating PAH in dredged materials. Total PAH is one of the most commonly occurring contaminants in dredged materials from San Pedro Bay (Steinberger and Schiff 2003). While some of these contaminants likely arise from port and industrial activities, they are colocated at the mouths of the Los Angeles River and Dominguez Channel watersheds, which is likely a contributing source.

The impact of PAH contributions on receiving waters from urbanized watersheds are not constrained to the southern California Bight. The National Status and Trends Program, which samples sediments and tissues in estuaries and coastal areas nationwide, repeatedly finds elevated PAHs near urban centers (Daskalakis and O'Conner 1995). San Pedro Bay (CA, USA) ranked third nationwide in total PAH concentration in mussel tissue during 2002. The top two locations are Elliott Bay (WA, USA) and Puget Sound (WA, USA), both located near urban centers. On the East Coast, Long Island Sound (NY, USA) adjacent to New York City was ranked fourth.

The annual watershed loading of PAHs estimated from this study are lower than those estimated from two studies in the eastern United States. Hoffman et al. (1984) estimated 680 kg/year of PAH loading from the 4,081 km^2 Narragansett Bay watershed in Rhode Island, USA. Similarly, Menzie et al. (2002) estimated 640 kg/year of PAH loading from the 758 km^2

Massachusetts Bay, USA, watershed. This difference may be explained by several factors. First, PAH loading relies on washoff of aerially deposited materials (the process by which airborne toxic contaminants enter coastal waters via aerial fallout). Watersheds in the western United States typically experience less than one-third rainfall and runoff volumes than comparably sized watersheds in the eastern United States. The lower volumes of annual runoff likely translate to lower loads. This may seem counter-intuitive; however, if the primary pollutant source to the land surface is aerial deposition (vs. being generated by activities within the land use), there is a practical limit to the amount of material that can accumulate. Previous studies have shown that physical processes such as wind or turbulence from traffic can limit pollutant accumulation on roads and other impervious surfaces (Pitt and Shawley 1981, Asplund et al. 1982, Kerri et al. 1985). Chemical processes, such as volatilization or oxidation can also limit the accumulation of potential pollutants on impervious surfaces (Hewitt and Rashad 1992). Because of the asymptotic nature of PAH buildup, loading to receiving waters is controlled by the frequency and magnitude of runoff events that "cleanse" impervious surfaces and provide subsequent opportunity for additional material to accumulate. A second reason for higher estimated PAH loading in the eastern United States is that PAHs are generated predominantly from concentrated point sources, such as coal-fired power plants. Southern California does not have coal-fired power plants; rather, PAHs are predominantly from mobile sources (cars, trucks, and trains), which discharge more diffusely across the region.

Concentrations in runoff from land use sites in this study were between 0.03 and 7.84 µg/L; these values are similar to those observed in previous studies by others. For example, Mahler et al. (2004) reported PAH concentrations between 5.1 and 8.6 µg/L in parking lot runoff, and Menzie et al. (2002) reported concentrations between 1 and 14 µg/L from a broad range of land uses.

In contrast to the results of this study, storm-water monitoring by local municipalities in southern California consistently report no detectable PAHs in storm water. This discrepancy is likely attributable to two factors. First, the practical PAH detection limit used by local municipalities is typically between 1 and 5 µg/L, which is acceptable by U.S. EPA regulatory guidelines. However, the mean FWM concentrations in storm water during this study were often lower than this level. The second factor is the sampling design used for regulatory-based monitoring. Most local municipalities are mandated to collect a storm composite sample that do not emphasize (and may completely miss) the first flush of total PAH that was observed. We almost always observed the greatest peaks in total PAH concentrations during initial storm flows, up to 8 h before peak flow. This pronounced first flush suggests that in highly urbanized watersheds, particle-bound PAHs may be rapidly mobilized from impervious land surfaces during the early portions of storms. Similar first-flush patterns in PAH concentrations during storms were observed by Hoffman et al. (1984) and (Smith et al. 2000). Furthermore, (Buffleben et al. 2002; University of California, Los Angeles, Los Angeles, CA, USA, unpublished data) also observed that peak PAH concentrations in Ballona Creek occur up to 14 h before peak flow.

Seasonal flushing at mass emissions sites was one phenomenon not previously reported by others. Seasonal flushing occurred when early-season storms consistently discharged higher PAH loads than storms of a similar size or larger later in the season. This seasonal effect was correlated with the length of antecedent dry condition but not with rainfall quantity. The lack of

a meaningful relationship between rainfall quantity and PAH loading has been reported in several other studies (Eaganhouse et al., Hoffman et al. 1984). Hoffman et al. (1984) suggested that the lack of a clear relationship was due to the complex spatial and temporal dynamics associated with rain patterns, which may affect runoff patterns more than the total amount of rainfall during a given storm. In addition, differential particle wash-off from land surfaces may mask any differences associated with total rainfall. The strong relationship between PAH flux and antecedent dry period suggests that storm-event PAH loads are a function primarily of the amount of time available for PAHs to build up on the land surfaces between subsequent rain events. The PAH loads from land surfaces during later-season storms (i.e., after 10 cm of accumulated rainfall) may reflect contributions from wet deposition or from localized accumulation; however, we currently lack the data to answer this question definitively. Analysis of PAH concentration in wet deposition would help improve our understanding of the sources of PAHs during the latter part of the storm season. Environmental managers can use this knowledge of temporal patterns of PAH loading to focus efforts on storm capture or treatment during the early portions of storms and during the earliest storms of the year.

Sources of PAHs in Storm water

Several lines of evidence implicate aerial deposition and subsequent wash-off of combustion by-products as the main source of PAH loading in storm water. First, the flux of total PAHs among large developed watersheds were similar throughout the urbanized region of Los Angeles, suggesting a similar regional source of PAHs. If urban land use distribution strongly influenced PAH loadings, then flux would have differed by watershed based on differential urban land use practices. In fact, no difference was observed in PAH concentrations in runoff between various urban land uses, which differs from the findings of previous studies from the eastern United States (Ngabe et al. 2000). Menzie et al. (2002) concluded that residential and commercial land uses generated higher PAH concentrations than other land use types because of secondary petrogenic sources that enhanced the regional pyrogenic source of PAHs. Hoffman et al. (1984) found that runoff from industrial and highway sites had higher PAH concentrations than residential runoff but accounted for these differences in runoff dynamics as opposed to unique sources.

Second, the relative abundance of individual PAHs in runoff indicates a strong pyrogenic source indicative of combusted fossil fuels. The typical distribution of PAHs observed from mass emission sites (Figure 4-7) was similar to the distribution of PAHs observed in dry deposition collected in Los Angeles by Sabin et al. (2004). Furthermore, in this study, HMW PAH consistently comprised approximately 73% of the total PAH concentration regardless of land use. Hoffman et al. (1984) reported comparable results in their study of urban runoff in Rhode Island's Narragansett Bay watershed, where HMW PAHs accounted for 71% of the total inputs to Narragansett Bay. A more recent study by Menzie et al. (2002) of PAHs in storm-water runoff in coastal Massachusetts identified similar HMW PAH compounds as observed in this study (chrysene, fluoranthene, phenanthrene, and pyrene) as the primary PAH compounds in storm water. Similarly, (Soclo et al. 2002) found that high PAH loads associated with storm-water runoff to the Cotonou Lagoon in Benin were characterized by HMW PAHs that appear to be derived from atmospheric deposition. The consistent predominance of HMW PAHs throughout all storms, even during the period of first flush, further indicates a consistent regional source, such as aerial deposition. If specific land uses were generating secondary petrogenic

wash-off as suggested by Menzie et al. (2002), the distribution of PAHs would have changed during the storm; however, we did not observe any differences within storms. The exception to this pattern was for the industrial oil refinery site, where the signature of petrogenic PAHs was more pronounced. This makes sense given the obvious petrogenic source associated with this land use type. Nevertheless, the pyrogenic signature was still prevalent at this land use, especially during the latter portions of the storm.

The PAH sources can also be inferred by examining ratios of particular PAHs in runoff samples. We used both the fluoranthene/pyrene (F/P) and phenanthrene/anthracene (P/A) ratios. Small F/P ratios close to 0.9 suggest that individual PAHs are associated with combustion products (Maher and Aislabie 1992); in contrast, large F/P ratios suggest petrogenic sources of PAHs (Colombo et al. 1989;Table 4-4). Both the F/P and the P/A ratios observed in this study indicate that aerial deposition of combustion by-products is likely the dominant source of PAHs in the watersheds that drain to the greater Los Angeles coastal region, and this source is consistent during all portions of storm-water runoff. Several additional ratios have been used to assess the different sources of PAHs. Takada et al. (1990) used methylated/parent PAH ratios as indicators of PAH sources. Results showed that PAHs in runoff from residential streets had a more significant contribution from atmospheric fallout of other combustion products. Zakaria et al. (2002) explained their low ratios of methylphenanthrene to phenanthrene (MP/P; <0.6) to mean that combustion- derived PAHs are transported atmospherically for a long distance and serve as background contamination. The ratios of methylphenanthrene to phenanthrene in our study (0–0.2) also suggest a strong contribution of aged urban aerosols to overall PAH loads (Nielsen 1996, Simo et al. 1997, Hwang et al. 2003). Watersheds in the greater Los Angeles area are heavily urbanized; therefore, ample opportunity exists for combustion-derived aerosols that generate particulate matter to be deposited on land surfaces. The petrogenic signature seen in the Dominguez Channel can be explained by the presence of slightly different sources in this watershed. The Dominguez watershed contains a high density of oil refineries and other industrial land uses that drain directly to the Ports of Los Angeles and Long Beach. The presence of multiple oil refineries discharging to a single stream explains the concentration of petrogenic PAHs in this area.

Conclusions based on ratios of specific PAH compounds should be used with some caution, especially because a relatively limited set of PAHs were analyzed in this study. Furthermore, if reference (or source) samples were not analyzed, it is always a good idea to use these ratios on a relative basis. Nevertheless, the preponderance of evidence from this study, combined with the well-documented fact that atmospheric deposition (both wet and dry) is the major source of contamination in arid and semiarid climates, such as that existing in southern California (Sabin et al. 2004, Gunther et al. 1987), supports the conclusions of this study: The predominant source of PAHs in urban storm water in the greater Los Angeles area is from aerial deposition and subsequent wash-off of PAHs associated with combustion byproducts.

Table 4-1. Storm-water polycyclic hydrocarbon mass emissions from in-river sampling locations. Annual loads are based on water year, as indicated in the foot notes. Cubic meters per second(cms); Standard deviation (SD); Polycyclic aromatic hydrocarbons (PAH); and Event mean concentration(EMC).

							Total PAHs					
Mass Emission Sites	Size km^2	Date of Storm Event	Rainfall (cm)	Antecedant Dry Days	Mean Flow (cms)	Peak Flow (cms)	EMC ng/L	SD	Flux (kg/km^2)	Mass Emissions (kg)	SD	Annual Total PAH (kg/year)
LA River above Arroyo Seco	1460	11/12 - 11/13/2001	1.73	127	62.6	262.5	3,256.80	846.70	0.0049	7.16	0.35	3.74[a]
LA River at Wardlow	2161	5/2 - 5/3/2003	3.56	4	209.9	756.7	470.70	453.20	0.0023	4.90	0.32	34.9[b]
		2/2/04	1.14	29	90.4	375.6	3,559.33	1,185.50	0.0	13.93	0.99	150.6[c]
										Mean Load:		92.8 ± 81.8
Verdugo Wash	65	11/12 - 11/13/2001	1.83	11	68.5	368.2	4,283.70	2,043.20	0.2236	14.54	0.83	NA[d]
		10/31 - 11/1/2003	1.74	30	56.5	155.0	4,992.30	1,093.30	0.1529	9.94	0.46	NA
Arroyo Seco	130	2/9 - 2/11/2001	3.56	12	2.9	13.5	788.80	177.80	0.0009	0.11	0.01	2.79[e]
		4/6 - 4/7/2001	1.78	30	7.8	21.8	816.50	258.50	0.0016	0.20	0.01	
Ballona Creek	338	4/6 - 4/7/2001	1.24	31	32.6	100.9	948.70	379.90	0.0054	1.81	0.13	20.5[e]
		11/24 - 11/25/2001	1.52	11	53.1	396.2	3,118.90	1,104.80	0.0246	8.30	1.78	17.3[a]
		5/2 - 5/3/2003	2.03	4	52.8	134.4	981.70	583.00	0.0032	1.08	0.12	20.0[b]
		10/31 - 11/1/2003	2.03	30	62.0	148.1	5,821.20	1,814.90	0.0233	7.87	0.54	72.9[c]
										Mean Load:		32.7 ± 26.8
Dominguez Channel	187	3/17 - 3/18/2002	0.28	10	4.8	14.0	3,293.40	791.80	0.0013	0.24	0.01	NA
		2/21 - 2/22/2004	1.52	18	14.7	35.5	2,182.10	745.20	0.0123	2.31	0.09	NA
Santa Monica Canyon	41	4/6 - 4/7/2001	3.05	50	0.6	3.0	766.8	247.2	0.0002	0.01	0.00	NA
Open Space Arroyo Sequit	31	2/25 - 2/26/2004	9.17	2	3.4	21.9	137.6	0.0	0.0013	0.04	0.00	NA

[a]Water year 2002 = October 2001-September 2002
[b]Water year 2003 = October 2002-September 2003
[c]Water year 2004 = October 2003-September 2004
[d]NA = annual storm volumes not available; consequently, annual loads could not be estimated
[e]Water year 2001 = October 2000-September 2001

Table 4-2. Total polycyclic aromatic hydrocarbons (PAHs) and selected polycyclic aromatic hydrocarbon ratios. Event mean concentration (EMC); High-molecular-weight compounds (HMW).

Mass Emission Sites	Date of Storm Event	EMC ∑PAHs (ng/L)	EMC Pyrene (ng/L)	Pyrene/ ∑PAHs (%)	Fluoranthene/ Pyrene Ratio	Phenanthrene/ Anthracene Ratio	EMC Phenanthrene (ng/L)	Phenanthrene/ ∑PAHs (%)	HMW (%)	Dominant Sources of Origin
LA River above Arroyo Seco	11/12 - 11/13/2001	3256.8	427.9	13.1	1.1	8.0	291.3	8.9	76.4	Pyrogenic
LA River at Wardlow	5/2 - 5/3/2003	470.7	133.5	28.4	1.1	20.9	97.3	20.7	69.7	Pyrogenic
	2/2/04	3559.3	401.0	11.3	1.0	7.5	278.1	7.8	71.8	Pyrogenic
Verdugo Wash	11/12 - 11/13/2001	4283.7	593.8	13.9	1.1	7.8	373.0	8.7	83.5	Pyrogenic
	10/31 - 11/1/2003	4992.3	677.9	13.6	0.9	11.6	341.8	6.8	82.0	Pyrogenic
Arroyo Seco	2/9 - 2/11/2001	788.8	131.9	16.7	1.0	8.6	101.2	12.8	81.7	Pyrogenic
	4/6 - 4/7/2001	816.5	135.0	16.5	1.1	7.2	101.9	12.5	84.6	Pyrogenic
Ballona Creek	4/6 - 4/7/2001	948.7	177.9	18.8	0.9	4.9	89.6	9.4	88.7	Pyrogenic
	11/24 - 11/25/2001	3118.9	428.8	13.8	1.0	8.1	302.9	9.7	71.8	Pyrogenic
	5/2 - 5/3/2003	981.7	237.4	24.2	1.0	4.3	122.3	12.4	74.6	Pyrogenic
	10/31 - 11/1/2003	5821.2	786.2	13.5	1.1	10.2	473.0	8.1	82.7	Pyrogenic
Dominguez Channel	3/17 - 3/18/2002	3293.4	534.6	16.2	0.9	74.9	508.2	15.4	77.5	Petrogenic
	2/21 - 2/22/2004	2182.1	308.8	14.2	1.1	6.4	210.5	9.6	69.7	Pyrogenic
Santa Monica Canyon	4/6 - 4/7/2001	766.8	134.9	17.6	1.0	4.1	73.8	9.6	86.5	Pyrogenic
Open Space Arroyo Sequit	2/25 - 2/26/2004	137.6	14.3	10.4	1.2	10.2	17.2	12.5	63.0	Pyrogenic
	Mean ∑PAHs (ng/L)	2,300.00								

Table 4-3. Event mean concentration (EMC) and mass loading of polycyclic aromatic hydrocarbons (PAHs) from land use sites. Site numbers indicate different sites within a given land use category. SD = standard deviation; NA = watershed size not available.

							Total PAHs		
Land-use Type	Size (km^2)	Date of Storm Event	Rainfall (cm)	Dry Days	Mean Flow (cms)	Peak Flow (cms)	Flux (kg/km^2)	EMC (ng/L)	SD
High Density Residential #1	0.02	2/17/02	0.89	21	0.001	0.006	1.8E-03	1.92E+03	7.03E+02
High Density Residential #1	0.02	2/2/04	1.19	2	0.0042	0.0251	2.0E-02	3.31E+03	1.00E+03
High Density Residential #2	0.52	3/17 - 3/18/2002	0.20	27	0.000	0.003	1.1E-05	7.84E+03	5.99E+03
High Density Residential #3	1.0	12/28/04	3.25	0	0.009	0.080	2.45E-06	7.11E+00	2.97E+00
High Density Residential #3	1.0	2/11/05	1.35	13	0.004	0.016	5.4E-08	5.06E-01	1.28E-01
Mean High Density Residential #1							***7.2E-03***	***4.4E+03***	***2.6E+03***
Mean High Density Residential #3							**1.2E-06**	**3.8E+00**	**1.6E+00**
Low Density Residential #1	0.98	3/4 - 3/5/2001	2.67	3	0.017	0.071	7.2E-05	1.55E+02	5.54E+01
Low Density Residential #1	0.98	2/2/04	2.26	2	0.030	0.143	3.3E-03	3.3E+03	1.6E+03
Low Density Residential #2	0.18	3/17 - 3/18/2002	2.13	9	0.008	0.116	1.7E-03	8.86E+02	1.82E+02
Mean Low Density Residential							***1.7E-03***	***1.4E+03***	***6.0E+02***
Commercial #1	NA	2/17/02	0.89	20	0.002	0.008	NA	2.27E+02	1.63E+02
Commercial #2	2.45	2/17/02	0.74	20	0.337	1.340	7.7E-03	4.43E+03	2.05E+03
Commercial #3	0.06	4/6 - 4/7/2001	2.03	31	0.008	0.018	8.2E-05	3.00E+01	1.95E+01
Commercial #3	0.06	3/17 - 3/18/2002	0.12	9	0.000	0.001	2.9E-06	2.08E+02	6.93E+01
Mean Commercial							***2.6E-03***	***1.2E+03***	***5.8E+02***
Industrial #1	0.004	4/6 - 4/7/2001	2.06	31	0.008	0.017	5.7E-03	1.36E+02	6.85E+01
Industrial #2	0.001	2/17/02	0.74	20	0.000	0.002	2.9E-03	6.31E+02	3.42E+02
Industrial #3	2.77	3/17 - 3/18/2002	0.25	9	0.000	0.003	6.6E-06	4.41E+03	2.29E+03
Industrial #4	0.01	3/15/03	4.50	9	0.117	0.375	5.6E-01	8.89E+02	7.55E+02
Mean Industrial							***1.4E-01***	***1.5E+03***	***8.6E+02***

Table 4-3. Continued

Land-use Type	Size (km^2)	Date of Storm Event	Rainfall (cm)	Dry Days	Mean Flow (cms)	Peak Flow (cms)	Total PAHs Flux (kg/km^2)	Total PAHs EMC (ng/L)	Total PAHs EMC SD
		3/4 - 3/5/2001	2.74	3	0.021	0.053	4.3E-04	6.83E+02	7.77E+02
Agricultural #1	0.98	3/17 - 3/18/2002	0.23	10	0.012	0.031	2.0E-05	4.55E+02	1.72E+02
		2/2/04	1.17	2	0.0228	0.128	5.3E-04	1.43E+03	2.09E+03
Mean Agricultural							***3.3E-04***	***8.6E+02***	***1.0E+03***
Recreational (horse)	0.03	3/4 - 3/5/2001	1.42	3	0.003	0.014	1.8E-03	4.58E+02	2.97E+02
Mean Recreational							***1.8E-03***	***4.6E+02***	***3.0E+02***
Transportation #1	0.01	4/6 - 4/7/2001	3.05	31	0.022	0.057	1.4E-02	3.63E+02	2.53E+02
Transportation #2	0.002	2/17/02	0.89	47	0.001	0.006	3.7E-03	5.95E+02	3.16E+02
Mean Transportation							***8.9E-03***	***4.8E+02***	***2.8E+02***

Table 4-4. Selected polycyclic aromatic hydrocarbon ratios and their source signature ranges.

Indicator	Pyrogenic	Petrogenic	Reference
Fluoranthene / Pyrene Ratio	0.9 - ≤1	>1	Maher and Aislabie, 1992
Phenanthrene / Anthracene Ratio	3 - 26	>26	Gschwend and Hites, 1981; Lake et al., 1979
Methylphenanthrene / Phenanthrene Ratio	<1.0	2 - 6	Hwang et al., 2003

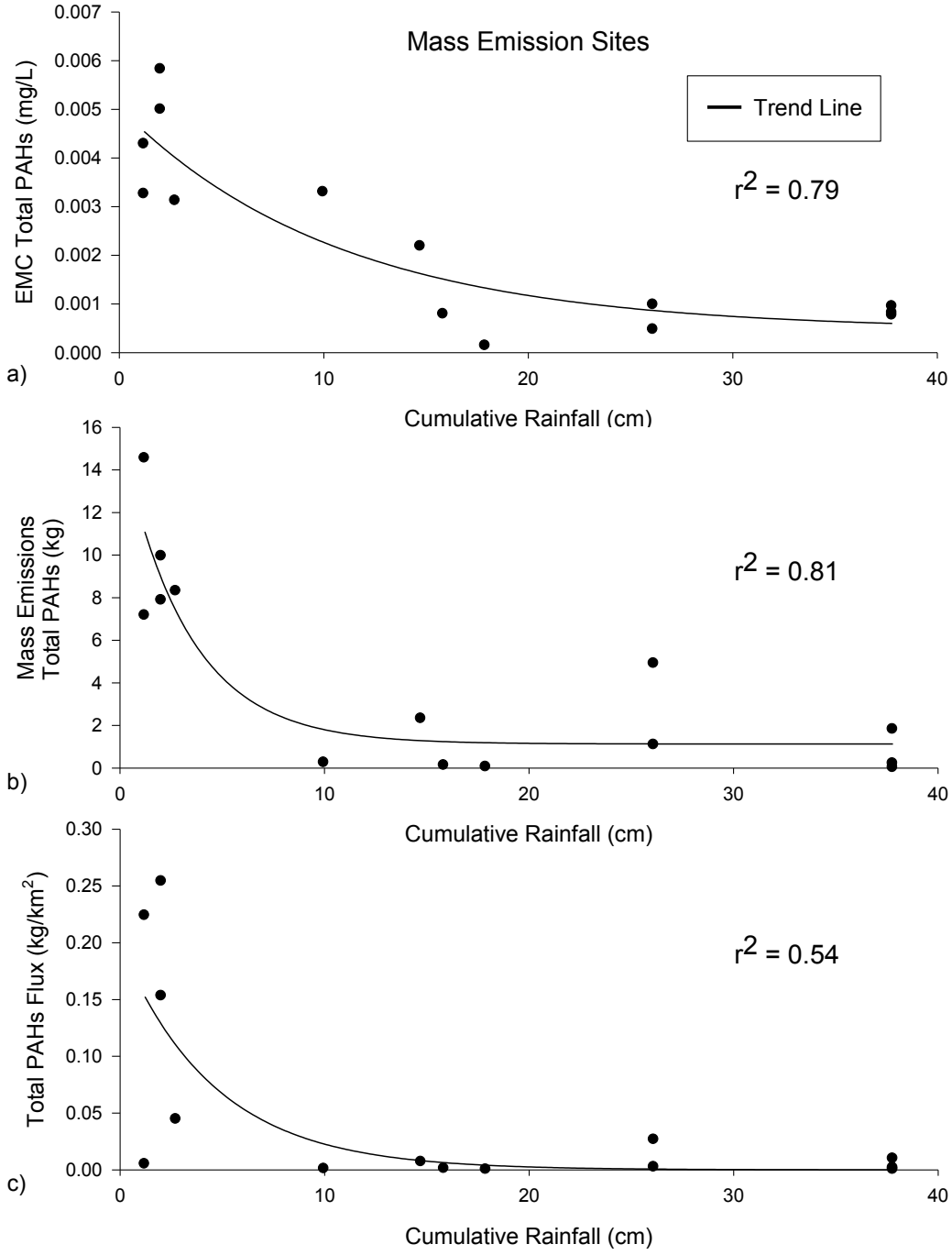

Figure 4-1. Cumulative annual rainfall versus polycyclic aromatic hydrocarbon (PAH) event mean concentration (EMC; a), load (b), and flux (c). Plots show data for mass emission sites only.

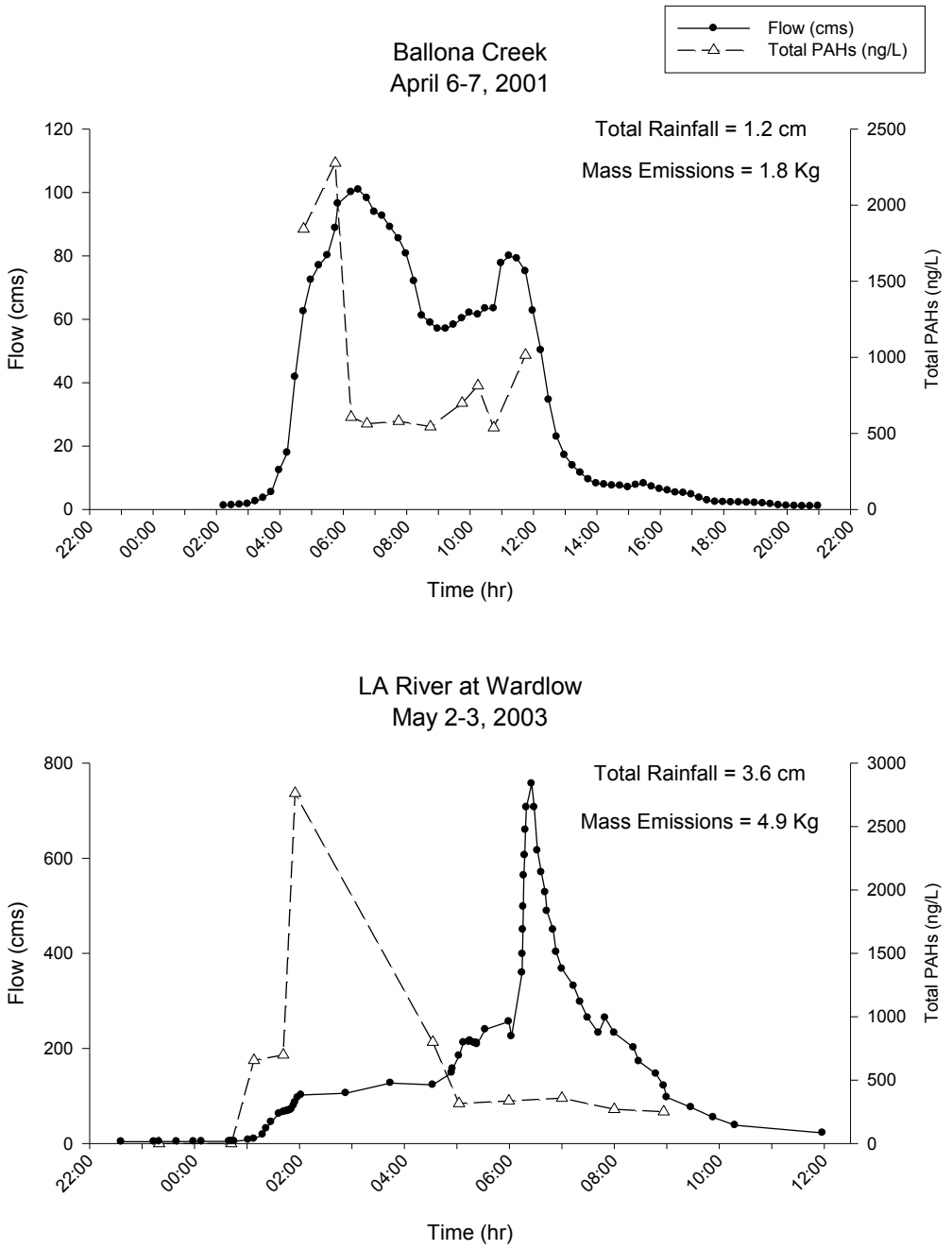

Figure 4-2. Variation in polycyclic aromatic hydrocarbon (PAH) concentrations with time for storm events in Ballona Creek (top) and Los Angeles River (bottom).

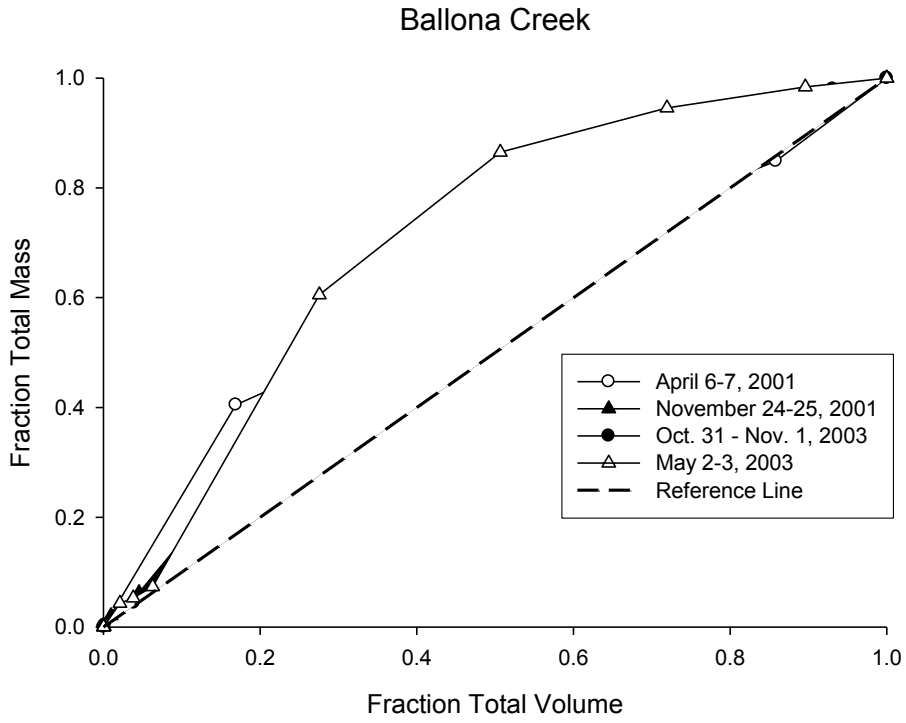

Figure 4-3. Cumulative polycyclic aromatic hydrocarbon mass loading for four storms in Ballona Creek. Plots show percent of mass washed off for a given fraction of the total runoff. Reference line indicates a 1:1 relationship between volume and mass loading. Portions of the curve above the line indicate proportionately higher mass loading per unit volume (i.e., first flush). Portions below the line (if any) indicate the reverse pattern.

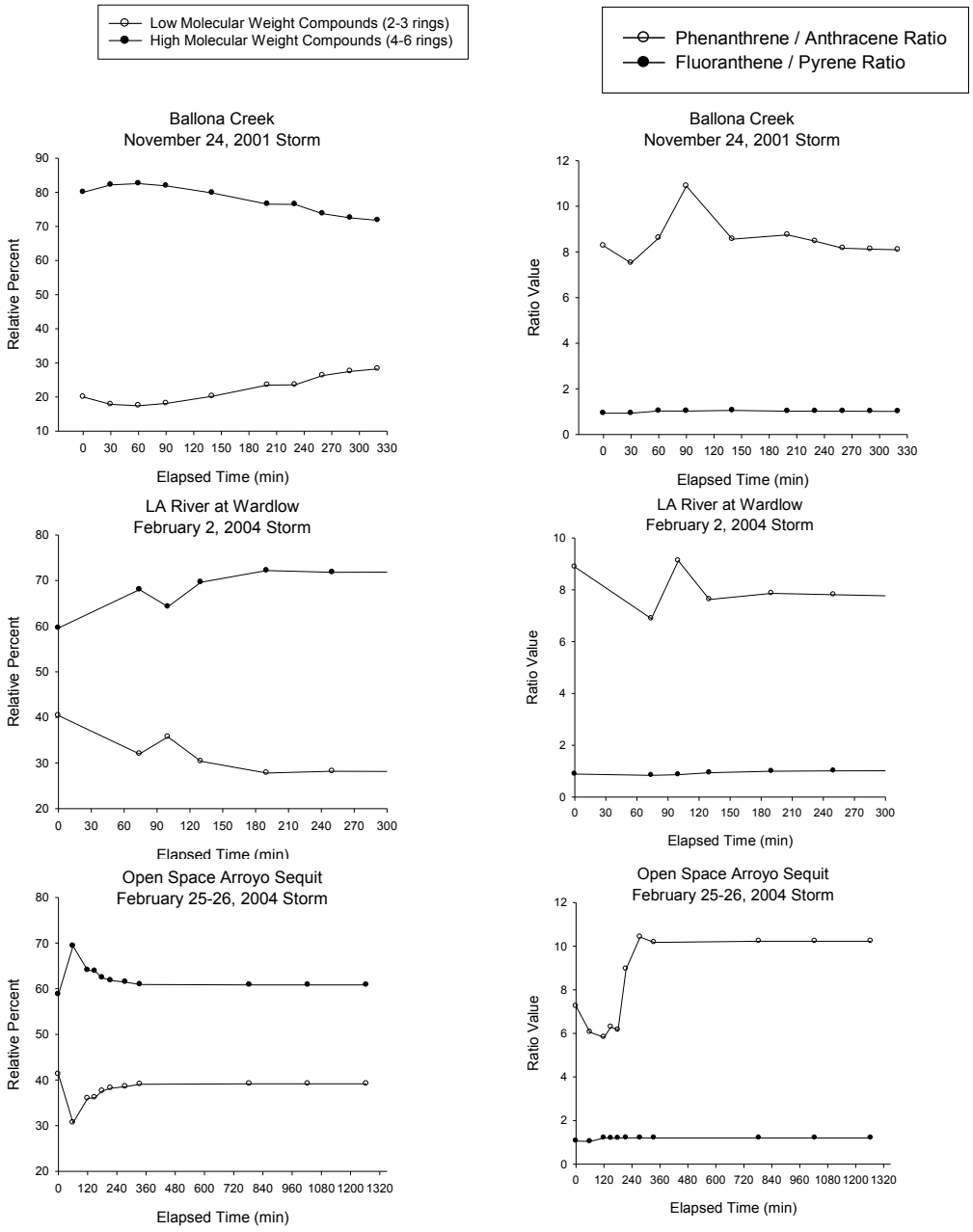

Figure 4-4. Distribution of polycyclic aromatic hydrocarbons (PAHs) within storms for mass emission sites. Plots on the left (a–c) show distribution of high- versus low-molecular-weight PAHs throughout individual storms. Plots on the right (d–f) show phenanthrene/anthracene (P/A) and fluoranthene/pyrene (F/P) ratios throughout individual storms. Peaks in the P/A ratio correspond to peak storm flows.

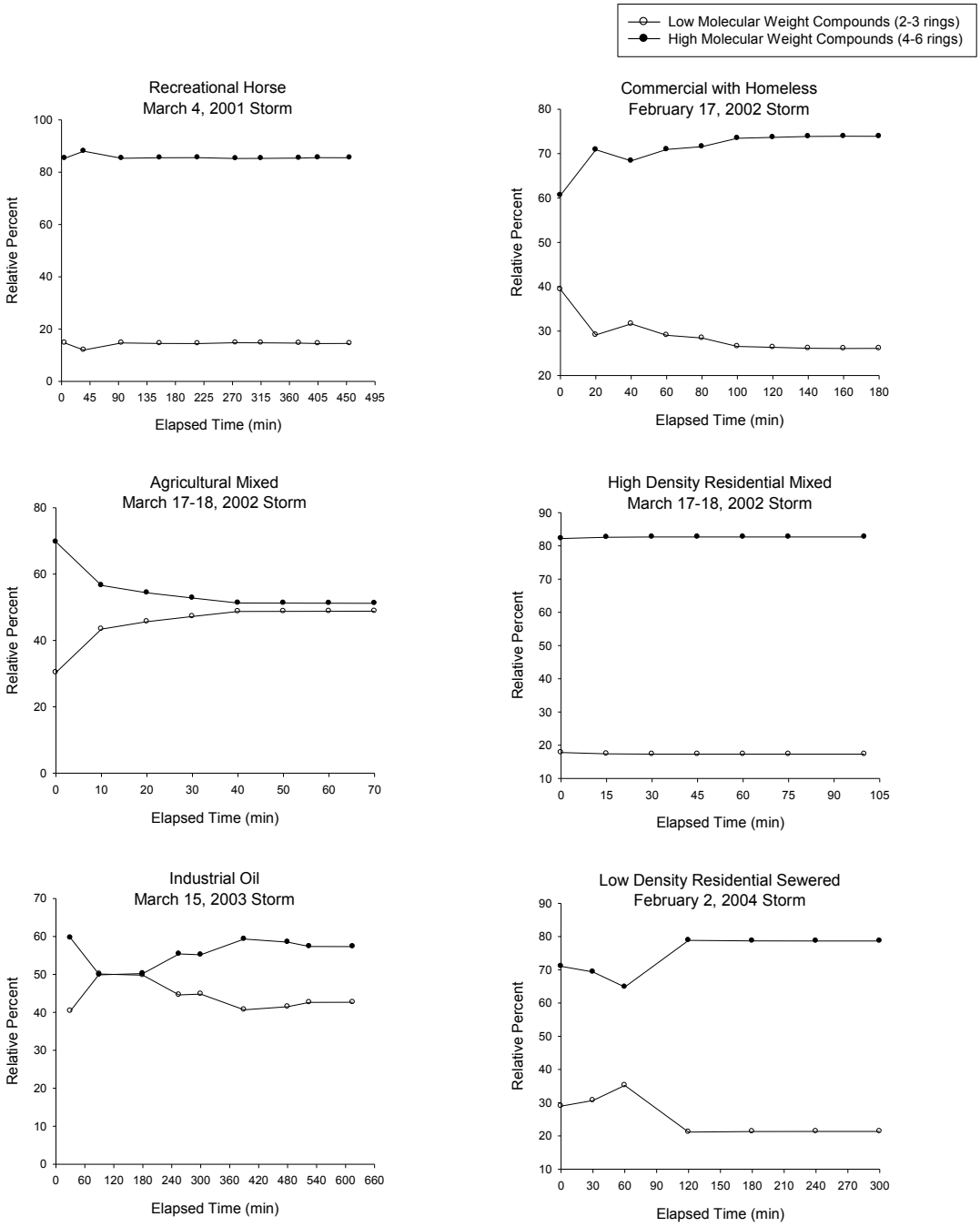

Figure 4-5. Distribution of polycyclic aromatic hydrocarbons (PAHs) within storms for representative land use sites (a–f). Plots show distribution of high- versus low-molecular-weight PAHs throughout individual storms. Data are shown for six sites that represent the results observed for the 15 land use sites where data were collected.

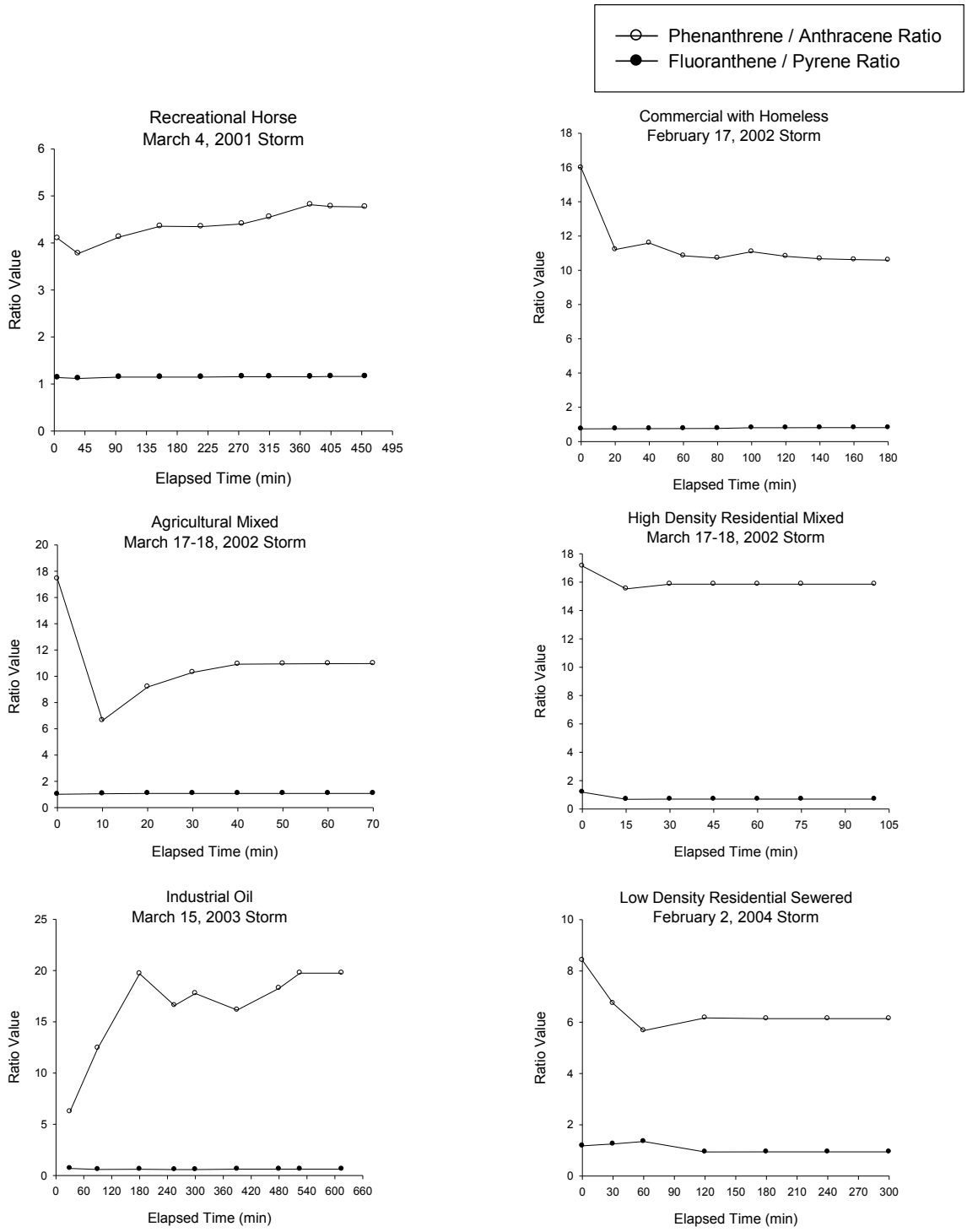

Figure 4-6. Distribution of polycyclic aromatic hydrocarbons within storms for representative land use sites. Plots (a–f) show phenanthrene/anthracene (P/A) and fluoranthene/pyrene (F/P) ratios throughout individual storms. Data are shown for six sites that represent the results observed for the 15 land use sites where data were collected.

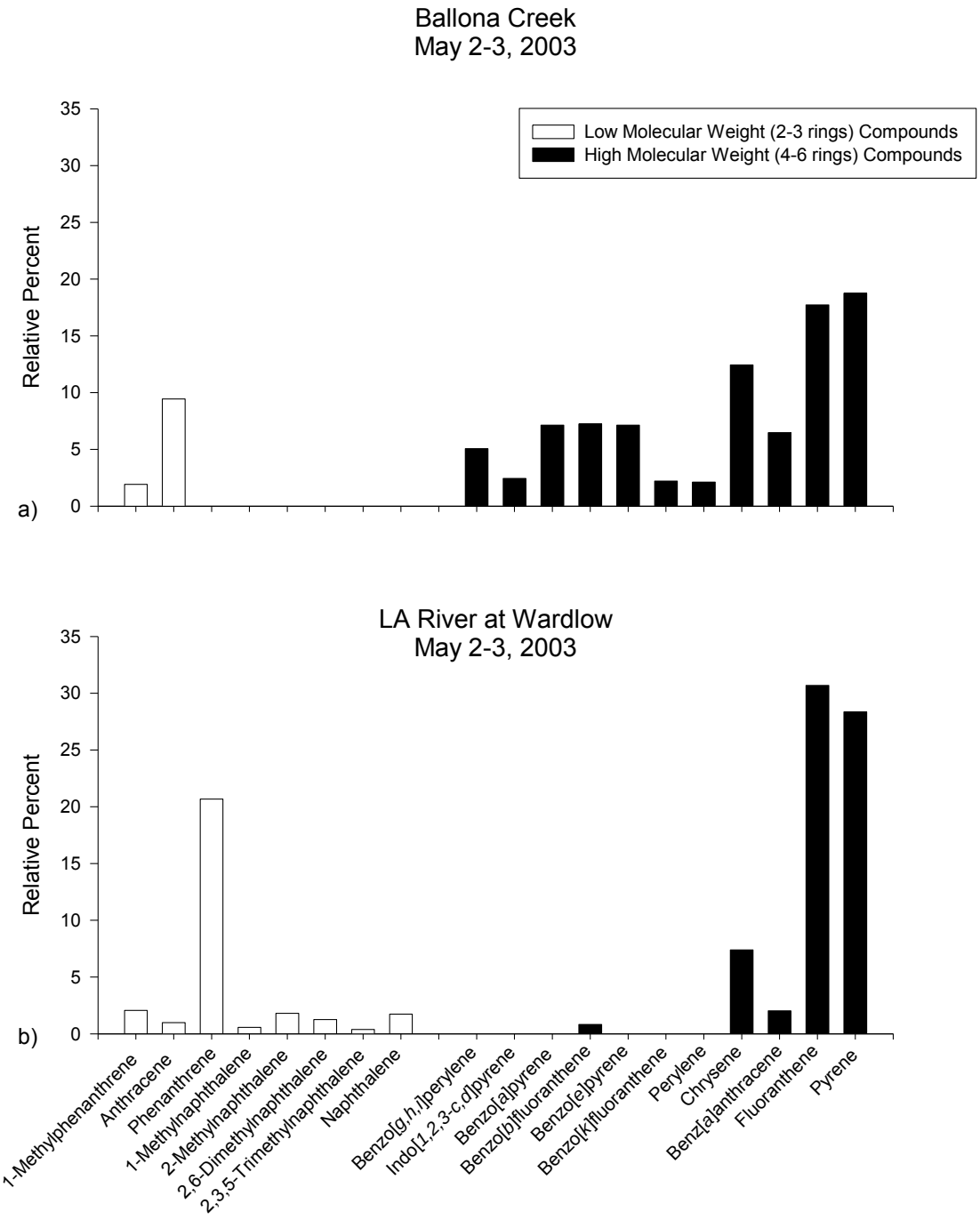

Figure 4-7. Relative distribution of individual polycyclic aromatic hydrocarbon compounds for Ballona Creek (a) and Los Angeles River (b) on May 2–3, 2003.

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SECTION 5. FECAL INDICATOR BACTERIA (FIB)

Results

FIB from Developed and Undeveloped Watersheds

E. coli, *Enterococcus* spp. and total coliforms occurred at all ME sites at concentrations that consistently and uniformly exceeded CA water quality standards (Figure 5-1). Mean *E. coli*, *Enterococcus* spp. and total coliforms EMCs and fluxes were significantly greater at ME sites from developed compared to undeveloped watersheds (ANOVA, p = 0.006). For example the mean EMC at the developed Ballona Creek watershed was two orders of magnitude higher than at the undeveloped Open Space Arroyo Sequit watershed (10^4 MPN/100 mL vs. 10^2 MPN/100 mL, respectively; Fig. 5-1a). Bacteria EMCs were typically higher in the Los Angeles River compared to the other watersheds sampled.

Bacterial flux from ME sites exhibited a similar pattern as that observed for the EMCs. For example, *E. coli* fluxes were two orders of magnitude higher at the developed Ballona Creek watershed versus the undeveloped Arroyo Sequit watershed (i.e., 10^{12} colonies/km^2 vs. 10^{10} colonies/km^2, respectively; ANOVA, p = 0.02, Figure 5-1b). Similarly, *Enterococcus* spp., and total coliforms fluxes were substantially higher for the developed watersheds versus the undeveloped watershed, but these differences were not statistically significant. Furthermore, the higher fluxes from developed watersheds were generated by substantially less rainfall than the lower fluxes from the undeveloped watersheds (2.07 ±1.22 cm for storms in developed watersheds vs 6.49 ±3.79 cm for storms in undeveloped watersheds).

FIB Concentration and Flux from Specific Land-use Types

Figure 5-2 shows the median FIB concentrations for the storm events sampled for each LU category. Mean *E. coli,* EMCs from the recreational LU site were significantly higher than the commercial, high density residential, industrial and transportation LU sites (i.e., $5.3\text{x}10^5$ MPN/100mL ± $1.7\text{x}10^5$, p=0.004, Appendix B-15) and were an order of magnitude higher than mean EMCs observed at ME sites. Agricultural LU sites contributed the second highest mean indicator bacteria EMCs but were not statistically different from all other LU sites (i.e., $4.0\text{x}10^4$ MPN/100mL ± $1.4\text{x}10^4$ *E. coli*, $1.2\text{x}10^5$ MPN/100mL ± $9.6\text{x}10^4$ *Enterococcus* spp. and $6.4\text{x}10^5$ MPN/100mL ± $9.6\text{x}10^4$ total coliforms).

Direct comparison of flux showed that storm water from agricultural, recreational and industrial LU sites had the highest mean FIB fluxes. Most of the developed LU types exhibited comparable fluxes of 10^{11} colonies/km^2 (Appendix B-15). In contrast, the agricultural LU contributed substantially higher flux of both *Enterococcus* spp. and total coliforms (e.g. mean *Enterococcus* flux = 10^{14} colonies/km^2 (Appendix B-15). Mean FIB fluxes at the open space LU were comparable to those observed at developed LU sites (e.g. 10^{12} colonies/km^2; Appendix B-15).

Correlation between FIB and TSS Concentration

A simple Spearman's correlation matrix (Table 5-1) of TSS, stream flow and FIB indicates that *E. coli* was significantly positively correlated (p <0.0001) with TSS from agricultural, recreational and open LU sites. *E. coli* concentration from low-density residential

and industrial LU sites were weakly correlated with TSS. *Enterococcus* spp. was significantly correlated (p <0.0001) with total suspended solids from low-density residential, agricultural, recreational and transportation LU sites and all correlations with the exception of the low-density residential site were positive. *Enterococcus* spp. counts from commercial and open LU sites were weakly and positively correlated with TSS. Both *E. coli* and *Enterococcus* spp. had correlation coefficients (Spearman's *rho* or ρ) between 0.5 and 0.8, indicating that similar processes may have controlled the effect of TSS on each of these parameters. FIB concentrations were only significantly correlated (p <0.0001) with stream flow at the commercial, high-density residential and agricultural LU sites.

California Bacterial Water Quality Standards

Bacteria concentrations exceeded the California beach water quality single-sample water quality standard in almost all of the samples collected during this study. Concentrations of FIB at many LU sites were as high as those found in primary wastewater effluent (10^6-10^7 MPN/100ml). Cumulative density frequency plots showed 98%, 94% and 92% of the in-river storm water samples for *Enterococcus* spp., *E. coli* and total coliforms bacteria exceeded CA ambient water quality standards (Figure 5-3). Similar results were observed at LU sites. Approximately 80% of all samples exceeded water quality thresholds at LU sites for at least one indicator (i.e., *E. coli* exceedence = 83%; Figure 5-3). The above comparisons are based on receiving water quality standards. If compared to the proposed freshwater standards, which are approximately 60% lower than the receiving water standards, the exceedances would be slightly higher.

Large-sized watersheds (>100 km^2) exhibited the greatest frequency of water quality threshold exceedences (Figure 5-4). More than any other indicator, concentrations of *Enterococcus* spp. were responsible for the majority of water quality threshold exceedences across all three watershed size categories, exceeding thresholds 98% of the time for both large and medium-sized watersheds (25-100 km^2), and 96% of the time for small-sized watersheds. *E. coli* and total coliform concentrations followed a decreasing frequency of exceedences in terms of watershed size (i.e., large > medium > small).

Temporal Patterns in Indicator Bacteria Loading

Effect of Rainfall Patterns: Indicator bacteria from LU sites showed little variation when evaluated for seasonal differences between early- and late- season storms. In contrast, antecedent dry period (expressed as cumulative annual rainfall) was strongly correlated with FIB concentrations from ME sites in an exponentially non-linear manner (r^2 = 0.67-0.92; Figure 5-5). Early-season storms generally had higher *Enterococcus* spp. and total coliforms EMCs than late-season storms both within and between watersheds with an inflection point at approximately 10 cm, even when rainfall quantity was similar. For example, the early-season storm from Ballona Creek in water year 2004 had an *Enterococcus* spp. EMC two times larger (3.0×10^4 MPN/100mL) than the storm that occurred at the end of the rainy season in water year 2003 (1.6×10^4 MPN/100mL), despite the early- and late-season storms resulting from comparable rainfall (approx. 3.0 cm). The results for *E. coli* EMCs from early- and late- season storms were comparable. When all watersheds are analyzed together *E. coli*, *Enterococcus* spp. and total coliforms concentrations decrease with increasing cumulative annual rainfall until approximately

10 cm (average annual rainfall is 33 cm), beyond which the effect is markedly less dramatic (Figure 5-5).

With-in Storm Variability

FIB concentrations varied with time and as a function of flow over the course of storm events. Figures 5-6 and 5-7 show the pattern of change throughout storm events for *E. coli* and *Enterococcus* spp.. In all cases, bacterial concentrations increased markedly preceding peak flow (compared to base flow level). *Enterococcus* spp. concentrations stayed high for a relatively short period at the developed Ballona Creek site (2.4×10^5 MPN/100mL) and then decreased back to base levels within two hours (Figure 5-6a). In contrast, *E. coli* concentrations were more variable exhibiting two separate peaks around 2.6×10^4 MPN/100mL and an order of magnitude lower than *Enterococcus* spp. concentrations (Figure 5-6b)[1]. Although the pattern of an early peak in concentration was comparable in both undeveloped and developed watersheds, in the undeveloped watersheds the peak concentration tended to occur later in the storm and persist for a longer duration (i.e., three to four hours; Figure 5-7). Furthermore, flow continued above base flow conditions for a longer duration in the undeveloped watersheds however FIB concentrations steadily decreased following the early peak in storm. We cannot make conclusions about the consistency of these patterns given the small number of storms sampled at undeveloped watersheds.

Discussion

The relatively higher bacteria concentrations from recreational and agricultural LU sites may be due to several sources. Sources of bacteria include domestic pet and wildlife wastes that are deposited, stored, or applied to the land, a fact that may account for the high *E. coli* and *Enterococcus* spp. EMCs, and overall mean flux of 1.4×10^{13} and 1.1×10^{14} colonies/km^2, respectively observed at the agricultural sites during this study. In contrast, land use sites, such as industrial areas and built-out residential areas, have proportionately less direct sources of fecal material and have lower sediment concentrations in storm water than do mixed LU and developing areas (i.e., recreational, Mallin 1998, Burnhart 1991). This difference in source material may be a factor that accounts for why these LU sites had lower indicator bacteria EMCs and fluxes.

The association of bacteria with storm water particles may also explain differences in *E. coli* and *Enterococcus* spp. concentrations from different LU sites. Correlations of FIB with TSS from recreational and agricultural LU sites indicate associations with particulate material, but it is unclear if that particulate material resulted from soils transported to the stream from these LU sources or from erosion and resuspension of sediment already in the streambed from upland sources. Other studies have implicated streambed sediment and its resuspension (Matson et al. 1978, Francy et al. 2000, Embrey 2001) as sources and principal transport vectors for bacteria. The higher indicator bacterial concentrations at the recreational and agricultural land use sites indicate that bacteria associated with these areas may be directly associated with sources at those sites. Another possible explanation for the high FIB concentrations at agricultural sites may be

[1] Unfortunately FIB samples were not collected prior to 3:30 AM due to failure to be on site when storm commenced.

due to the regular application of fertilizers, algaecides and fungicides (Niemi 1991, Cook and Baker 2001). Assessing particle size distribution over the entire storm duration at these LU sites may provide a clearer or consistent particle source association. Interestingly, indicator bacteria concentrations were only significantly correlated ($p<0.0001$) with stream flow at the commercial and high-density residential LU sites even though bacteria in streams are commonly associated with suspended particles (Schillinger and Gannon, 1985, Hunter et al. 1999), either because they were transported to the streams attached to the particles, they were bound to streambed sediment (Matson et al.1978) that has been resuspended (Grimes 1975, Matson et al.1978, Hunter et al. 1999) or because of specific bacterial affinities for sediment particles (Scholl and Harvey 1992, Bolster et al. 2001) that may occur in the water column. Although bacterial transport has been correlated with stream stage (Hunter et al. 1992) and stream flow during storms and also tends to be associated with the transport of suspended sediment (Davis et al. 1977), these associations are not always evident (Qureshi and Dutka 1979). In the Los Angeles River watershed the lack of correlations at specific LU sites with stream flow may indicate that contributing sources or processes for bacteria were different from storm to storm.

Comparison of FIB concentrations in runoff from LU sites from this study reveal median *E. coli* EMCs that are comparable to current U.S. averages reported in the National Stormwater Quality Database (NSQD; Maestre et al. 2003), but lower than concentrations reported in the Nationwide Urban Runoff Program (NURP) database (U.S. EPA 1983a; Figure 5-8). The exception is that median total coliform values from all LU sites in Los Angeles, CA are substantially higher than those observed in the rest of the U.S. (Table 5-2). The similarities in median event-mean *E. coli* concentrations from LU sites across the U.S. measured since 1992 (reported by the NSQD) and those observed 13 years later during this study demonstrate that the issue of fecal bacteria contamination in urban watersheds is not improving over time.

Seasonal comparisons of wet weather FIB concentrations to dry weather concentrations from the urbanized Ballona Creek watershed during 2002-03 revealed that contributions from wet weather far exceeded those from dry weather (Table 5-3). Freshwater outlets such as storm drains are found to be especially high contributors of indicator bacteria contamination (Stein and Tiefenthaler 2005, Noble et al. 2000, Bay and Schiff 1998). Nevertheless, the highest mean dry-weather *E. coli* concentrations (7,457 MPN/100 ml) found in samples from Ballona Creek were still an order of magnitude lower than the lowest mean *E. coli* storm EMC from this study (43,305 MPN/100 ml; Table 5-3, $p<0.03$ E. coli; $p<0.04$ *Enterococcus* spp.; $p<0.02$ total coliforms). Wet versus dry sampling events have been compared by other studies in the southern California region (Noble et al. 2006, Schiff et al. 2003, Noble et al. 2003). These studies also found a higher number of exceedences of water quality standards during wet weather for all indicators, especially at storm water outflows and storm drains.

Consistently higher bacteria levels during early season storms likely reflect bacteria buildup during dry periods that "flushes" to rivers during early season storms. Bacteria concentrations in rivers were strongly influenced by the length of antecedent dry condition but not with amount of rainfall. The strong relationship between indicator bacteria EMC and antecedent dry period suggests that the magnitude of bacterial load associated with storm water runoff depends on the amount of time available for build up on land surfaces, and that storm size is a less reliable predictor of the magnitude of bacterial loading. Since indicator bacteria

continue to reproduce in the environment and reproduction is favored in aerobic temperate waters, low flow and high temperature conditions that typically occurs in southern California between May and October likely allows indicator bacteria concentrations to build-up on land surfaces, resulting in proportionally higher bacteria concentrations and loads during the initial storms of the season. A similar seasonal pattern (i.e., 10 cm cumulative annual rainfall threshold) was observed for polycyclic aromatic hydrocarbons (PAHs) and trace metals in the Los Angeles region (Stein et al. 2006, Sabin et al. 2004, Tiefenthaler et al. in press). Han et al. (2006) also reported that antecedent dry period was the best predictor of the magnitude of pollutant runoff from highways. Other researchers (Anderson and Rounds 2003, Ngoye and Machiwa 2004) have reported corresponding temporal trends for other particle-bound contaminants. This seasonal pattern suggests that focusing management actions on early season storms may provide relatively greater efficiency than distributing lower intensity management actions throughout the season.

FIB concentrations in storm water were highly variable, with concentrations often ranging by factors of 10 to 100 during a single storm. The greatest bacteria concentrations occurred at or just before the peak in flow of the storm hydrograph for nearly every storm sampled. This hydrograph/pollutograph pattern was also observed for PAHs (Stein et al. 2006) and trace metals (Tiefenthaler et al. in press) in the greater Los Angeles area. Tiefenthaler et al. (2001) observed similar pollutographs that showed peak suspended-sediment concentrations preceding the peak in discharge for the Santa Ana River. Similar time vs. concentration relationships were observed by Characklis and Wiesner (1997), who reported that the maximum concentrations of zinc, organic carbon and solids coincided with early peak storm water flows. Bacterial counts typically vary by up to five orders of magnitude on daily, seasonal, and inter-annual scales. The extreme variability in FIB makes storm water bacteria concentrations difficult to accurately estimate. Furthermore, as living organisms, many processes that do not influence other constituents, such as re-growth of environmentally adapted strains, die-off, and random fluctuations in population size, may affect bacterial counts (Ferguson et al. 2005). Therefore, more frequent monitoring over longer time periods and for the entire duration of storms is necessary in order to make assessments of "typical" bacterial counts and accurate estimates of EMC and FIB loading.

Further research is needed to directly assess the relationship between indicator bacteria concentrations and particle-size distributions in storm water runoff from mass emission and LU sites to better understand the fate, transport and treatment of indicator bacteria in urban runoff. Storm water borne bacteria are typically associated with particulates to varying degrees depending on the indicator bacteria and the size distribution of suspended solids in the storm water runoff. Furthermore, the particle size distribution, and bacteria partitioning can change over the course of a storm event (Furumai et al. 2002). Understanding the dynamic partitioning of indicator bacteria to various size particles is important to being able to estimate temporal and spatial patterns of bacterial deposition in estuaries and harbors, and should be an area of future investigation. Our understanding of the mechanisms of indicator bacteria loading from urban LU sites could also be improved by estimating the percent watershed impervious surface coverage in each LU category (i.e., percent rooftop, sidewalks, paved driveways and streets) and its impacts on storm water runoff concentrations and loads. This could allow identification of critical source areas and allow for more targeted application of best management practices.

Table 5-1. Correlations between total suspended solids (TSS) and stream flow with respect to fecal indicator bacteria (FIB) during storm condition. Within a table cell, the upper row shows Spearman's correlation coefficient (ρ), the middle row shows probability (p) that the null hypothesis of no correlation is true, and the lower row shows number of samples (n). Numbers in bold indicate correlations that are significant (p <0.04).

	Total Suspended Solids			Stream Flow		
	E. coli	*Enterococcus* spp.	Total Coliforms	*E. coli*	*Enterococcus* spp.	Total Coliforms
High Density Residential	-0.0815 0.6060 42	0.0226 0.8860 42	-0.0196 0.9010 42	0.6110 **<0.0001** 42	-0.0564 0.7050 42	0.0656 0.66 42
Low Density Residential	-0.3640 **0.0268** 37	-0.6030 **<0.0001** 37	-0.1800 0.2850 37	0.2390 0.1280 37	0.0400 0.8000 37	-0.2690 0.0851 37
Commercial	0.2460 0.0958 47	0.3540 **0.0149** 47	0.4160 **0.0038** 47	0.7720 **<0.0001** 47	0.8190 **<0.0001** 47	0.7960 **<0.0001** 47
Industrial	-0.3890 **0.0035** 55	-0.3040 **0.0244** 55	-0.1300 0.3440 55	-0.2510 **0.0421** 55	-0.2480 **0.0447** 55	-0.1330 0.285 55
Agricultural	0.5530 **<0.0001** 44	0.6160 **<0.0001** 44	0.3560 **0.0178** 44	0.2810 **0.0440** 44	0.4360 **0.0015** 44	0.6880 **<0.0001** 44
Recreational (horse)	0.6940 **<0.0001** 20	0.7670 **<0.0001** 20	0.7320 **<0.0001** 20	-0.0162 0.9370 20	0.5870 **0.0027** 20	-0.0921 0.664 20
Transportation	0.5190 **0.0190** 20	0.7410 **<0.0001** 20	0.6720 **0.0011** 20	-0.7120 **0.0080** 20	0.3920 0.1970 20	-0.3470 0.253 20
Open	0.6700 **<0.0001** 30	0.4610 **0.0106** 30	0.1740 0.3550 30	0.2550 0.0980 30	0.2230 0.1490 30	-0.1990 0.198 30

Table 5-2. Comparison of Nationwide Urban Runoff Program (NURP) and National Stormwater Quality Database data to fecal indicator bacteria concentrations from specific land uses in the Los Angeles, California, USA region. Median event mean concentration (EMCs) are in (MPN/100mL). NA = not analyzed.

Land Use Type	Median EMC (MPN/100mL)	
	E. coli	Total Coliform
Overall		
LARW[1]	3,922	40,559
NSQD[2]	5,091	11,000
NURP[3]	20,000	NA
Residential		
LARW[1]	6,331	55,426
NSQD[2]	8,345	5,467
NURP[3]	17,000	NA
Commercial		
LARW[1]	3,939	22,291
NSQD[2]	4,300	NA
NURP[3]	16,000	NA
Industrial		
LARW[1]	1,546	39,595
NSQD[2]	2,500	12,500
NURP[3]	14,000	NA
Open Space		
LARW[1]	5,374	25,565
NSQD[2]	7,200	NA
NURP[3]	NA	NA

[1]2001-2005 Los Angeles River Watershed Wet Weather Study
[2]The National Stormwater Quality Database (NSDQ), Pitt et al. (2003)
[3]Nationwide Urban Runoff Program (U.S. EPA 1983a)

Table 5-3. Comparison of seasonal concentrations of fecal indicator bacteria (FIB) from the Ballona Creek watershed. Event mean concentration (EMCs) in MPN/100mL.

	Ballona Creek					
Fecal Indicator Bacteria	Dry Weather[1] EMC (MPN/100 mL)			Wet Weather EMC (MPN/100 mL)		
	Min	Max	n	Min	Max	n
E. coli	693	7,457	3	8,304	43,305	6
Enterococcus spp.	727	2,173	3	14,438	78,368	6
Total Coliforms	21,763	40,556	3	127,635	678,973	6

[1]Data summarized from Water, Air and Soil Pollution, 2005. Vol. 164 (Stein E, Tiefenthaler L)

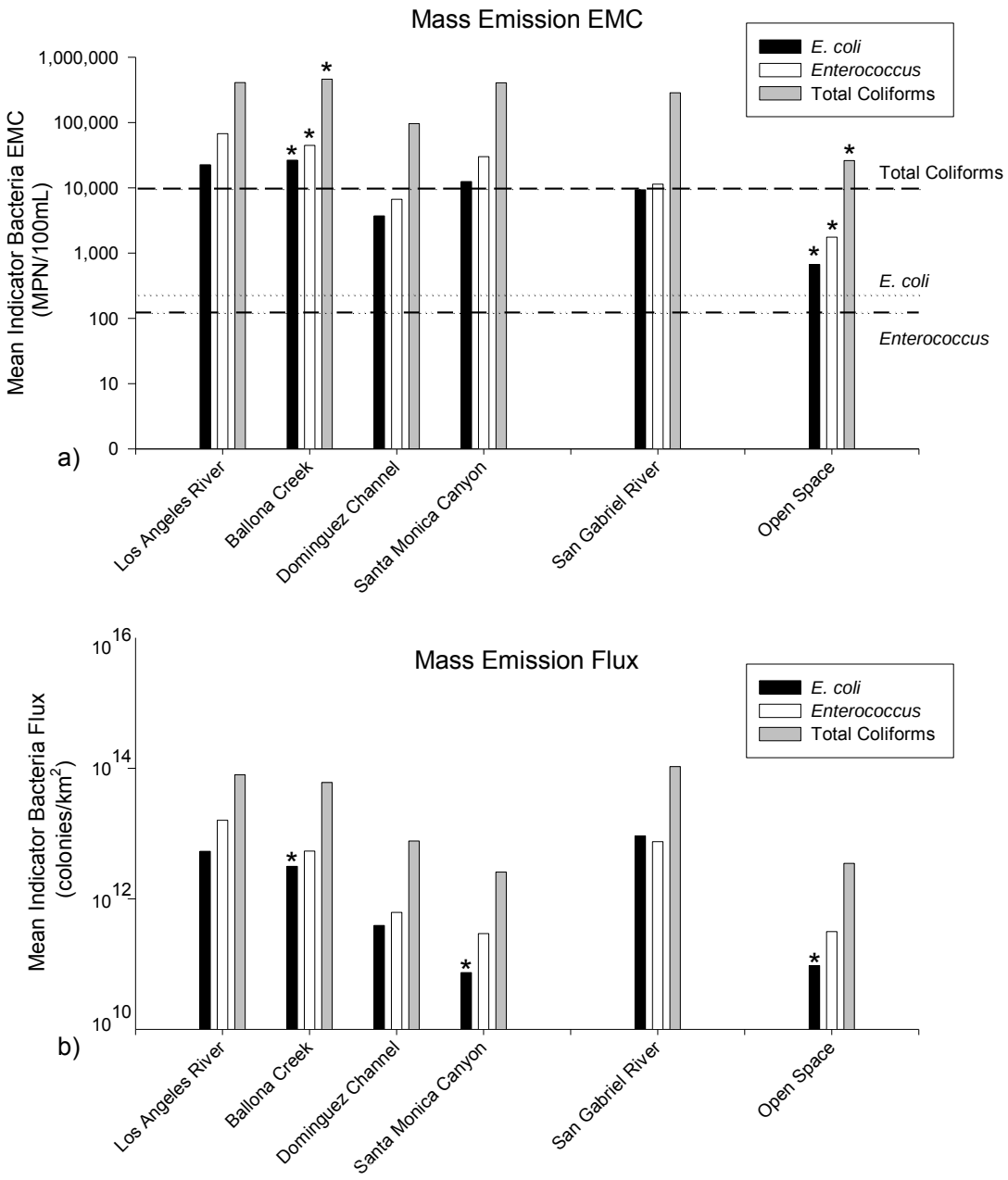

Figure 5-1. Mean storm EMCs (a) and fluxes (b) of *E. coli*, *Enterococcus* spp. and total coliform concentrations at specific southern California watersheds during the 2000/01-2004/05 storm seasons. Dotted lines indicate California beach water quality standards.

~~Will be updated~~

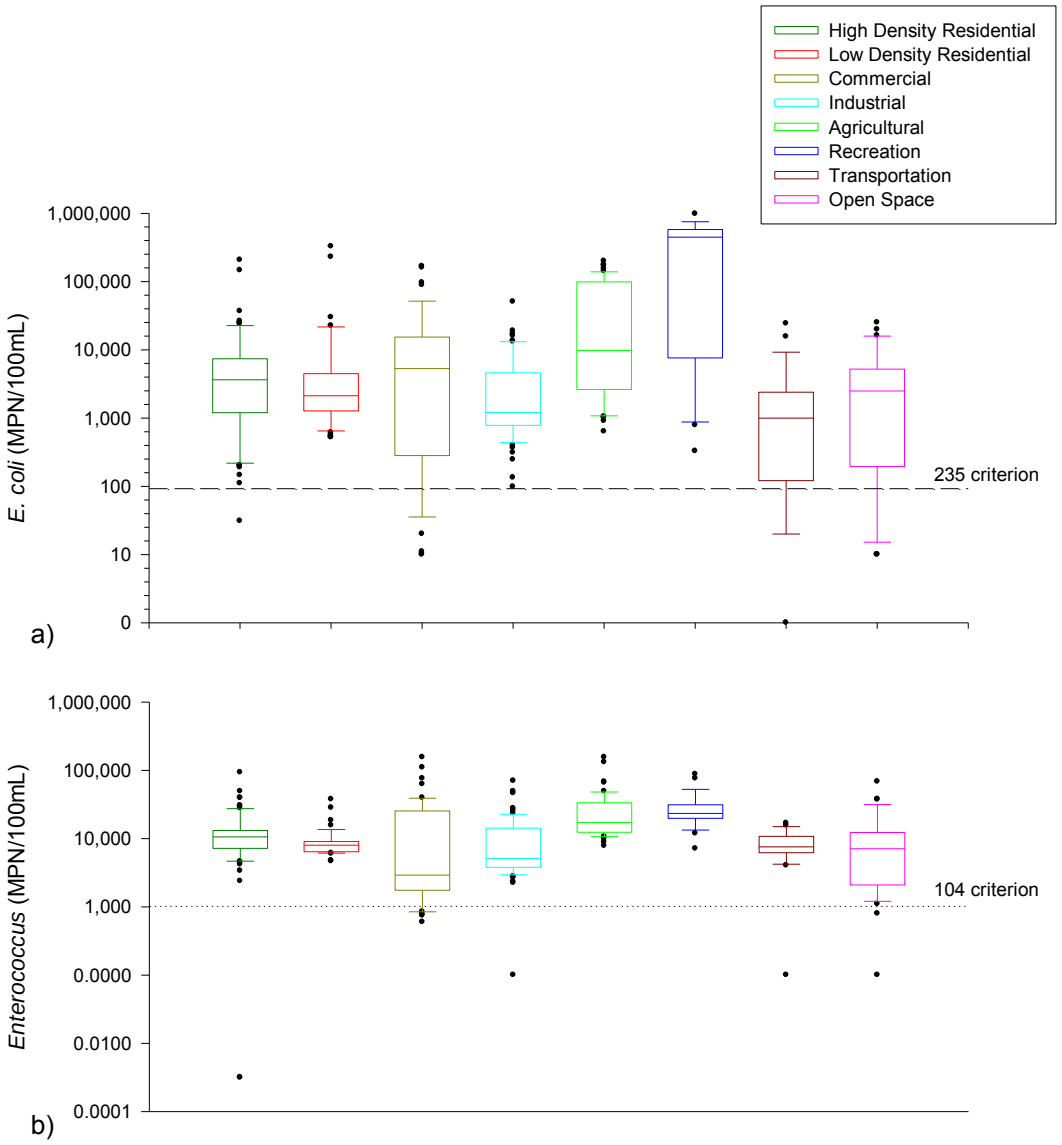

Figure 5-2. Distribution of *E. coli* (a) and *Enterococcus* spp. (b) concentrations during the 2000-2005 wet seasons from land use (LU) sites.

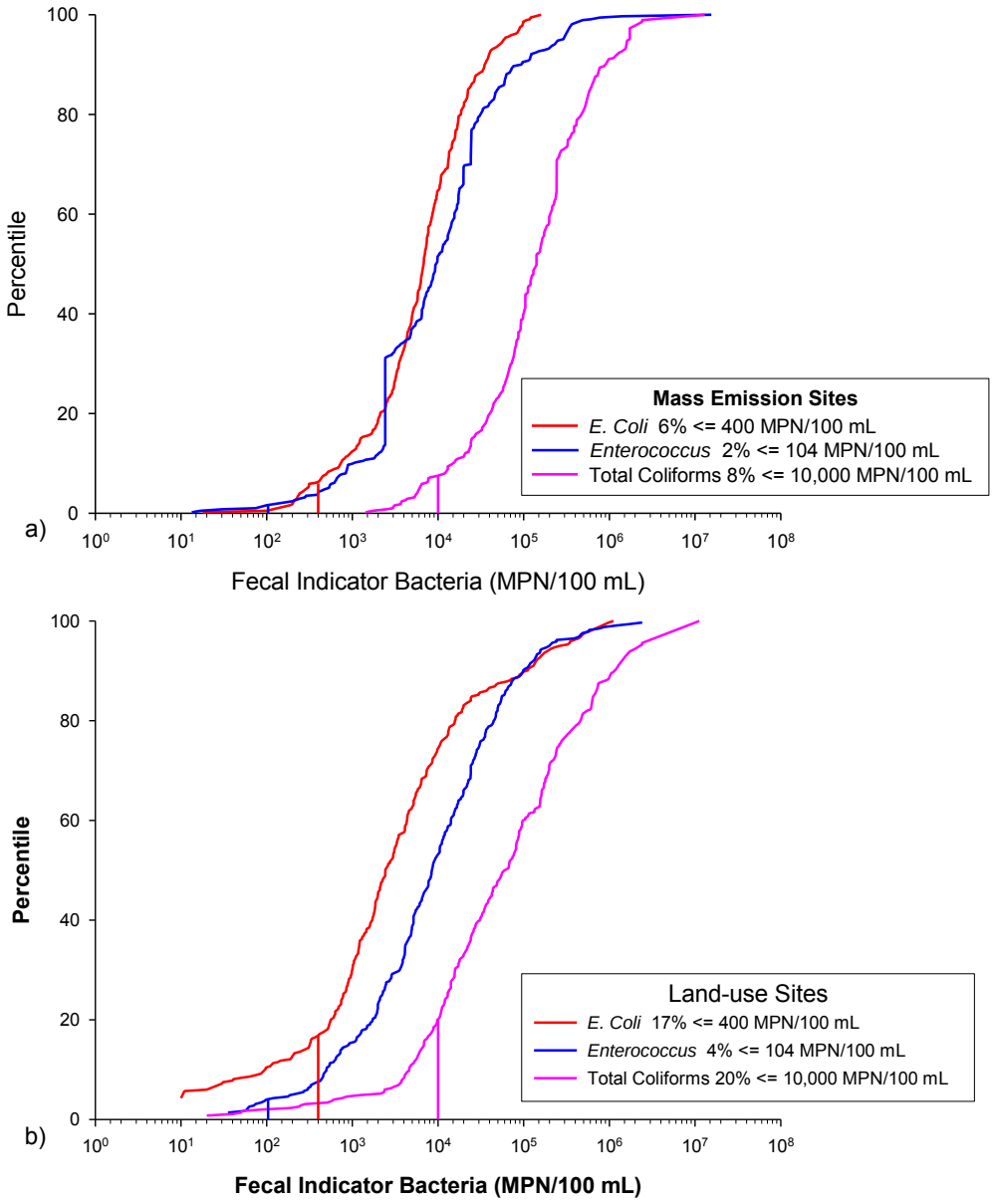

Figure 5-3. Cumulative density frequency plots (CDFs) of mass emission (ME; a) and land use (LU) sites (b) relative to beach water quality standards.

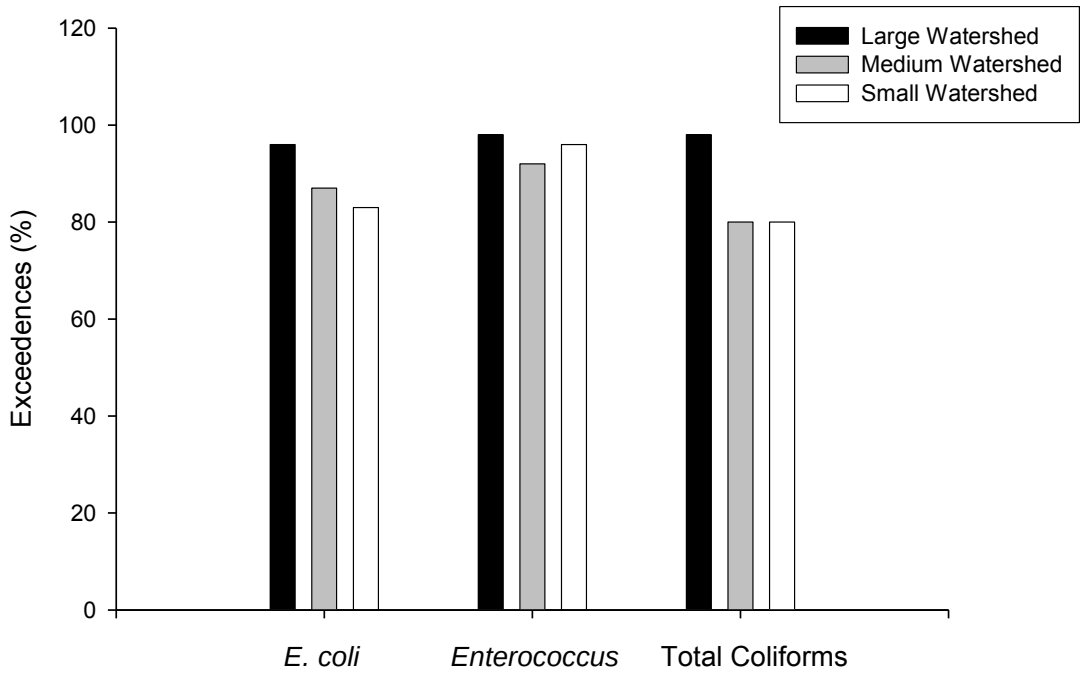

Figure 5-4. Comparison of water quality threshold exceedences at mass emission (ME) and land use (LU) sites with watershed size (small: <25 km^2, 25-100 km^2, >100 km^2).

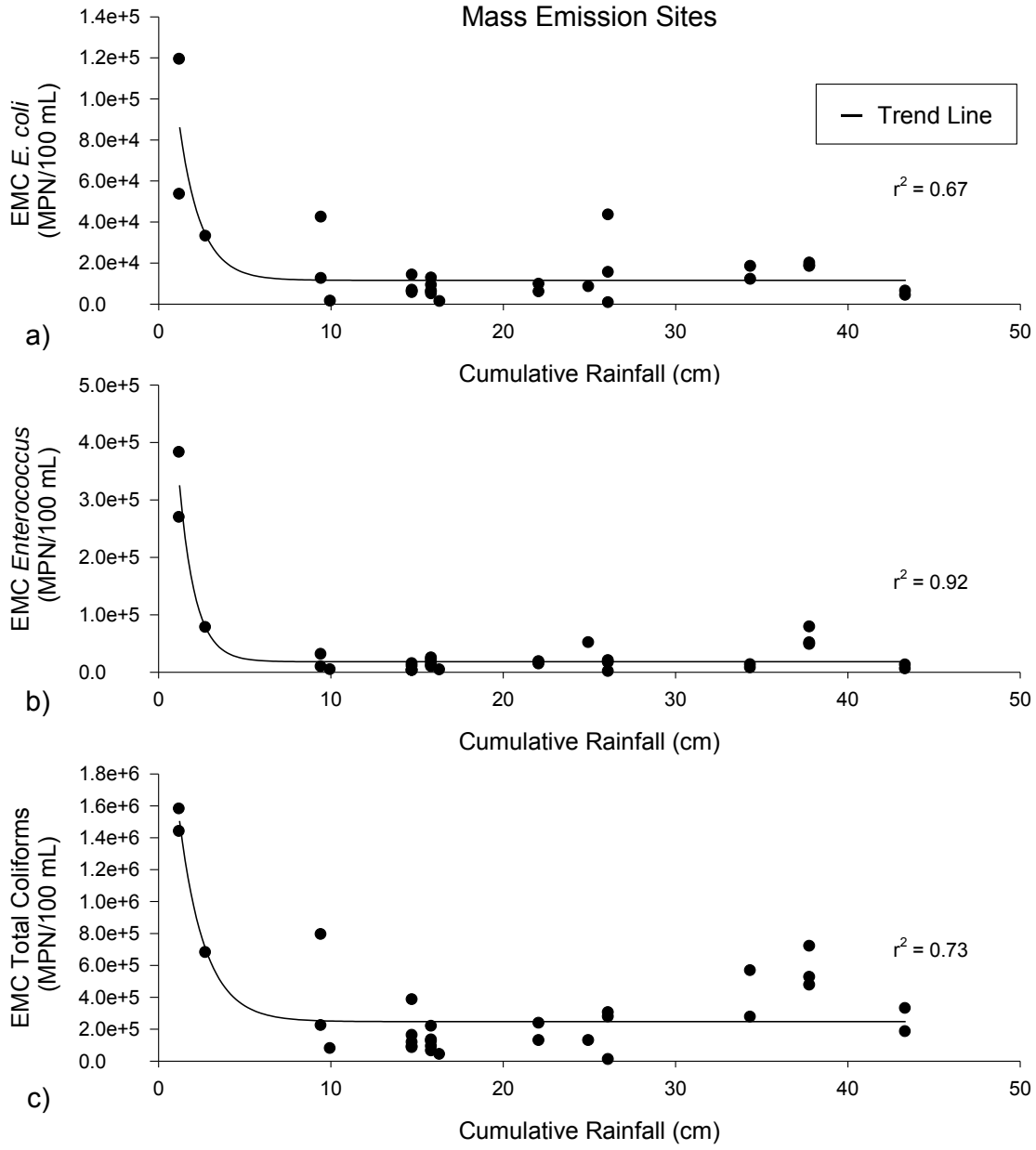

Figure 5-5. Cumulative annual rainfall versus event mean concentration (EMC) for *E. coli* (a), *Enterococcus* spp. (b), and total coliforms (c) during 2000-2005 storm seasons for mass emission (in-river) sites only.

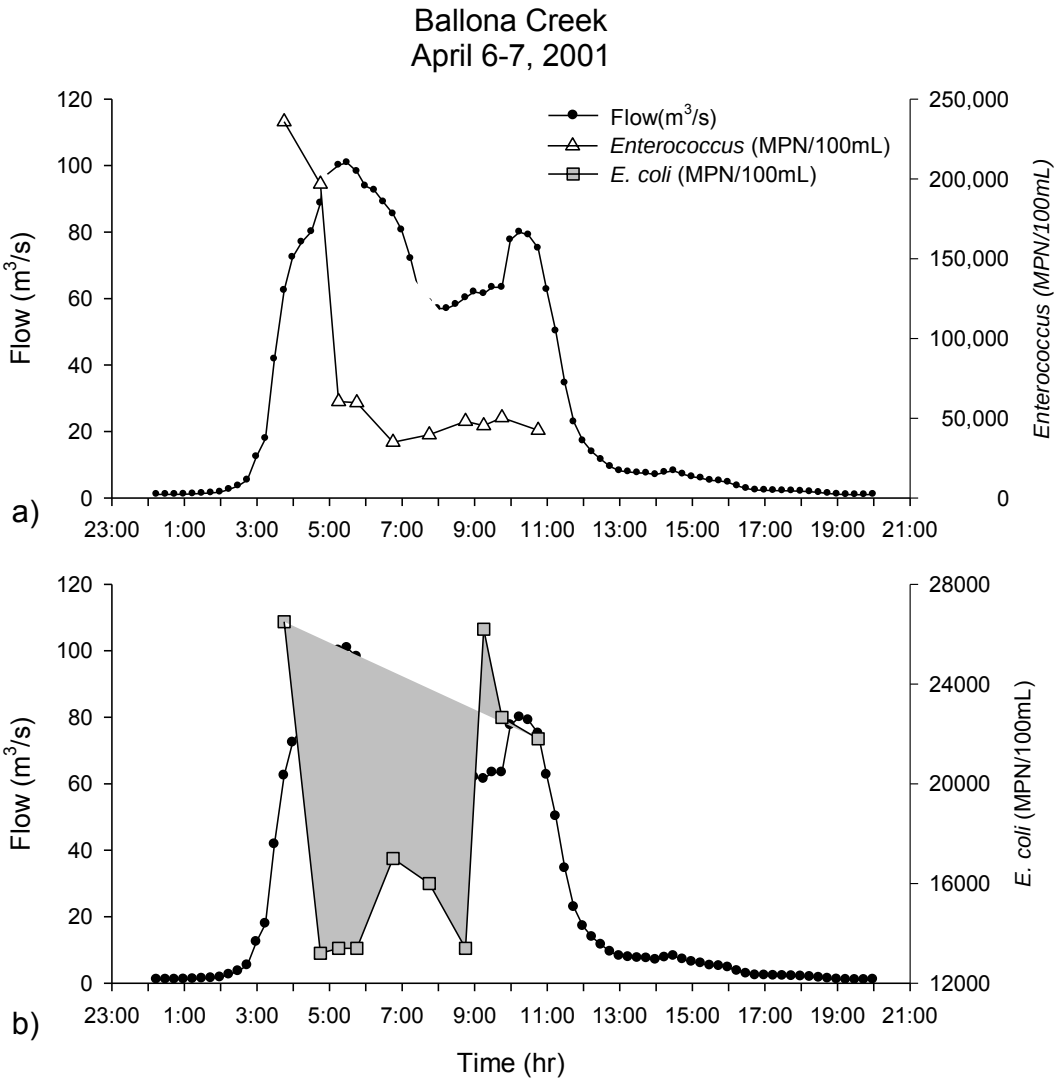

Figure 5-6. ***Enterococcus*** **spp. (a) and** ***E. coli*** **(b) concentrations with time for a storm event from the developed Ballona Creek watershed.**

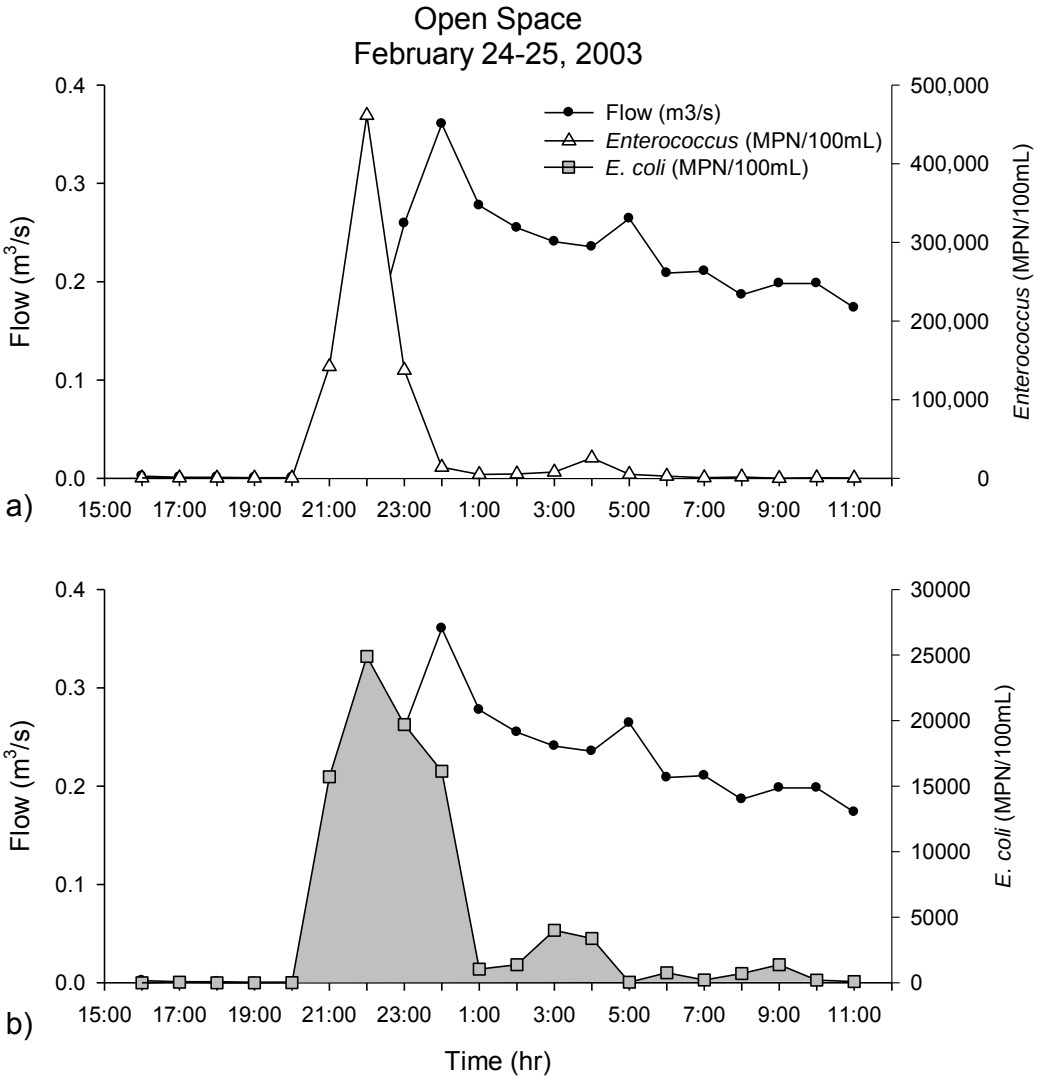

Figure 5-7. *Enterococcus* spp. (a) and *E. coli* (b) concentrations with time for a storm event from the open space land use site.

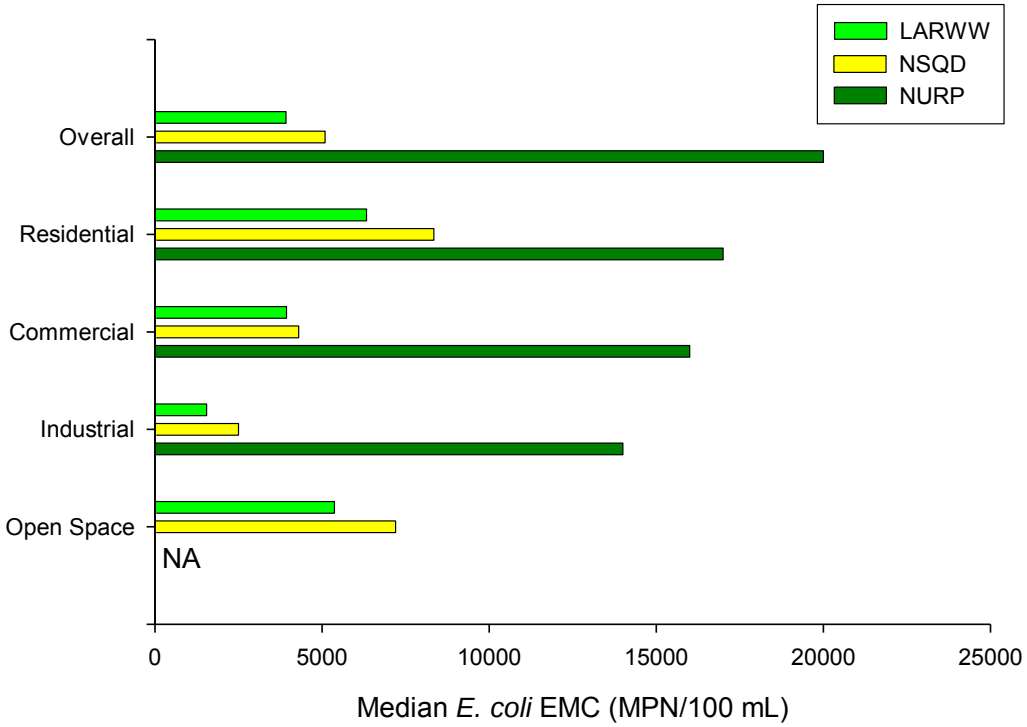

Figure 5-8. Comparison of median *E. coli* event mean concentration (EMCs) at specific land use (LU) sites during the 1983 Nationwide Urban Runoff Program (NURP, U.S. EPA 1983a), to the 1990 National Stormwater Quality Database (NSQD, Pitt et al. 2003) monitoring study and the 2001-2005 Los Angeles River Wet Weather (LARWW) study. Median EMCs are in (MPN/100mL). NA = not analyzed.

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APPENDIX A: Detailed Description of Land Use Categories

ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/TechnicalReports/510_APPENDIX_A.pdf

APPENDIX B: Trace Metal, TSS and Bacteria EMCs, Fluxes and Loadings at Mass Emission and Land Use Sites

ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/TechnicalReports/510_APPENDIX_B.pdf

APPENDIX C: First Flush Patterns of Trace Metals at Mass Emission and Land Use Sites

ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/TechnicalReports/510_APPENDIX_C.pdf

APPENDIX D: Probability Density Frequency Plots (PDFs) of Fecal Indicator Bacteria at Land Use Sites in the Greater Los Angeles, CA Region

ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/TechnicalReports/510_APPENDIX_D.pdf

From: Richard Watson <rwatson@rwaplanning.com>
To: "Renee A. Purdy" <rpurdy@waterboards.ca.gov>
CC: Sam Unger <sunger@waterboards.ca.gov>, Deborah Smith <Dsmith@waterboards.ca.gov>, Ivar Ridgeway <IRidgeway@waterboards.ca.gov>
Date: 7/12/2012 6:09 PM
Subject: Fwd: DPR Acts to Curb Water Pollution from Pyrethroid Insecticides

Renee:

If DPR's new regulations and the new labeling of pyrethroids will be as effective in cutting the amount of pyrethroids in urban runoff as indicated in this email, do we really need the pyrethroid study specified in the Tentative Order?

Rich

Richard Watson
Richard Watson & Associates, Inc.
Development Services. Stormwater Quality. Strategic Planning.
21922 Viso Lane
Mission Viejo, CA 92691-1318 U.S.A.
949.855.6272 Phone
949.394.8495 Cell
949.855.0403 Fax
www.rwaplanning.com

Begin forwarded message:

> From: Kelly Moran <kmoran@tdcenvironmental.com>
> Subject: Fwd: DPR Acts to Curb Water Pollution from Pyrethroid Insecticides
> Date: July 12, 2012 4:45:04 pm PDT
> To: Richard Watson <rwatson@rwaplanning.com>
>
>
>
> Begin forwarded message:
>
>> From: Kelly Moran <kmoran@tdcenvironmental.com>
>> Date: July 5, 2012 10:48:09 AM PDT
>> To: Kelly Moran <kmoran@tdcenvironmental.com>
>> Subject: DPR Acts to Curb Water Pollution from Pyrethroid Insecticides
>>
>> New California Department of Pesticide Regulation (DPR) requirements that become effective July 19 will modify the way that professional applicators apply pyrethroid insecticides around buildings. In parallel, new pyrethroid product labeling being implemented voluntarily by manufacturers at DPR's request--including special labels for the most persistent pyrethroid, bifenthrin--will provide further water quality protection. Both the regulations and the labeling will reduce treatments of outdoor impervious surfaces, thus reducing the quantity of pyrethroids that can be washed directly into gutters and storm drains when it rains or when water like irrigation overflow runs across treated surfaces. Together, the regulations and the new labeling will reduce the amount of pyrethroid insecticides in urban stormwater runoff by 80-90%.
>>
>> DPR developed the regulations and requested manufacturers modify product labels in response to the finding that pyrethroid insecticides are causing water and sediments in California urban creeks to be toxic to sensitive aquatic organisms. California Water Boards and the California Stormwater Quality Association (CASQA), using information assembled by the government-funded Urban Pesticides Pollution Prevention Project (UP3 Project), worked with DPR toward development of a solution to this water pollution problem.
>>
>> University of California scientific research played a key role in the characterization of the pyrethroid insecticide water pollution problem and in identification of application practices that reduce pyrethroid use while continuing to control pests. California's professional structural pest control applicators provided DPR and other agencies invaluable information about pyrethroid application practices and the practical aspects of controlling insects around buildings.
>>
>> UP3 Project analysis--based on pyrethroid monitoring data, pyrethroid use data, and urban runoff modeling by U.C. Davis-- suggests that the regulations will largely--but not completely--end widespread water and sediment toxicity from pyrethroids in California's urban watersheds. In some watersheds, lower levels of toxicity may continue. In a larger number of watersheds, pyrethroid concentrations will continue to exceed aquatic life protection benchmarks such as the water quality criteria developed by UC Davis with funding from the Central Valley Water Board.
>>
>> In coming months, some professional pest control operators are likely to switch to other insecticides, some of which may create new water pollution problems. A recent CASQA monitoring data summary suggests that one substitute insecticide, fipronil, may already be washing into urban creeks at levels sufficient to harm sensitive aquatic organisms.
>>
>> California government agencies will be monitoring urban creeks and working together toward making further adjustments as necessary to

protect water quality.
>>
>> Businesses and residents can prevent pesticide-related water pollution by employing effective pest control practices that minimize the need to use pesticides. Professional applicators certified by Ecowise or Green Pro provide this type of pest control. Do-it-yourselfers can learn how to implement these practices from Our Water Our World or University of California's Integrated Pest Management Program.
>>
>> DPR's Enforcement Branch will be working with California's Agricultural Commissioners and California professional pest control applicators to implement the new regulations. For implementation questions, DPR recommends contacting George Farnsworth, Chief of DPR's Enforcement Branch at gfarnsworth@cdpr.ca.gov
>>
>> Kelly Moran
>> TDC Environmental
>> UP3 Project
>

United States Environmental Agency
Office of Wastewater Management (4203M)
EPA 833-R-10-004 (June/2010)
http://www.epa.gov/npdes/permitbasics

National Pollutant Discharge Elimination System Test of Significant Toxicity Technical Document

June 2010

NATIONAL POLLUTANT DISCHARGE ELIMINATION SYSTEM
TEST OF SIGNIFICANT TOXICITY
TECHNICAL DOCUMENT

An Additional Whole Effluent Toxicity Statistical Approach for Analyzing Acute and Chronic Test Data

U.S. Environmental Protection Agency
Office of Wastewater Management
Water Permits Division
1200 Pennsylvania Avenue, NW
Mail Code 4203M
EPA East Building – Room 7135
Washington, DC 20460

June 2010

NOTICE AND DISCLAIMER

This document provides the technical basis for the Test of Significant Toxicity (TST) approach under the National Pollutant Discharge Elimination System (NPDES) for permitting authorities (states and Regions) and persons interested in analyzing valid whole effluent toxicity (WET) test data using the traditional hypothesis testing approach as part of the NPDES Program under the Clean Water Act (CWA). This document describes what the U.S. Environmental Protection Agency (EPA) believes is another statistical option to analyze valid WET test data for NPDES WET reasonable potential and permit compliance determinations. The document does not, however, substitute for the CWA, an NPDES permit, or EPA or state regulations applicable to permits or WET testing; nor is this document a permit or a regulation itself. The TST approach does not result in changes to EPA's WET test methods promulgated at Title 40 of the *Code of Federal Regulations* Part 136. The document does not and cannot impose any legally binding requirements on EPA, states, NPDES permittees, or laboratories conducting or using WET testing for permittees (or for states in evaluating ambient water quality). EPA could revise this document without public notice to reflect changes in EPA policy and guidance. Finally, mention of any trade names, products, or services is not and should not be interpreted as conveying official EPA approval, endorsement, or recommendation.

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FIGURES

EXECUTIVE SUMMARY

The U.S. Environmental Protection Agency (EPA or the Agency) has developed a new statistical approach that assesses the whole effluent toxicity (WET) measurement of wastewater effects on specific test organisms' ability to survive, grow, and reproduce. This new approach is called the Test of Significant Toxicity (TST) and is a statistical method that uses hypothesis testing techniques based on research and peer-reviewed publications. The hypothesis test under the TST approach examines whether an effluent, at the critical concentration (e.g., in-stream waste concentration or IWC), as recommended in EPA's Technical Support Document (TSD; USEPA 1991) and implemented under EPA's WET National Pollutant Discharge Elimination System (NPDES) permits program, and the control within a WET test differ by an unacceptable amount (the amount that would have a measured detrimental effect on the ability of aquatic organisms to thrive and survive).

Since the inception of EPA's NPDES WET program in the mid 1980s, the Agency has striven to advance and improve its application and implementation under the NPDES WET Program. The TST approach explicitly incorporates test power, which, using the TST approach, is the ability to correctly classify the effluent as acceptable under the NPDES WET Program (i.e., non-toxic). The TST approach also provides a positive incentive to generate high quality, valid WET data to make informed decisions regarding NPDES WET reasonable potential (RP) and permit compliance determinations. Once the WET test has been conducted (using multiple effluent concentrations and other requirements as specified in the WET test methods), the TST approach can be used to analyze valid WET test results to assess whether the effluent discharge is toxic. The TST approach is designed to be used for a two concentration data analysis of the IWC or a receiving water concentration (RWC) as compared to a control concentration.

Background

In the NPDES WET Program, an effluent sample is declared toxic relative to a permitted WET limit if the no observed effect concentration (NOEC) is less than the permitted IWC using a hypothesis statistical approach. In such an approach, the question being answered is, "Is the mean response of the organisms the same or worse in the control than at the IWC?" The hypothesis testing approach has four possible outcomes: (1) the IWC is truly toxic and is declared toxic, (2) the IWC is truly non-toxic and is declared non-toxic, (3) the IWC is truly toxic but is declared non-toxic, and (4) the IWC is truly non-toxic but is declared toxic. The latter two possible outcomes represent decision errors that can occur with any hypothesis testing approach. In the NPDES WET Program, those two types of errors occur when either test control replication is poor (i.e., the within-test variability is high) so that even large differences in organism response between the IWC and control are incorrectly classified as non-toxic (outcome [3] above) or, test control replication is very good (i.e., the within-test variability is low) so that a very small difference between IWC and control is declared toxic (outcome [4] above). That former outcome stems from the fact that in the NPDES WET Program, the hypothesis approach established and controls the false positive error rate (i.e., Type I or alpha) but not the false negative error rate (i.e., Type II or beta). Establishing the beta error rate determines the power of the test (power = 1-beta), which is the probability of correctly detecting an actual toxic effect using the traditional hypothesis testing approach (i.e., declaring an effluent toxic when, in fact, it is toxic). By not establishing an appropriate beta error rate and test power in the NPDES WET

Program, the permittee has no incentive to generate more precise data within a test using the traditional hypothesis approach, and, in fact, is at a disadvantage for achieving a high level of precision.

What is the Test of Significant Toxicity Approach?

Organism responses to the effluent and control are unlikely to be exactly the same, even if no toxicity is present. They might differ by such a small amount that even if statistically significant, it would be considered negligible biologically. A more useful approach could be to rephrase the null hypothesis, "Is the mean response in the effluent less than a defined biological amount?" the Food and Drug Administration has successfully used that approach for many years to evaluate drugs, as have many researchers in other biological fields. In that approach, the null hypothesis is stated as the organism response in the effluent is less than or equal to a fixed fraction (*b*) of the control response (e.g., 0.75 of the control mean response):

$$\text{Null hypothesis: Treatment mean} \leq b \times \text{Control mean}$$

In the NPDES WET Program, to reject the null hypothesis above means the effluent is considered non-toxic. To accept the null hypothesis means the effluent is toxic. That test has been adapted for the NPDES WET Program and is referred to as the *Test of Significant Toxicity* (TST).

Before the TST null hypothesis expression could be used in the NPDES WET Program, certain decisions were needed, including what effect level in the effluent is considered unacceptably toxic and the desired frequency of declaring a truly negligible effect within a test non-toxic. Such decisions are referred to as Regulatory Management Decisions (RMDs).

What are the RMDs for TST?

In the TST approach, the *b* value in the null hypothesis represents the threshold for unacceptable toxicity. For *chronic* testing in EPA's NPDES WET Program, the *b* value in the TST analysis is set at 0.75, which means that a 25 percent effect (or more) at the IWC is considered evidence of unacceptable *chronic* toxicity. IWC responses substantially less than a 25 percent effect would be interpreted to have a lower risk potential. The RMD for *acute* WET methods is set at 0.80, which means that a 20 percent effect (or more) at the IWC is considered evidence of unacceptable *acute* toxicity. The acute RMD toxicity threshold is higher (i.e., more strict) than that for chronic WET methods because of the severe environmental implications of acute toxicity (lethality or organism death).

EPA's RMDs using the TST approach are intended to identify unacceptable toxicity in WET tests most of the time when it occurs, while also minimizing the probability that the IWC is declared toxic when in fact it is truly acceptable. This objective requires additional RMDs regarding acceptable maximum false positive (**β using a TST approach)** and false negative rates (**α using a TST approach)**. In the TST approach, the RMDs are defined as (1) declare a sample toxic between 75–95 percent of the time ($0.05 \leq \alpha \leq 0.25$) when there is unacceptable toxicity (20 percent effect for acute and 25 percent effect for chronic tests), and (2) declare an effluent non-toxic no more than 5 percent of the time ($\beta \leq 0.05$) when the effluent effect at the critical effluent concentration is 10 percent. Table ES-1 summarizes the difference in Type I and II error

expressions between the TST approach and the traditional hypothesis approach currently used in the NPDES WET Program.

Table ES-1. Definition of the Type I and Type II error under the traditional hypothesis testing approach and the TST approach.

	Traditional hypothesis approach	TST
Type I (alpha)	Set at 0.05	Set at 0.05 to 0.25 given a *b* value of 0.80 or 0.75 depending on whether the WET test method is acute or chronic, respectively
	Effluent is considered safe but declared *toxic*	Effluent is considered toxic, but declared *safe*
	Permittee concern	Regulatory concern
Type II (beta)	Not established	Set at 0.05
	Effluent is considered toxic but declared *safe*	Effluent is considered safe but declared *toxic*
	Regulatory concern	Permittee concern

How was the TST approach developed?

EPA used valid WET data from approximately 2,000 WET tests to develop and evaluate the TST approach. The TST approach was tested using nine different WET test methods comprising twelve biological endpoints (e.g., reproduction, growth, survival) and representing most of the different types of WET test designs in use. More than one million computer simulations were used to select appropriate alpha error rates for each test method that also achieved EPA's other RMDs for the TST approach.

Once the alpha error rates were established, the results of the TST approach were compared to those obtained using the traditional hypothesis testing approach for a range of test results. The alpha values identified in this project build on existing information (such as data sources and analyses examining ability to detect toxic effects) on WET published and peer reviewed by EPA, including *Understanding and Accounting for Method Variability in WET Applications Under the NPDES Program* (USEPA 2000).

This document outlines the recommended TST approach and presents the following:

- How an appropriate alpha value was identified for several common WET test methods on the basis of desired beta error rates, various effect levels, and within-test control variability.

- The degree of protectiveness of TST compared to the traditional hypothesis testing approach. In this report, *as protective as* is defined as an equal ability to declare a sample toxic at or above the regulatory management level.

Because TST is a form of hypothesis testing, analyses in this document focus on comparing results of TST to the traditional hypothesis testing approach and not to point estimate techniques such as linear interpolation (i.e., IC25). Therefore, this document does not discuss point estimate procedures.

Data analysis approach

EPA assembled a comprehensive database to analyze the utility of the TST approach with data obtained from EPA Regions, several states, and private laboratories, which represent a widespread sampling of typical laboratories and test methods for approximately 2,000 tests. Nine commonly tested WET methods were examined. For each test method, control precision (coefficient of variation [CV]) was calculated on the basis of valid WET test data compiled in the project. Cumulative frequency plots were used to identify percentiles of observed method-specific CVs (e.g., 25^{th}, 50^{th}, 75^{th} percentiles). The measures were calculated to update previous EPA analyses (USEPA 2000) using more recent valid WET test data and to characterize typical, achievable test performance in terms of within-test control variability. A similar analysis was performed for the control response for each of the nine test methods (e.g., mean offspring per female in the *Ceriodaphnia dubia* test method) to characterize typical achievable test performance in terms of control response.

Monte Carlo simulation analysis was used to estimate the percentage of WET tests that would be declared toxic using TST as a function of different α levels, within-test control variability, and mean percent effect level. The simulation analysis identified expected beta error rates (i.e., declaring an effluent toxic when in fact it is acceptable under TST) for a broad range of possible test scenarios. Using the RMDs above, an appropriate α level was then identified for a given WET test design that also yielded a β error rate ≤ 0.05 when there was a 10 percent mean effect. By simulating thousands of WET tests for a given scenario (mean percent effect and control CV), the percentage of tests declared toxic could be calculated and compared among scenarios, and between TST and the traditional hypothesis approach.

Results of the analysis

Results of all analyses indicate that TST is a suitable alternative to the traditional hypothesis approach for analyzing two-concentration WET data (i.e., IWC and control) in the NPDES WET Program. A demonstrated benefit of the TST approach is that increasing the precision and power of the test increases the chances of declaring an effluent non-toxic when there is ≤ 10 percent mean effect in the effluent. Increasing test replication (and thereby the power of the test) results in a *lower* rate of tests declared toxic using TST but a *higher* rate of tests declared toxic using the traditional hypothesis approach (see Figure ES-1). Using TST, a permittee has the ability to demonstrate that its effluent is acceptable, by improving the quality of test data (e.g., decreasing within-test variability, and/or increasing replication), if indeed the mean effect at the IWC is less than the regulatory management decision (25 percent [chronic] or 20 percent [acute]).

On the basis of EPA's analyses, the alpha levels shown in Table ES-2 are recommended for the nine EPA WET test methods examined using the TST approach. An important feature of the TST approach is that the TST's alpha is analogous to beta under the traditional hypothesis testing approach, which had not been established by EPA previously for the NPDES WET Program.

Table ES-2. Summary of alpha (α) levels or false negative rates recommended for different EPA WET test methods using the TST approach.

EPA WET test method	*b* value	Probability of declaring a toxic effluent non-toxic False negative (α) error[a]
Chronic Freshwater and East Coast Methods		
Ceriodaphnia dubia (water flea) survival and reproduction	0.75	0.20
Pimephales promelas (fathead minnow) survival and growth	0.75	0.25
Selenastrum capricornutum (green algae) growth	0.75	0.25
Americamysis bahia (mysid shrimp) survival and growth	0.75	0.15
Arbacia punctulata (Echinoderm) fertilization	0.75	0.05
Cyprinodon variegatus (Sheepshead minnow) and *Menidia beryllina* (inland silverside) survival and growth	0.75	0.25
Chronic West Coast Marine Methods		
Dendraster excentricus and *Strongylocentrotus purpuratus* (Echinoderm) fertilization	0.75	0.05
Atherinops affinis (topsmelt) survival and growth	0.75	0.25
Haliotis rufescens (red abalone), *Crassostrea gigas (oyster), Dendraster excentricus, Strongylocentrotus purpuratus* (Echinoderm) and *Mytilus sp* (mussel) larval development methods	0.75	0.05
Macrocystis pyrifera (giant kelp) germination and germ-tube length	0.75	0.05
Acute Methods		
Pimephales promelas (fathead minnow), *Cyprinodon variegatus* (Sheepshead minnow), *Atherinops affinis* (topsmelt), *Menidia beryllina* (inland silverside) acute survival[b]	0.80	0.10
Ceriodaphnia dubia, *Daphnia magna, Daphnia pulex*, *Americamysis bahia* acute survival[b]	0.80	0.10

Notes:
a. α levels shown are the probability of declaring an effluent toxic when the mean effluent effect = 25% for chronic tests or 20% for acute tests and the false positive rate (β) is ≤ 0.05 (5%) when mean effluent effect = 10%.
b. Based on a four replicate test design

Results obtained from the TST analyses using the nine EPA WET test methods should be applicable to other EPA WET methods not examined. For example, results generated under this project for the fish *Pimephales promelas* survival and growth test is extrapolated to other EPA fish survival and growth tests (e.g., *Menidia* sp., *Cyprinus variegatus, Atherinops affinis*) because the test methods use a similar test design (e.g., number of replicates, number of organisms tested) and measure the same endpoints.

Figure ES-1 illustrates that conducting tests with more replicates (a priori) can assist a permittee to demonstrate that the effluent is acceptable. Conversely, increasing the number of replicates in a test does not assist a permittee using the current hypothesis testing approach.

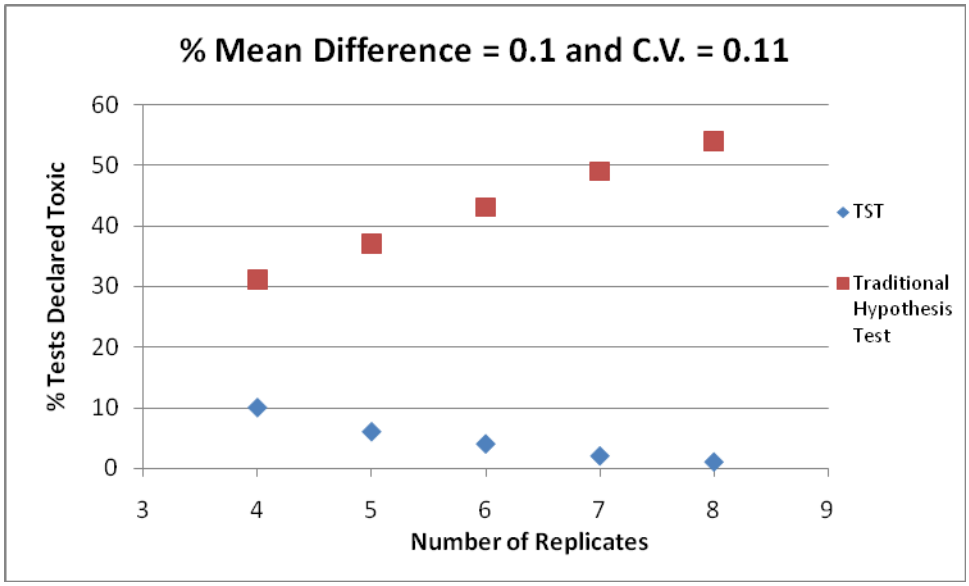

Figure ES-1. Percent of chronic fathead minnow WET tests declared toxic using TST having a mean effluent effect of 10 percent, above average control variability (CV = 0.11 or 11 percent) and an α = 0.25, as a function of the number of within-test replicates. Results using the traditional hypothesis test are shown as well.

Summary

Results of nearly 2,000 valid WET tests and thousands of simulations were conducted to develop the technical basis for the TST approach. That approach builds on the strengths of the traditional hypothesis testing approach, including use of robust statistical analyses, to determine whether an effluent sample is acceptable in WET testing. Specific benefits of using TST in WET analysis include the following:

- Provides transparent RMDs, which are incorporated into the data analysis process
- Incorporates statistical power directly into the statistical process by controlling for both alpha and beta errors, thereby, increasing the confidence in the WET test result
- Provides a positive incentive for the permittee to generate valid, high quality WET data
- Applicable to both NPDES WET permitting and 303(d) watershed assessment programs

Results of this project indicate that the TST is a viable additional statistical approach for analyzing valid acute and chronic WET test data. Using the explicit RMD and test method-specific alpha values, TST provides similar protection as the traditional hypothesis testing approach when there is unacceptable toxicity while also providing a transparent methodology for demonstrating whether an effluent is acceptable under the NPDES WET Program.

In summary, the TST approach provides another option for permitting authorities and permittees to use for analyzing WET test data. The TST approach provides a positive incentive to generate valid, high quality WET data to make informed decisions regarding NPDES WET reasonable

potential (RP) and permit compliance determinations. Using TST, permitting authorities will be better able to identify toxic or acceptable samples.

ACRONYMS AND ABBREVIATIONS

CETIS®	Comprehensive Environmental Toxicity Information System
CFR	Code of Federal Regulations
CV	coefficient of variation
WDNR	Wisconsin Department of Natural Resources
EPA	U.S. Environmental Protection Agency
IC25	25 percent inhibition concentration
IWC	in-stream waste concentration
LOEC	lowest observed effect concentration
LC50	50 percent lethal concentration
MSD	minimum significant difference
NOEC	no observed effect concentration
NPDES	National Pollutant Discharge Elimination System
QA/QC	quality assurance/quality control
RMD	regulatory management decision
RP	reasonable potential
RWC	receiving water concentration
SWAMP	Surface Water Ambient Monitoring Program (California)
TAC	Test acceptability criteria
TMDL	total maximum daily load
TSD	Technical Support Document for Water Quality-Based Toxics Control
TST	Test of Significant Toxicity
WET	whole effluent toxicity

GLOSSARY

Acute Toxicity Test is a test to determine the concentration of effluent or ambient waters that causes an adverse effect (usually mortality) on a group of test organisms during a short-term exposure (e.g., 24, 48, or 96 hours). Acute toxicity is determined using statistical procedures (e.g., point estimate techniques or a t-test).

Ambient Toxicity is measured by a toxicity test on a sample collected from a receiving waterbody.

Chronic Toxicity Test is a short-term test in which sublethal effects (e.g., reduced growth or reproduction) are usually measured in addition to lethality.

Coefficient of Variation (CV) is a standard statistical measure of the relative variation of a distribution or set of data, defined as the standard deviation divided by the mean. The CV can be used as a measure of precision within and between laboratories, or among replicates for each treatment concentration.

Effect Concentration (EC) is a point estimate of the toxicant concentration that would cause an observable adverse effect (e.g., mortality, fertilization). EC_{25} is a point estimate of the toxicant concentration that would cause observable 25% adverse effect as compared to the control test organisms.

False Negative is when the in-stream waste concentration is declared non-toxic but in fact is truly toxic. In the traditional hypothesis approach, false negative error rate is denoted by Beta (β). In the TST approach, false negative error rate is denoted as Alpha (α), which applies when the percent effect in the critical effluent concentration is $\geq$ 25% for a given test.

False Positive is when the in-stream waste concentration is declared toxic but in fact is truly non-toxic. In the traditional hypothesis approach, false positive error rate is denoted by Alpha (α). In the TST approach, false positive error rate is denoted as Beta (β), which applies when the percent effect in the critical effluent concentration is $\leq$ 10% for a given test.

Hypothesis Testing is a statistical approach (e.g., Dunnett's procedure) for determining whether a test concentration is statistically different from the control. Endpoints determined from hypothesis testing are no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC). The two hypotheses commonly tested in WET are

- **Null hypothesis (H_o)**: The effluent is non-toxic.
- **Alternative hypothesis (H_a)**: The effluent is toxic.

Inhibition Concentration (IC) is a point estimate of the toxicant concentration that would cause a given, percent reduction in a non-lethal biological measurement (e.g., reproduction or growth), calculated from a continuous model (i.e., Interpolation Method). E.g., IC_{25} is a point estimate of the toxicant concentration that would cause a 25 percent reduction in a non-lethal biological measurement.

In-stream Waste Concentration (IWC) is the concentration of a toxicant or effluent in the receiving water after mixing. The IWC is the inverse of the dilution factor. It is sometimes referred to as the receiving water concentration (RWC).

LC50 (lethal concentration, 50 percent) is the toxicant or effluent concentration that would cause death to 50 percent of the test organisms.

Lowest Observed Effect Concentration (LOEC) is the lowest concentration of an effluent or toxicant that results in statistically significant adverse effects on the test organisms (i.e., where the values for the observed endpoints are statistically different from the control).

Minimum Significant Difference (MSD) is the magnitude of difference from control where the null hypothesis is rejected in a statistical test comparing a treatment with a control. MSD is based on the number of replicates, control performance, and power of the test.

No Observed Effect Concentration (NOEC) is the highest tested concentration of an effluent or toxicant that causes no observable adverse effect on the test organisms (i.e., the highest concentration of toxicant at which the values for the observed responses are not statistically different from the control).

National Pollutant Discharge Elimination System (NPDES) is the national program for issuing, modifying, revoking and reissuing, terminating, monitoring and enforcing permits, and imposing and enforcing pretreatment requirements, under sections 307, 318, 402, and 405 of Clean Water Act.

Power is the probability of correctly rejecting the null hypothesis (i.e., declaring an effluent toxic when, in fact, it is toxic using the traditional hypothesis test approach).

Precision is a measure of reproducibility within a data set. Precision can be measured both within a laboratory (within-laboratory) and between laboratories (between-laboratory) using the same test method and toxicant.

Quality Assurance (QA) is a practice in toxicity testing that addresses all activities affecting the quality of the final effluent toxicity data. QA includes practices such as effluent sampling and handling, source and condition of test organisms, equipment condition, test conditions, instrument calibration, replication, use of reference toxicants, recordkeeping, and data evaluation.

Quality Control (QC) is the set of more focused, routine, day-to-day activities carried out as part of the overall QA program.

Reasonable Potential (RP) is where an effluent is projected or calculated to cause an excursion above a water quality standard on the basis of a number of factors including the four factors listed in Title 40 of the *Code of Federal Regulations* (CFR) 122.44(d)(1)(ii).

Reference Toxicant Test is a check of the sensitivity of the test organisms and the suitability of the test methodology. Reference toxicant data are part of a routine QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

Regulatory Management Decision (RMD) is the decision that represents the maximum allowable error rates and thresholds for toxicity and non-toxicity that would result in an acceptable risk to aquatic life.

Replicate is two or more independent organism exposures of the same treatment (i.e., effluent concentration) within a whole effluent toxicity test. Replicates are typically separate test chambers with organisms, each having the same effluent concentration**.**

Sample is a representative portion of a specific environmental matrix that is used in toxicity testing. For this document, environmental matrices could include effluents, surface waters, groundwater, stormwater, and sediment.

Significant Difference is a statistically significant difference (e.g., 95 percent confidence level) in the means of two distributions of sampling results.

Statistic is a computed or estimated quantity such as the mean, standard deviation, or Coefficient of Variation.

Test Acceptability Criteria (TAC) are test method-specific criteria for determining whether toxicity test results are acceptable. The effluent and reference toxicant must meet specific criteria as defined in the test method (e.g., for the *Ceriodaphnia dubia* survival and reproduction test, the criteria are as follows: the test must achieve at least 80 percent survival and an average of 15 young per surviving female in the control and at least 60% of surviving organisms must have three broods).

t-test (formally Student's t-Test) is a statistical analysis comparing two sets of replicate observations, in the case of WET, only two test concentrations (e.g., a control and IWC). The purpose of this test is to determine if the means of the two sets of observations are different (e.g., if the 100-percent effluent or ambient concentration differs from the control [i.e., the test passes or fails]).

Type I Error (alpha α) is the error of rejecting the null hypothesis (H_o) that should have been accepted.

Type II Error (beta β) is the error of accepting the null hypothesis (H_o) that should have been rejected.

Toxicity Test is a procedure to determine the toxicity of a chemical or an effluent using living organisms. A toxicity test measures the degree of effect on exposed test organisms of a specific chemical or effluent.

Welch's t-test is an adaptation of Student's t-test intended for use with two samples having unequal variances.

Whole Effluent Toxicity (WET) is the total toxic effect of an effluent measured directly with a toxicity test.

1.0 INTRODUCTION

1.1 Summary of Current EPA Recommended WET Analysis Approaches

Within the National Pollutant Discharge Elimination System (NPDES) Program, freshwater and marine acute and chronic whole effluent toxicity (WET) tests are used in conjunction with other analyses to evaluate and assess compliance of wastewater and surface waters with water quality standards of the Clean Water Act. In the NPDES WET Program, WET tests examine organism responses to effluent, typically along a dilution series (USEPA 1995, 2002a, 2002b). Acute WET test methods measure the lethal response of test organisms exposed to effluent (USEPA 2002c). The principal response endpoints for such methods are the effluent concentration that is lethal to 50 percent of the test organisms (LC50) or the effluent concentration at which survival is significantly lower than the control (e.g., t-test). Chronic WET test methods often measure both lethal and sublethal responses of test organisms. The statistical endpoints that are used in chronic WET testing in the NPDES WET Program are the no observed effect concentration (NOEC), and the 25 percent inhibition concentration (IC25). The NOEC endpoint is determined using a traditional hypothesis testing approach that identifies the maximum effluent concentration tested at which the response of test organisms is not significantly worse from the control. From a regulatory perspective, an effluent sample is declared toxic relative to a permitted WET limit if the NOEC is less than the permitted in-stream waste concentration (IWC), as recommended in EPA's Technical Support Document (TSD) (USEPA 1991) and implemented under EPA's WET NPDES permits program. The IC25, by contrast, is a point-estimation approach. It identifies the concentration at which the response of test organisms is 25 percent below that observed in the control concentration and interpolates the effluent concentration at which this magnitude of response is expected to occur. From a regulatory perspective, an effluent sample is declared toxic relative to a permitted WET limit if the IC25 is less than the permitted IWC. This document focuses on another statistical option with respect to the traditional hypothesis testing approach for analyzing and interpreting valid WET data.

1.2 Advantages and Disadvantages of Recommended Traditional Hypothesis Testing Approach

The hypotheses traditionally used in WET statistical comparisons of a biological measure (survival, growth, reproduction) in control water versus a particular effluent sample are the following:

$$\begin{aligned} &\textit{Null Hypothesis}: && \mu_T \geq \mu_C \\ &\textit{Alternative Hypothesis}: && \mu_T < \mu_C \end{aligned}$$

where μ_C refers to the true mean for the biological measure in the control water and μ_T refers to the true mean for this measure in the effluent sample. *True mean* here refers to the mean for a theoretical statistical population of results from indefinite repetition of toxicity tests on the same control water and effluent sample. In contrast, the mean for the biological measure for a single toxicity test would be referred to as the *sample mean*, and random variation among organisms might cause a sample mean for an effluent to be less than the control even if the effluent is actually non-toxic. The traditional WET hypothesis thus assumes that the effluent sample is non-toxic. For an individual test, there must be a statistical test to determine if the null hypothesis is

rejected in favor of the alternative hypothesis; i.e., that any apparent toxicity based on the sample means is real and not simply reflective of random variation. Such a statistical test is part of current recommended practice in WET testing.

Table 1-1 summarizes the correctness of results from such statistical testing, contrasting the true condition of whether the effluent sample is toxic to the result of the statistical test. Two types of errors can occur in the statistical test result. A false positive occurs when the effluent is actually non-toxic, but the statistical test infers that it is toxic. For the statistical hypotheses here, that is a Type I error (the null hypothesis is rejected when it is true) and the probability of this error is typically designated by the variable α, so that the correct decision occurs with probability 1 - α. The other type of error, a false negative, occurs when the effluent truly is toxic, but the statistical test infers that it is non-toxic. For the statistical hypotheses here, that is a Type II error (the null hypothesis is accepted when it is false) and the probability of the error is typically designated by the variable β, so that the probability of the correct decision is 1 - β, which is also referred to as the test power.

Table 1-1. Error terminology for traditional WET hypothesis methodology

Statistical test result	**True condition**	
	$\mu_T \geq \mu_C$ (sample is non-toxic)	**$\mu_T < \mu_C$ (sample is toxic)**
$\mu_T \geq \mu_C$ (Sample is non-toxic)	Correct Decision (probability=1–α)	False Negative Type II Error (probability=β)
$\mu_T < \mu_C$ (Sample is toxic)	False Positive Type I Error (probability=α)	Correct decision Test Power (1-β)

It is important to note that β does not have a single value but rather is a function of how toxic the sample actually is (i.e., there is a greater chance of incorrectly saying an effluent is non-toxic if it is only slightly toxic than if it is highly toxic). Similarly, given that the null hypothesis is an inequality, α also does not have a single value, because if effluent characteristics actually improve the biological measure, the probability with which a non-toxic effluent is called toxic will be a function of the extent of this beneficial effect. Although there is a designated single value for α in the statistical test calculations (e.g., 0.05), this error probability applies only when the true condition is exactly at $\mu_T = \mu_C$.

This variation of α and β can be better understood using Figure 1-1, which depicts the probability of declaring an effluent toxic versus the true toxicity of the effluent, expressed as the ratio of the true biological measure in the effluent to the true biological measure in the control (μ_T / μ_C). The curves on this figure are for a hypothetical statistical analysis of hypothetical toxicity tests, but exemplify performance curves that could be drawn for *any* statistical analysis of *any* toxicity test under the traditional WET hypotheses provided above. The solid line is for a toxicity test with large variability so that it is less likely that the statistical test will detect toxicity, and the dashed line is for a toxicity test with low variability. Such curves provide a useful and complete summary of the basic information desired from WET testing. How

effectively will the testing detect toxicity for different levels of true toxicity? How often will non-toxic effluents mistakenly be declared toxic? Although test performance can be appreciated from such curves without addressing specific types of statistical errors, the behavior of those errors can be illustrated using the curves. The portion of the curve with $\mu_T / \mu_C \geq 1$ gives values for α (i.e., the effluent is truly non-toxic so that calling it toxic, a false positive, is a Type I error under the traditional null hypothesis). In accordance with WET hypothesis test procedures, the example curves have $\alpha = 0.05$ when μ_T / μ_C is exactly at 1.0. The portion of the curve with $\mu_T / \mu_C < 1$ is the *power curve* for the test (i.e., 1–β, the probability of calling an effluent toxic when it truly is toxic). This illustrates how test power is very low (approaching 0.05) when the effluent is only slightly toxic, but it increases as the true toxicity increases. The two different curves illustrate how this increase in test power depends on test uncertainty—i.e., higher within-test variability in the toxicity test results in less power for the statistical analysis.

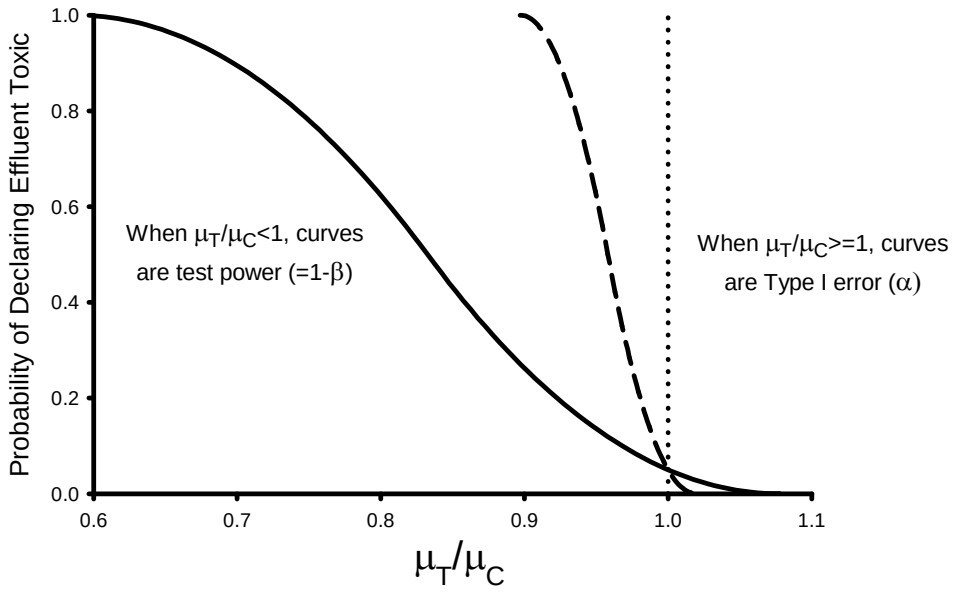

Figure 1-1. Example test performance curves for traditional WET hypothesis tests. The dotted line marks where the true mean biological measure in the effluent equals that in the control. The solid curve is for a high variability test, while the dashed curve is for a low variability test.

Various researchers have reported several advantages and disadvantages of the traditional hypothesis testing approach as currently used in the NPDES WET Program (Grothe et al. 1996). Two common limitations cited are (1) if the test control replication is very good (i.e., test is very precise), an effluent might be considered toxic when in fact its toxicity is low enough to be considered acceptable, and (2) if test control replication is poor (i.e., the test is very imprecise), a highly toxic effluent might be incorrectly classified as non-toxic. For example, the more precise test in Figure 1-1 would declare an effluent with only 5 percent toxicity to be toxic about 60 percent of the time, whereas the less precise test in Figure 1-1 would declare 20 percent toxicity to be non-toxic about 40 percent of the time. The first limitation arises because the null hypothesis is defined around $\mu_T = \mu_C$, so the goal is to call an effluent toxic if $\mu_T < \mu_C$, no matter

how small the difference. The second limitation arises from the fact that the NPDES WET Program hypothesis testing approach does not address the false negative error rate (i.e., Type II error, β) and thus does not address requirements regarding the power of the test to detect substantial levels of toxicity. By not establishing an appropriate β and test power in the NPDES WET Program, the permittee has no incentive to increase the precision of a WET test when using the traditional hypothesis approach. As illustrated in Figure 1-1, greater precision simply results in more samples being declared toxic and can lead to high rejection rates for effluents with low levels of toxicity that might be considered acceptable. Although EPA has made improvements in statistical procedures, such as including a test review step of the percent minimum significant differences (i.e., to minimize within-test variability), it is desirable to further improve the hypothesis testing approach. Such improvement is the focus of this report and a general approach for this, the Test of Significant Toxicity (TST), is discussed next.

1.3 Test of Significant Toxicity

The TST is an alternative statistical approach for analyzing and interpreting valid WET test data that also uses a hypothesis testing approach but in a different way, building on previous work conducted by EPA in the NPDES WET Program (USEPA 2000) as well as other researchers (Erickson and McDonald 1995; Shukla et al. 2000; Berger and Hsu 1996). The TST approach is based on a type of hypothesis testing referred to as *bioequivalence testing*. Bioequivalence is a statistical approach that has long been used in evaluating clinical trials of pharmaceutical products (Anderson and Hauck 1983) and by the Food and Drug Administration (Hatch 1996; Aras 2001; Streiner 2003). The approach has also been used to evaluate the attainment of soil cleanup standards for contaminated sites (USEPA 1988, 1989) and to evaluate effects of pesticides in experimental ponds (Stunkard 1990).

For the NPDES WET Program, the TST approach changes the hypotheses to the following:

$$\textit{Null Hypothesis:} \qquad \mu_T \leq b \times \mu_C$$

$$\textit{Alternative Hypothesis:} \qquad \mu_T > b \times \mu_C$$

The TST hypotheses thus incorporate two important differences from the traditional WET hypotheses. First, a specific value for the ratio μ_T / μ_C, designated *b*, is included to delineate unacceptable and acceptable levels of toxicity, allowing a risk management decision about what level of toxicity should be allowed if the true means were known, other than the absence of any toxicity as specified by the traditional hypothesis. Second, the inequalities are reversed so that it is assumed that the effluent sample has an unacceptable level of toxicity until demonstrated otherwise. As a result of this reversal of the inequalities, the meanings of α and β under the TST hypotheses (Table 1-2) are reversed from those under the traditional hypothesis approach (Table 1-1). Under the TST approach, α is associated with false negatives, β is associated with false positives, and statistical test power using the TST approach in the NPDES WET Program is the ability to correctly conclude that true toxicity levels are acceptable. In addition, an effluent sample would be considered acceptable under the TST approach when the null hypothesis is rejected; in contrast, a sample is considered unacceptable under the traditional hypothesis approach when the null hypothesis is rejected.

Table 1-2. Error terminology for TST WET hypothesis methodology

Statistical test result	**True condition**	
	$\mu T \leq b \times \mu C$ (Toxicity is unacceptable)	**$\mu T > b \times \mu C$ (Toxicity is acceptable)**
$\mu_T \leq b \times \mu_C$ (Toxicity is unacceptable)	Correct Decision (1–α)	False Positive Type II Error (β)
$\mu_T > b \times \mu_C$ (Toxicity is acceptable)	False Negative Type I Error (α)	Correct Decision Test Power (1–β)

Figure 1-2 provides illustrative examples of test performance under the TST approach and illustrates advantages of this approach over the traditional hypotheses. This figure shows the same basic type of performance curve as in Figure 1-1: the probability of calling an effluent unacceptably toxic versus the true toxicity in the effluent. Incorporating *b* in the hypotheses explicitly recognizes that the true mean for the organism response in an effluent can be less than that in the control by a certain amount and still be considered acceptable, and it keeps the false negative rate for this amount of toxicity constant regardless of test variability (Figure 1-2). As mentioned previously, the current NPDES WET Program does not control the false negative rate, which varies markedly at any given level of toxicity as test precision varies (Figure 1-1). By reversing the inequalities and referencing them to *b*, the TST approach also results in more precise tests having lower false positive errors (Figure 1-2); i.e., effluents with true levels of toxicity that are acceptably low are declared toxic with less frequency as precision increases, a desirable attribute for the method. That provides permittees with a clear incentive to improve the precision of test results. Thus, using the TST approach, a permittee has to demonstrate with some confidence that their effluent has toxicity in an acceptable range, but can also improve testing procedures as needed to do so (i.e., increase replicates or decrease within-test variability or both).

1.4 Regulatory Management Decisions for TST

Regulatory management decisions (RMDs) are incorporated into the TST methodology by selecting values for *b*, the dividing point between acceptable and unacceptable toxicity, and α, the false negative error rate when $\mu_T = b \times \mu_C$.

The selection of *b* should reflect what is considered acceptable if the true biological response means for the effluent and control were actually known, especially because precise tests might have performances closely approaching this ideal. For all chronic WET test methods, the RMD is to set *b* to 0.75. This *b* value (25 percent toxic effect) is consistent with EPA's use of the IC25 in point estimation methods for examining chronic WET data. Chronic effects less than 25 percent would be considered to have an acceptably low risk potential. Because of the more severe environmental implications of acute toxicity (organism death), the RMD for acute WET test methods is to set *b* higher than that for chronic WET test methods, at 0.80.

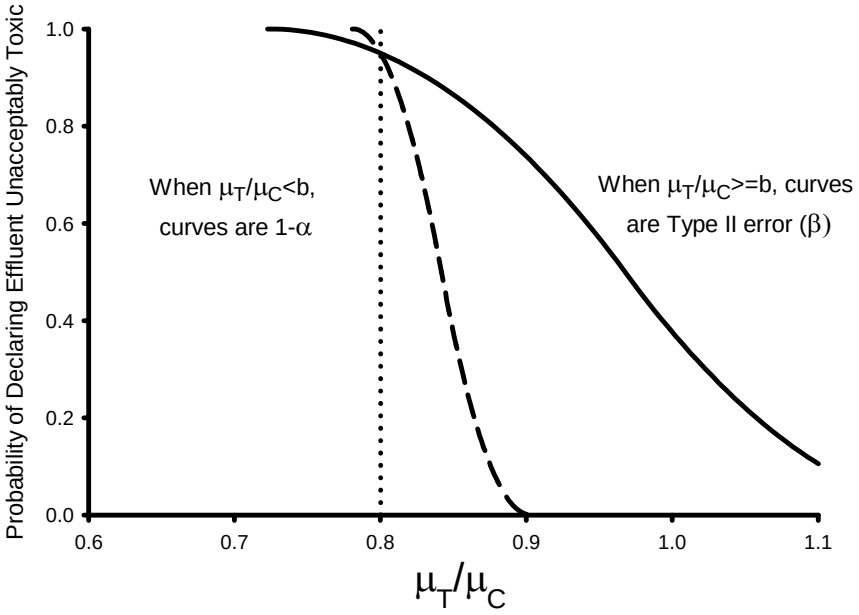

Figure 1-2. Example test performance curves for TST WET hypothesis tests. For this example, *b* is set to 0.8 (denoted by dotted line), with $\alpha = 0.05$. The two curves represent test performance for tests with high (solid line) and low (dashed line) variability.

For a given test precision and value for *b*, selecting a value for α completely determines both false negative and false positive error rates at all toxicity levels, such as the curves in Figure 1-2. However, the value selected for α does not have to be based just on consideration of the desired error rate when $\mu_T = b \times \mu_C$. Rather, α can be selected on the basis of balancing goals regarding this false negative error rate with goals for false positive error rates at lower levels of toxicity. Therefore, a different α can be assigned for different types of WET toxicity tests based on test precision and on specific goals regarding false positive and false negative rates.

With regard to false negative rates, EPA's general goal is to identify unacceptable toxicity in WET tests most of the time when it occurs. It would be preferred to set α at the typical 0.05 level (i.e., if $\mu_T = b \times \mu_C$, the effluent will be declared unacceptable 95 percent of the time). However, for tests with low precision, this could result in a high rate of false positives (declaring effluents unacceptable) when toxicity is low or absent (e.g., Figure 1-2). Therefore, values of α up to 0.25 will be allowed, as needed to meet the goal regarding false positive rates discussed in the next paragraph. Thus, the false negative rate RMD is $0.05 \leq \alpha \leq 0.25$, so that there is at least a 0.75 probability that an effluent with unacceptable toxicity ($\mu_T \leq b \times \mu_C$) will be declared toxic.

With regard to false positive error probabilities, EPA's general goal is that they be low when toxicity is negligible. It is necessary to define *negligible* as a second, smaller level of effect than *acceptable* because the latter includes toxicity as high as that represented by *b*, at which point the false positive error rate always will approach $1 - \alpha$, so cannot be low. With regard to this, EPA defines negligible as 10 percent toxicity or less, and specifies that the false positive error

probability be no higher than 0.05 at 10 percent toxicity. Thus, the false positive RMD is $\beta \leq 0.05$ at $\mu_T/\mu_C=0.90$, provided this is achievable with $\alpha \leq 0.25$ (if α is at this maximum, this false positive RMD no longer applies). It should be emphasized that this RMD relates to only one point in the range of toxicity considered acceptable, and that false positives will vary widely within this range (e.g. Figure 1-2). False positive rates will be lower when toxicity is lower than 10 percent, dropping to near zero when toxicity is absent, and will be higher when toxicity values are greater than negligible but still acceptable, rising to 1-α as the toxicity approaches the unacceptable level.

Therefore, the overall RMD for α (the false negative rate when $\mu_T / \mu_C = b$) is to set it to the lowest value that results in $\beta \leq 0.05$ (the false positive rate) when the true toxicity is at $\mu_T / \mu_C = 0.90$, but that α will be no lower than 0.05 and no higher than 0.25. This selection will be primarily a function of test method within-test variability (e.g., control coefficient of variation or CV), but cannot and should not be done on an individual test basis. Rather, TST alphas are assigned for different types of WET tests on the basis of simulations that address how TST method performance is affected by the test design and types of endpoints measured, and the associated CVs.

1.5 Document Objectives

This document presents TST as a useful alternative data analysis approach for valid WET test data that may be used in addition to the approaches currently recommended in EPA's Technical Support Document (USEPA 1991) and EPA's WET test method manuals. In adapting the TST for use in evaluating WET test data, analyses were conducted to identify an appropriate Type I error rate (α) for several common EPA WET methods given certain RMDs. Once alpha error rates were established, results of the TST approach were compared to those obtained using the traditional hypothesis testing approach and a range of test results.

This document outlines the recommended TST approach and presents the following:

- How an appropriate alpha value was identified for several common EPA WET test methods on the basis of desired alpha and beta error rates using explicit RMDs (i.e., effect levels) and considering a range of within-test control variability observed in valid WET tests.
- The degree of protectiveness of TST compared to the traditional hypothesis testing approach. In this report, *as protective as* is defined as an equal ability to declare a sample toxic at or above the regulatory management decision.

In this project, emphasis was placed on comparing results of TST to traditional hypothesis testing approaches and not to point estimate techniques such as linear interpolation (i.e., IC25). Therefore, this document does not discuss linear interpolation techniques. In addition, this document discusses the TST approach only with regard to comparing individual effluent samples to a control, and does not evaluate extensions of the TST approach to simultaneous multiple comparisons such as in Erickson and McDonald (1995).

The focus of this document is on chronic WET test methods and sublethal endpoints because many different types of alternative analysis procedures have been proposed for these tests.

Applying the TST methodology to the acute fish and *Ceriodaphnia* WET test method is also included. This document provides a summary of the recommended TST method, α values for several common WET methods, and results of comprehensive analyses supporting EPA recommendations.

2.0 METHODS

Methods used to evaluate the TST approach and determine how it should be applied for WET test analysis in the NPDES WET Program proceeded using several general steps as follows:

Step 1: WET test methods and endpoints were selected for analysis in the TST evaluation. A range of the more common EPA WET test methods were identified in this step.

Step 2: WET data were compiled from several state and EPA sources to determine current WET test method performance in terms of control response and within-test control variability.

Step 3: Simulation analyses were conducted using data characteristics obtained from Step 2 to guide the types of simulated data analyzed in this project and to set test method-specific α levels.

The following sections describe in more detail each of the steps.

2.1 Test Methods and Endpoints Evaluated

Table 2-1 summarizes the nine EPA WET test methods evaluated in this project. Preference was given to valid WET data generated using the EPA 1995 WET test methods for the EPA West Coast marine species (USEPA 1995) and for all other species the 2002 EPA WET test methods (USEPA 2002a, 2002b). Examining the inter-laboratory reference toxicant data for *C. dubia* by year indicated significantly more precise data from 1996 on as compared to pre-1995 (Figure 2-1). Similar results were observed for the fathead minnow and chronic mysid test methods as well. This result is not unexpected because the EPA chronic WET test methods were substantially refined as of 1995 and laboratories had more experience with the chronic test methods by this time. Within-test control 90th percentile CVs were not significantly different among years following 1995. Therefore, only post-1995 data were used in analyses for all EPA WET test methods.

All of the WET test methods listed in Table 2-1 are commonly used by regulatory authorities in making regulatory decisions such as determining WET reasonable potential (RP) or to determine compliance with acute and chronic WET limits or monitoring triggers. These nine test methods are representative of the range of EPA WET test methods commonly required of permittees in terms of types of toxicity endpoints written into NPDES permits and test designs followed by permittee's testing laboratories. Results obtained using these nine EPA test methods should be applicable to other EPA WET test methods not examined. For example, results of this project for the fish *Pimephales promelas* survival and growth test is extrapolated to other EPA fish survival and growth tests (e.g., *Menidia sp*., *Cyprinus variegatus*, *Atherinops affinis*) because those test methods use a similar test design (e.g., number of replicates, number of organisms tested) and measure the same endpoints. Previous analyses conducted by EPA (Denton and Norberg-King 1996; Denton et al. 2003) found comparable effect sizes for a given power among similar experimental designs and test endpoints. Similarly, the acute freshwater fish WET test analyzed in this project can be extrapolated to other fish acute test methods because they use a similar test design and measure mortality or immobility. The use of both EPA saltwater and freshwater WET tests ensured that there was adequate representation of different types of discharge situations and laboratories.

Table 2-1. Summary of test condition requirements and test acceptability criteria for each EPA WET test method evaluated in TST analyses

EPA method	Organism with scientific name	Endpoint type	Test type	Minimum # per test chamber	Minimum # of rep per conc.	Minimum # effluent conc.	Test duration	Test acceptance criteria (TAC)
2000.0	Fathead minnow *(Pimephales promelas)*	Survival	Acute	10	2	5	48–96 hours	≥ 90% survival in controls
1000.0	Fathead minnow *(Pimephales promelas)*	Survival and growth (larval)	Chronic	10	4	5	7 days	≥ 80% survival in controls; average dry weight per surviving organism in control chambers equals or exceeds 0.25 mg
1002.0	Water flea *(Ceriodaphnia dubia)*	Survival and reproduction	Chronic	1	10	5	Until 60% of surviving control organisms have 3 broods (6–8 days)	≥ 80% survival and an average of 15 or more young per surviving female in the control solutions. 60% of surviving control organisms must produce three broods
1007.0	Mysid shrimp *(Americamysis bahia)*	Survival and growth	Chronic	5	8	5	7 days	≥ 80% survival; average dry weight ≥ 0.20 mg in controls
1016.0	Purple urchin *(Strongylocentro-tus purpuratus)* or Sand dollar *(Dendraster excentricus)*	Fertilization	Chronic	100	4	4	40 min (20 min plus 20 min)	≥ 70% egg fertilization in controls; %MSD < 25%; and appropriate sperm counts
1017.0	Giant kelp *(Macrocystis pyrifera*	Germination and germ-tube length	Chronic	100 for germination 10 for germ-tube length	5	4	48 hours	≥ 70% germination in controls; ≥ 10 μm germ-tube lengths in controls; %MSD of < 20% for both germination and germ-tube length NOEC must be below 35 μg/L in reference toxicant test
1014.0	Red abalone (*Haliotis rufescens*)	Larval development	Chronic	100	5	4	48 hours	≥ 80% normal larval development in controls Statistical significance @ 56 μg/L zinc % MSD < 20%

Table 2-1. continued.

EPA method	Organism with scientific name	Endpoint type	Test type	Minimum # per test chamber	Minimum # of rep per conc.	Minimum # effluent conc.	Test duration	Test acceptance criteria (TAC)
2002.0	Water flea (*Ceriodaphnia dubia)*	Survival	Acute	5	4	5	24, 48, or 96 hours	≥ 90% survival in controls
1003.0	Green algae (*Selenastrum capricornutum*)	Growth (cell counts, chlorophyll fluorescence, absorbance, or biomass)	Chronic	10,000cells/ mL	4	5	96 hour	Mean cell density of at least 1 X 10^6 cells/mL in the controls; variability (CV%) among control replicates less than or equal to 20%

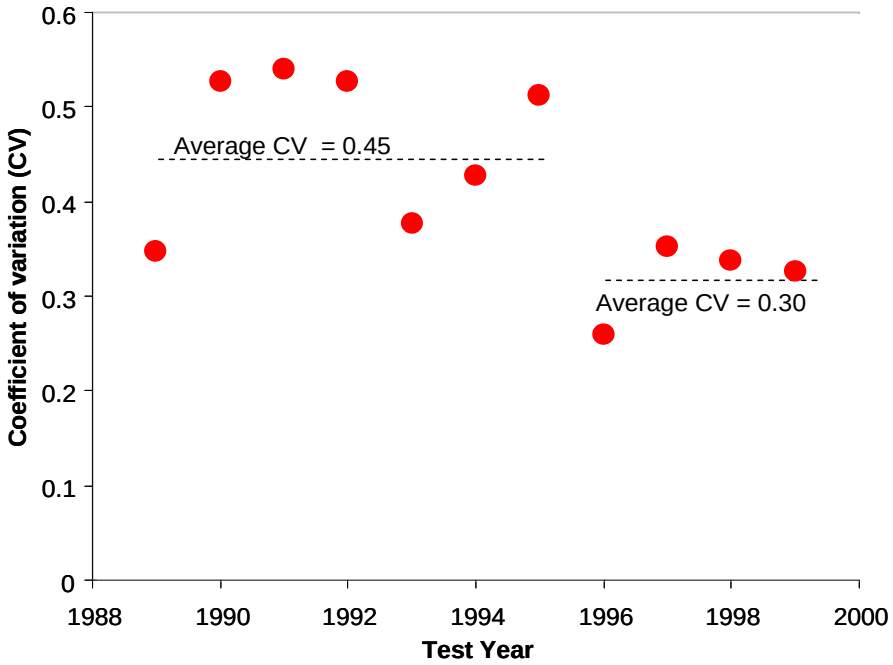

Figure 2-1. Summary of test variability (expressed as the control 90th percentile coefficient of variation or CV) observed between 1989 and 2000 for the chronic *Ceriodaphnia dubia* EPA WET test. This figure illustrates and supports the basis for using test data post 1995, as test precision improved from an average 90th percentile CV of 0.47 to 0.30.

2.2 Data Compilation

Data Sources

WET data were received from several reliable sources to identify baseline test method statistics (e.g., control CV percentiles, mean response percentiles) that were used in simulation analyses (see Section 2.4) and to help identify appropriate α values for each test method. The sources included Washington State Department of Ecology, EPA's Office of Science and Technology, North Carolina Department of the Environment and Natural Resources, California State Water Resources Control Board, and Virginia Department of Environmental Quality. Data acceptance criteria and types of WET test data desired were identified and documented in the Data Management Plan and the Quality Assurance Project Plan for this project. Nearly 2,000 valid WET tests of interest were incorporated, representing many permittees and laboratories (Table 2-2). Only data from WET tests meeting EPA's test acceptability criteria were used in the analyses.

For each set of test data received, additional metadata information was required including the following:

- Permittee name and NPDES permit number (coded for anonymity)
- Laboratory name and location (coded for anonymity)
- Design effluent concentration in the receiving water (expressed as percent effluent upon complete mix) used by the regulatory authority
- EPA test method version used (cited EPA number)
- Information indicating that all EPA test method's test acceptability criteria were met

In addition to the above effluent test data and metadata, two other sources of toxicity data were compiled in this project, which were used to help calculate the range of control organism response by endpoint for each EPA WET test method in Table 2-1. The first source of data was reference toxicant test data previously compiled for the EPA document, *Understanding and Accounting for Method Variability in Whole Effluent Toxicity Application Under the NPDES Program* (USEPA 2000). A second source of additional WET test data used in this project was data generated in ambient toxicity tests by the California State Water Resources Control Board. These data were useful in supplying information on control responses for the freshwater test methods in Table 2-1. Many states routinely conduct ambient toxicity tests as part of 305(b) monitoring; Total Maximum Daily Loads (TMDLs), and other programs (e.g., California's Surface Water Ambient Monitoring program (SWAMP), Washington Department of Ecology's ambient program, Wisconsin Department of Natural Resources' (WDNR) ambient monitoring program).

Table 2-2. Summary of WET test data analyzed

EPA WET test method	Number of tests		Number of laboratories	Number of permittees
	Effluent	Ref Tox		
Ceriodaphnia dubia (water flea) Survival and Reproduction[a]	554	238	44	68
Pimephales promelas (fathead minnow) Acute Survival[b]	347	0	15	101
Pimephales promelas (fathead minnow) Survival and Growth[b]	275	197	28	50
Americamysis bahia (mysid shrimp) Survival and Growth[c]	74	136	20	6
Dendraster excentricus and *Strongylocentrotus purpuratus* (Echinoderm) Fertilization[c]	83	94	11	10
Macrocystis pyrifera (giant kelp) Germination and Germ-tube length[d]	0	135	11	--
Haliotis rufescens (red abalone) Larval Development[c]	0	136	10	--
Ceriodaphnia dubia (water flea) Survival	7	232	27	2
Selenastrum capricomutum (green algae)	139	84	14	44

Notes:
a. Freshwater invertebrate
b. Freshwater vertebrate
c. Saltwater invertebrate
d. Saltwater algae

Representativeness of WET Data

The usefulness of the results obtained in this project depended on having valid, representative WET test data for each of the EPA WET test methods examined. Representativeness was characterized in this project as having data that met the following:

- Cover a range of NPDES permitted facility types, including both industrial and municipal permittees

- Represent many facilities for a given EPA WET test method (i.e., no one facility dominates the data for a given WET test method)
- Cover a range of target (design) effluent dilutions upon which WET RP and compliance are based, ranging from perhaps 10 percent to 100 percent effluent
- Generated by several laboratories for a given EPA WET test method
- Cover a range of observed effluent toxicity for each EPA WET test method (e.g., NOECs range from < 10 percent to 100 percent effluent)

Efforts were made to ensure that no one laboratory or permittee had > 10 percent of the test data for a given test type. The summary information presented in Table 2-2 demonstrates that WET test data were received from numerous laboratories and facilities for all EPA WET test methods analyzed under this project.

Data Processing

Processing of raw WET test data began with identifying the contents of each data package and recording the data source, test type, and related information as described in the previous section. Each valid WET test was assigned a unique code, and each laboratory was uniquely coded. A tracking system was used to help evaluate whether WET test data were needed for certain types of EPA WET test methods and to help increase representativeness of laboratories or types of facilities for a method.

Data were received in a variety of formats and compiled by test type in the database program CETIS® (Comprehensive Environmental Toxicity Information System; Tidepool Software, v. 1.0). The CETIS program is designed to analyze, store, and manage WET test data. WET test data received in either ToxCalc® or CETIS were imported directly into the CETIS database dedicated to this project. WET test data received in Excel or other spreadsheet formats were also directly imported into CETIS. In cases where the source organizations had not yet entered its WET test data electronically, they were supplied with a template so the data could be readily transferred to CETIS to minimize transcription errors. Data in CETIS were checked on 10 percent of the tests received from each source to document proper data transfer.

WET test data received as copies of bench sheets were first checked to ensure that all EPA WET method test acceptance criteria were met, as well as several other requirements discussed in the previous section. Those tests meeting all requirements were input into the CETIS database directly using the double entry mode and a comparison of entries to ensure accuracy of data input. All WET test data used in analyses originated from tests conducted with the minimum number of treatment replicates as required according to the specific EPA WET test methods (e.g., 10 replicates in chronic *Ceriodaphnia* tests). Tests using a different number of replicates per treatment were not used in analyses to generate percentiles of CV or mean response.

2.3 Setting the Test Method-Specific α Level

Monte Carlo simulation analysis was used to estimate the percentage of WET tests that would be declared toxic using TST as a function of different α levels, within-test control variability, and mean percent effect level. This analysis identified probable beta error rates (i.e., declaring an effluent toxic when in fact it is acceptable) as a function of α, mean effect at the IWC, and control CV. Using the RMDs discussed in Section 1.4, the lowest α level (with 0.05 being the

lowest α level used) was then identified for a given WET test design that also resulted in a β = 0.05 at a 10 percent mean effect in the effluent sample.

For each of the nine test methods examined, control CV was calculated on the basis of WET test data compiled as described in Section 2.2. Cumulative frequency plots were used to identify various percentiles of observed method-specific CVs (e.g., 25th, 50th, 75th percentiles). These measures were calculated to characterize typical achievable test performance in terms of control variability. A similar analysis was performed for the control endpoint responses for each of the nine test methods (e.g., mean offspring per female in the chronic *Ceriodaphnia dubia* test method) to characterize typical achievable test performance in terms of control response. The following describes the simulation analysis used to help identify appropriate alpha levels for each WET test method examined.

2.3.1 Simulation Analyses

In simulation analyses, sets of effluent and control WET test data were constructed having known properties with respect to different mean effect percentages and control CV as described below. Control CVs examined were based on CV percentiles observed in actual WET test data for a given WET test method. All simulation analyses were based on normally distributed WET test data and equal variances between the effluent and control for each scenario examined. These data were then analyzed using the one-tailed t-test published by Erickson and McDonald (1995) for bioequivalence testing (and mathematically defended in Erickson 1992 for normally distributed equal variance data) and the one-tailed traditional hypothesis t-test formulation (see Equations 1 and 2 below) to determine whether a given effluent was declared toxic using each approach at a specified α value. By simulating thousands of WET tests for a given scenario (mean percent effect and control CV and α level), the percentage of tests declared toxic could be calculated and compared among scenarios, and between the TST and the traditional hypothesis testing approach.

Equation 1: TST t-test assuming equal variances

$$t = \frac{\overline{Y_t} - b \times \overline{Y_c}}{S_p \sqrt{\frac{1}{n_t} + \frac{b^2}{n_c}}}$$

$$S_p = \sqrt{\frac{S_t{}^2 \times (n_t - 1) + S_c{}^2 \times (n_c - 1)}{(n_t + n_c - 2)}}$$

Equation 2: Traditional t-test assuming equal variances

$$t = \frac{\overline{Y_c} - \overline{Y_t}}{S_p \sqrt{\frac{1}{n_t} + \frac{1}{n_c}}}$$

$$S_p = \sqrt{\frac{S_t{}^2 \times (n_t - 1) + S_c{}^2 \times (n_c - 1)}{(n_t + n_c - 2)}}$$

It is understood that using normally distributed data and equal variances is a simplification for some WET test methods that are prone to non-normally distributed data and heterogeneous variances (e.g., acute fathead minnow test method). Additional analyses suggested that the bioequivalence t-test of Erickson and McDonald (1995) results in a very small (< 0.01) departure of the nominal α error rate using TST with data that have even a nine-fold difference between control and effluent variances (which is greater than most variance ratios observed in nearly 2,000 WET tests) and with data that were non-normally distributed (Appendix A). Thus, results of simulation analyses should be applicable to the types of non-normality and variance heterogeneity encountered in WET tests. This was further supported by additional research showing that WET test data distributions are typically not highly skewed or long-tailed because of the way in which the tests are designed and because there are boundaries on test acceptability criteria that truncate the data within a test and the difference in variance one observes between control and an effluent treatment. A review of the statistical literature as well as additional analyses in developing the TST approach confirmed that Welch's t-test is appropriate for the types of non-normal data distributions encountered in actual effluent WET tests as well as for normally distributed data (see Appendix A).

Probabilities of accepting the null hypothesis for the traditional and TST approaches will differ according to different settings for a number of parameters, including population variances, test sample size, and effect size (i.e., fraction of the control response). Each of these factors was varied in simulation analysis as follows:

Population Variances: Population variances were defined by test method (control CVs in a large number of actual WET tests for a given method). The population mean was set to the median value of observed control mean values from actual effluent tests, and the CV value ranged from approximately the 10^{th} to 90^{th} percentile of the observed control CV range. N samples (representing the minimum number of replicates required in the test method) from the control population were selected for each simulation.

Effect Size: Population mean for the treatment group was defined by a specified effect size. Five different effect sizes (from 10 percent to 30 percent of the control mean) were evaluated for each treatment group. For example, when the control mean = 25 and the effect size = 10 percent, N samples (corresponding to the minimum number of replicates required in the test method) were picked at random from a population with mean = 25 × ([100 – 10] percent).

Sample Size (N): For certain WET test methods, sample size for each test method was increased up to double the minimum number of replicates required for a given test method. For example, number of replicates for the chronic *C. dubia* test ranged from 10 to 20 in simulation analyses. This analysis provided useful information indicating potential benefits to a permittee if they conducted a WET test method with additional replicates, given a specified mean percent effect level and control CV observed, and a specified α level.

Alpha Error: The maximum allowable Type I error (α) in TST was specified at different levels ranging from 0.05 to 0.30 (6 values). Results of these analyses indicated potential β error rates (probability of declaring a sample toxic when it is acceptable) given a specified mean percent effect in the effluent and control CV. These results were also compared with results using the traditional hypothesis testing approach and an $\alpha = 0.05$ (the EPA-recommended α level using the

traditional hypothesis testing approach) to compare β error rates using both approaches. While comparison of results between TST and the traditional approach were not used to set test method α levels, this analysis was useful in documenting whether the TST approach was as protective as the traditional approach using a given α level.

After N samples of control and effluent were randomly selected from specified populations, the traditional hypothesis testing approach and TST were conducted as specified in equations 1 and 2 above. The one tail probabilities of declaring the test toxic using the traditional hypothesis testing approach and the TST approach were calculated and saved. This simulation was repeated 10,000 times for each combination of effect levels, CV, and alpha level. The percent of tests declared toxic was then calculated for each simulation setting.

Once β error rates were identified for a WET method given different α levels, control CVs, and percent mean effect levels, bivariate plots were used to compare the percentage of tests declared toxic as a function of α and the ratio of effluent mean: control mean at various within-test variability percentiles (e.g., 25^{th}, 50^{th}, 75^{th}) and the RMD effect thresholds identified as either toxic (25 percent effect for chronic and 20 percent for acute) or negligible (10 percent mean effect). The results were then used to identify an appropriate α error rate for a test method given the RMDs noted in Section 1.4.

Finally, where there was sufficient effluent test data available, an analysis of actual effluent data was conducted using TST and the α level identified for the test method, and using the traditional hypothesis testing approach. Results of that analysis were used to estimate potential results if TST was used in the NPDES WET Program and to compare those results with those using the traditional hypothesis testing approach.

3.0 RESULTS

3.1 Chronic *Ceriodaphnia dubia* Reproduction Test

On the basis of actual WET data (N = 792 tests), the mean control reproduction ranged from 15.0 to 51.7, with a median mean value of 25.5 (Table 3-1). Control CVs ranged from 0.04 to 1.22 with a median value of 0.15 (Table 3-1). Using these data, simulation analyses were conducted to evaluate the percentages of tests declared toxic (i.e., failure to reject the null hypothesis) by TST at various alpha error rates (between 0.05 and 0.30), CVs, and percent mean effect in reproduction between the control and effluent concentration.

Table 3-1. Summary of mean control reproduction and control CV derived from analyses of 792 chronic *Ceriodaphnia dubia* WET tests

Percentile	Mean control reproduction	Control CV	Control SD
10th	17.7	0.08	2.07
25th	21.2	0.10	2.64
50th	25.5	0.15	3.79
70th	28.4	0.22	5.27
75th	29.4	0.24	5.82
85th	31.6	0.31	7.24
90th	33.3	0.35	8.41
95th	35.6	0.40	10.25

Identifying Test Method-Specific α

A summary of the simulation results is shown graphically in Figure 3-1. An alpha level of 0.20 satisfies both RMDs of (1) ensuring at least a 75 percent probability of declaring a 25 percent mean effect as toxic regardless of within-test control variability (denoted as effluent mean: control mean value of 0.75 on the x-axis of each graph in Figure 3-1), and (2) ensuring that a negligible effect (10 percent mean effect denoted as effluent mean: control mean value of 0.90) is declared toxic $\leq$ 5 percent of the time. Lower α levels (e.g., $\alpha = 0.10$) resulted in > 5 percent tests declared toxic when there was a 10 percent effect under average within-test CV values (i.e., $\beta > 0.05$). Note that using an $\alpha = 0.20$, a *Ceriodaphnia* test having a 20 percent mean effect at the IWC (effluent mean:control mean = 0.8) and median control variability (control CV = 0.15) will be declared toxic approximately 50 percent of the time using TST (Figure 3-1). Thus, as discussed in Section 1.3 and shown in Figure 1.2, some percentage of tests having an effluent mean effect less than the RMD threshold of 25 percent will be declared toxic using TST, even when the test control responds acceptably. Likewise, at an $\alpha = 0.20$, a *Ceriodaphnia* test exhibiting a 10 percent mean effect in the effluent (0.9 on the x-axis in Figure 3-1) and relatively high control variability (control CV = 0.25, 75th percentile for this WET test method) will have approximately a 25 percent probability of being declared toxic (Figure 3-1), even though a 10 percent mean effect is considered acceptable using TST.

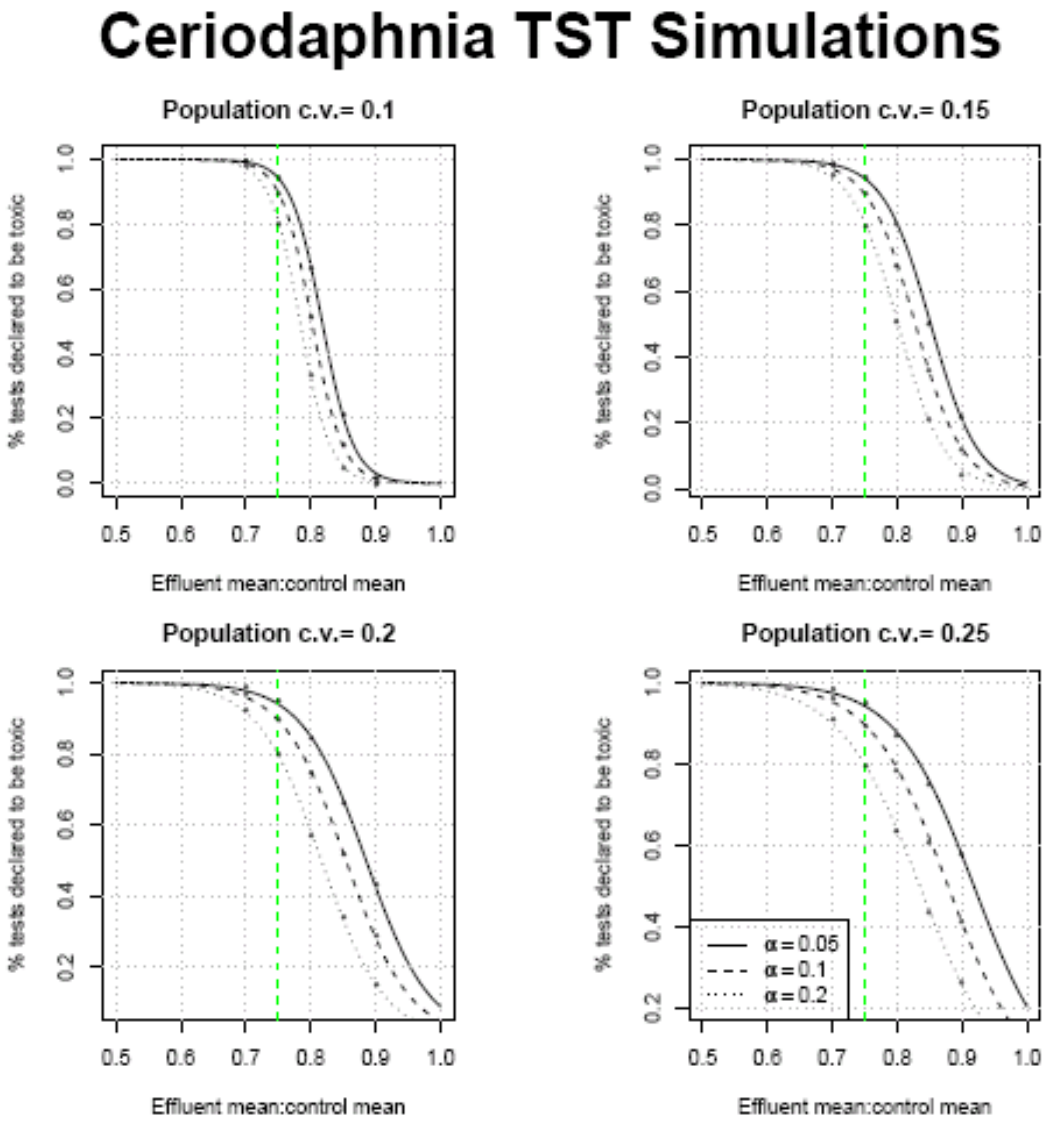

Figure 3-1. Power curves showing the percentage of tests declared toxic as a function of the ratio of effluent mean to control mean response and α level categorized by the level of control within-test variability. CVs of 0.1, 0.15, 0.2, and 0.25 correspond to the approximate 25th, 50th, 70th, and 75th percentiles for the chronic *Ceriodaphnia* WET method. The dashed line indicates the 75 percent mean effect level, which is the decision threshold for chronic tests.

The above results illustrate two features of the TST approach that should be understood: (1) At mean effect levels < the RMD toxicity threshold, there are differing probabilities of an effluent being declared toxic (i.e., different actual α error rates) depending on within-test variability and the difference in mean responses observed between control and IWC (see Figure 1-2). An effluent with a mean effect substantially lower than the RMD threshold of 25 percent will have some probability of being declared toxic. (2) For this WET test method and some others examined in this project, there is some probability of declaring a test non-toxic when the mean effect in the effluent exceeds the RMD threshold of 25 percent; e.g., at an α = 0.20 and relatively

high within-test variability, a 30 percent mean effect in the effluent might not be declared toxic as much as 10 percent of the time.

The following examples give representative results of the simulation analysis, illustrating the effect of different alpha levels in terms of meeting RMDs for TST.

In the first example, there is a 10 percent mean effect in the effluent and a median level of within-test control precision (50th percentile CV of 0.15). Use of alpha levels ranging from 0.05 to 0.30 resulted in failure to reject the null hypothesis in ~20 percent to ~5 percent of tests, respectively, with α levels $\geq$ 0.20 meeting the RMD of $\beta \leq 0.05$ at a 10 percent mean effect level (Figure 3-2).

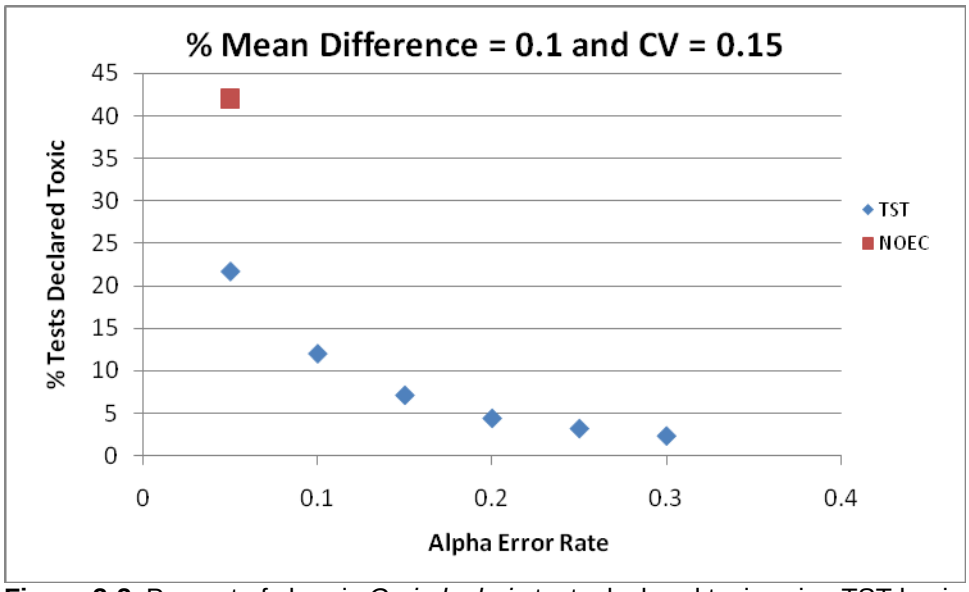

Figure 3-2. Percent of chronic *Ceriodaphnia* tests declared toxic using TST having a mean effluent effect of 10 percent and average control variability as a function of α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

In a second example, the effluent has a mean effect of 25 percent and above average control CV (75th percentile). At α levels < 0.25, the percentage of tests declared toxic is $\geq$ 75 percent, meeting the RMD for false negative rate (α).

The rate at which tests were declared toxic was evaluated using both the traditional hypothesis testing approach with an alpha error rate of 0.05 (as recommended in the EPA WET test methods) and the TST approach with different alpha error rates. At a 50th percentile CV (0.15) and a mean effect of 10 percent, use of the TST approach results in fewer declared toxic tests relative to the traditional hypothesis approach at all alpha error rates examined (Figure 3-2). For tests with the same mean effect (10 percent) but higher control variability (CV = 0.25), TST yields a higher rate of tests declared toxic at alpha error rates of 0.05, 0.10, and 0.15 and approximately equivalent percent toxic tests at alpha error rates of 0.20 and 0.25 (Figure 3-2). Those results are in keeping with the RMD that tests with negligible (10 percent) mean effect in

the effluent are declared non-toxic most of the time but are declared to be toxic more frequently as test precision is poorer.

Tests with a mean effect of 25 percent and above average precision (CV = 0.25) result in a higher rate of tests declared toxic using TST than using the traditional hypothesis approach (Figure 3-3). This result is a direct consequence of the RMDs defined for TST but illustrate disincentives to collect more precise data using the traditional hypothesis approach currently used.

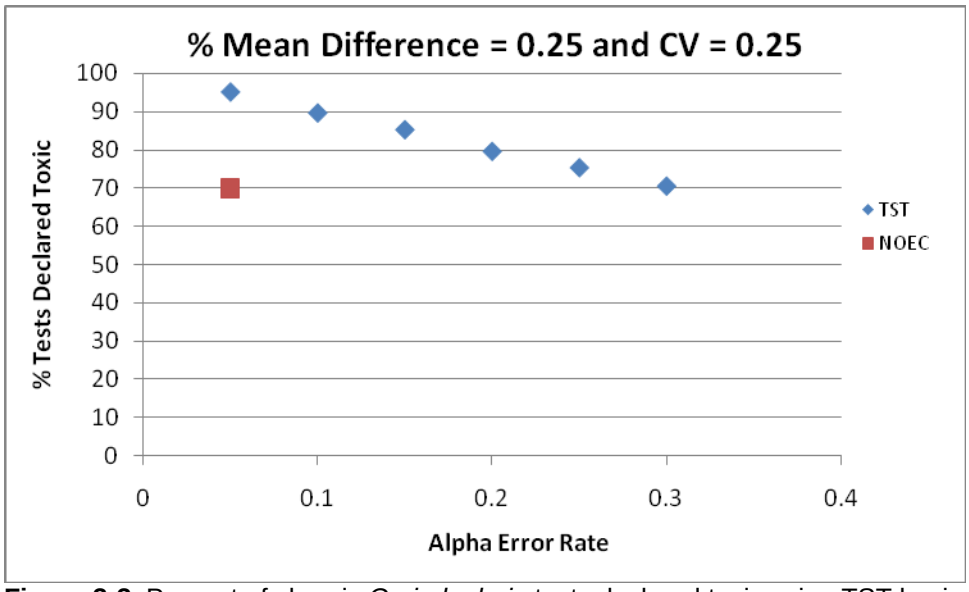

Figure 3-3. Percent of chronic *Ceriodaphnia* tests declared toxic using TST having a mean effluent effect of 25 percent and high control variability as a function of α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

Effect of Increased Number of Within-Test Replicates

One of the intended benefits of the TST approach is that increasing the precision and power of the test increases the chances of rejecting the null hypothesis and declaring a sample non-toxic when it meets the RMD for acceptability. This increases the ability of the permittee to *prove the negative* that a sample is acceptable. To demonstrate this benefit, the effect of increasing test replication on the TST β error rate (declaring a sample toxic when it is not) was explored using simulated data.

Increasing test replication with this method (and thereby the power of the test) results in a *higher* rate of tests declared toxic using the traditional hypothesis testing approach and a *lower* rate of tests declared toxic using the TST approach (e.g., Figure 3-4). For tests with a mean effect of 10 percent and a control CV of 0.25 (approximately 75th percentile for this method), slightly more tests will be declared toxic using the TST approach as compared to the traditional hypothesis testing approach when the minimum test design of 10 replicates is used for this WET method. If the number of within-test replicates is increased, the TST approach demonstrates an improved ability to declare such a test as acceptable. As the mean effect at the effluent approaches 25 percent, the percentage of tests declared toxic is less affected by increased replication using TST because the *b* value and α value were selected to identify a 25 percent mean effect in the IWC as

toxic ≥ 75 percent of the time. However, the percentage of tests declared toxic continues to increase using the traditional hypothesis approach even when there is a negligible effect (10 percent effect) of the effluent as defined by TST (Figure 3-5). Thus, increasing test replication increases TST's ability to confirm that an effluent is acceptable in tests with mean effect less than 25 percent.

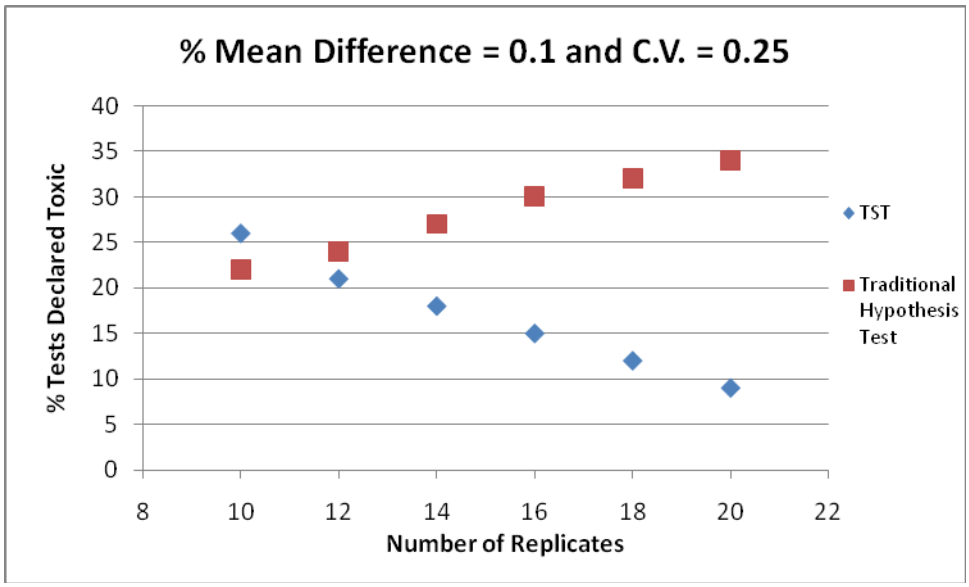

Figure 3-4. Percent of chronic *Ceriodaphnia* tests declared toxic using TST having a mean effluent effect of 10 percent and above average control variability and α = 0.20, as a function of the number of test replicates. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

Effluent Data Results

Results from actual effluent tests were compared between TST and the traditional hypothesis testing approach for those tests having control CV between 0.15–0.24 (Table 3-2). At a mean effect of 10–15 percent at the IWC (N = 48), TST declared a lower percentage of tests toxic than the traditional hypothesis testing approach. This result is consistent with the RMD that a 10 percent mean effect should be declared acceptable much (95 percent) of the time. However, when the mean effect was greater than 25 percent (N = 303), TST declared 100 percent of the tests toxic while the traditional hypothesis testing approach did not. This result is also consistent with the TST goal that as the mean effect approaches 25 percent at least 75 percent of the tests should be declared toxic. This result also indicates that given the effluent data available, TST is at least as protective as the traditional hypothesis approach currently used.

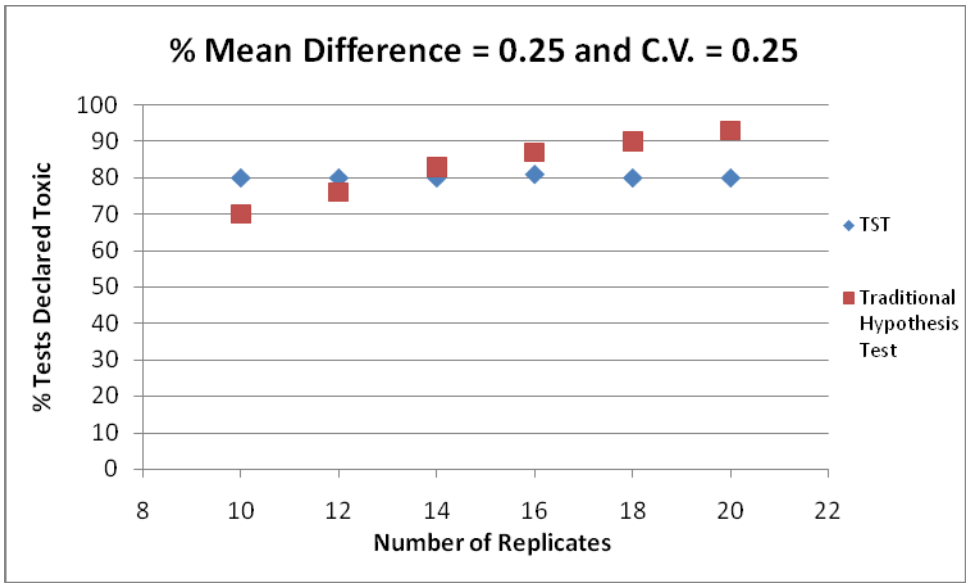

Figure 3-5. Percent of *Ceriodaphnia* tests declared toxic using TST having a mean effluent effect of 25 percent and above average control variability (α = 0.20) as a function of the number of test replicates. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

Table 3-2. Comparison of the percentage of chronic effluent *Ceriodaphnia* tests declared toxic using TST versus the traditional hypothesis testing approach

% Mean effect	N	% tests toxic using TST	% Tests toxic using traditional hypothesis testing approach
10–15	48	6.2	18.7
20–30	48	100	87.5
> 25	303	100	95.2

3.2 Chronic *Pimephales promelas* Growth Test

On the basis of actual WET data (N = 472 tests), the mean control growth ranged from 0.31 to 1.30, with a median mean value of 0.62 (Table 3-3). Control CVs ranged from 0.03 to 0.50 with a median value of 0.09 (Table 3-3). Using these data, simulation analyses were conducted to evaluate the percentages of tests declared toxic (i.e., failure to reject the null hypothesis) by TST at various alpha error rates (between 0.05 and 0.30), CVs, and percent mean effect in growth between the control and effluent concentration.

Identifying Test Method-Specific α

On the basis of all simulation results (Figure 3-6), an alpha error rate of 0.25 is appropriate for use in applying the TST approach to analysis of two concentration chronic *P. promelas* data because using that alpha error rate satisfies both RMDs of (1) ensuring at least an 75 percent probability of declaring a 25 percent mean effect as toxic and (2) ensuring that a negligible effect ($\leq$ 10 percent mean effect) is declared toxic $\leq$ 5 percent of the time.

Table 3-3. Summary of mean control growth and control CV derived from analyses of 472 chronic *Pimephales promelas* WET tests

Percentile	Mean control growth	Control CV	Control SD
10th	0.34	0.04	0.02
25th	0.43	0.06	0.03
50th	0.62	0.09	0.05
70th	0.76	0.12	0.07
75th	0.79	0.13	0.08
85th	0.86	0.16	0.10
90th	0.89	0.17	0.11
95th	0.94	0.21	0.13

As noted for the *Ceriodaphnia* chronic test in Section 3.1, the Type I error rate will vary from the RMD Type I error rate of 0.25 depending on the level of toxicity observed in the effluent and control variability within a test. When toxicity is > 25 percent mean effect in the effluent, the Type I error rate is lower. However, as noted in Section 1.3, there is some probability (< 10 percent) that a mean effect > 25 percent in the IWC will be declared non-toxic depending on within-test variability. Likewise, a reasonable percentage (as much as 50 percent) of tests having a mean effect = 15 percent in the effluent will be declared toxic using the TST approach, again depending on within-test variability: the greater the within-test variability the greater the probability of declaring toxicity at mean effect levels below the toxicity decision threshold of 25 percent.

For example, at a 10 percent mean effect in the effluent and above average within-test control variability (between the 50th and 75th percentile, CV of 0.11), use of an alpha level of 0.25 results in failure to reject the null hypothesis ~5 percent of the time (Figure 3-7). Lower alpha levels resulted in a higher percentage of tests declared toxic at that mean effect level and CV range (Figure 3-6). That indicates that using an alpha = 0.25 for this test method, TST achieves the RMD of correctly identifying an acceptable sample (based on the RMD that a 10 percent mean effect is negligible). However, less precise tests (but still well within normal test method performance) result in less ability to reject the null hypothesis that the sample is toxic and the rate of tests declared toxic increases even at a percent mean effect of 10 percent (Figure 3-6). For tests with a mean effect of 25 percent (the RMD toxicity threshold) and alpha error rate of 0.25, 75 percent of the tests are declared toxic as expected (Figure 3-8).

Effect of Increased Number of Within-Test Replicates

As expected, increasing test replication (and thereby the power of the test) results in a *higher* rate of tests declared toxic using the traditional hypothesis testing approach and a *lower* rate of tests declared toxic using the TST approach and chronic *P. promelas* test data (e.g., Figure 3-9). For tests with a mean effect of 10 percent in the effluent and a control CV of 0.15 (slightly greater than the 75th percentile for this method), slightly more tests are declared toxic using the TST approach as compared to the traditional hypothesis testing approach when the minimum test design of four replicates is used for this WET endpoint. If replicates are added to the test design, the TST approach demonstrates an increased ability to declare the results acceptable. As the mean effect approaches 25 percent, the percentage of tests declared toxic is less affected by

increased replication using TST because a 25 percent effect is the RMD used to define *b* and the null hypothesis. However, the percentage of tests declared toxic continues to increase using the traditional hypothesis testing approach even when there is a 10 percent effect of the effluent. Thus, increasing test replication increases TST's ability to confirm an acceptable effluent when the mean effect is less than 25 percent in the effluent.

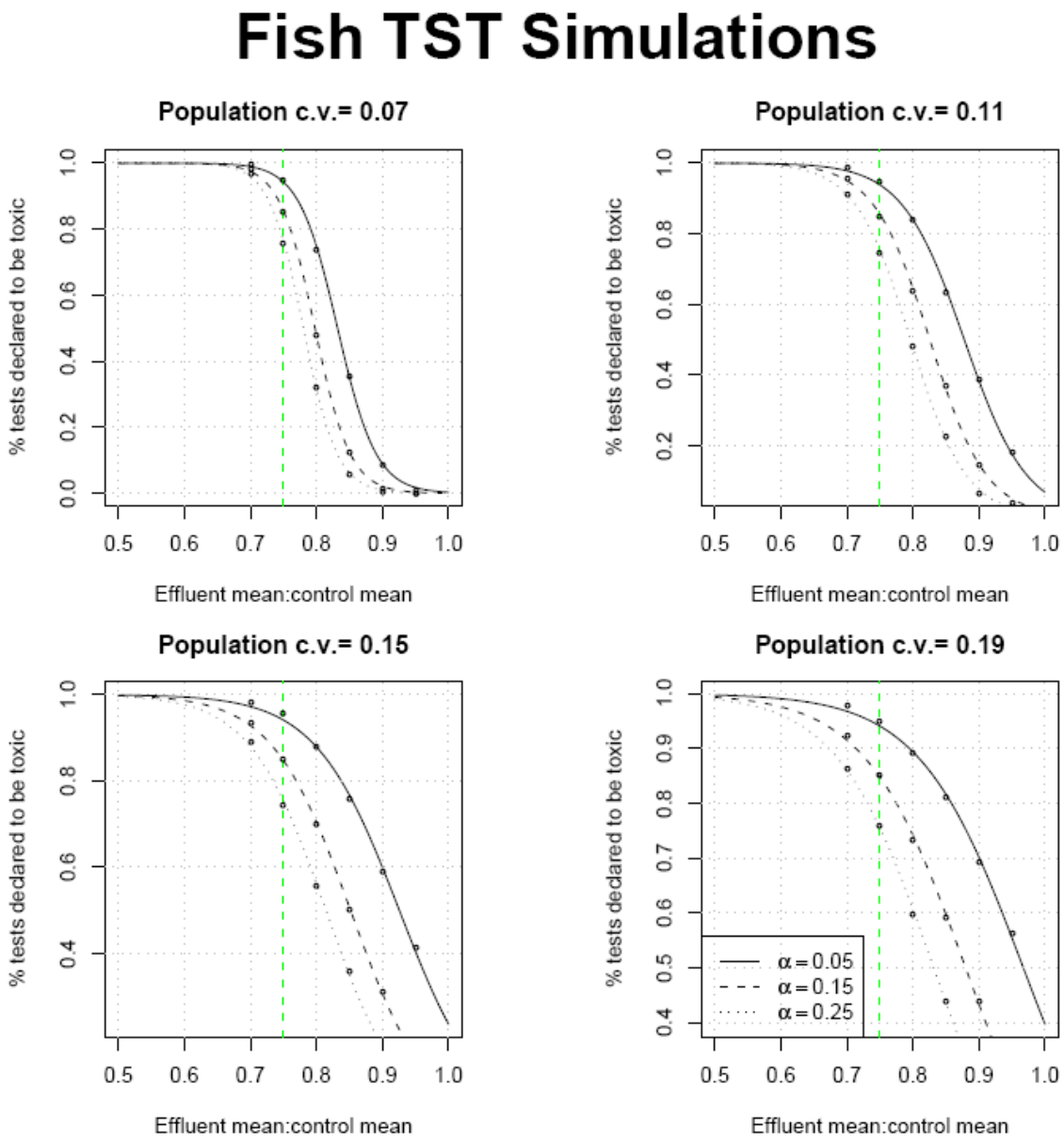

Figure 3-6. Power curves showing the percentage of tests declared toxic as a function of the ratio of effluent mean to control mean response and α level categorized by the level of control within-test variability. CVs correspond to the 25th, 50th, 75th, and 90th percentiles for the chronic fathead minnow WET method. The dashed line indicates the 75 percent mean effect level, which is the decision threshold for chronic tests.

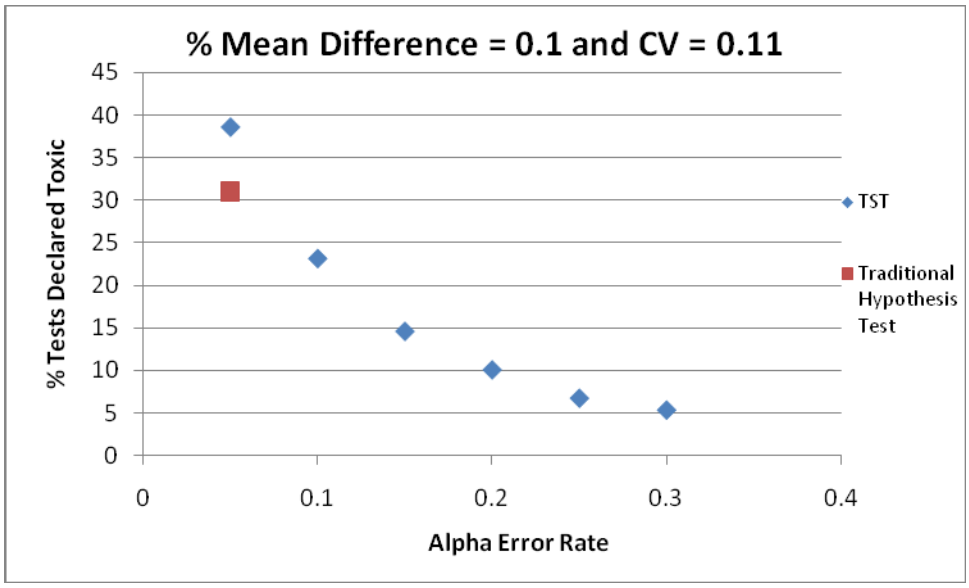

Figure 3-7. Percent of chronic fathead minnow tests declared toxic using TST having a mean effluent effect of 10 percent and average control variability as a function of α error rate. Result using the traditional approach (α = 0.05) is shown as well.

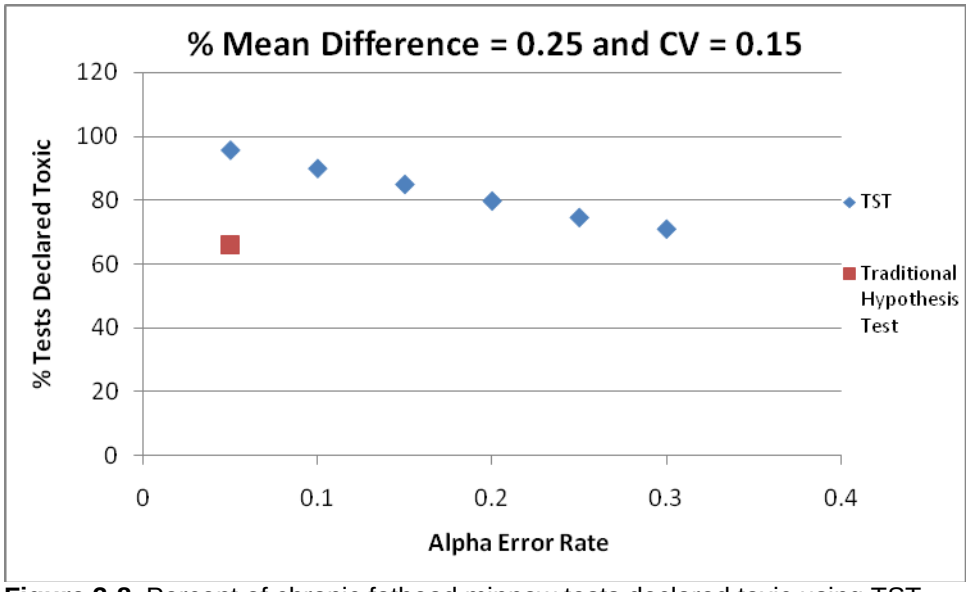

Figure 3-8. Percent of chronic fathead minnow tests declared toxic using TST having a mean effluent effect of 25 percent and above average control variability as a function of α error rate. Result using the traditional approach (α = 0.05) is shown as well.

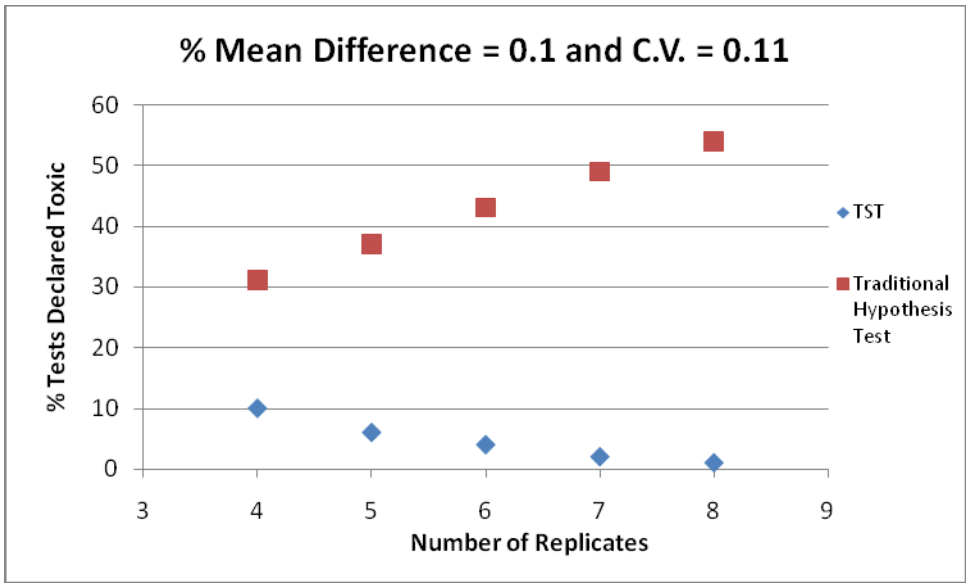

Figure 3-9. Percent of chronic fathead minnow tests declared toxic using TST having a mean effluent effect of 10 percent and average control variability and an α = 0.25, as a function of the number of test replicates. Result using the traditional approach (α = 0.05) is shown as well.

Effluent Data Results

Results from actual effluent tests were compared between TST and the traditional hypothesis testing approach for those tests having control CV between 0.09–0.13 (Table 3-4). At a mean effect of 10–15 percent (N = 58), TST declared none of the tests toxic while the traditional hypothesis testing approach declared nearly all of the tests toxic. However, if the mean effect is greater than 25 percent (N = 136), both approaches declared 100 percent of the tests toxic. Those results indicate that TST is as protective as the current hypothesis testing approach for those tests when the TST RMD threshold for toxicity is exceeded.

Table 3-4. Comparison of the percentage of chronic effluent fathead minnow tests declared toxic using TST versus the traditional hypothesis testing approach

% Mean effect	N	% tests toxic using TST	% tests toxic using traditional hypothesis testing approach
10–15	58	0	98
> 25	136	100	100

3.3 Chronic *Americamysis bahia* Growth Test

On the basis of actual WET data (N = 210 tests), the mean control growth ranged from 0.20 to 0.66, with a median value of 0.30 (Table 3-5). Control CVs ranged from 0.07 to 0.87 with a median value of 0.14 (Table 3-5). Using those data, simulation analyses were conducted to evaluate the percentages of tests declared toxic (i.e., failure to reject the null hypothesis) by TST at various alpha error rates (between 0.05 and 0.30), CVs, and percent mean effect in growth between the control and effluent concentration.

Table 3-5. Summary of mean control growth and control CV derived from analyses of 210 chronic *Americamysis bahia* WET tests

Percentile	Mean control growth	Control CV	Control SD
10th	0.22	0.08	0.02
25th	0.25	0.10	0.03
50th	0.30	0.14	0.04
70th	0.36	0.17	0.06
75th	0.38	0.18	0.06
85th	0.41	0.22	0.07
90th	0.43	0.27	0.08
95th	0.47	0.35	0.11

Identifying Test Method-Specific α

On the basis of all simulation results (Figure 3-10), an alpha error rate of 0.15 is appropriate for use in applying the TST approach to analysis of chronic mysid data because using this alpha error rate satisfies both RMDs of (1) ensuring at least an 75 percent probability of declaring a 25 percent mean effect as toxic and (2) ensuring that a negligible effect ($\leq$ 10 percent mean effect) is declared toxic $\leq$ 5 percent of the time under average or better than average test performance.

For example, at a 10 percent mean effect in effluent and an approximate median level of precision (50^{th} percentile CV of 0.14), an alpha level of 0.15 or greater resulted in failure to reject the null hypothesis in ≤ 5 percent of tests (Figure 3-11). For tests with a mean effect of 25 percent, the rate of tests declared toxic > 75 percent is achieved for alpha values ≤ 0.25 (Figure 3-12).

At a ~50^{th} percentile CV (0.13) and a mean effect of 10 percent, use of the TST approach results in significantly fewer toxic tests relative to the traditional hypothesis testing approach at all alpha error rates (Figure 3-11). For tests with the same mean effect (10 percent) but lower control precision (CV = 0.18), TST yields a higher rate of tests declared toxic at an alpha error rate of 0.05 and approximately equivalent percent toxic tests at a alpha error rate of 0.10.

Tests with a mean effect of 25 percent and above average precision (CV = 0.18) result in a high rate of tests declared toxic (Figure 3-12). The results are in agreement with the RMDs of the TST: As the mean effect approaches 25 percent, a greater proportion of the tests are determined to be toxic. Further, the less precise the test control data, the greater the rate of tests declared toxic (i.e., fail to reject the null hypothesis).

Effect of Increased Number of Within-Test Replicates

As expected, increasing test replication (and thereby the power of the test) results in a *higher* rate of tests declared toxic using the traditional hypothesis testing approach and a *lower* rate of tests declared toxic using the TST approach at a negligible effect of 10 percent, as shown in the example using chronic *A. bahia* test data (e.g., Figure 3-13). If replicates are added to the test design, the TST approach demonstrates an increased ability to declare such a test as non-toxic. As the mean effect approaches 25 percent, the percentage of tests declared toxic is less affected by increased replication using TST because a 25 percent effect is the RMD toxicity threshold identified in TST. However, the percentage of tests declared toxic continues to increase using the

traditional hypothesis testing approach even when there is a negligible effect (10 percent effect as defined by TST) of the effluent. Thus, increasing test replication increases TST's ability to confirm an acceptable level of toxicity in tests with mean effect less than 25 percent.

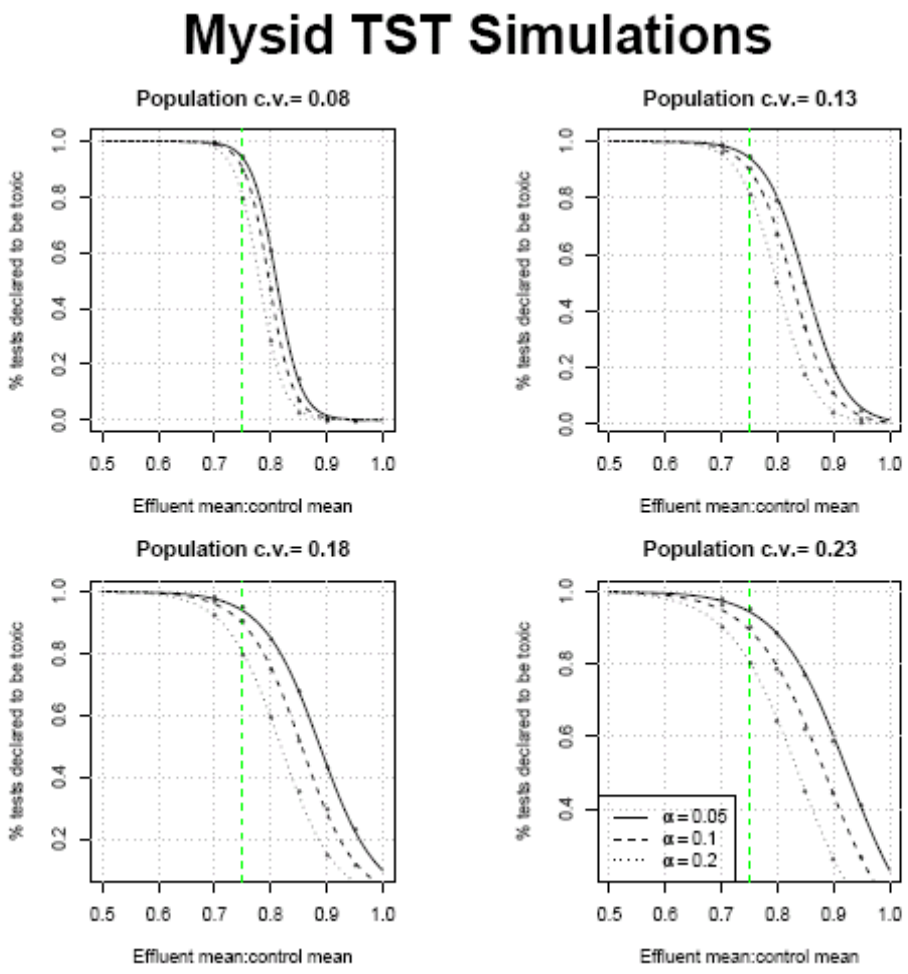

Figure 3-10. Power curves showing the percentage of tests declared toxic as a function of the ratio of effluent mean to control mean response and α level categorized by the level of control within-test variability. CVs correspond to the 25th, 50th, 70th, and 90th percentiles for the chronic mysid WET method. The dashed line indicates the 75 percent mean effect level, which is the decision threshold for chronic tests.

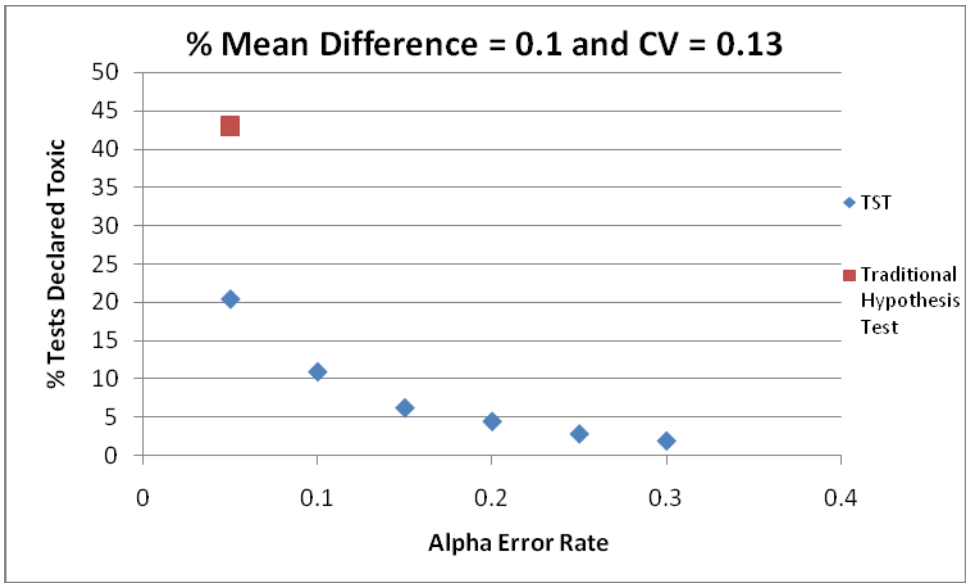

Figure 3-11. Percent of chronic mysid tests declared toxic using TST having a mean effluent effect of 10 percent and average control variability as a function of the α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

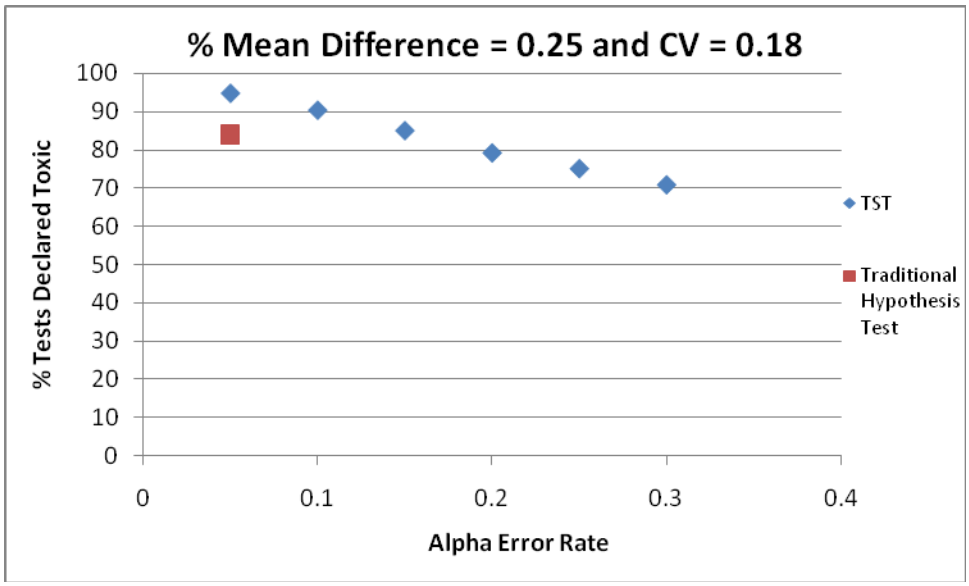

Figure 3-12. Percent of chronic mysid tests declared toxic using TST having a mean effluent effect of 25 percent and average control variability as a function of the α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

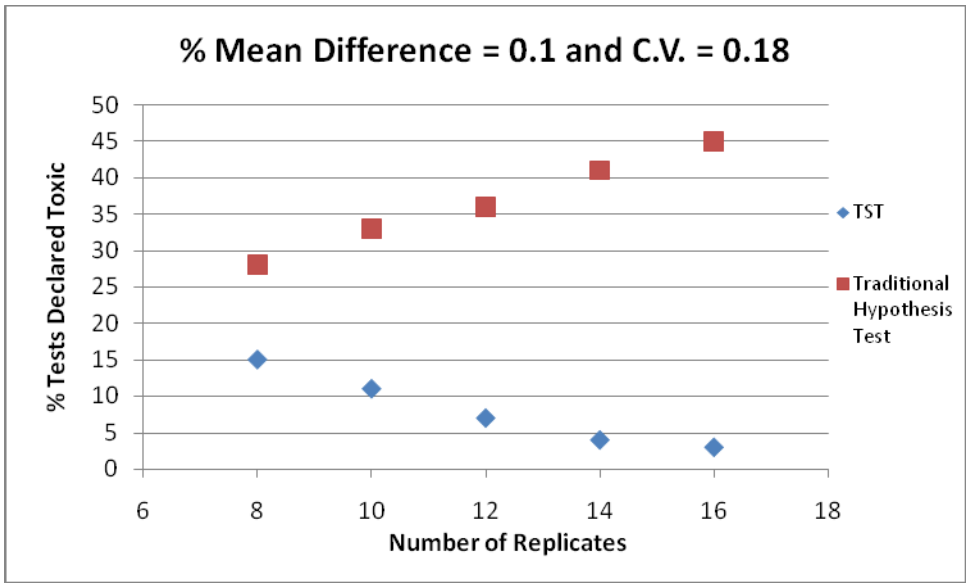

Figure 3-13. Percent of chronic mysid tests having a mean effluent effect of 10 percent and above average control variability declared toxic using TST and an α = 0.15, as a function of the number of test replicates. Results using the traditional hypothesis approach (α = 0.05) are shown as well.

Effluent Data Results

Results from actual effluent tests were compared between TST and the traditional hypothesis testing approach for those tests having control CV between 0.14–0.26 (75^{th} – 90^{th} percentile; Table 3-6). At a mean effect of 5–15 percent (N = 52), TST declared a lower percentage of tests toxic than the traditional hypothesis approach. That is expected because 10 percent mean effect in the effluent is considered negligible. However, when the mean effect in the effluent is greater than 25 percent (N = 95), both approaches declared 100 percent of the tests toxic.

Table 3-6. Comparison of percentage of chronic effluent mysid shrimp tests declared toxic using TST versus the traditional hypothesis testing approach

% Mean effect	N	% tests toxic using TST	% tests toxic using traditional hypothesis testing approach
5-15	52	1.9	11.5
> 25	95	100	100

3.4 Chronic *Haliotis rufescens* Larval Development Test

From actual WET data (N = 136 reference toxicant tests), mean control larval development ranged from 0.800 to 1.000, with a median mean value of 0.938 (Table 3-7). Control CVs ranged from 0.000 to 0.333 with a median value of 0.03 (Table 3-7). Using those data, simulation analyses were conducted to evaluate the percentages of tests declared toxic (i.e., failure to reject the null hypothesis) by TST at various alpha error rates (between 0.05 and 0.30), CVs, and percent mean effect in larval development between the control and effluent concentration.

Identifying Test Method-Specific α

On the basis of simulation results and power analyses (Figure 3-14), an alpha error rate of 0.05 is appropriate for use in applying the TST approach to analysis of chronic *H. rufescens* data because using this alpha error rate satisfies both RMDs of (1) ensuring at least an 75 percent probability of declaring a 25 percent mean effect as toxic and (2) ensuring that a negligible effect ($\leq$ 10 percent mean effect) is declared toxic $\leq$ 5 percent of the time (Figure 3-14). Note that higher alpha levels would also satisfy the above RMDs; however, as noted in Section 1.4, the Type I error rate is set as close to 0.05 as practicable given routine control performance.

Table 3-7. Summary of mean control larval development and control CV derived from analyses of 136 chronic red abalone WET tests

Percentile	Mean control larval development	Control CV	Control SD
10th	0.839	0.02	0.01
25th	0.900	0.02	0.02
50th	0.938	0.03	0.03
70th	0.961	0.04	0.04
75th	0.968	0.05	0.04
85th	0.977	0.06	0.05
90th	0.982	0.06	0.06
95th	0.988	0.07	0.07

At a 10 percent mean effect in the effluent, for example, and ~80th percentile CV of 0.05, alpha levels ranging from 0.05 to 0.30 result in failure to reject the null hypothesis in none of the tests (Figure 3-15). The rate of rejection of the null hypothesis using TST decreases only slightly with increasing CV. This result is indicative of the low within-test control variability routinely achieved using this WET test method.

For tests with a mean effect of 25 percent, the rate of tests declared toxic ranges from ~95 to ~70 percent, at approximately the 80th percentile CV value for alpha levels ranging from 0.05 to 0.30, respectively (Figure 3-16). Thus, at an alpha = 0.05, the rate of tests declared toxic at a 25 percent mean effect in the effluent meets the RMD.

At ~80th percentile CV (0.05) and a mean effect of 10 percent, use of the TST approach results in significantly fewer toxic tests relative to the traditional hypothesis approach at all alpha error rates (Figure 3-15). Those results are in keeping with the RMD of the TST approach; tests with a negligible (10 percent) mean effect of the effluent are declared non-toxic 95 percent of the time when test control data have average precision.

Tests with a mean effect of 25 percent and above average precision (CV = 0.05) resulted in an equivalent rate of tests declared toxic as the traditional hypothesis approach when the TST α = 0.05 (Figure 3-16). The results further support the selection of TST α = 0.05 for this test method.

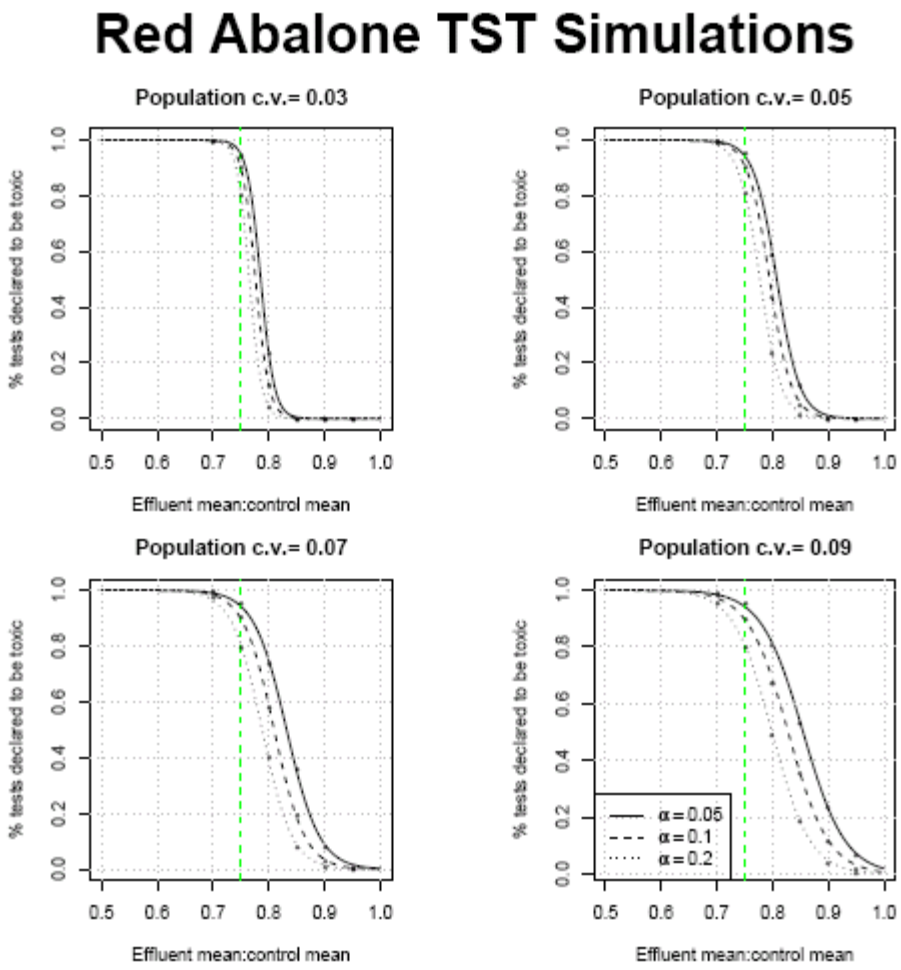

Figure 3-14. Power curves showing the percentage of tests declared toxic as a function of the ratio of effluent mean to control mean response and α level categorized by the level of control within-test variability. CVs correspond to the 25th, 50th, 75th, and 98th percentiles for the chronic red abalone WET method. The dashed line indicates the 75 percent mean effect level, which is the decision threshold for chronic tests.

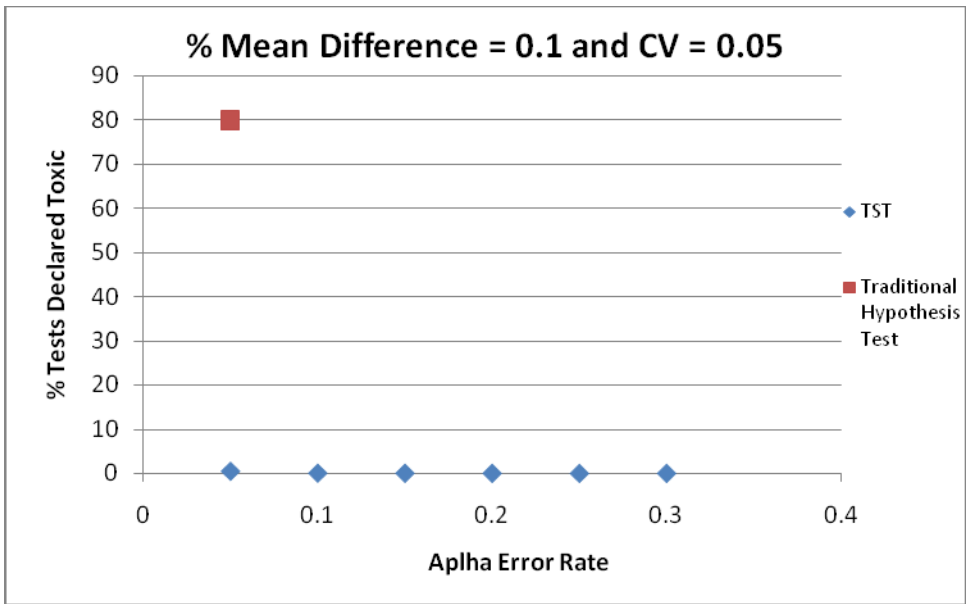

Figure 3-15. Percent of chronic red abalone tests declared toxic using TST having a mean effluent effect of 10 percent and average control variability as a function of the α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

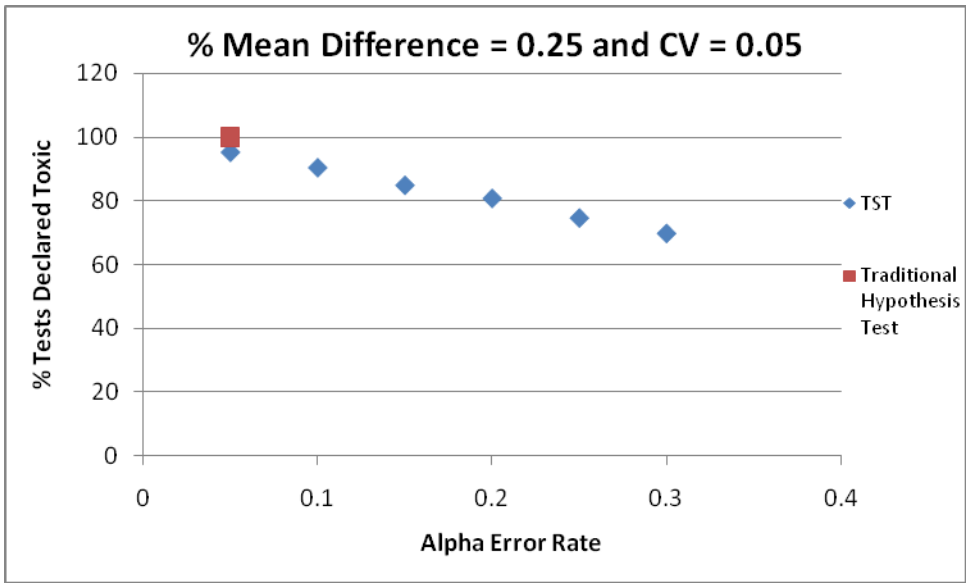

Figure 3-16. Percent of chronic red abalone tests declared toxic using TST having a mean effluent effect of 25 percent and average control variability as a function of the α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

3.5 Chronic *Macrocystis pyrifera* Germination Test

On the basis of actual WET data (N = 135 reference toxicant tests), mean control germination ranged from 0.700 to 0.985, with a median mean value of 0.908 (Table 3-8). Control CVs ranged

from 0.006 to 0.560 with a median value of 0.04 (Table 3-8). Using that data, simulation analyses were conducted to evaluate the percentages of tests declared toxic (i.e., failure to reject the null hypothesis) by TST at various alpha error rates (between 0.05 and 0.30), CVs, and percent mean effect in germination between the control and effluent concentrations.

Table 3-8. Summary of mean control germination and control CV derived from analyses of 135 chronic giant kelp WET tests

Percentile	Mean control germination	Control CV	Control SD
10th	0.783	0.02	0.02
25th	0.859	0.03	0.02
50th	0.908	0.04	0.03
70th	0.936	0.05	0.04
75th	0.940	0.05	0.05
85th	0.958	0.07	0.06
90th	0.965	0.07	0.06
95th	0.973	0.10	0.09

Identifying Test Method-Specific α

On the basis of all simulation results (Figure 3-17), an alpha error rate of 0.05 is appropriate for use in applying the TST approach to analysis of chronic *M. pyrifera* germination data because using this alpha error rate satisfies both RMDs of (1) ensuring at least an 75 percent probability of declaring a 25 percent mean effect as toxic and (2) ensuring that a negligible effect ($\leq$ 10 percent mean effect) is declared toxic $\leq$ 5 percent of the time under average test performance. As noted above for the Abalone test method, higher alpha levels also satisfy the above RMDs; however, an alpha level of 0.05 is selected because it is more protective at effect levels > 25 percent.

At a 10 percent mean effect in the effluent for example, and routine, achievable control precision (~75th percentile CV of 0.05), alpha levels ranging from 0.05 to 0.30 resulted in failure to reject the null hypothesis in none of tests (Figure 3-18). Thus, for this test endpoint, low within-test control variability is routinely achieved.

For tests with a mean effect of 25 percent, the rate of tests declared toxic ranges from ~95 percent to ~70 percent, at alpha levels ranging from 0.05 to 0.30, respectively, and approximately the 75th percentile CV level (Figure 3-19). All alpha levels < 0.25 achieved the RMD that a 25 percent mean effect is declared toxic at least 75 percent of the time.

At ~75th percentile CV (0.05) and a mean effect of 10 percent, use of the TST approach results in significantly fewer tests declared toxic relative to the traditional hypothesis approach at all alpha error rates (Figure 3-18). Those results are because the RMD for effluent acceptability (10 percent mean effect) is designed to be met ≥ 95 percent of the time.

Tests with a mean effect of 25 percent and above average precision (CV = 0.05) result in a similar rate of tests declared toxic (Figure 3-19) as the traditional hypothesis approach when the TST $\alpha = 0.05$. The results further support the selection of TST $\alpha = 0.05$ for this test endpoint.

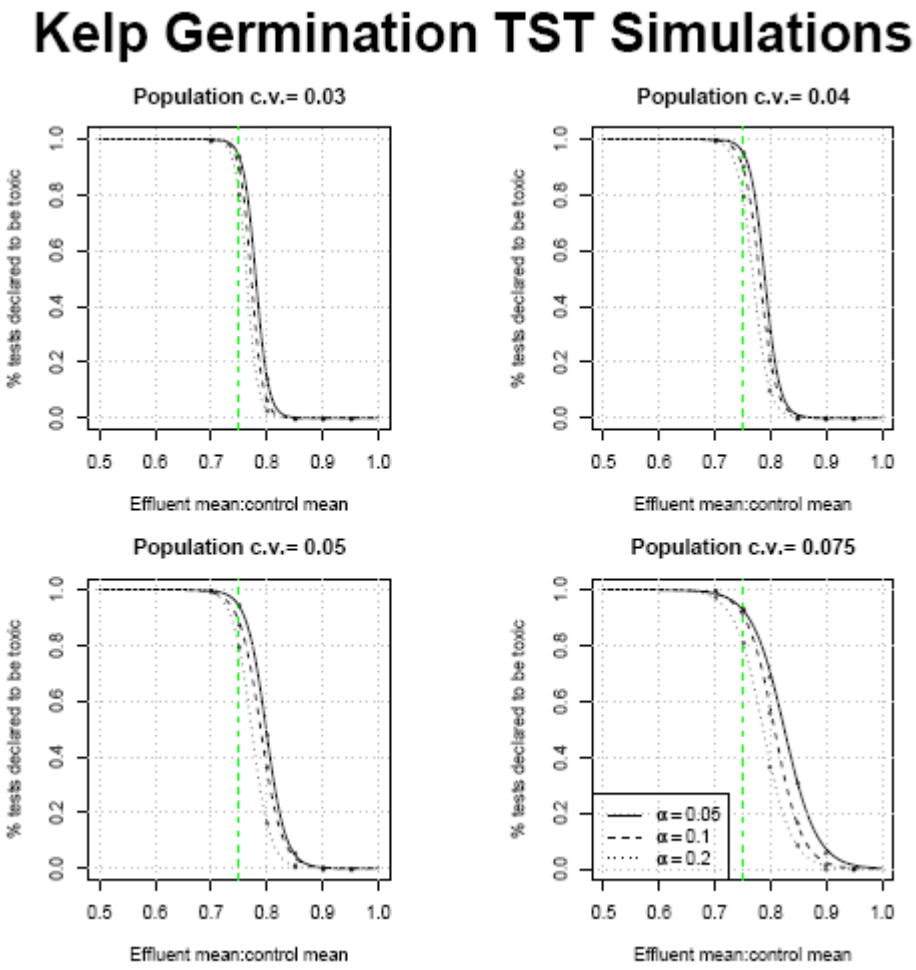

Figure 3-17. Power curves showing the percentage of tests declared toxic as a function of the ratio of effluent mean to control mean response and α level categorized by the level of control within-test variability. CVs correspond to the 25th, 50th, 75th, and 95th percentiles for the chronic giant kelp germination WET method. The dashed line indicates the 75 percent mean effect level, which is the decision threshold for chronic tests.

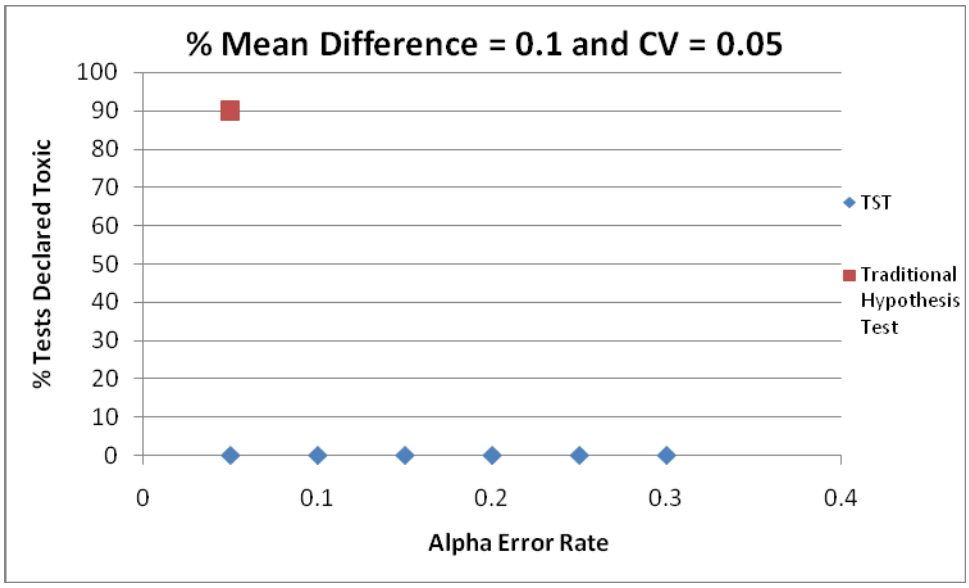

Figure 3-18. Percent of chronic giant kelp germination tests declared toxic using TST having a mean effluent effect of 10 percent and average control variability as a function of the α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

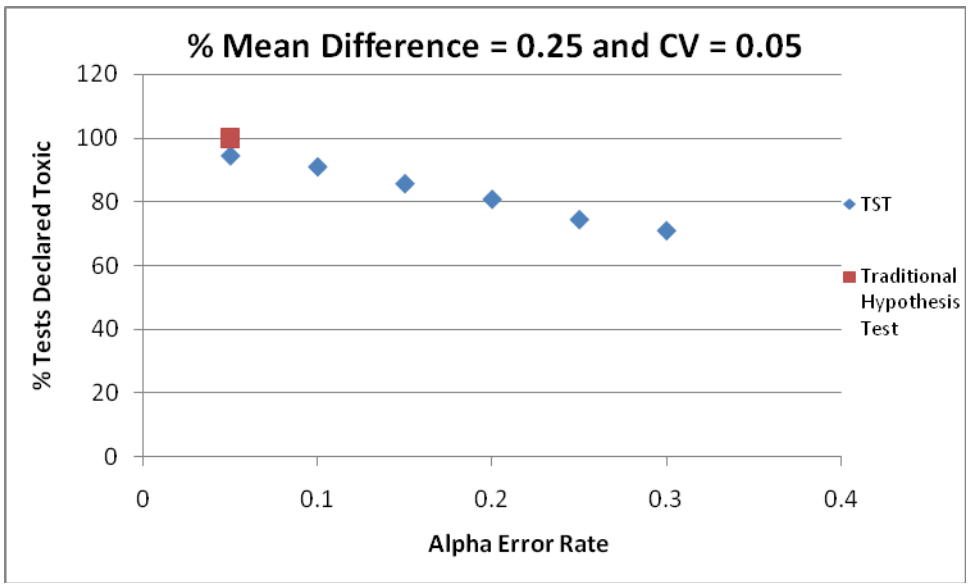

Figure 3-19. Percent of chronic giant kelp germination tests declared toxic using TST having a mean effluent effect of 25 percent and average control variability as a function of the α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

3.6 Chronic *Macrocystis pyrifera* Germ-tube Length Test

On the basis of actual WET data (N = 135 reference toxicant tests), the mean control germ-tube length ranged from 10.200 to 20.778, with a median mean value of 14.014 (Table 3-9). Control CVs ranged from 0.009 to 0.189 with a median value of 0.073 (Table 3-9). Using that data, simulation analyses were conducted to evaluate the percentages of tests declared toxic (i.e., failure to reject the null hypothesis) by TST at various alpha error rates (between 0.05 and 0.30), CVs, and percent mean effect in germ-tube length between the control and effluent concentration.

Table 3-9. Summary of mean control germ-tube length and control CV derived from analyses of 135 chronic *Macrocystis pyrifera* WET tests

Percentile	Mean control germ-tube length	Control CV	Control SD
10th	11.965	0.03	0.46
25th	12.704	0.05	0.71
50th	14.014	0.07	1.04
70th	15.210	0.09	1.22
75th	15.554	0.09	1.29
85th	16.848	0.11	1.54
90th	17.568	0.12	1.74
95th	18.694	0.14	1.89

Identifying Test Method-Specific α

On the basis of all simulation results (Figure 3-20), an alpha error rate of 0.05 is appropriate for use in applying the TST approach to analysis of chronic *M. pyrifera* tube-length data because using that alpha error rate satisfies both RMDs of (1) ensuring at least a 75 percent probability of declaring a 25 percent mean effect as toxic and (2) ensuring that a negligible effect ($\leq$ 10 percent mean effect) is declared toxic $\leq$ 5 percent of the time under average test performance. As noted for the germination endpoint of this species above, higher alpha levels would also satisfy these RMDs; however, in such cases, the lowest alpha $\geq$ 0.05 is selected.

At a 10 percent mean effect in the effluent for example and ~50th percentile CV of 0.07, alpha levels ranging from 0.05 to 0.30 resulted in failure to reject the null hypothesis in almost none of the tests (Figure 3-21). For tests with a mean effect of 25 percent, the rate of tests declared toxic ranged from ~95 to ~70 percent, at alpha error rates ranging from 0.05 to 0.30, respectively, and the 75th percentile CV value (Figure 3-22). Thus, alpha levels < 0.25 achieved the RMD that a 25 percent mean effect is declared toxic at least 75 percent of the time.

At ~50th percentile CV (0.07) and a mean effect of 10 percent, use of the TST approach results in significantly fewer tests declared toxic relative to the traditional hypothesis approach at all alpha error rates examined (Figure 3-21). These results are because of the RMDs of the TST approach; tests with a small (10 percent) mean effect of the effluent are declared non-toxic most of the time when test control data are average or better.

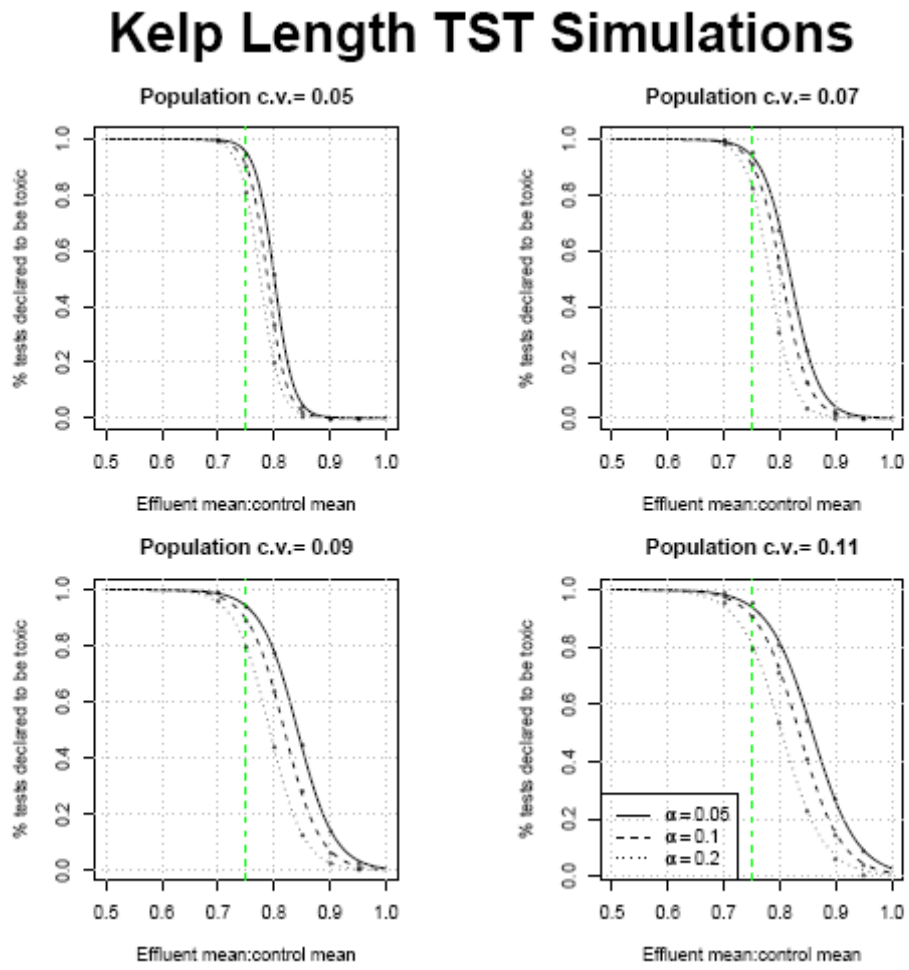

Figure 3-20. Power curves showing the percentage of tests declared toxic as a function of the ratio of effluent mean to control mean response and α level categorized by the level of control within-test variability. CVs correspond to the 25th, 50th, 75th, and 90th percentiles for the chronic giant kelp germ-tube length WET method. The dashed line indicates the 75 percent mean effect level, which is the decision threshold for chronic tests.

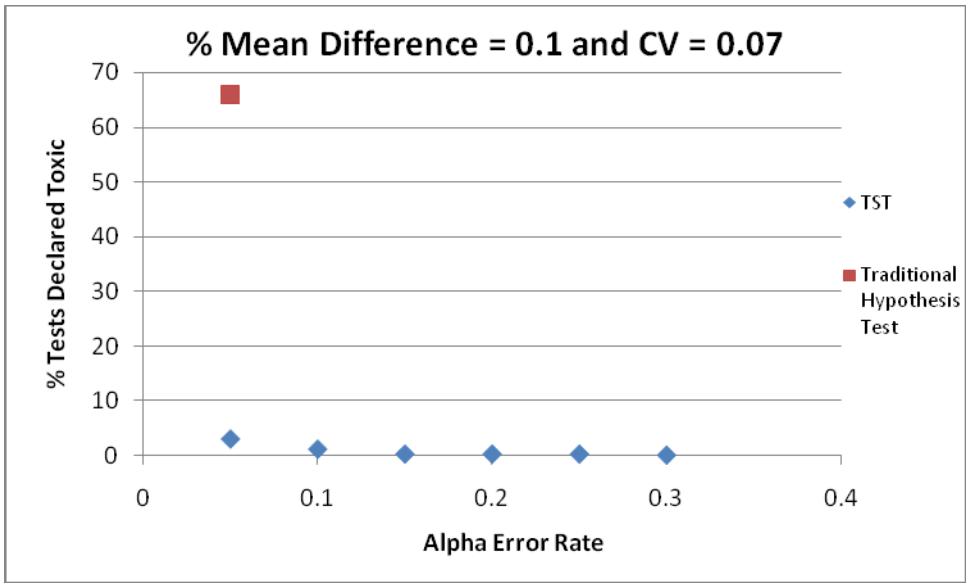

Figure 3-21. Percent of chronic giant kelp germ-tube length tests declared toxic using TST having a mean effluent effect of 10 percent and average control variability as a function of the α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

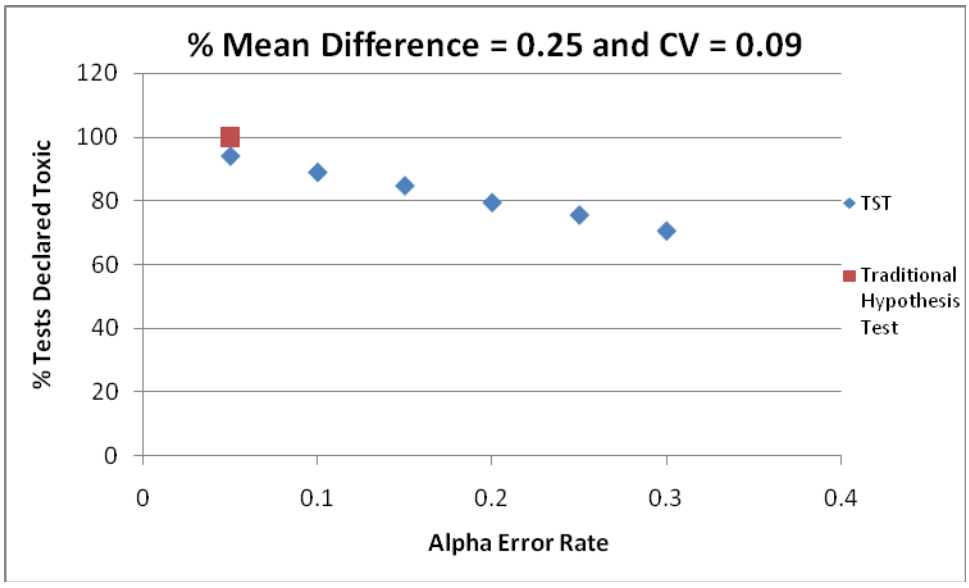

Figure 3-22. Percent of chronic giant kelp germ-tube length tests declared toxic using TST having a mean effluent effect of 25 percent and above average control variability as a function of the α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

Tests with a mean effect of 25 percent and above average precision (CV = 0.09) result in a similar rate of tests declared toxic as the traditional approach when alpha = 0.05 (Figure 3-22). These results further support the selection of 0.05 as the alpha value under TST for this WET endpoint.

3.7 Chronic Echinoderm Fertilization Test

On the basis of actual WET data (N = 177 tests), mean control fertilization ranged from 0.538 to 1.000, with a median mean value of 0.953 (Table 3-10). Control CVs ranged from 0.000 to 0.667 with a median value of approximately 0.03 (Table 3-10). Using that data, simulation analyses were conducted to evaluate the percentages of tests declared toxic (i.e., failure to reject the null hypothesis) by TST at various alpha error rates (between 0.05 and 0.3), CVs, and percent mean effect in reproduction between the control and effluent concentration of concern.

Table 3-10. Summary of mean control fertilization and control CV derived from analyses of 177 chronic *Dendraster excentricus and Strongylocentrotus purpuratus* WET tests

Percentile	Mean control fertilization	Control CV	Control SD
10th	0.826	0.01	0.58
25th	0.875	0.01	1.16
50th	0.953	0.03	2.45
70th	0.975	0.05	4.32
75th	0.978	0.07	5.97
85th	0.990	0.09	7.44
90th	0.993	0.11	9.32
95th	0.996	0.14	11.00

Identifying Test Method-Specific α

On the basis of all simulation results (Figure 3-23), an alpha error rate of 0.05 is appropriate for use in applying the TST approach to analysis of chronic *D. excentricus* and *S. purpuratus* data because using this alpha error rate satisfies both RMDs of (1) ensuring at least an 75 percent probability of declaring a 25 percent mean effect as toxic and (2) ensuring that a negligible effect ($\leq$ 10 percent mean effect) is declared toxic $\leq$ 5 percent of the time under average test performance. As with the other West Coast chronic WET test methods, higher alpha values also satisfy the above RMDs. In these cases, the alpha value $\geq$ 0.05 that satisfies the RMDs is used.

At a 10 percent mean effect in the effluent for example, and ~50th percentile CV of 0.03, alpha levels ranging from 0.05 to 0.30 result in failure to reject the null hypothesis in none of the tests (Figure 3-24). For tests with a mean effect of 25 percent, the rate of tests declared toxic ranged from ~95 to ~70 percent, at alpha error rates ranging from 0.05 to 0.30, respectively, and approximately the 80th percentile CV value (Figure 3-25). Thus, alpha levels < 0.25 achieved the RMD that a 25 percent mean effect in the effluent is declared toxic at least 75 percent of the time regardless of within-test variability.

At ~50th percentile CV for this test endpoint (0.03) and a mean effect of 10 percent in the effluent, TST resulted in significantly fewer tests declared toxic relative to the traditional hypothesis approach at all alpha error rates (Figure 3-24). This results from the fact that the RMD is that tests with a negligible (10 percent) mean effect in the effluent are declared non-toxic most of the time when test control data are average or better.

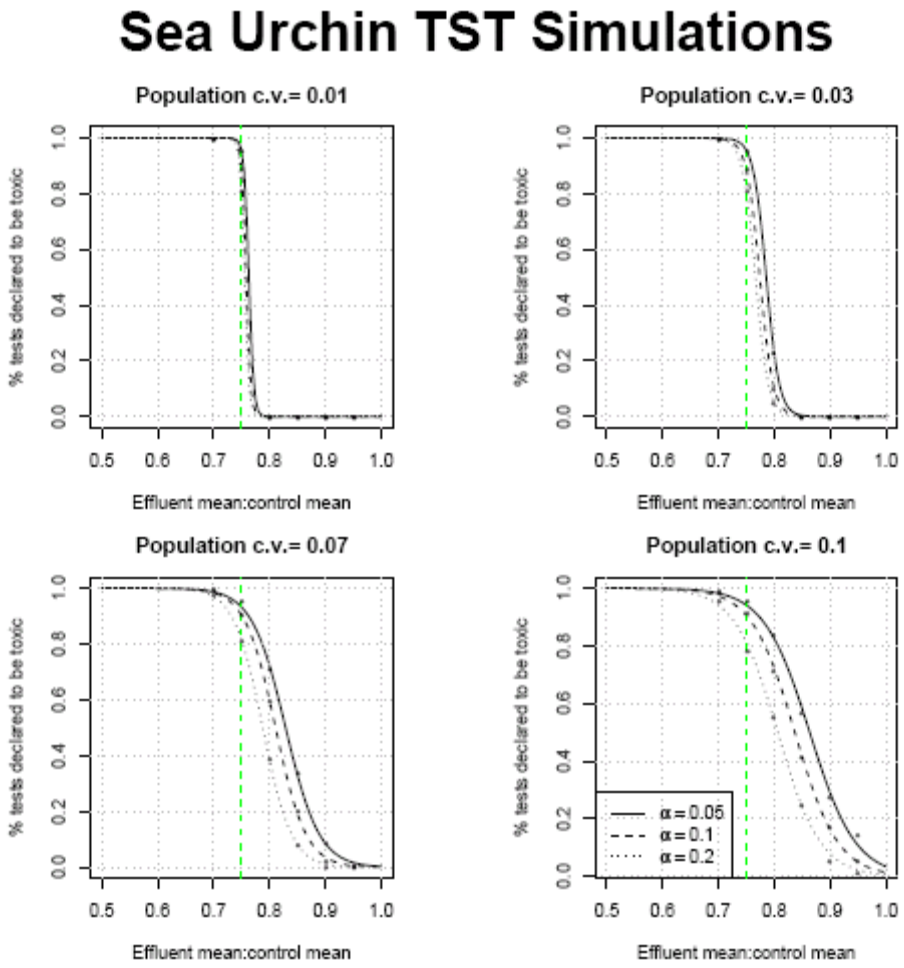

Figure 3-23. Power curves showing the percentage of tests declared toxic as a function of the ratio of effluent mean to control mean response and α level categorized by the level of control within-test variability. CVs correspond to the 25th, 50th, 75th, and 90th percentiles for the chronic echinoderm fertilization WET method. The dashed line indicates the 75 percent mean effect level, which is the decision threshold for chronic tests.

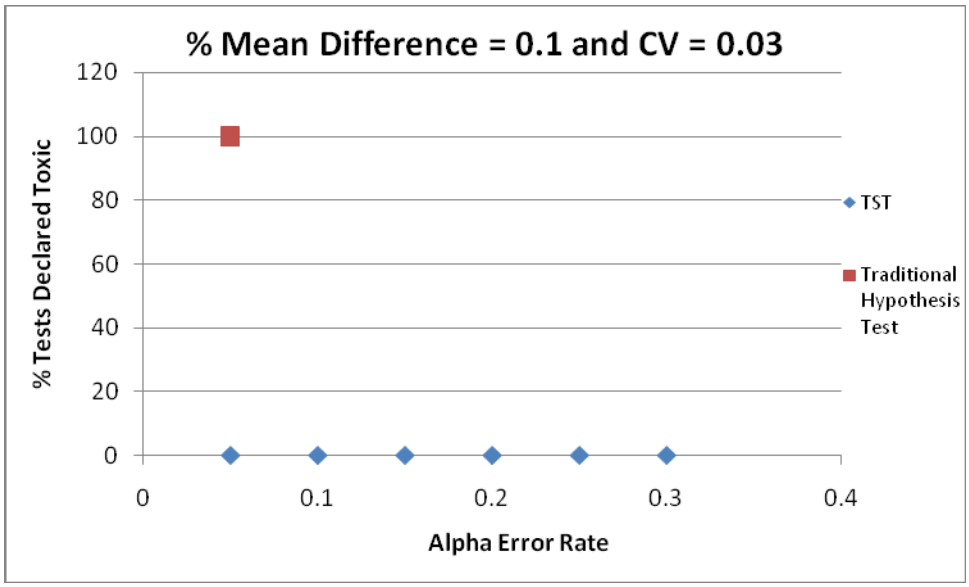

Figure 3-24. Percent of chronic echinoderm tests declared toxic using TST having a mean effluent effect of 10 percent and average control variability as a function of the α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

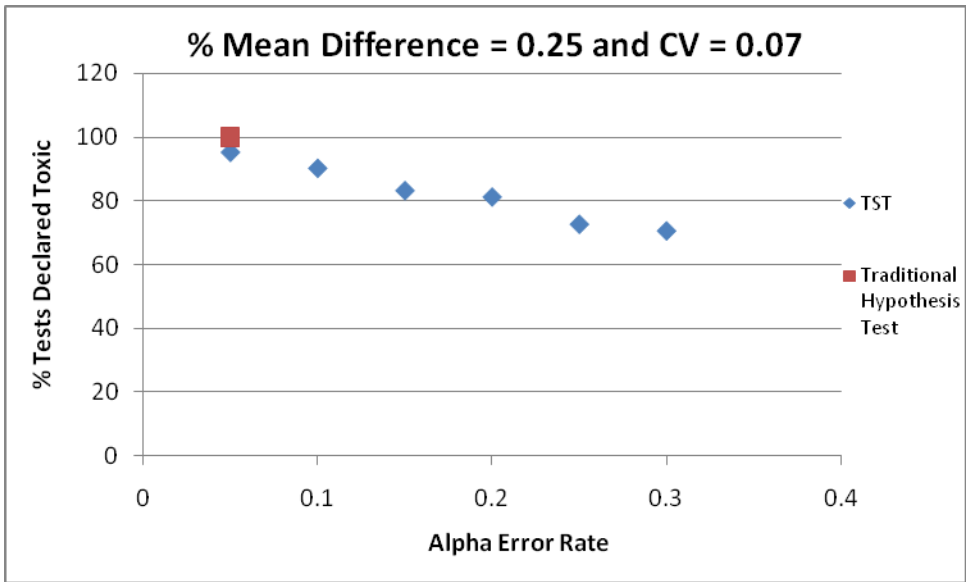

Figure 3-25. Percent of chronic echinoderm tests declared toxic using TST having a mean effluent effect of 25 percent and above average control variability as a function of α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

Tests with a mean effect of 25 percent and above average precision (CV = 0.07) result in a similar rate of tests declared toxic as the traditional hypothesis approach when alpha = 0.05 (Figure 3-25). The results further support the selection of alpha = 0.05 for this WET test endpoint.

3.8 Acute *Pimephales promelas* Survival Test

As noted in the RMD discussion in Section 2.1, acute toxicity (i.e., mortality or immobility of organisms) needs to be tightly controlled because of the potential environmental implications of acute toxicity. Therefore, the RMD toxicity threshold for acute WET methods is set higher than that for the chronic WET methods, with the acute WET method *b* value = 0.80, rather than 0.75 as in the chronic methods. Consequently, the following analyses and results incorporated a *b* value of 0.80.

On the basis of actual WET data (N = 347 tests), mean control survival ranged from 0.900 to 1.000, with a median mean value of 1.000 (Table 3-11). Control CVs ranged from 0.000 to 0.185 with a median value of 0.00 (Table 3-11). The very low control variability observed is expected because of the strength and repeatability of the test endpoint (survival) and the fact that test acceptability criteria for acute WET methods require no less than 90 percent survival in controls. Using that data, simulation analyses were conducted to evaluate the percentages of tests declared toxic (i.e., failure to reject the null hypothesis) by TST at various alpha error rates (between 0.05 and 0.20), a range of CVs corresponding to between the 75th to the 90th percentiles, and percent mean effect in reproduction between the control and effluent concentration.

Table 3-11. Summary of mean control survival and control CV derived from analyses of 347 acute *Pimephales promelas* WET tests

Percentile	Mean control survival	Control CV	Control SD
10th	0.95	0.00	0.00
25th	1.00	0.00	0.00
50th	1.00	0.00	0.00
70th	1.00	0.00	0.00
75th	1.00	0.00	0.00
85th	1.00	0.09	0.15
90th	1.00	0.12	0.18
95th	1.00	0.19	0.23

Identifying Test Method-Specific α

On the basis of all simulation results (Figure 3-26), an alpha error rate of 0.10 is appropriate for use in applying the TST approach to analysis of acute *P. promelas* data because using this alpha error rate satisfies both RMDs of (1) ensuring at least a 75 percent probability of declaring a 20 percent mean effect as toxic and (2) ensuring that a negligible effect ($\leq$ 10 percent mean effect) is declared toxic $\leq$ 5 percent of the time under average control performance.

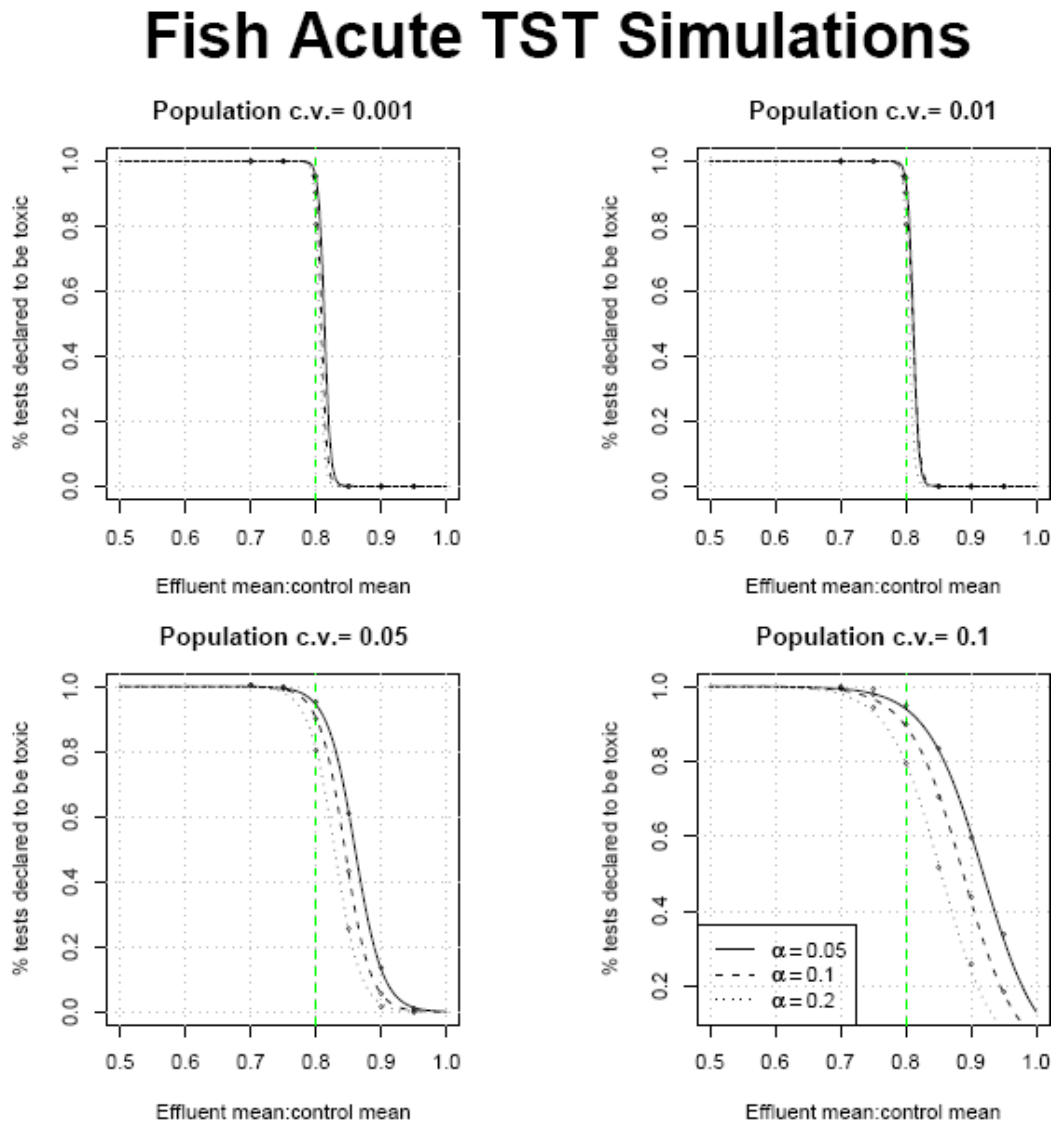

Figure 3-26. Power curves showing the percentage of tests declared toxic as a function of the ratio of effluent mean to control mean response and α level categorized by the level of control within-test variability. CVs correspond to the 75th, 80th, 85th, and 88th percentiles for the acute fathead minnow WET method. The dashed line indicates the 80 percent mean effect level, which is the decision threshold for acute tests.

At a 10 percent mean effect in the effluent and a CV of 0.001 (slightly higher than the 75th percentile), alpha levels ranging from 0.05 to 0.20 resulted in failure to reject the null hypothesis in none of the tests (Figure 3-27). At the 88th percentile CV of 0.10 and a mean effect of 10 percent, alpha levels ranging from 0.05 to 0.20 resulted in declaring between 60 and 25 percent of the tests toxic, respectively. At more moderate CVs (85th percentile), an alpha of 0.10 results in 5 percent of the tests declared toxic. A lower alpha has a higher percentage of tests declared toxic.

For tests with a mean effect of 20 percent, the rate of tests declared toxic ranged from ~100 percent to ~80 percent, at alpha levels ranging from 0.05 to 0.20, respectively, and above average

CV values (Figure 3-28). The rates of tests declared toxic are consistent with the RMD that a 20 percent mean effect in the effluent is declared toxic at least 75 percent of the time. With more routine test performance, an alpha = 0.10 results in 95 percent of the tests declared toxic at a mean effect of 20 percent.

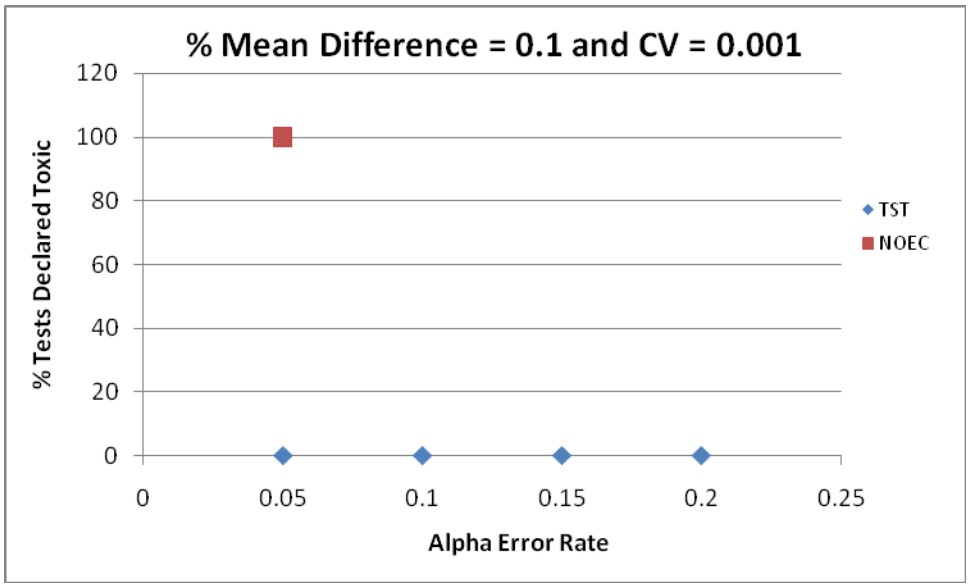

Figure 3-27. Percent of acute fathead minnow tests declared toxic using TST having a mean effluent effect of 10 percent and average control variability as a function of α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

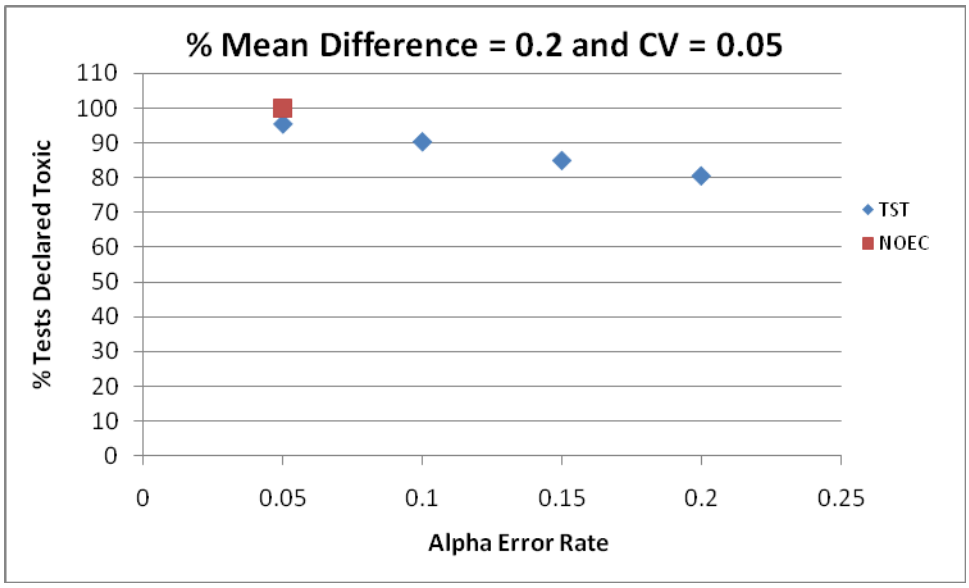

Figure 3-28. Percent of acute fathead minnow tests declared toxic using TST having a mean effluent effect of 20 percent and above average control variability as a function of α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

At a CV of 0.001 and a mean effect of 10 percent, use of the TST approach results in significantly fewer toxic tests relative to the traditional hypothesis approach at all alpha levels (Figure 3-27). These results are due to the RMD that tests with a 10 percent mean effect at the IWC are declared non-toxic most of the time.

Tests with a mean effect of 20 percent and a CV of 0.05 (85th percentile) result in a similar rate of tests declared toxic at alpha = 0.05 and 10 percent fewer tests declared toxic (90 percent of tests) at alpha = 0.10 (Figure 3-28). Because all the results noted above, an alpha = 0.10 is considered appropriately protective for this WET test method.

Effect of Increased Number of Within-Test Replicates

As expected, increasing test replication from two (the minimum allowed in the EPA WET test methods for acute fish tests) to four replicates results in a *higher* rate of tests declared toxic using the traditional hypothesis testing approach and a *lower* rate of tests declared toxic using the TST approach at a 10 percent effect using *P. promelas* acute test data. For tests with a mean effect of 10 percent and a control CV of 0.05 (corresponding to between the 75th and 90th percentile), if replicates are added to the test design, the TST approach demonstrates an increased ability to declare such a test as non-toxic (Table 3-12). As the mean effect approaches 20 percent, the percentage of tests declared toxic is less affected by increased replication using TST because a 20 percent effect in the effluent is the toxicity threshold using TST. However, the percentage of tests declared toxic continues to increase with increased replication using the traditional hypothesis approach, even when there is a negligible effect (10 percent effect as defined by TST) of the effluent. Thus, increasing test replication increases TST's ability to confirm an acceptable effluent test with mean effect less than 20 percent.

Table 3-12. Percent of fathead minnow acute tests declared toxic using TST and a *b* value = 0.8 as a function of percent mean effect, number of replicates (2 or 4 replicates), and different alpha or Type I error levels

				Alpha			
***B* value**	**CV**	**% effect**	**# reps**	**0.05**	**0.1**	**0.15**	**0.2**
0.8	0.05	0.10	2	57	33	21	13
0.8	0.05	0.20	2	95	91	85	80
0.8	0.05	0.10	4	14	5	3	1
0.8	0.05	0.20	4	95	90	85	80

3.9 Chronic *Selenastrum capricornutum* Growth Test

On the basis of actual WET data (N = 223 tests), the mean control growth ranged from 1,019,250 cells to 14,109,450 cells, with a median value of 3,331,250 cells (Table 3-13). Control CVs ranged from 0.00 to 0.20 with a median value of 0.06 (Table 3-13). Using those data, simulation analyses were conducted to evaluate the percentages of tests declared toxic (i.e., failure to reject the null hypothesis) by TST at various alpha error rates (between 0.05 and 0.25), CVs, and percent mean effect in growth between the control and effluent concentration. In addition, WET

test data (N = 173), in which EDTA was added to the controls, as required in the 2002 *Selenastrum* method, were evaluated independently and compared to the simulation results. For those tests the mean control growth ranged from 1,019,250 cells to 14,109,450 cells, with a median value of 3,430,000 cells (Table 3-13). Control CVs from those tests ranged from 0.00 to 0.20 with a median value of 0.06, similar to the results observed for all 223 tests (Table 3-13).

Table 3-13. Summary of mean control growth, CV and standard deviation derived from the analyses of all chronic *Selenastrum capricornutum* WET test data and compared with the analysis of only the chronic *Selenastrum capricornutum* WET test in which it was assumed that EDTA was added to the controls.

All Tests (N = 223)				Only Tests With EDTA Addition (N = 173)			
Percentile	Mean Cell Density	Control CV	Control SD	Percentile	Mean Cell Density	Control CV	Control SD
10th	1233050.0	0.02	44928.62	10th	1554500.0	0.02	43664.06
25th	2245833.5	0.04	108449.85	25th	2502500.0	0.03	135154.20
50th	3331250.0	0.06	277653.90	50th	3430000.0	0.06	309232.90
70th	4869000.0	0.10	407505.12	70th	5581650.0	0.10	417361.66
75th	6179667.0	0.11	444887.25	75th	8220000.0	0.11	447446.50
85th	9265500.0	0.13	545764.05	85th	9785000.0	0.14	543717.8
90th	9888000.0	0.16	599644.32	90th	10048000.0	0.16	583299.40
95th	10149500.0	0.18	751884.62	95th	10279000.0	0.18	669780.04

Identifying Test Method-Specific α

On the basis of all simulation results (Figure 3-29), an alpha error rate of 0.25 is appropriate, for both tests with EDTA addition and tests with no EDTA addition, for use in applying the TST approach to analysis of chronic *Selenastrum* data. Using this alpha error rate addresses both RMDs of (1) ensuring at least a 75 percent probability of declaring a 25 percent mean effect as toxic and (2) ensuring that a negligible effect ($\leq$ 10 percent mean effect) is declared toxic $\leq$ 5 percent of the time under average or better than average test performance.

For example, at a 10 percent mean effect and a low level of precision (~70th percentile for all tests, CV of 0.10), an alpha level of 0.25 resulted in failure to reject the null hypothesis in $\leq$ 5 percent of tests with or without EDTA addition (Figure 3-29). For all tests with a mean effect of 25 percent, and a similar precision, the rate of tests declared toxic is 75 percent at an alpha value of 0.25, consistent with RMDs (Figure 3-29).

At ~70th percentile CV (0.10) and a mean effect of 10 percent, for both tests with and without EDTA addition, use of the TST approach results in fewer toxic tests relative to the traditional hypothesis testing approach at all alpha error rates, including the alpha error rate of 0.25 which declared less than 5 percent of the tests toxic (Figure 3-30).

Tests with a mean effect of 25 percent, regardless of precision (CV = 0.10 or 0.15), result in a 75 percent or greater rate of tests declared toxic, which is significantly more than that using the traditional hypothesis testing approach using any alpha value between 0.05 and 0.25 (Figure 3-31). The percent of tests found to be toxic using the TST approach with a mean effect of 25 percent was not significantly affected by the change in CV values.

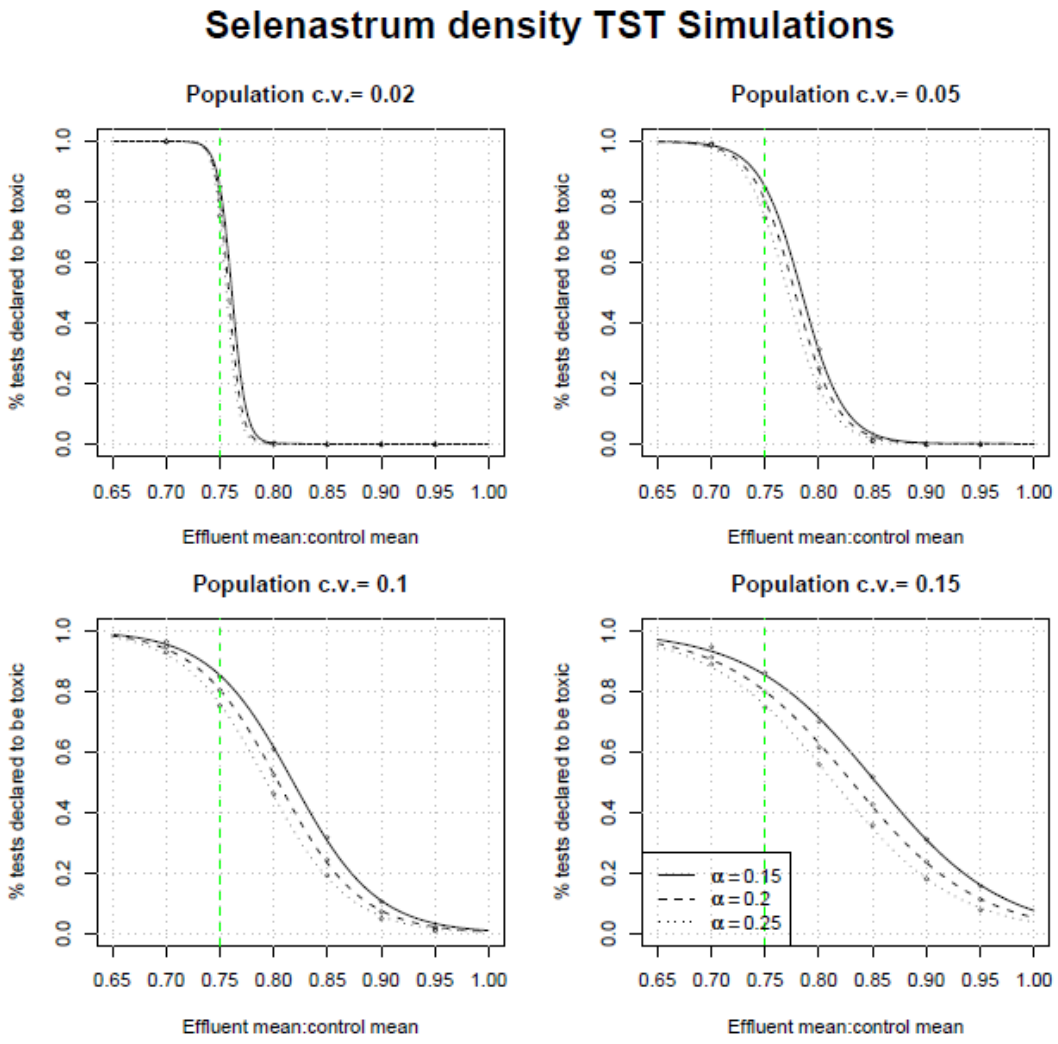

Figure 3-29. Power curves showing the percentage of tests declared toxic as a function of the ratio of effluent mean to control mean response and α level categorized by the level of control within-test variability. CVs correspond to the 10th, 40th, 70th, and 85th percentiles for the chronic *Selenastrum* WET method. The dashed line indicates the 75 percent mean effect level, which is the decision threshold for chronic tests.

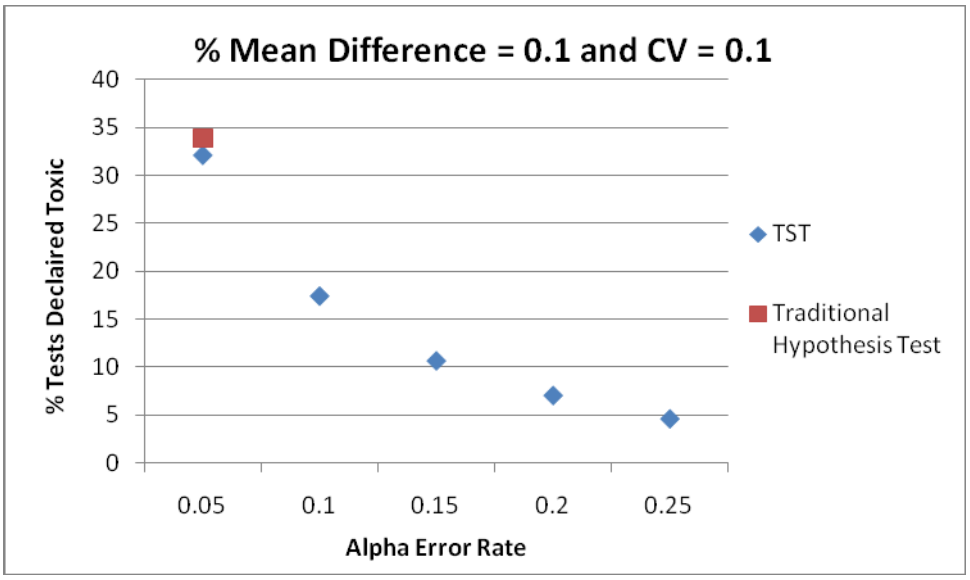

Figure 3-30. Percent of *Selenastrum* tests declared toxic using TST having a mean effluent effect of 10 percent and average control variability as a function of α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

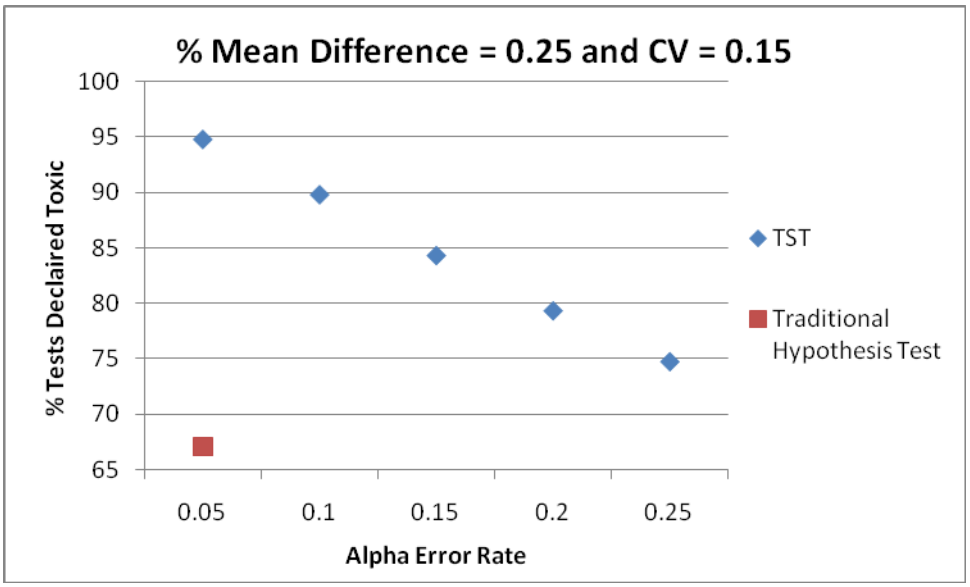

Figure 3-31. Percent of *Selenastrum* tests declared toxic using TST having a mean effluent effect of 25 percent and above average control variability as a function of α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

Effluent Data Results

Results from actual effluent tests were compared between TST and the traditional hypothesis testing approach for all control CV's (Table 3-14). At a mean effect of 10–15 percent (N = 25),

TST declared none of the tests toxic while the traditional hypothesis testing approach declared 67 percent of the tests toxic. However, if the mean effect is greater than 25 percent (N = 97), TST declared 100 percent of the tests toxic, while the traditional hypothesis testing approach declared 98 percent of the tests toxic. These results indicate that TST is as protective as the current hypothesis testing approach for those tests when the TST RMD threshold for toxicity is exceeded.

Table 3-14. Comparison of the percentage of chronic *Selenastrum* tests declared toxic using TST versus the traditional hypothesis testing approach

% Mean effect	N	% tests toxic using TST	% tests toxic using traditional hypothesis testing approach
10–15	25	0	67
> 25	97	100	98

3.10 Acute *Ceriodaphnia dubia* Survival Test

Acute toxicity (i.e., mortality or immobility of organisms) needs to be tightly controlled because of the potential environmental implications of acute toxicity. Therefore, the RMD toxicity threshold for acute WET methods is set higher than that for the chronic WET methods, with the acute WET method *b* value = 0.80, rather than 0.75 as in the chronic methods. Consequently, the following analyses and results incorporated a *b* value of 0.80.

On the basis of actual WET data (N = 239 tests), mean control survival ranged from 0.900 to 1.000, with a median mean value of 1.000 (Table 3-15). Control CVs ranged from 0.00 to 0.22 (the minimum and maximum levels obtainable using the test acceptability criteria) with a median value of 0.00 (Table 3-15). The very low control variability observed is expected because of the strength and repeatability of the test endpoint (survival) and the fact that test acceptability criteria for acute WET methods stipulate no less than 90 percent survival in the controls. Using that data, simulation analyses were conducted to evaluate the percentages of tests declared toxic (i.e., failure to reject the null hypothesis) by TST at various alpha error rates (between 0.05 and 0.30), a range of CVs, and percent mean effect in survival between the control and effluent concentration.

Table 3-15. Summary of mean control growth, CV and standard deviation derived from analyses of 239 acute *Ceriodaphnia dubia* WET tests.

Percentile	Mean Survival (%)	Control CV	Control SD
10th	0.95	0.00	0.00
25th	1.00	0.00	0.00
50th	1.00	0.00	0.00
70th	1.00	0.00	0.00
75th	1.00	0.00	0.00
85th	1.00	0.00	0.00
90th	1.00	0.11	0.10
95th	1.00	0.11	0.10

Identifying Test Method-Specific α

On the basis of all simulation results (Figure 3-32), an alpha error rate of 0.10 is appropriate for use in applying the TST approach to analysis of acute *Ceriodaphnia dubia* data because using this alpha error rate best satisfies both RMDs of (1) ensuring at least a 75 percent probability of declaring a 20 percent mean effect as toxic and (2) ensuring that a negligible effect (≤ 10 percent mean effect) is declared toxic ≤ 5 percent of the time under average control performance.

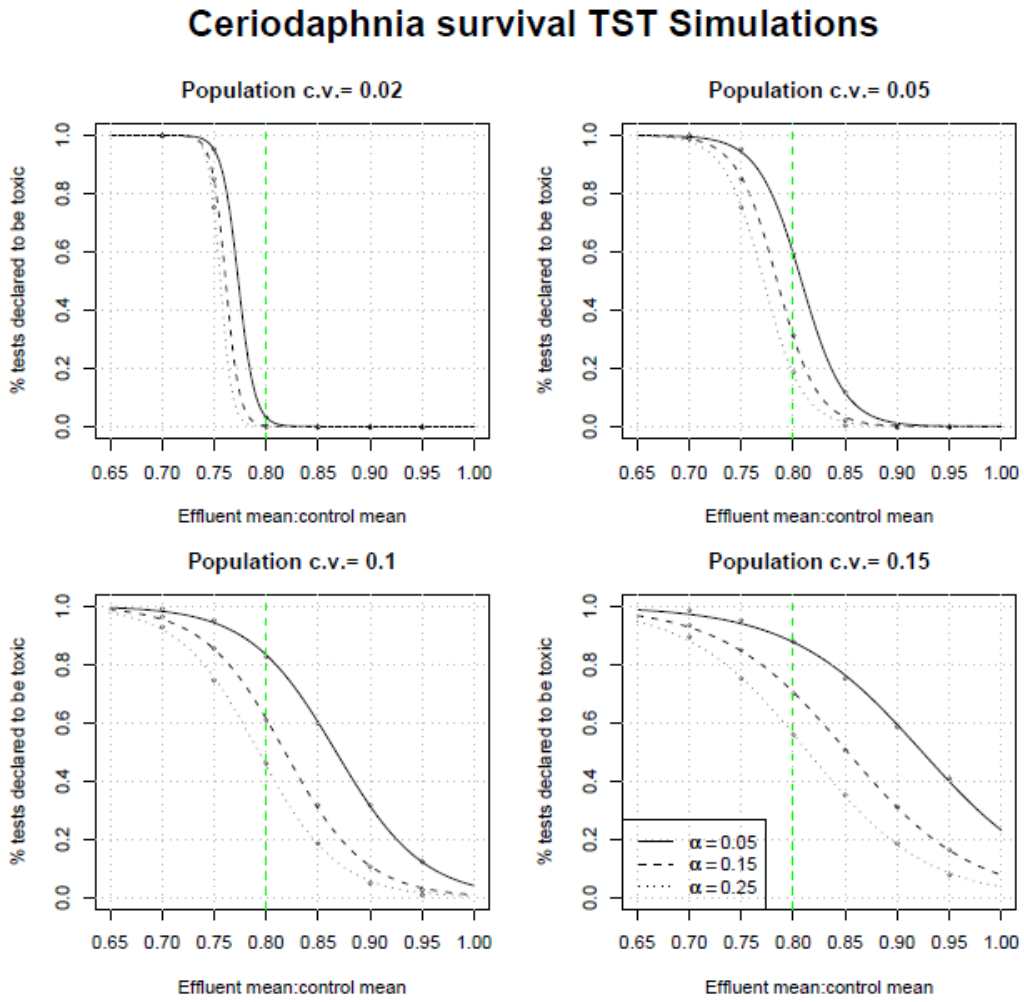

Figure 3-32. Power curves showing the percentage of tests declared toxic as a function of the ratio of effluent mean to control mean response and α level categorized by the level of control within-test variability. The first two CVs correspond to the 85th percentile, and the following two correspond to the 95th and ~98th, respectively for the acute *Ceriodaphnia dubia* WET method. The dashed line indicates the 80 percent mean effect level, which is the decision threshold for acute tests.

For example, at a 10 percent mean effect in the effluent and a CV of 0.02 (slightly higher than the 85th percentile), alpha levels ranging from 0.05 to 0.25 resulted in failure to reject the null hypothesis in ≤ 5 percent of the tests (Figure 3-32). However, at the 90th and 95th percentile CVs of 0.10 and a mean effect of 10 percent, the alpha level of 0.25 resulted in 19 percent of the tests

found toxic. For tests with a mean effect of 20 percent, and ~85th percentile precision (CV of 0.02), 75 percent of the tests are declared toxic, achieving the RMD using an alpha value of 0.25 (Figure 3-32).

For tests with a mean effect of 20 percent, the rate of tests declared toxic ranged from ~95 percent to ~75 percent, at alpha levels ranging from 0.05 to 0.25, respectively, using all CV values that correspond to $\leq$ 95th percentile. (Figure 3-32). The rates of tests declared toxic are consistent with the RMD that a 20 percent mean effect in the effluent is declared toxic at least 75 percent of the time. With more routine test performance, an alpha of 0.10 results in 90 percent of the tests declared toxic at a mean effect of 20 percent.

At a CV of 0.02 (~85th percentile) and a mean effect of 10 percent, use of the TST approach results in no toxic tests, while the traditional hypothesis approach results in 100 percent toxic tests at all alpha levels (Figure 3-33).

Tests with a mean effect of 20 percent and a range of within-test control precision values (CV of 0.02 to 0.15) result in at least 75 percent of the tests declared toxic using an alpha = 0.10 (Figure 3-34). In contrast fewer tests are declared toxic at a 20% effect when using the traditional hypothesis testing approach and any alpha value between 0.05 and 0.25 (Figure 3-34). Thus, the percent of tests found to be toxic using the TST approach with a mean effect of 20 percent was not significantly affected by the change in CV values.

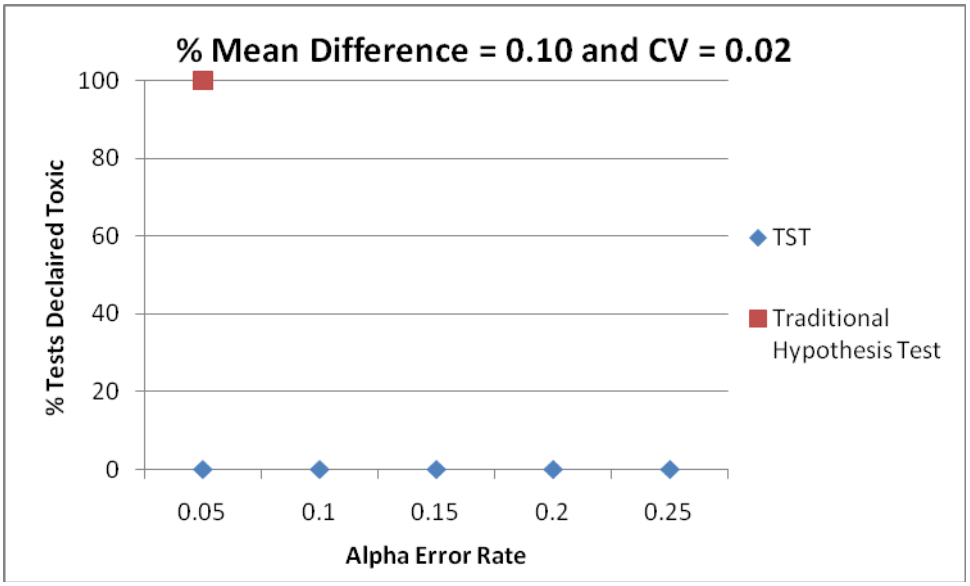

Figure 3-33. Percent of acute *C. dubia* tests declared toxic using TST having a mean effluent effect of 10 percent and average control variability as a function of α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

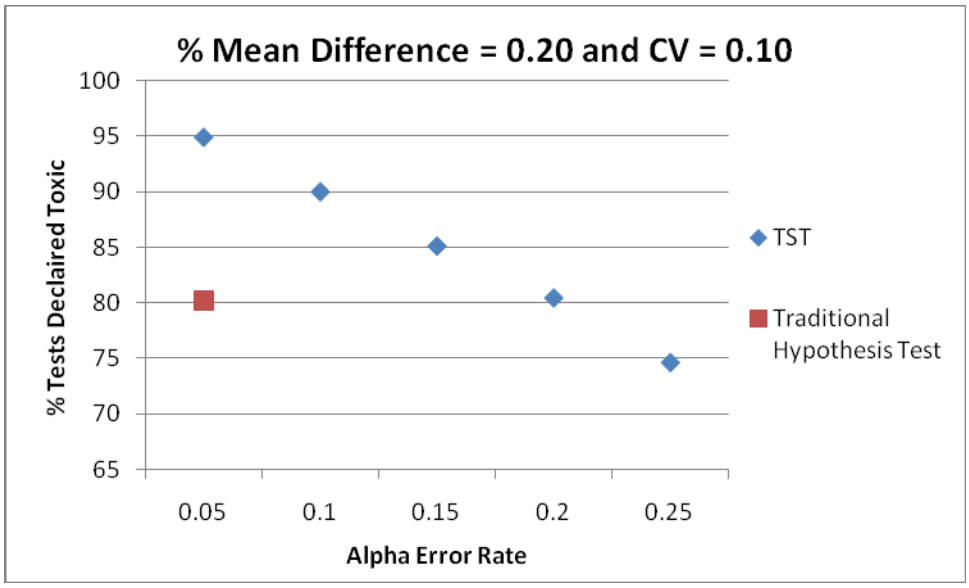

Figure 3-34. Percent of acute *C. dubia* tests declared toxic using TST having a mean effluent effect of 20 percent and above average control variability as a function of α error rate. Result using the traditional hypothesis approach (α = 0.05) is shown as well.

Effect of Increased Number of Within-Test Replicates

As with the fathead minnow acute method, increasing test replication from four (the minimum allowed in the EPA WET test methods for acute *Ceriodaphnia dubia* tests) to six replicates results in a *higher* rate of tests declared toxic using the traditional hypothesis testing approach and a *lower* rate of tests declared toxic using the TST approach at a 10 percent mean effect using *C. dubia* acute test data. For tests with a mean effect of 10 percent and a control CV of 0.06 (corresponding to between the 85th and 90th percentile), if replicates are added to the test design, the TST approach demonstrates an increased ability to declare such a test as non-toxic (Table 3-16). As the mean effect approaches 20 percent, the percentage of tests declared toxic is less affected by increased replication using TST because a 20 percent effect in the effluent is the RMD using TST. However, the percentage of tests declared toxic continues to increase with increased replication using the traditional hypothesis approach, even when there is a negligible effect (10 percent effect as defined by TST) of the effluent. Thus, increasing test replication increases TST's ability to confirm an acceptable effluent test with mean effect less than 20 percent.

Table 3-16. Percent of *Ceriodaphnia dubia* acute tests declared toxic using TST and a *b* value = 0.8 as a function of percent mean effect, number of replicates (4 or 6 replicates), and different alpha or Type I error levels

B value	CV	% effect	# reps	Alpha			
				0.05	0.1	0.15	0.2
0.8	0.06	0.10	4	23	12	7	5
0.8	0.06	0.20	4	95	90	85	80
0.8	0.06	0.10	6	8	4	3	2
0.8	0.06	0.20	6	95	90	85	80

4.0 SUMMARY OF RESULTS AND IMPLEMENTING TST

4.1 Summary of Test Method-Specific Alpha Values

On the basis of all the analyses conducted in this project, the test method-specific alpha levels summarized in Table 4-1 are used with the TST approach. The method-specific alpha values apply to all test endpoints for a given EPA WET test method (e.g., survival and reproduction for the *Ceriodaphnia* chronic WET test method). As noted in Section 2.3.1, alpha values were selected on the basis of simulation analyses using normally distributed data and equal variances in the control and the effluent. While additional analyses indicate that the alpha levels identified are robust to the type of heterogeneous variances and non-normal data observed in WET test data (see Appendix A), this issue is still acknowledged as a potential uncertainty.

The alpha values identified above provide as much protection under most circumstances as the current approved WET test analysis methods when the mean effect at the IWC exceeds the toxicity threshold of the TST approach.

At the chronic toxicity regulatory management threshold of 25 percent mean effect of the effluent and lower within-test control CVs ($< 50^{th}$ percentile), TST declares a greater percentage of tests non-toxic than the traditional hypothesis approach for some of the chronic WET test methods examined (e.g., fathead minnow chronic WET test) because of the higher alpha levels assigned to those test methods. At either higher within-test CVs or higher mean effect levels, results are more similar between the two approaches, as explained in Section 1.4 of this document. With more extreme within-test variability ($\geq 80^{th}$ percentile CV), results tend to be reversed with TST declaring a higher percentage of tests toxic at 25 percent mean effect of the effluent as compared to the traditional hypothesis approach; e.g., for the *Ceriodaphnia* reproduction endpoint, at the 80^{th} percentile CV, TST declares ~20 percent of the tests non-toxic at a 25 percent mean effect, while the traditional approach declares 24 percent of the tests non-toxic. If test data are non-normal (a somewhat frequent condition for some WET endpoints such as acute and chronic survival, or when a high level of toxicity is observed in certain effluent concentrations within a test), additional research has indicated that use of Welch's t-test results in a lower rejection rate (i.e., is more conservative) using the TST approach, resulting in a higher percentage of tests declared toxic when the effluent effect $\geq b \times$ control mean (Appendix A). For the acute fathead minnow test method, at the acute toxicity regulatory management threshold of 20 percent mean effect of the effluent, both approaches had a similarly low percentage of tests declared non-toxic over all within-test CVs. Results of this comparison also demonstrate that for all WET test methods, the TST approach declares a lower percentage of tests as toxic at a 10 percent mean effect in the effluent, for most WET tests (i.e., within-test CV $\leq 75^{th}$ percentile for a given WET test method). If within-test variability is lower (control data has greater precision), the result is further accentuated; i.e., an even greater percentage of tests are declared toxic at a 10 percent effect using the traditional hypothesis approach and an even lower percentage of tests declared toxic using TST.

Table 4-1. Summary of alpha (α) levels or false negative rates recommended for different EPA WET test methods using the TST.

EPA WET test method	*b* value	Probability of declaring a toxic effluent non-toxic: False negative (α) error[a]
Chronic Freshwater and East Coast Methods		
Ceriodaphnia dubia (water flea) survival and reproduction	0.75	0.20
Pimephales promelas (fathead minnow) survival and growth	0.75	0.25
Selenastrum capricornutum (green algae) growth	0.75	0.25
Americamysis bahia (mysid shrimp) survival and growth	0.75	0.15
Arbacia punctulata (Echinoderm) fertilization	0.75	0.05
Cyprinodon variegatus (Sheepshead minnow) and *Menidia beryllina* (inland silverside) survival and growth	0.75	0.25
Chronic West Coast Marine Methods		
Dendraster excentricus and *Strongylocentrotus purpuratus* (Echinoderm) fertilization	0.75	0.05
Atherinops affinis (topsmelt) survival and growth	0.75	0.25
Haliotis rufescens (red abalone), *Crassostrea gigas (oyster), Dendraster excentricus, Strongylocentrotus purpuratus* (Echinoderm) and *Mytilus sp* (mussel) larval development methods	0.75	0.05
Macrocystis pyrifera (giant kelp) germination and germ-tube length	0.75	0.05
Acute Methods		
Pimephales promelas (fathead minnow), *Cyprinodon variegatus* (Sheepshead minnow), *Atherinops affinis* (topsmelt), *Menidia beryllina* (inland silverside) acute survival[b]	0.80	0.10
Ceriodaphnia dubia, *Daphnia magna, Daphnia pulex*, *Americamysis bahia* acute survival[b]	0.80	0.10

Notes:
a α levels shown are the probability of declaring an effluent toxic when the mean effluent effect = 25% for chronic tests or 20% for acute tests and the false positive rate (β) is ≤ 0.05 (5%) when mean effluent effect = 10%.
b. Based on a four replicate test design

4.2 Calculating Statistics for Valid WET Data Using the TST Approach

Appendix B includes a step-by-step guide for using the TST approach to analyze valid WET data. The appendix also includes a statistical flowchart. Note that the WET test method should follow the test condition requirements as specified in EPA's approved WET methods (USEPA 1995, 2002a, 2002b, 2002c).

The TST approach is used to statistically compare organism responses from two treatments of the WET test, the IWC and the control. Percent data (quantal data), such as percent survival or percent germination from a WET test, is first transformed as recommended in the EPA WET test manuals. Other types of WET data (e.g., growth or reproduction data) are not transformed (for

the rationale, see Appendix A). Data are then analyzed using Welch's t-test, a well-known modification of the traditional t-test (Zar 1996), which is appropriate for the TST approach (see Appendix A).

Appendix C lists the critical t values that apply to WET testing using the TST approach given the number of degrees of freedom and the α level that applies for a given WET test method from Table 4-1 of this document. If the calculated t value for the WET test is greater than the critical t value (given in Appendix C), the null hypothesis is rejected, i.e., the test result is a *pass* and **the effluent is declared *non-toxic***. If the calculated t value is less than the critical t value in Appendix C, the null hypothesis is not rejected, i.e., the test result is a *fail* and **the effluent *is* declared toxic**.

4.3 Benefits of Increased Replication Using TST

One of the intended benefits of the TST approach is that increasing the precision and power of the test increases the chances of rejecting the null hypothesis and declaring a truly acceptable sample as non-toxic. This increases the permittee's ability to demonstrate that a sample is acceptable. Results for the *Ceriodaphnia*, fathead minnow, and mysid chronic test methods presented in Section 3 indicate the benefits of increased replication within a test, especially when the mean effect of the sample is below about 20 percent in the case of chronic tests and about 15 percent for acute tests. As expected, increasing test replication (and thereby the power of the test) results in a *higher* rate of tests declared toxic using the traditional hypothesis testing approach but a *lower* rate of tests declared toxic using the TST approach.

Conducting tests with more replicates can help a permittee demonstrate that the effluent is acceptable if the mean effect at the IWC is truly less than the RMDs as defined by TST (25 percent effect for chronic and 20 percent for acute). Conversely, increasing replicates does not assist a permittee using the traditional hypothesis testing approach.

4.4 Applying TST to Ambient Toxicity Programs

In ambient and stormwater toxicity testing, a laboratory control and a single concentration (i.e., 100 percent ambient water or stormwater) are often tested. In those two-concentration WET tests, the objective is to determine if a sample or site water is toxic, as indicated by a significantly worse organism response compared to the control. In this WET testing design, the determination of pass or fail (i.e., toxic or non-toxic) is ascertained using a traditional t-test (USEPA 2002c). EPA WET test methods recommend that the statistical significance (i.e., pass/fail) of a two-sample test design for ambient and stormwater toxicity testing be determined by using only a modified t-test (if homogeneity of variance is not achieved) or a traditional t-test (if homogeneity of variance is achieved).

To demonstrate the value of the TST approach in ambient toxicity programs, ambient toxicity test data from California's SWAMP was used for 409 chronic tests for *Ceriodaphnia dubia* and 256 chronic tests for *Pimephales promelas* using EPA's 2002 WET test methods (USEPA 2002a). WET test data for each WET test method were subjected to the same statistical analyses as described in Section 2 of this document.

Chronic *Ceriodaphnia dubia* Ambient Toxicity Tests

Table 4-2 summarizes results from the 409 *Ceriodaphnia dubia* ambient toxicity tests analyzed and a $\alpha = 0.20$ for this test method. Although the majority of the tests examined resulted in the same decision using either the TST or the traditional t-test approach, approximately 6 percent of the tests (24 tests) would have been declared non-toxic using the traditional t-test approach with mean effect levels > 25 percent. In addition, 2 percent of the tests (7 tests) would have been declared toxic at mean effect levels < 15 percent and as low as 7 percent.

Table 4-2. Comparison of results of chronic *Ceriodaphnia* ambient toxicity tests using the TST approach and the traditional t-test analysis. $\alpha = 0.2$ and *b* value = 0.75 for the TST approach. $\alpha = 0.05$ for the traditional hypothesis testing approach

Both approaches declare toxic	Only TST declares toxic	Only traditional approach declares toxic	Both approaches declare non-toxic
19.8%	5.9%	1.7%	72.6%

Figure 4-1 shows ranges of CV values observed in *Ceriodaphnia dubia* ambient toxicity tests for those samples declared toxic using either the TST approach or the traditional t-test but not both approaches. As expected, within-test variability was relatively high (higher CVs) for those tests found non-toxic using a t-test but toxic using the TST approach. The results again demonstrate a limitation of the traditional hypothesis testing approach when control variability is relatively high. Under those conditions, the t-test did not have the power to detect toxicity when it was present. Figure 4-1 also demonstrates that the TST approach is superior to the traditional t-test when within-test variability is relatively low and the mean percent effect is well below the risk management level of 25 percent. Under such conditions, the traditional t-test declared some samples toxic using this WET test method, even when the mean effect was as little as 7 percent. The TST approach, however, declared all such samples non-toxic using the recommended $\alpha = 0.20$. Thus, the TST approach reduces the number of tests classified as toxic when effects are actually well below risk management levels of concern.

Similar to the *Ceriodaphnia* ambient test data, within-test variability was higher in those chronic fathead minnow ambient tests found non-toxic using a t-test but toxic using the TST approach (Figure 4-2). Similarly, those tests declared non-toxic by the TST approach but toxic using t-test had lower within-test variability and mean effect levels < 25 percent (Figure 4-2). Thus, as with the chronic *Ceriodaphnia* ambient tests, data from chronic fathead minnow ambient tests demonstrate that the TST approach provides better protection than the traditional t-test approach while also identifying those samples that are truly acceptable from a regulatory management perspective.

4.5 Implementing TST in WET Permitting under NPDES

The TST approach is an alternative statistical approach for analyzing and interpreting valid WET data; it is not an alternative approach to developing NPDES permit WET limitations. Using the TST approach does not result in any changes to EPA's WET test methods.

Chronic *Ceriodaphnia* ambient WET tests that are identified as non-toxic (pass) using the traditional hypothesis approach (t-test) generally have poor test sensitivity (high control CVs), masking effects, as compared to using the TST approach.

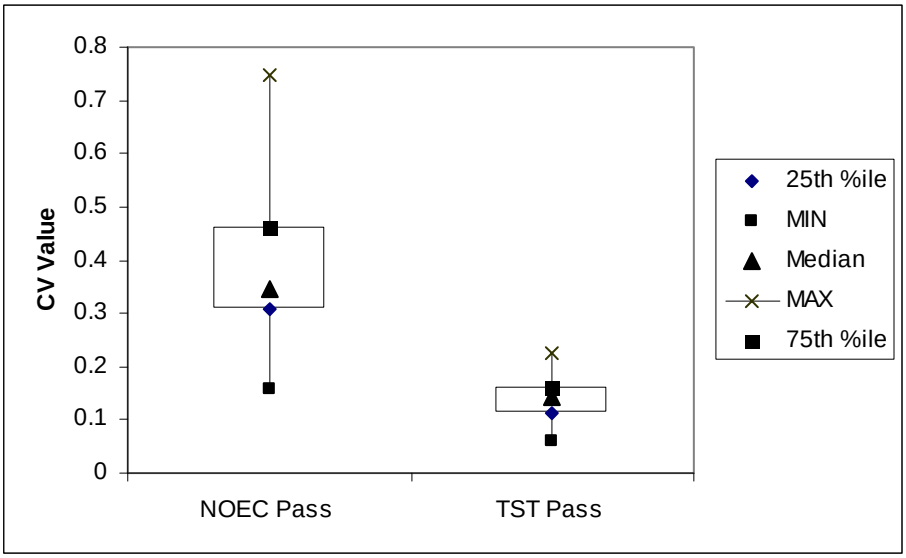

Figure 4-1. Range of CV values observed in chronic *C. dubia* ambient toxicity tests for samples that were found to be non-toxic using the traditional t-test but toxic using the TST approach (*NOEC Pass*) and for those samples declared toxic using t-test but not the TST approach (*TST Pass*). California's SWAMP WET test data.

Fish ambient WET tests that are identified as non-toxic using the traditional hypothesis approach (t-test) generally have poor test sensitivity (high control CVs), masking effects, as compared to using the TST approach.

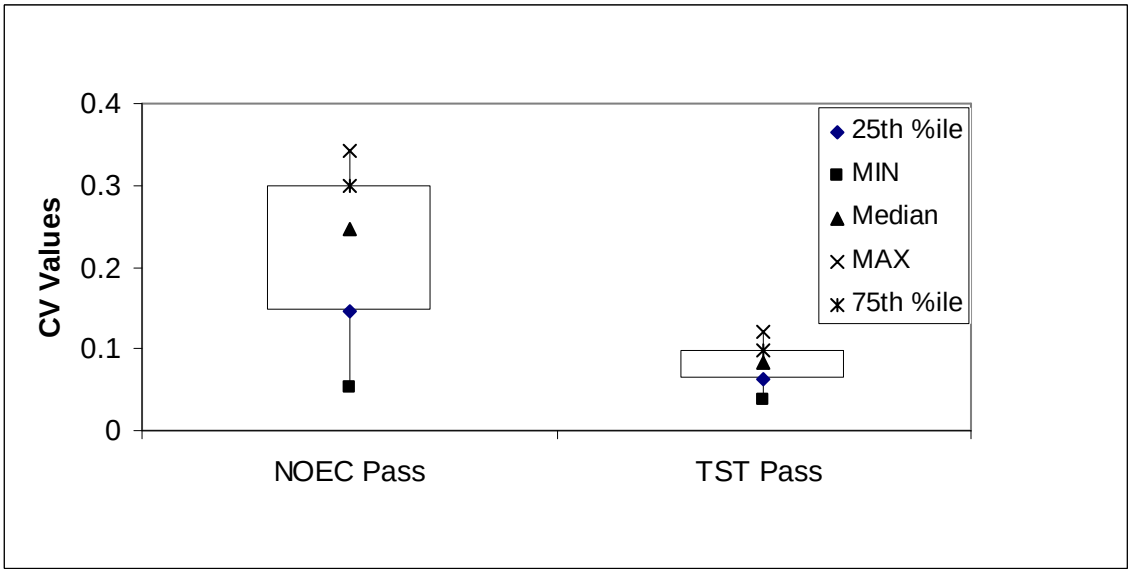

Figure 4-2. Range of CV values observed in chronic *P. promelas* ambient toxicity tests for samples that were declared to be non-toxic using the traditional t-test but toxic using the TST approach (*NOEC Pass*) and for those samples declared toxic using t-test but not the TST approach (*TST Pass*). California's SWAMP WET test data.

4.6 Reasonable Potential (RP) WET Analysis

NPDES permitting authorities conducting an RP analysis must follow 40 CFR 122.44(d)(1) to determine whether a discharge will, "cause, have the reasonable potential to cause, or contribute to" an excursion of a numeric criterion or a narrative WET criterion. Some states have state-specific WET RP approaches in their water quality control plan or other NPDES policy or guidance.

For RP calculations using the TST approach, EPA recommends that permitting authorities use all valid WET test data generated during the current permit term and any additional valid data that are submitted as part of the permit renewal application. The TST RP approach necessitates having at least a minimum of four valid WET tests to address effluent representativeness (see EPA's TSD, Chapter 3, pg. 57, under Step 2 in the section *Steps in Whole Effluent Characterization Process*). EPA also recommends that states request that their permittees provide the actual test endpoint responses for the control (i.e., control mean) and IWC concentration (i.e., IWC mean) for each WET test conducted to make it easier for permit writers to find the necessary WET test results when determining WET RP. WET test data are then analyzed according to the TST approach using the IWC and control test concentrations for all the valid WET test data available. If fewer than four valid WET test data points are available, permitting authorities should follow EPA's TSD RP approach because it addresses small WET data sets by incorporating an RP multiplying factor (see section 3.3.2 of the TSD, pg. 54) to account for effluent variability in small WET data sets. If sufficient, valid WET test data are available and the TST statistical approach indicates that the IWC is toxic in any WET test, RP has been demonstrated (40 CFR 122.44(d)(1)(i)). To address concerns regarding the "potential to cause or contribute to toxicity," an analysis of the mean effect at the IWC is also conducted to determine whether the effluent has RP, even if all test results are declared a *pass* using the TST approach (for more details, see EPA's *TST Implementation Document* EPA 833-R-10-003).

Note that using the TST approach might be to the permittee's advantage. If the permittee decides to incorporate additional test replicates for the control and the IWC when conducting the WET test, above the minimum required in the EPA WET test methods, the test power is increased. More test replicates increases test power, which means a lower probability of a false positive using the TST approach *if the effluent is truly non-toxic based on the RMDs in the TST approach*. Thus, using the TST approach, a permittee has a greater ability to *prove the negative* (i.e., its effluent does not have RP).

In those cases where the WET RP outcome is *yes*, a WET limit is expressed in the permit. In situations where the RP outcome is *no*, WET monitoring requirements should still be incorporated in the permit. A *fail* test result during monitoring could trigger additional steps if described in the permit. In either of those situations, if toxicity is demonstrated, states should specify an approach to address toxicity in the permit. This often includes initially accelerated toxicity tests (i.e., increased frequency of testing) and permit requirements to perform a toxicity reduction evaluation.

4.7 NPDES WET Permit Limits

Using the TST approach, WET NPDES permit limits would be expressed as *no significant toxicity of the effluent at the IWC using the TST analysis approach*. A test result of *Pass* is when

the calculated *t* value is greater than *the critical t value*. A test result of *Fail* is when the calculated *t* value is less than *the critical t value*.

Beyond assessing WET data for the NPDES Program, WET tests are used to assess toxicity of receiving water (watershed assessment for CWA section 303(d) determinations) and stormwater samples. Often as a first assessment of receiving or stormwater toxicity, researchers test a control and a single concentration (e.g., 100 percent receiving water or stormwater). In such cases, the TST approach can be used in the same way a t-test is used. Such analysis is used to determine whether organism response in a specified ambient concentration is significantly different than the control organism response.

5.0 CONCLUSIONS

Results of this project indicate that the TST is a viable additional option for analyzing valid acute and chronic WET test data. Given the RMDs and test-method specific alpha values specified in the TST approach, TST provides a transparent methodology for demonstrating whether an effluent truly is acceptable under the NPDES WET Program. The advantage of the TST approach is that it provides a structure in which it is easier to express, understand, and implement regulatory management goals. The alpha values identified in this project build on existing statistical information (such as data sources and analysis examining ability to detect toxic effects) on WET previously published by EPA, including *Understanding and Accounting for Method Variability in WET Applications Under the NPDES Program* (USEPA 2000).

More than 2,000 valid WET test results and thousands of simulations were conducted to develop the technical basis for the TST approach. This approach builds on the strengths of the traditional hypothesis testing approach, including using robust statistical analyses to determine whether an effluent is toxic (i.e., Welch's t-test), as well as published EPA documents regarding WET analysis and interpretation and the statistical literature. The TST approach yields a rigorous statistical interpretation of valid WET data by incorporating the transparent RMDs, established alpha and beta error rates, and thereby test power. Because this approach incorporates statistical test power, using TST will result in greater confidence in WET regulatory decisions. Additional benefits of using TST in WET analysis include the following:

- It provides a positive incentive for the permittee to generate high quality WET data to the permitting authority.
- It provides the ability to analyze a two-concentration test design (e.g., IWC versus control; stormwater and watershed assessments) using a streamlined statistical analysis flowchart. It is applicable to both NPDES WET permitting and section 303(d) watershed assessment programs.

In summary, the TST approach provides another option for permitting authorities and permittees to use in analyzing valid WET test data. The TST provides a positive incentive to generate high quality WET data to make informed decisions regarding NPDES WET RP and permit compliance determinations. By using TST, permitting authorities will be better able to identify toxic or non-toxic samples.

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APPENDIX A

RATIONALE FOR USING WELCH'S T-TEST IN TST ANALYSIS OF WET DATA FOR TWO-SAMPLE COMPARISONS

APPENDIX A

RATIONALE FOR USING WELCH'S T-TEST IN TST ANALYSIS OF WET DATA FOR TWO-SAMPLE COMPARISONS

This appendix demonstrates that the Welch modification of the t-test is suitable for WET test data and applicable to the TST approach. It also provides the evaluation and justification for certain WET test data that do not strictly adhere to the assumptions of the Welch t-test.

The Welch t-test accounts for different variances in two groups and assumes data are normally distributed (Welch 1938, 1947; Moser et al. 1989; Coombs et al. 1996; Zar 1996). For non-normal data that have skewed, long-tailed distributions, the Welch's t-test is known to have poor coverage (Zimmerman 2006). (By poor coverage, EPA means that the realized error rate, alpha, under the null hypothesis, is greater than the intended, nominal value of alpha). It is demonstrated below that WET data to which the TST will be applied typically have moderately unequal variances in the control and the IWC. That fact motivates use of the Welch t-test rather than the t-test (which assumes equal variances). It is also demonstrated that WET test data are typically non-normal but in a way that does not substantially compromise coverage of the Welch test—the data are leptokurtic and typically held within some range by the test design of the EPA WET test methods. Such data are known to have little effect on coverage for the Welch t-test (Zimmerman 2006; Zar 1996).

So as not to rely on previous literature alone, simulations were conducted to demonstrate that the Welch t-test applied to the TST is suitable for WET test data. Simulated data were generated, having variances and non-normal distributions similar to WET test data for control and IWC groups. It is demonstrated that (a) moderately unequal variances (similar to WET data) have little effect on coverage of the t-test or Welch t-test (for normally-distributed data), and (b) for non-normally distributed data (similar in distribution to WET data) representing control and IWC groups, the TST using the Welch t-test has close to nominal coverage, on the basis of simulations with up to a nine-fold difference in variance between IWC and control (a relatively high difference in variances on the basis of observed WET test data).

Therefore, published studies provide ample evidence, the analysis of WET data and simulations described here, that the Welch t-test can be applied with confidence using the TST approach.

Characterization of WET Data

Because various WET test methods have a different experimental design, and thus could represent different distribution functions, a range of WET test methods (six) was examined to determine the frequency and magnitude of unequal variances between control and IWC as well as the frequency and type of non-normality in these methods. In addition, standard data transformations were used for tests when data were non-normal to see whether transformed data would meet assumptions of normality.

Unequal Variances

Standard F-tests ($p = 0.01$) were conducted for each valid WET test (IWC and control) to determine whether variances were unequal. Some WET test methods and endpoints demonstrated a higher frequency of unequal variances than other test methods (Table A-1).

Table A-1. Number (and percent) of tests with non-normal distribution and unequal variances for different types of WET tests, as well as the effect of data transformation on distribution, including skew and kurtosis

Test name	Number of tests	Data transformation	# (%) of non-normal tests ($p < 0.01$)	# (%) tests failing f-test for unequal variances ($p \leq 0.01$)	Range of skewness statistic for non-normal tests	# (%) tests failing D'Agostino test for skewness ($p \leq 0.01$)	Range of kurtosis statistic for non-normal tests	# (%) tests failing Anscombe test for kurtosis ($p \leq 0.01$)
C. dubia reproduction	1,382	Raw	285 (20.6)	390 (28.2)	-1.529 – -0.26	33 (2.4)	3.821 – 6.571	159 (11.5)
		Sqrt trans	418 (30.2)	545 (39.4)	-1.790 – -0.385	89 (6.4)	4.013 – 7.45	268 (19.4)
		Log +1	525 (37.9)	630 (45.6)	-2.058 – -0.564	143 (10.3)	4.06 – 8.43	343 (24.9)
Fish growth	108	Raw	2 (1.9)	18 (16.7)	-1.253 – 1.250	0 (0)	3.261 – 4.213	0 (0)
Mysid growth	907	Raw	10 (1.1)	37 (4.0)	-0.423 – 1.443	1 (0.1)	2.52 – 4.912	7 (0.77)
Kelp growth	100	Raw	9 (9.0)	22 (22)	-1.478 – 1.548	0 (0)	4.025 – 5.456	6 (6)
		Log+1	8 (8.0)	30 (30)	-1.571 – 1.234	0 (0)	4.25 – 6.080	8 (8)
		sqrt	9 (9.0)	29 (29)	-1.625 – 1.381	0 (0)	4.238 – 6.068	8 (8)
Kelp germination	100	Raw	3 (3.0)	15 (15)	-0.9 – 1.281	0 (0)	3.465 – 4.697	3 (3)
		arcsin(sqrt)	1 (1.0)	9 (9)	-0.872 – 1.04	0 (0)	3.465 – 4.698	0 (0)
Fish survival	108	percent	44 (40.7)	61 (56.5)	-1.633 – 0.654	0 (0)	2 – 4.67	3 (2.8)
		arcsin(sqrt)	42 (38.9)	61 (56.5)	-1.633 – 0	0 (0)	2 – 4.67	3 (2.8)

For example, over half of the *P. promelas* (fish) acute survival tests had unequal variances. That result is expected because control acute survival typically has little or no variance (i.e., all control replicates display 100 percent survival). *Ceriodaphnia* reproduction had the next highest frequency of tests with unequal variances (28.2 percent). The giant kelp growth or germination, and *P. promelas* (fish) chronic growth WET endpoints each had a lower frequency of tests with unequal variances (15–22 percent) while the mysid growth endpoint had the lowest frequency of unequal variances of the six test endpoints evaluated (4 percent). Using the *Ceriodaphnia* test method as an example of a WET method having a higher frequency of heterogeneous variances, the variance ratio between IWC and control was generally < 9:1 (95^{th} percentile ratio) with a median variance ratio of 2.5. Examination of data using other growth/reproduction methods indicates that most tests have a variance ratio < 10:1 (95^{th} percentile) and median variance ratio < 3.0. Percent data (germination) are subject to higher variance ratios (20~30:1); however, the fish acute test method has a variance ratio generally < 6.2:1 (95^{th} percentile).

Non-Normality

Shapiro's normality test was used to evaluate if WET test data were normally distributed. A measure of skewness was then used and Pearson's measure of kurtosis (R moments package) to examine if skewness or kurtosis or both are the major sources of non-normality. The critical values of those moments for a normal distribution are shown in Table A-2. A skewness measure significantly less than 0 indicates that the sample comes from a population that is skewed to the left, and a skewness measure significantly larger than 0 indicates that the distribution is skewed to the right. A kurtosis measure significantly larger than the median value (50^{th} percentile) for a given test design in Table A-2 indicates an underlying leptokurtic distribution. EPA also used the D'Agostino test of skewness (D'Agostino 1970) and Anscombe–Glynn test of kurtosis (Anscombe and Glynn 1983) for hypothesis testing.

Table A-2. Distribution of critical skewness and kurtosis ranges for different sample size (N) based on 1,000,000 simulation runs. N = 20 corresponds to *C. dubia* reproduction test (10 replicates in IWC and control); N = 16 corresponds to the Mysid chronic test (8 replicates per treatment); N = 10 corresponds to the two giant kelp chronic test endpoints (5 replicates per treatment); N = 8 corresponds to fathead minnow acute and chronic tests (four replicates per treatment)

N	Statistic	Percentiles						
		1%	5%	10%	50%	90%	95%	99%
20	Skewness	-1.152	-0.771	-0.587	0	0.588	0.772	1.155
	Kurtosis	1.645	1.831	1.951	2.551	3.667	4.151	5.361
16	Skewness	-1.244	-0.834	-0.635	0	0.635	0.833	1.247
	Kurtosis	1.562	1.746	1.866	2.477	3.629	4.126	5.351
10	Skewness	-1.407	-0.956	-0.729	0	0.726	0.953	1.404
	Kurtosis	1.387	1.563	1.679	2.289	3.463	3.940	4.972
8	Skewness	-1.453	-0.998	-0.766	0	0.766	0.997	1.450
	Kurtosis	1.318	1.470	1.583	2.173	3.319	3.731	4.567

The number of tests failing the hypothesis tests at 1 percent probability is reported in Table A-1. About 21 percent of the *Ceriodaphnia* reproduction tests (285 out of 1,382 cases) failed Shapiro's normality test (Table A-1). Both square root transformation and logarithm

transformation did not correct the non-normal distribution problem and instead increased the total number of tests failing the normality test (Table A-1). The D'Agostino test of skewness indicated that 33 tests (< 3 percent) were highly skewed. A test of kurtosis found 11 percent of tests (160) had significantly leptokurtic distribution (Table A-1). Apparently, most of the *Ceriodaphnia* test data failed the normality test because of kurtosis (leptokurtic distribution) and that occasional asymmetric distribution was mostly from outliers (Figure A-1). In general, most WET test growth data (i.e., *Pimephales promelas* growth, mysid growth, or kelp growth) were normally distributed. Both fish and mysid growth data exhibited non-normal distribution in only a very few cases (< 2 percent) and those were generally related to leptokurtic distributions that were short-tailed. Almost half of the acute fish survival tests had non-normally distributed data. Zero variance in many tests for either the control (34 cases) or IWC (26 cases) were the main cause of failing the normality test. Non-normality in acute fish survival data was because of leptokurtic data distribution (Table A-1).

The above analyses indicate that WET data in general do not have the distribution characteristics indicative of when Welch's t-test would be inappropriate (long-tail, highly skewed distribution).

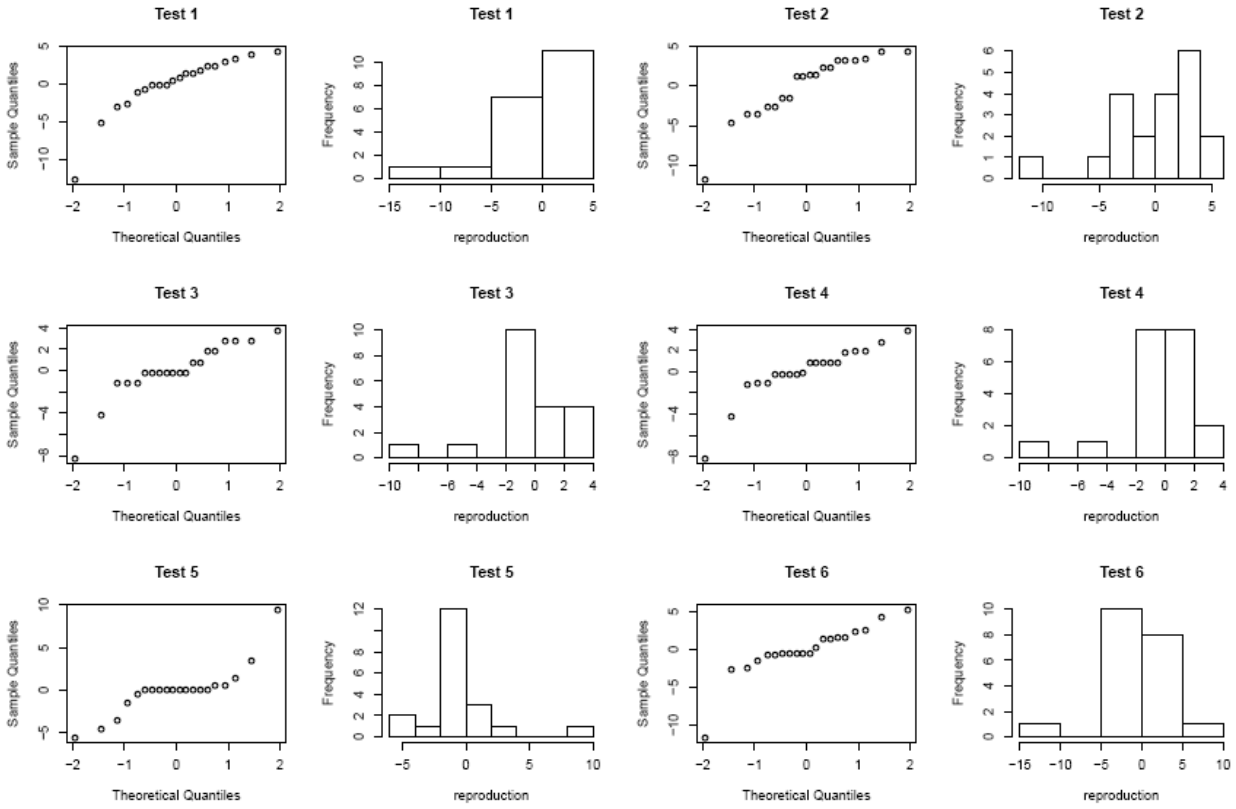

Figure A-1. Probability plots and histograms of examples of *Ceriodaphinia* chronic reproduction test data showing non-normal distribution and especially leptokurtic distribution.

Simulations

Unequal Variances

Various simulations were conducted using the chronic *Ceriodaphnia* test method as an example, to examine alpha error rate using either the traditional hypothesis t-test or Welch's t-test with data having different relationships between control and effluent variance. From analyses of more than 2,000 WET tests presented in Table A-1, a variance ratio (IWC/control) of 9:1 (95th percentile of variance ratio) is a reasonable upper limit. Therefore, simulation scenarios examined included (1) equal variances and no mean difference between control and effluent; (2) IWC with 9 times the control variance and no mean difference; (3) equal variance and a 25 percent mean effect of the IWC; and (4) IWC with 9 times the control variance and a 25 percent mean effect. Equal sample size (N = 10 using *Ceriodaphnia* chronic test method as the example) was assumed for both control and treatment group which is most often the case in WET analyses. Results are shown in Table A-3.

Table A-3. Results of Monte Carlo simulations evaluating alpha error rate using either the traditional t-test or Welch's t-test with data having different relationships between control and effluent variances. S_c^2 = control variance, S_t^2 = IWC variance, μ_c = control mean, and μ_t = IWC mean. Results are based on 1,000,000 simulation runs per scenario.

		$\mu_c = \mu_t$		$\mu_t = 0.75\ \mu_c$	
	Alpha	**T-test**	**Welch t-test**	**T-test**	**Welch t-test**
$S_c^2 = S_t^2$	0.010	0.0098	0.0093	0.0099	0.0095
	0.050	0.0498	0.0490	0.0497	0.0491
	0.100	0.0996	0.0988	0.1000	0.0992
	0.150	0.1493	0.1486	0.1501	0.1506
	0.200	0.1996	0.1991	0.2000	0.1997
	0.250	0.2498	0.2493	0.2502	0.2498
$S_c^2 = S_t^2/9$	0.010	0.0132	0.0105	0.0204	0.0103
	0.050	0.0550	0.0503	0.0725	0.0503
	0.100	0.1050	0.1001	0.1269	0.1002
	0.150	0.1543	0.1501	0.1774	0.1499
	0.200	0.2037	0.2003	0.2260	0.1999
	0.250	0.2526	0.2499	0.2732	0.2499

When there are equal variances and the true difference is equal to 0, the observed error rates from both the traditional t-test and Welch's t-test are very close to the expected error rates. When control and treatment groups have unequal variance, (effluent variance = 9 times the control variance), the traditional t-test has a slightly higher Type I error rate, but Welch's t-test has a Type I error rate similar to the expected value. When the true response at the IWC is 0.75 × control mean, and both populations have equal variances, alpha error rates are very similar to expected using both the traditional t-test and Welch's t-test. When the true response at the IWC is 0.75 × control mean and population variances are not equal (i.e., effluent variance is 9 times

the control variance), the error rates are about 2–3 percent higher than expected using the traditional t-test but are similar to expected alphas using Welch's t-test.

While the specific results pertain to the *Ceriodaphnia* reproduction endpoint, the general conclusions of this analysis would apply to all WET methods and endpoints. Such results confirm that Welch's t-test has better coverage than the traditional t-test using the TST approach when variances are unequal.

Non-Normality

The objective of the simulations was to confirm that the alpha error rate is relatively stable against deviations from non-normal distribution when variances are unequal as well for both the traditional hypothesis test and Welch's t-test.

EPA examined the distribution of control and effluent reproduction data from 281 *C. dubia* multiple concentration tests (Figure A-2). While most tests indicate that control reproduction follows a normal distribution (mean = 24.5, standard deviation = 5.56), effluent data tend to deviate from a normal distribution: effluents with low toxicity have less skewed data, while effluents with data that have high toxicity are more likely to deviate from normal distribution. To address this observation, two populations were simulated on the basis of the shape of the frequency distribution in the highest effluent concentration in each *C. dubia* test (Figure A-3). The first simulated effluent population had a mean = 25 (equal to the population mean for the control group) and a standard deviation = 7.7, while the second one had a population mean of $b \times 25$ (where $b = 0.75$ for chronic test methods), resulting in an effluent mean of 18.75. The variance of those two effluent populations was the same. Random samples taken from these two populations were used to compare with the control population data (mean = 25, standard deviation = 5.56).

Simulation results (Table A-4) indicate that when the two populations had the same mean but had a different distribution shape as compared to a normal distribution (control population), the alpha error rate using the traditional t-test was about 1 percent higher than expected. Welch's modified t-test slightly corrected the error rate (Table A-4). When the true population mean difference between control and effluent is 25 percent of the control mean and when the effluent population is not normally distributed, the alpha error rate is almost identical to the expected value using traditional t-test (Table A-4). Welch's t-test resulted in a decrease in the nominal alpha error rate by 2–3 percent using the TST approach. That is, when data are extremely non-normal (for WET test data) and variances are heterogeneous between control and effluent, Welch's t-test is less likely to reject the null hypothesis and slightly more likely to declare a sample toxic than expected (i.e., the analysis will be more conservative). As data approach a normal distribution, α error rates using Welch's t-test will be closer to nominal values.

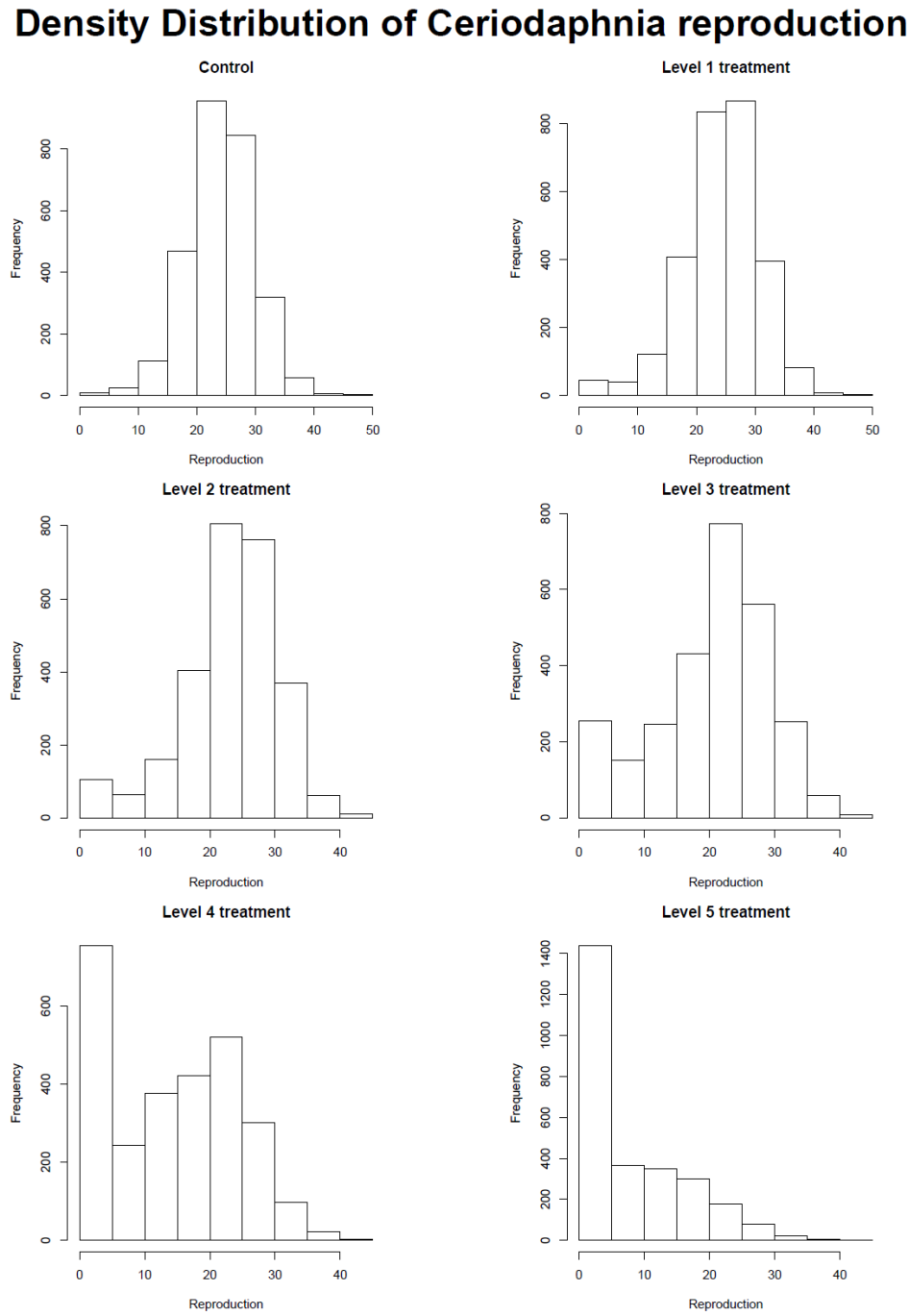

Figure A-2. Histogram of observed *Ceriodaphnia* reproduction at different level of effluent concentrations based on 281 multiple concentration tests.

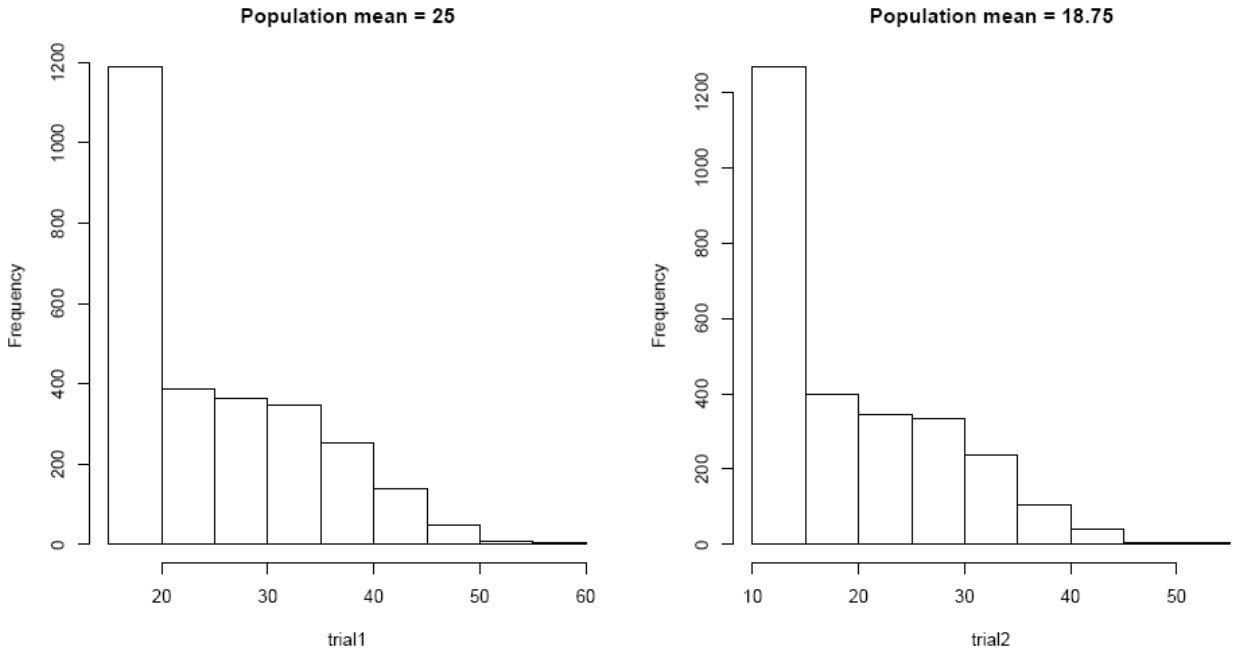

Figure A-3. Simulated frequency distributions of Ceriodaphnia reproduction data with two populations having non-normal data and different means. Both populations have a standard deviation of 7.7.

Table A-4. Results of Monte Carlo simulation analyses (100,000 simulations per scenario) indicating alpha error rates based on comparisons between two non-normally distributed populations and a normal distribution (control population, mean = 25, standard deviation = 5.65). The population means are 25 and 18.75, respectively, and the standard deviation is 7.7 in both populations.

Alpha	Welch's (μ = 25)	Traditional t (μ = 25)	TST t-test (μ = 18.75, b = 0.75)	TST Welch's (μ = 18.75, b = 0.75)
0.05	0.053	0.059	0.043	0.031
0.10	0.104	0.108	0.090	0.074
0.15	0.151	0.155	0.140	0.122
0.20	0.199	0.203	0.191	0.173

Although the simulated population does not necessarily represent the true population of effluent groups, EPA's examination of sample distribution indicates that effluent populations with low toxicity are less likely to deviate from normal distribution. The simulation also indicates that the alpha error rate using Welch's t-test under severely non-normal distributions and heterogeneous variances is less than the expected/critical values. That is, Welch's t-test is more conservative when toxicity is high (a desirable attribute for WET analysis) than when effluent toxicity is low. When effluent toxicity is low, results of analyses using *Ceriodaphnia* reproduction WET test data indicate that the effluent data are less likely to be non-normally distributed, and the observed alpha error rate approaches the expected error rate. On the basis of the foregoing results, the type of non-normal distribution observed in WET tests should not affect the overall performance of simulation analyses used to derive test method alpha values for the TST approach.

Rationale/Conclusions

When population variances are not equal or test samples are non-normally distributed (or both), concerns could be raised in using the two concentration t-test or the bioequivalence t-test (Erickson and McDonald 1995) because statistical assumptions might not be met. EPA WET test methods specify that if the data fail Shapiro-Wilks's normality test or Bartlett's homoscedasticity test (or both), a non-parametric test such as Wilcoxon Rank sum test should be used in such situations. Extension of such nonparametric tests to TST is, however, complicated because the null hypothesis for those tests is that results from control and effluent are from same population. This is stated as the null hypothesis of no difference among treatments. Because an effect size $1–(b \times \mu_0)$ is specified in the TST approach that is related to the control population mean, a non-parametric equivalent to a t-test approach using a bioequivalence formulation (such as with the TST approach), has been difficult to demonstrate (Zimmerman and Zumbo 1993; Manly 2004).

Data compiled from more than 2,000 valid WET tests in this project confirmed that the type of distributions exhibited by most test data do not seriously compromise the use of a t-test. The data can be dealt with appropriately using Welch's t-tests for unequal variances, as shown in simulation analyses. Use of Welch's t-test for TST analysis is supported on the basis of analysis of actual WET test data, which indicate that the majority of WET test data are normally distributed or have a leptokurtic distribution with short tails such that the use of Welch's t-test produces Type I error rates very close to expected error rates. Statistical literature indicates that actual power of the t-test (and by extension Welch's t-test) is *greater* when populations are leptokurtic, especially for small sample sizes (Zar 1996).

WET test data are biologically expected to have short-tailed distributions supporting the use of Welch's t-test because of the test method's required test acceptability criteria and test termination times, which constrain the range of endpoint responses encountered. For example, a chronic *Ceriodaphnia dubia* test must have 80 percent or greater survival and an average of 15 or more young per surviving female in the control for the test to meet the required test acceptability criteria (i.e., a valid test). Additionally, test termination is prescribed in the method as the time at which at least 60 percent or more of the surviving control females generate at least three broods, which can be 6–8 days (maximum is 8 days), also a test requirement. That results in a lower distribution bound (e.g., reproduction responses in controls start at 15). In addition, the upper part of the distribution cannot go to infinity, even if populations were to survive and reproduce beyond the prescribed test requirements because of biological constraints. Similar test method and biological constraints apply to all other WET test endpoints (e.g., growth, survival).

Furthermore, Welch's t-test is robust to non-normal distributions when the underlying distribution is symmetric and skewness is low, especially with sample sizes > 10 (Tiku 1971; Lee and D'Agostino 1976; Tiku and Akkaya 2004). For the West Coast WET methods examined and the *Ceriodaphnia* and Mysid chronic WET method evaluated, those conditions are met. Therefore, at least for those WET methods and others with similarly large sample sizes, Welch's t-test should not result in a substantial underestimation of the Type I error rate.

In addition, the Type I error rate using TST for several WET methods is set ≥ 0.05. The higher α levels include WET test methods that have smaller sample sizes such as the fathead minnow acute test. For those methods, the slight overestimation of the nominal Type I error rate that can occur using Welch's t-test when WET test data are not normally distributed is insignificant given

the higher nominal α levels established. For the West Coast WET test methods that have α levels set at 0.05, effect size examined in those test methods is large and, in many cases, data are normally distributed even without data transformation (e.g., giant kelp germination and tube-length endpoints, Table A-1).

The observed sample distribution from 281 *C. dubia* multiple concentration tests indicates that test populations at low effluent concentrations are less likely to deviate from normal distribution. A similar trend is expected for other WET endpoints such as growth. The simulation based on the distribution shape of the high effluent concentration population also indicates that the alpha error rate using Welch's t-test is less than expected. That is, Welch's t-test is more conservative when toxicity is high. Therefore, the type of non-normal distribution observed in WET tests should not negatively affect the outcome of TST analyses.

Analyses used to develop the TST analysis approach indicate that data transformation (log or square root) does not help the non-normality issue for WET test data (Table A-1). That is usually because of the leptokurtic distribution observed rather than because of skewness of data (Table A-2). Therefore, data transformation before TST analysis is not recommended except for percent data, which should be arcsine square root transformed before TST analysis (consistent with current EPA analysis recommendations). This precaution is suggested because percent data (especially acute percent survival) is most prone to non-normality.

In conclusion, given the leptokurtic and short-tailed distribution of most WET test data, as well as the other factors noted above, Welch's t-test is appropriate to use for one-tailed, two-sample comparisons using TST. Furthermore, because Welch's t-test performs as effectively as the t-test in terms of Type I error when data are normally distributed and variances are equal (Moser et al. 1989; Coombs et al. 1996), Welch's t-test should be used for all WET test data analysis using TST. Furthermore, many researchers have shown that the combination of using a preliminary variance test (e.g., F-test) plus a t-test does not control Type I error rates as well as simply always performing an unequal variance t-test such as Welch's t-test (Gans 1992; Moser and Stevens 1992). That is one reason why it is generally unwise to decide whether to perform one statistical test on the basis of the outcome of another (Smith 1936; Markowski and Markowski 1990; Zimmerman 2004).

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APPENDIX B

STEP-BY-STEP PROCEDURES FOR ANALYZING VALID WET DATA USING THE TST APPROACH

APPENDIX B

STEP-BY-STEP PROCEDURES FOR ANALYZING VALID WET DATA USING THE TST APPROACH

The following is a step-by-step guide for using the TST approach to analyze valid WET data for the NPDES WET Program. This guide is applicable for a two-concentration data analysis of an IWC or a receiving water concentration compared to a control concentration. For further information regarding conducting WET tests and proper quality assurance/quality control needed, see the EPA WET method manuals. As you proceed through this guide, refer to the flowchart shown in Figure B-1 of this appendix.

Step 1: Conduct WET test following procedures in the appropriate EPA WET test method manual. This includes following all test requirements specified in the method (USEPA 1995 for chronic West Coast marine methods, USEPA 2002a for chronic freshwater WET methods, USEPA 2002b for chronic East Coast marine WET methods, and USEPA 2002c for acute freshwater and marine methods).

Step 2: For each test endpoint specified in the WET test method manual (e.g., survival and reproduction for the *Ceriodaphnia* chronic WET test method), follow Steps 3–7 below. Note that the guide refers to an effluent concentration tested, which is assumed to be the IWC as specified in the permit or a receiving water concentration for ambient testing. For example, if no mixing zone is allocated, the IWC is 100 percent effluent.

Note: If there is no variance (i.e., zero variance) in the endpoint in both concentrations being compared (i.e., all replicates in each concentration have the same exact response), then skip the remaining steps in the flowchart and do the following. Compute the percent difference between the control and the other concentration (e.g., IWC) and compare the percent difference against the RMD values of 25% for chronic and 20% for acute endpoints. Percent mean effect is calculated as:

$$\%\ \text{Effect at IWC} = \frac{\text{Mean Control Response} - \text{Mean Response at IWC}}{\text{Mean Control Response}} \times 100$$

If the percent mean response is $\geq$ the RMD, the sample is declared toxic and the test is "*Fail*". If the percent mean response is < the RMD, the sample is declared non-toxic and the test is "*Pass*".

Step 3: For data consisting of proportions from a binomial (response/no response; live/dead) response variable, the variance within the *i*th treatment is proportional to $P_i (1 - P_i)$, where P_i is the expected proportion for the treatment. That clearly violates the homogeneity of variance assumption required by parametric procedures such as the TST procedure because the existence of a treatment effect implies different values of P_i for different treatments, *i*. Also, when the observed proportions are based on small samples, or when P_i is close to zero or one, the normality assumption might be invalid. The arcsine square root (arcsine $\sqrt{P}$) transformation is used for such data to stabilize the variance and satisfy the normality requirement. The square root of percent data (e.g., percent survival, percent fertilization), expressed as a decimal fraction (where 1.00 = 100 percent) for each treatment, is first calculated. The square root value is then

arcsine transformed before analysis in Step 4. Note: Excel and most statistical software packages can calculate arcsine values.

Step 4: Conduct Welch's t-test (Zar 1996) using Equation 1:

Equation 1

$$t = \frac{\overline{Y_t} - b \times \overline{Y_c}}{\sqrt{\frac{S_t^{\,2}}{n_t} + \frac{b^2 S_c^{\,2}}{n_c}}}$$

where

$\overline{Y_c}$ = Mean for the control

$\overline{Y_t}$ = Mean for the IWC

$s_c^{\,2}$ = Estimate of the variance for the control

$s_t^{\,2}$ = Estimate of the variance for the IWC

n_c = Number of replicates for the control

n_t = Number of replicates for the IWC

b = 0.75 for chronic tests; 0.80 for acute tests

Note on the use of Welch's t-test: Welch's t-test is appropriate to use when there are an unequal number of replicates between control and the IWC. When sample sizes of the control and treatment are the same (i.e., $n_t = n_{c)}$, Welch's t-test is equivalent to the usual Student's t-test (Zar 1996).

Step 5: Adjust the degrees of freedom (df) using Equation 2:

Equation 2

$$\upsilon = \frac{\left(\frac{S_t^{\,2}}{n_t} + \frac{b^2 S_c^{\,2}}{n_c}\right)^2}{\frac{\left(\frac{S_t^{\,2}}{n_t}\right)^2}{n_t - 1} + \frac{\left(\frac{b^2 S_c^{\,2}}{n_c}\right)^2}{n_c - 1}}$$

Using Welch's t-test, df is the value obtained for *v* in Equation 2 above. Because *v is* most likely a non-integer, round *v* to the next smallest integer, and that number is the df.

Step 6: Using the calculated t value from Step 4, compare that *t* value with the critical *t* value table in Appendix C using the test method-specific alpha values shown in Table 4-1. To obtain the correct *t* value, look across the table for the alpha value that corresponds to the WET test method (for the appropriate alpha value, see Table 4-1 of this document) and then look down the table for the appropriate df.

Step 7: If the calculated *t* value is less than the critical *t* value, the IWC is declared toxic, and the test result is *Fail*. If the calculated *t* value is greater than the critical *t* value, the IWC is not declared toxic and the test result is *Pass*.

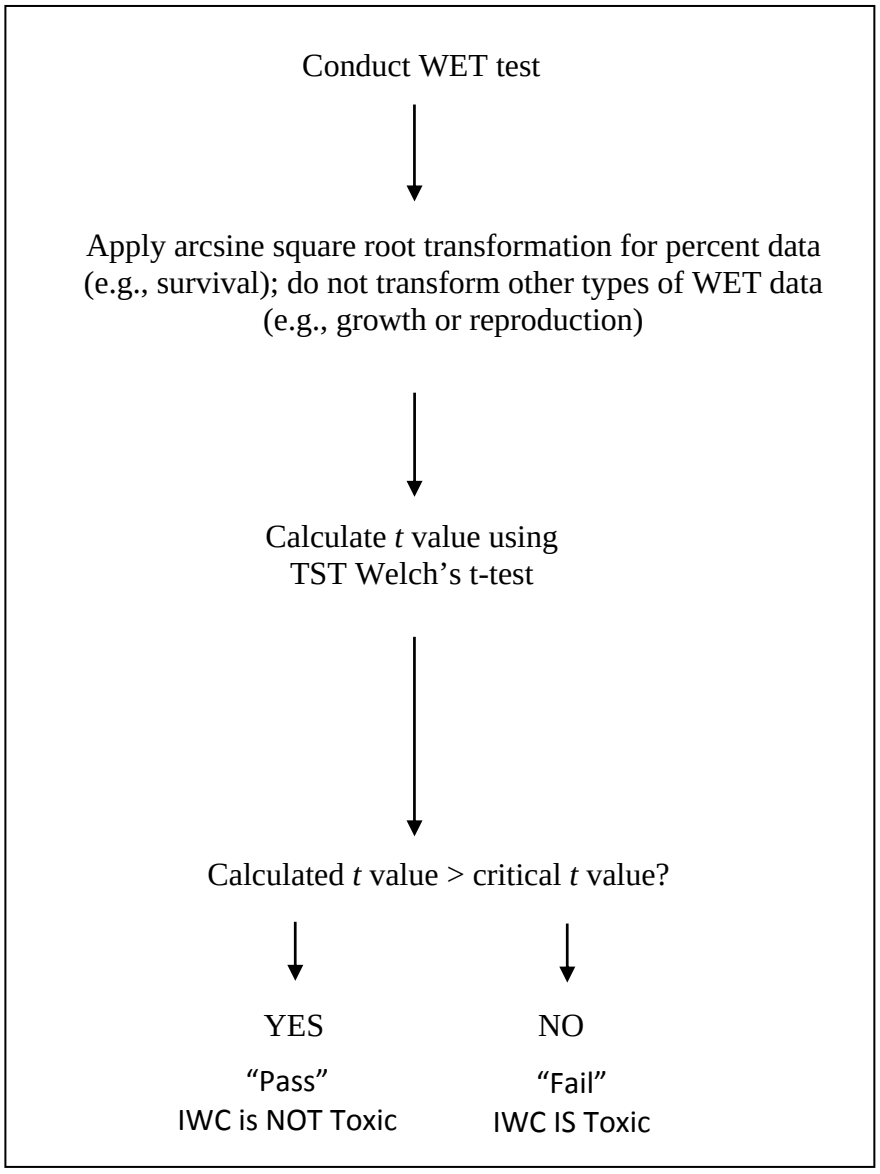

Figure B-1. Statistical flowchart for analyzing valid WET data using the TST approach for control and the IWC, receiving water, or stormwater.

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APPENDIX C

CRITICAL *t* VALUES FOR THE TEST OF SIGNIFICANT TOXICITY APPROACH

Table C-1. Critical values of the *t* distribution. One tail probability is assumed.

	Alpha				
Degrees of freedom	**0.25**	**0.20**	**0.15**	**0.10**	**0.05**
1	1	1.3764	1.9626	3.0777	6.3138
2	0.8165	1.0607	1.3862	1.8856	2.92
3	0.7649	0.9785	1.2498	1.6377	2.3534
4	0.7407	0.941	1.1896	1.5332	2.1318
5	0.7267	0.9195	1.1558	1.4759	2.015
6	0.7176	0.9057	1.1342	1.4398	1.9432
7	0.7111	0.896	1.1192	1.4149	1.8946
8	0.7064	0.8889	1.1081	1.3968	1.8595
9	0.7027	0.8834	1.0997	1.383	1.8331
10	0.6998	0.8791	1.0931	1.3722	1.8125
11	0.6974	0.8755	1.0877	1.3634	1.7959
12	0.6955	0.8726	1.0832	1.3562	1.7823
13	0.6938	0.8702	1.0795	1.3502	1.7709
14	0.6924	0.8681	1.0763	1.345	1.7613
15	0.6912	0.8662	1.0735	1.3406	1.7531
16	0.6901	0.8647	1.0711	1.3368	1.7459
17	0.6892	0.8633	1.069	1.3334	1.7396
18	0.6884	0.862	1.0672	1.3304	1.7341
19	0.6876	0.861	1.0655	1.3277	1.7291
20	0.687	0.86	1.064	1.3253	1.7247
21	0.6864	0.8591	1.0627	1.3232	1.7207
22	0.6858	0.8583	1.0614	1.3212	1.7171
23	0.6853	0.8575	1.0603	1.3195	1.7139
24	0.6849	0.8569	1.0593	1.3178	1.7109
25	0.6844	0.8562	1.0584	1.3163	1.7081
26	0.684	0.8557	1.0575	1.315	1.7056
27	0.6837	0.8551	1.0567	1.3137	1.7033
28	0.6834	0.8546	1.056	1.3125	1.7011
29	0.683	0.8542	1.0553	1.3114	1.6991
30	0.6828	0.8538	1.0547	1.3104	1.6973
inf	0.6745	0.8416	1.0364	1.2816	1.6449

United States Environmental Agency
Office of Wastewater Management (4203M)
EPA 833-R-10-003 (June/2010)
http://www.epa.gov/npdes/permitbasics

National Pollutant Discharge Elimination System Test of Significant Toxicity Implementation Document

June 2010

NATIONAL POLLUTANT DISCHARGE ELIMINATION SYSTEM
TEST OF SIGNIFICANT TOXICITY
IMPLEMENTATION DOCUMENT

An Additional Whole Effluent Toxicity Statistical Approach for Analyzing Acute and Chronic Test Data

U.S. Environmental Protection Agency
Office of Wastewater Management
Water Permits Division
1200 Pennsylvania Avenue, NW
Mail Code 4203M
EPA East – Room 7135
Washington, DC 20460

June, 2010

NOTICE AND DISCLAIMER

This document provides the basis for implementing the Test of Significant Toxicity (TST) approach under the National Pollutant Discharge Elimination System (NPDES) for permitting authorities (states and Regions) and persons interested in analyzing whole effluent toxicity (WET) test data using the traditional hypothesis testing approach as part of the NPDES Program under the Clean Water Act (CWA). This document describes what the U.S. Environmental Protection Agency (EPA) believes is another statistical option to analyze valid WET test data for NPDES WET reasonable potential and permit compliance determinations. The document does not, however, substitute for the CWA, an NPDES permit, or EPA or state regulations applicable to permits or WET testing; nor is this document a permit or a regulation itself. The TST approach does not result in changes to EPA's WET test methods promulgated at Title 40 of the *Code of Federal Regulations* Part 136. The document does not and cannot impose any legally binding requirements on EPA, states, NPDES permittees, or laboratories conducting or using WET testing for permittees (or for states in evaluating ambient water quality). EPA could revise this document without public notice to reflect changes in EPA policy and guidance. Finally, mention of any trade names, products, or services is not and should not be interpreted as conveying official EPA approval, endorsement, or recommendation.

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EXECUTIVE SUMMARY

The U.S. Environmental Protection Agency (EPA or the Agency) has developed a new statistical approach that assesses the whole effluent toxicity (WET) measurement of wastewater effects on specific test organisms' ability to survive, grow, and reproduce. The new approach is called the Test of Significant Toxicity (TST) and is a statistical method that uses hypothesis testing techniques based on research and peer-reviewed publications. The TST approach examines whether an effluent, at the critical concentration (e.g., in-stream waste concentration or IWC, as recommended in EPA's Technical Support Document (TSD) (USEPA 1991) and implemented under EPA's WET National Pollutant Discharge Elimination System (NPDES) permits program and the control within a WET test differ by an unacceptable amount (the amount that would have a measured detrimental effect on the ability of aquatic organisms to thrive and survive). EPA Regions and their NPDES states can still use EPA's TSD approaches. The TST approach is another statistical option to analyze valid WET test data.

Since the inception of EPA's NPDES WET Program in the mid 1980s, the Agency has striven to advance and improve its application and implementation under the NPDES Program. The TST approach explicitly incorporates test power (the ability to correctly classify the effluent as non-toxic, also see reference in the glossary under power) and provides a positive incentive to generate valid, high quality WET data to make informed decisions regarding NPDES WET reasonable potential (RP) and permit compliance determinations. Once the WET test has been conducted (using multiple effluent concentrations and other requirements as specified in the EPA WET test methods), the TST approach can be used to analyze the WET test results to assess whether the effluent discharge is toxic at the critical concentration. Performing the EPA WET test where the minimum five required test concentrations (pursuant to the EPA WET test methods) can establish a concentration-response curve. The TST approach is designed to be used for a two concentration data analysis of the IWC or a receiving water concentration (RWC) compared to a control concentration. Using the TST approach, permitting authorities will have more confidence when making NPDES determinations as to whether a permittee's effluent discharge is toxic or non-toxic. Use of the TST approach does not result in any changes to EPA's WET test methods; however, a facility might desire to modify its future WET tests by increasing the number of replicates over the minimum required (USEPA 1995, 2002a, 2002b, 2002c) by the approved EPA WET test method to increase test power, which is the probability of declaring an effluent *non-toxic* if the organism response at the IWC is truly acceptable. If WET tests have already been performed, the WET data generated cannot be modified to increase the number of test replicates because the TST analysis is done on valid WET data generated within a WET test.

The TST approach was developed on the basis of extensive analyses and detailed research. EPA used valid WET data from *more than 2,000* WET tests to develop and evaluate the TST approach. The TST approach was tested using *nine different* WET test methods comprising *twelve biological endpoints* (e.g., reproduction, growth, survival) and representing most of the different types of WET test designs currently in use. *More than one million* computer simulations were also used to select error rates achieving EPA's regulatory management decisions for the TST approach.

Background

In the NPDES Program, an effluent sample is declared toxic relative to a permitted WET limit if the no observed effect concentration (NOEC) is less than the permitted IWC using a hypothesis statistical approach. In that traditional hypothesis approach, the question being answered is, ~~"I~~s the mean response of the organisms the same in the control and at the IWC?" The hypothesis testing approach has four possible outcomes: (1) the IWC is truly toxic and is declared toxic, (2) the IWC is truly non-toxic and is declared non-toxic, (3) the IWC is truly toxic but is declared non-toxic, and (4) the IWC is truly non-toxic but is declared toxic. The latter two possible outcomes represent decision errors that can occur with any hypothesis testing approach. In the NPDES WET Program, those two types of errors can occur when test control replication is very good (i.e., test is very precise) so that a very small difference between IWC and control is declared toxic (outcome [4] above), and when test control replication is poor (i.e., the test is very imprecise) so that *even large differences in organism response between the IWC and control cannot be distinguished as statistically different, and the effluent is incorrectly classified as non-toxic (outcome [3] above)*.

Organism responses to the IWC and control are unlikely to be exactly the same. The difference might be so small that even if statistically significant, it would be considered biologically negligible. Another approach for assessing an effluent's toxicity on the basis of collected WET data might be to rephrase the question, ~~"D~~oes the mean WET test response in the control and the IWC differ by a defined biological amount?" That approach is known as the *test of bioequivalence*, which the Food and Drug Administration has successfully used to evaluate drugs, as have many researchers in other biological fields. Using the TST approach, the question is, ~~"I~~s the organism response at the IWC less than or equal to a fixed fraction of the control response (e.g., 75 percent of the control mean response)?" That fixed fraction, expressed as a decimal between 0.00 and 1.00, is termed ~~*"b"*~~ in the TST approach. Thus, the hypothesis being tested is written as follows: mean response [IWC] $\leq b \times$ mean response [control].

The TST approach requires defining what is considered toxic. For *chronic* testing (i.e., for both lethal and sublethal toxicity test endpoints) in EPA's NPDES WET Program, the *b* value in the TST analysis is set at 0.75, which means that a 25 percent effect (or more) is considered evidence of unacceptable *chronic* toxicity. IWC responses substantially less than a 25 percent effect would be interpreted to have a lower risk potential. The regulatory management decision (RMD) for *acute* WET methods is set at 0.80, which means that a 20 percent effect (or more) is considered evidence of unacceptable *acute* toxicity. The acute RMD toxicity threshold is higher than that for chronic WET methods because of the severe environmental implications of acute toxicity (lethality or organism death). For more discussion on the *b* values of 0.75 (chronic toxicity) and 0.80 (acute toxicity), see Section 2.1 of this document.

EPA's RMDs using the TST approach identify true toxicity in WET tests most of the time when it occurs, while also minimizing the probability that the IWC is declared toxic when in fact it is not. That objective requires additional RMDs regarding acceptable maximum false positive *(β or beta using a TST approach) and false negative rates (α or alpha using a TST approach)*. In the TST approach, the RMDs are defined as (1) declare a sample toxic at least 75 percent of the time (alpha, $\alpha \leq 0.25$) when there is unacceptable toxicity (20 percent effect for acute and 25 percent effect for chronic test methods), and (2) declare an effluent non-toxic no more than 5 percent

(beta, $\beta \leq 0.05$) of the time when the mean effect at the critical effluent concentration is ≤ 10 percent for both acute and chronic WET tests (including for sublethal endpoints). For more discussion on the RMDs, see Section 2.1 of this document.

On the basis of EPA's analyses, the alpha levels shown in Table ES-1 are recommended for the nine WET test methods examined using the TST approach. An important feature of the TST approach is that the false negative error rate (rate of declaring a toxic effluent to be non-toxic) is established, which, under the traditional hypothesis testing approach, had not been established by EPA previously. For more discussion on the inclusion of the beta error rate in the TST approach, see Section 1.2 of this document and Section 1.1 on the current approach in EPA's 1991 TSD. A demonstrated benefit of the TST approach is that increasing within-test replication (the test power) results in a *lower* rate of WET tests being declared toxic using the TST approach when the IWC is truly non-toxic.

Results obtained from the TST analyses using the *nine* EPA test methods should be applicable to other EPA WET methods not examined. For example, results generated under this project for the fish *Pimephales promelas* survival and growth test is extrapolated to other EPA fish survival and growth tests (e.g., *Menidia* sp., *Cyprinus variegatus*, *Atherinops affinis*) because those test methods use a similar test design (e.g., number of replicates, number of organisms tested) and measure the same endpoints.

Summary

More than 2,000 WET test results and more than one million simulations were conducted to develop the technical basis for the TST approach. The approach builds on the strengths of the traditional hypothesis testing approach, including use of robust statistical analyses and published EPA documents regarding WET data analysis and interpretation. The TST approach yields a rigorous statistical interpretation of valid WET data by incorporating transparent RMDs and established alpha and beta error rates, which can provide incentives to generate test results having greater test power. Because the approach considers statistical test power, its use will result in greater confidence in WET regulatory decisions. In addition, the TST approach provides a positive incentive for the permittee to generate valid, high quality WET data by either increasing the number of test replicates for the IWC and the control within a test and/or achieving better precision within a test through improved WET test method performance (e.g., a high level of quality assurance and quality control).

Permitting authorities should consider the practical programmatic shift from the traditional hypothesis testing approach to the TST approach by opening a dialogue with their regulated community. In addition, they might want to begin to identify what changes might be needed to assimilate the TST approach into any regulations, policy, guidance, and training in their respective NPDES WET Programs. Again, the traditional hypothesis testing approach under EPA's TSD is still considered valid as applied; however, that approach can now be advanced through the TST approach by providing new incentives to permittees to provide valid, high quality WET data.

Table ES-1. Summary of alpha (α) levels or false negative rates recommended for different WET test methods using the TST approach

EPA WET test method	*b* value	Probability of declaring a toxic effluent non-toxic False negative (α) error[a]
Chronic Freshwater and East Coast Methods		
Ceriodaphnia dubia (water flea) survival and reproduction	0.75	0.20
Pimephales promelas (fathead minnow) survival and growth	0.75	0.25
Selenastrum capricornutum (green algae) growth	0.75	0.25
Americamysis bahia (mysid shrimp) survival and growth	0.75	0.15
Arbacia punctulata (Echinoderm) fertilization	0.75	0.05
Cyprinodon variegatus (Sheepshead minnow) and *Menidia beryllina* (inland silverside) survival and growth	0.75	0.25
Chronic West Coast Marine Methods		
Dendraster excentricus and *Strongylocentrotus purpuratus* (Echinoderm) fertilization	0.75	0.05
Atherinops affinis (topsmelt) survival and growth	0.75	0.25
Haliotis rufescens (red abalone), *Crassostrea gigas (oyster), Dendraster excentricus, Strongylocentrotus purpuratus* (Echinoderm) and *Mytilus sp* (mussel) larval development methods	0.75	0.05
Macrocystis pyrifera (giant kelp) germination and germ-tube length	0.75	0.05
Acute Methods		
Pimephales promelas (fathead minnow), *Cyprinodon variegatus* (Sheepshead minnow), *Atherinops affinis* (topsmelt), *Menidia beryllina* (inland silverside) acute survival[b]	0.80	0.10
Ceriodaphnia dubia, *Daphnia magna, Daphnia pulex*, *Americamysis bahia* acute survival[b]	0.80	0.10

Notes:

a. (1) declare a sample toxic at least 75 percent of the time (alpha ≤ 0.25) when there is unacceptable toxicity (20 percent effect for acute and 25 percent effect for chronic test methods) and (2) declare an effluent non-toxic no more than 5 percent of the time (beta ≤ 0.05) when the mean effect at the critical effluent concentration is 10 percent for both acute and chronic WET tests (including sublethal endpoints). For more discussion on the RMDs, see Section 2.1 of this document.

b. Based on four replicate test design

In addition, EPA recommends the following:

- Permitting authorities should decide up front which approach (the EPA's 1991 TSD approach, the TST approach, or another scientifically defensible approach that is sufficient to meet the statutory and regulatory requirements) they will follow (including for their RP procedures) and use the selected approach consistently in all their state NPDES permits. Permitting authorities should ensure that the most environmentally protective approach is consistently used across all permits when assessing valid WET data (e.g., WET RP) for

NPDES permit requirements (e.g., WET limits, monitoring frequencies, toxicity identification evaluation/toxicity reduction evaluation) and avoid selecting the approach that underestimates the true toxicity of the permitted effluent discharge.

- Where a *small data set* exists (fewer than four valid WET tests performed and reported in the previous 5 years), permitting authorities should use the TSD approach for determining RP. With small WET data sets, the TSD's RP multiplying factor is more conservative for environmental water quality protection purposes than the TST. The TST approach is intended for larger data sets (four or more) because it does not use an RP multiplying factor.
- If WET tests have already been performed, the WET data generated cannot be modified to increase the number of test replicates within a test. The decision to increase the number of within test replicates is a decision that needs to be made before conducting the WET tests.
- Where a permittee has concerns about WET data quality, EPA recommends increasing the number of replicates in tests, even if the permitting authority has not yet adopted the TST approach.

ACRONYMS AND ABBREVIATIONS

CFR	Code of Federal Regulations
CV	coefficient of variation
CWA	Clean Water Act
DMR	discharge monitoring report
EC	effect concentration
EPA	U.S. Environmental Protection Agency
IC25	25 percent inhibition concentration
IWC	in-stream waste concentration
LC50	50 percent lethal concentration
LOEC	lowest observed effect concentration
MDL	maximum daily limit
NOEC	no observed effect concentration
NPDES	National Pollutant Discharge Elimination System
QA/QC	quality assurance/quality control
RMD	regulatory management decision
RP	reasonable potential
RPMF	reasonable potential multiplying factor
RWC	receiving water concentration
SWAMP	Surface Water Ambient Monitoring Program (California)
TAC	test acceptability criteria
TIE	toxicity identification evaluation
TRE	toxicity reduction evaluation
TSD	Technical Support Document for Water Quality-Based Toxics Control
TST	Test of Significant Toxicity
TU	toxicity unit
WET	whole effluent toxicity

GLOSSARY

Acute Toxicity Test is a test to determine the concentration of effluent or ambient waters that causes an adverse effect (usually mortality) on a group of test organisms during a short-term exposure (e.g., 24, 48, or 96 hours). Acute toxicity is determined using statistical procedures (e.g., point estimate techniques or a t-test).

Ambient Toxicity is measured using a toxicity test on a sample collected from a receiving waterbody.

Chronic Toxicity Test is a short-term test in which sublethal effects (e.g., reduced growth or reproduction) are usually measured in addition to lethality.

Coefficient of Variation (CV) is a standard statistical measure of the relative variation of a distribution or set of data, defined as the standard deviation divided by the mean. The CV can be used as a measure of precision within and between laboratories, or among replicates for each treatment concentration.

Confidence Interval is the numerical interval constructed around a point estimate of a population parameter.

Effect Concentration (EC) is a point estimate of the toxicant concentration that would cause an observable adverse effect (e.g., mortality, fertilization). EC25 is a point estimate of the toxicant concentration that would cause an observable adverse effect in 25 percent of the test organisms.

False Negative is when the in-stream waste concentration is declared non-toxic but in fact is truly toxic. In the traditional hypothesis approach, false negative error rate is denoted by Beta (β). In the TST approach, false negative error rate is denoted as Alpha (α), which applies when the percent effect in the critical effluent concentration is $\geq$ 25% for a given test.

False Positive is when the in-stream waste concentration is declared toxic but in fact is truly non-toxic. In the traditional hypothesis approach, false positive error rate is denoted by Alpha (α). In the TST approach, false positive error rate is denoted as Beta (β), which applies when the percent effect in the critical effluent concentration is $\leq$ 10% for a given test.

Hypothesis Testing is a statistical approach (e.g., Dunnett's procedure) for determining whether a test concentration is statistically different from the control. Endpoints determined from hypothesis testing are no observed effect concentration and lowest observed effect concentration (LOEC). The two hypotheses commonly tested in WET are:

Null hypothesis (Ho): The effluent is non-toxic.

Alternative hypothesis (Ha): The effluent is toxic.

Inhibition Concentration (IC) is a point estimate of the toxicant concentration that would cause a given percent reduction in a nonlethal biological measurement (e.g., reproduction or growth), calculated from a continuous model (i.e., Interpolation Method). IC25 is a point estimate of the toxicant concentration that would cause a 25 percent reduction in a nonlethal biological measurement.

In-stream Waste Concentration (IWC) is the concentration of a toxicant or effluent in the receiving water after mixing. The IWC is the inverse of the dilution factor. It is sometimes referred to as the receiving water concentration (RWC).

Lethal Concentration, 50 percent (LC50) is the toxicant or effluent concentration that would cause death to 50 percent of the test organisms.

Lowest Observed Effect Concentration (LOEC) is the lowest concentration of an effluent or toxicant that results in statistically significant adverse effects on the test organisms (i.e., where the values for the observed endpoints are statistically different from the control).

No Observed Effect Concentration (NOEC) is the highest tested concentration of an effluent or toxicant that causes no observable adverse effect on the test organisms (i.e., the highest concentration of toxicant at which the values for the observed responses are not statistically different from the control).

National Pollutant Discharge Elimination System (NPDES) is the national program for issuing, modifying, revoking and reissuing, terminating, monitoring and enforcing permits, and imposing and enforcing pretreatment requirements, under the Clean Water Act sections 307, 318, 402, and 405.

Power (or test power) in the context of the Test of Significant Toxicity approach, is the probability of correctly declaring an effluent non-toxic when, in fact, it has an acceptably low level of toxicity.

Precision is a measure of reproducibility (which is a statistical term about the ability to reproduce similar results across test replicates with in a test treatment) within a data set. Precision can be measured both within a laboratory (within-laboratory) and between laboratories (between-laboratory) using the same test method and toxicant.

Quality Assurance (QA) is a practice in toxicity testing that addresses all activities affecting the quality of the final effluent toxicity data. QA includes practices such as effluent sampling and handling, source and condition of test organisms, equipment condition, test conditions, instrument calibration, and replication, use of reference toxicants, recordkeeping, and data evaluation.

Quality Control (QC) is the set of more focused, routine, day-to-day activities carried out as part of the overall QA program.

Reasonable Potential (RP) is where an effluent is projected or calculated to cause an excursion above a water quality standard based on a number of factors including the four factors listed in Title 40 of the *Code of Federal Regulations* Part 122.44(d)(1)(ii).

Reference Toxicant Test is a check of the sensitivity of the test organisms and suitability of the test methodology using the reference toxicant required by the EPA WET test methods. Reference toxicant data are part of a routine QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

Regulatory Management Decision (RMD) is the decision that represents the maximum allowable error rates and thresholds for toxicity and non-toxicity that would result in an acceptable risk to aquatic life.

Replicate is two or more independent organism exposures of the same treatment (i.e., effluent concentration) within a WET test. Replicates are typically separate test chambers with organisms, each having the same effluent concentration**.**

Sample is defined as a representative portion of a specific environmental matrix that is used in toxicity testing. For this document, environmental matrices could include effluents, surface waters, groundwater, stormwater, and sediment.

Significant Difference is defined as a statistically significant difference (e.g., 95 percent confidence level) in the means of two distributions of sampling results.

Statistic is a computed or estimated quantity such as the mean, standard deviation, or coefficient of variation.

Test Acceptability Criteria (TAC) are test method-specific criteria for determining whether toxicity test results are acceptable. The effluent and reference toxicant must meet specific criteria as defined in the test method (e.g., for the *Ceriodaphnia dubia* survival and reproduction test, the criteria are as follows: the test must achieve at least 80 percent survival and an average of 15 young per surviving female in the control and at least 60% of surviving organisms must have three broods).

t-test (formally Student's t-Test) is a statistical analysis comparing two sets of replicate observations—in the case of WET, only two test concentrations (e.g., a control and IWC). The purpose of this test is to determine if the means of the two sets of observations are different (e.g., if the IWC or ambient concentration differs from the control [i.e., the test result is pass or fail]).

Type I Error (alpha α) is the error of rejecting the null hypothesis (H_o) that should have been accepted.

Type II Error (beta β) is the error of accepting the null hypothesis (H_o) that should have been rejected.

Toxicity Test is a procedure to determine the toxicity of a chemical or an effluent using living organisms. A toxicity test measures the degree of effect on exposed test organisms of a specific chemical or effluent.

Welch's t-test is an adaptation of Student's t-test intended for use with two samples having unequal variances.

Whole Effluent Toxicity (WET) is the total toxic effect of an effluent measured directly with a toxicity test.

1.0 INTRODUCTION

Whole effluent toxicity (WET) test methods are laboratory procedures that measure biological effects (e.g., survival, growth, reproduction) on aquatic organisms exposed to effluents or storm water discharged to receiving waters in implementing the National Pollutant Discharge Elimination System (NPDES) Program under the Clean Water Act (CWA) section 402. Since the publication of EPA's *Technical Support Document for Water Quality-based Toxics Control* (TSD) (USEPA 1991), permitting authorities have requested alternative approaches for analyzing WET test data that would provide increased confidence in the data assessment and simplify the NPDES permit decision-making process with respect to WET. In response to those requests, EPA developed the TST approach as another statistical option to analyze valid WET test data. This document presents the NPDES programmatic features of the TST statistical approach for analyzing valid WET data and how it can be used to support permitting authorities and permittees when analyzing and interpreting WET test data. Use of the TST approach does not result in any changes to EPA's WET test methods, nor does it preclude the use of EPA's TSD approaches for analyzing valid WET data, or another scientifically defensible approach that is sufficient to meet the statutory and regulatory requirements.

1.1 Terminology and Concepts

This section briefly summarizes the major statistical concepts and terminology involved in WET analysis so as to give the reader a context with which to understand the TST approach and how it differs from current statistical approaches used to analyze valid WET data. This TST implementation document is not intended to provide a detailed discussion of WET test methods, data interpretation, or statistics, and it is assumed that the reader will consult EPA's TSD, WET test method documents, and other WET-related documents (e.g., *Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications*, USEPA 2000).

In the NPDES Program, WET tests examine organism responses to effluent, typically along a dilution series (USEPA 1995, 2002a, 2002b, 2002c). Acute WET methods measure the lethal response of test organisms exposed to effluent (USEPA 2002c). The principal response endpoints for those methods are the effluent concentration that is lethal to 50 percent of the test organisms (LC50) or the effluent concentration at which survival is significantly lower than the control. Chronic WET methods often measure both lethal and sublethal responses of test organisms. The statistical endpoints used in chronic WET testing are the no observed effect concentration (NOEC) and the 25 percent inhibition concentration (IC25). The NOEC endpoint is determined using a hypothesis testing approach that identifies the maximum effluent concentration at which the response of test organisms is not significantly different from the control. From a regulatory perspective, an effluent sample is declared toxic if the NOEC is less than the in-stream waste concentration (IWC) specified through the WET limitations in the permit. The IC25, by contrast, is a point estimation approach. It identifies the concentration at which the response of test organisms is 25 percent below that observed in the control concentration, and it interpolates the effluent concentration at which this magnitude of response is expected to occur. From a regulatory perspective, an effluent sample is declared toxic if the IC25 is less than the IWC specified through the WET limitations in the permit. This document focuses only on the hypothesis testing approach and not on point estimation approaches for analyzing and interpreting WET data.

In any hypothesis testing approach, two hypotheses are stated: the null hypothesis and the alternative hypothesis. The statistical concepts associated with the traditional hypothesis testing approach currently used in WET analysis are summarized in Table 1. Using that approach, the null hypothesis is that the IWC is non-toxic (i.e., the organism response at the IWC is equal to or better than the response in the test control). The alternative hypothesis is that the IWC is toxic (i.e., the organism response is worse in the IWC than in the control). With any hypothesis testing approach, two types of decision errors occur: (1) conclude that the null hypothesis is correct when in fact it is not or (2) conclude that the null hypothesis is incorrect (i.e., reject the null hypothesis) and thereby declare that the alternative hypothesis is correct, when in fact the null hypothesis is correct. In WET testing, the first type of error above is referred to as a *false negative*, meaning that the *IWC is declared non-toxic when in fact it is toxic*. The second type of error above is referred to as a *false positive* in WET testing, meaning that the *IWC is declared toxic when in fact it is not*.

In the traditional hypothesis testing approach summarized in Table 1, statisticians have assigned Greek letters to the two types of errors identified above. Alpha (or α) refers to the false positive error rate. Beta (or β) refers to the rate of false negatives. In the EPA WET test methods supporting the NPDES WET Program (USEPA 1995, 2002a, 2002b), α was established but β was not. Therefore, the application of α from the EPA test methods and implemented under EPA's TSD, recommended that the maximum rate of false positives that should be observed should be low (no more than 5 percent or $\alpha = 0.05$), but the rate of false negatives was not similarly controlled and is not currently evaluated in WET testing. As a result, the rate of false negatives in the NPDES WET Program has not been controlled. Put another way, the statistical power of these tests, the ability to correctly classify the IWC as toxic (where power is defined as $1-\beta$, Table 1) has not been controlled.

As noted previously in this section, a hypothesis testing approach determines whether the organism response at the IWC is significantly worse than that in the control. In practice, this statistical approach relies on two properties of the data: the average values in the control and the IWC (e.g., average fish weight in each test concentration), and the variability observed among replicates (i.e., organisms' responses from multiple replicates) within the IWC and the control. Whether the IWC is considered toxic depends on both of those data properties, which in many cases results in a well-established, statistically rigorous way to evaluate WET data. However, there are two types of situations in which the traditional hypothesis testing approach can yield equivocal results in WET testing: (1) in tests where within-test variability is high and (2) in tests where within-test variability is exceptionally low. In the first case, because within-test variability is high, it will be difficult to determine statistically whether the organism response to the IWC is worse than the control. That could result in more false negatives than would otherwise be the case. In the second case above, because within-test variability is very low, it will be relatively easy to show statistically significant differences in organism response between the IWC and the control. That could result in more false positives (as defined in the TST approach) than would otherwise be the case.

Table 1. Expression of null and alternative hypotheses used in traditional hypothesis testing and relationships between error rates and resulting decisions based on this approach. Entries correspond to the probability decision given in parentheses. The probability of a false positive (i.e., rejecting a null hypothesis that should not have been rejected) is represented by α and the probability a false negative (i.e., failing to reject the null hypothesis when it should have been rejected) is represented by β.

	True condition	
Decision	**Null hypothesis** Treatment mean ≥ Control mean **Sample is non-toxic**	**Alternative hypothesis** Treatment mean < Control mean **Sample is toxic**
Treatment mean ≥ Control mean **Sample is non-toxic**	Correct decision (1-α)	False negative (β)
Treatment mean < Control mean **Sample is toxic**	False positive (α)	Correct decision (1 – β) (power)

1.2 Background on the TST Approach

The TST is an alternative statistical approach for analyzing and interpreting valid WET data that also uses a hypothesis testing approach but in a different way, building on previous work conducted by EPA in the NPDES WET Program (USEPA 2000) and other researchers (Erickson and McDonald 1995; Shukla et al. 2000; Berger and Hsu 1996). The TST approach is based on a type of hypothesis testing referred to as *bioequivalence testing*. Bioequivalence is a statistical approach that has long been used in evaluating clinical trials of pharmaceutical products (Anderson and Hauck 1983) and by the Food and Drug Administration (Hatch 1996; Aras 2001; Streiner 2003). The approach has also been used to evaluate the attainment of soil cleanup standards for contaminated sites (USEPA 1989) and to evaluate effects of pesticides in experimental ponds (Stunkard 1990). In the context of the NPDES WET Program, the TST approach assesses whether the response of test organisms at the IWC (e.g., fish weight or number of neonates per female) is less than a predetermined proportion of the control response that is considered unacceptably toxic. *Once the WET test has been conducted (using multiple effluent concentrations and other requirements have been met as specified in the EPA methods), the TST approach is designed to be used for a two concentration data analysis of the in-stream waste concentration (IWC) or a receiving water concentration (RWC) compared to a control concentration.*

The null hypothesis using the TST approach is that the IWC is significantly more toxic (i.e., results in a worse organism response) compared to the control (see Table 2). The alternative hypothesis using the TST approach is that the IWC is non-toxic. Thus, the null and alternative hypotheses using the TST approach are opposite of what they are under the traditional hypothesis testing approach described in Section 1.1. In addition, the meaning of α and β are also opposite from what they represent in the traditional hypothesis approach. Under the TST approach, α is associated with false negatives, and β is associated with false positives. Statistical power using the TST approach is the ability to correctly classify the IWC as non-toxic (Table 2). The proportion or fraction of the control response that represents the toxicity threshold is denoted as *b* in the equations in Table 2 and is expressed as a decimal between 0.00 and 1.00. For example,

a *b* value set at 0.85 would mean that a response at the IWC that is at least 85 percent of the control response in the test (i.e., no more than a 15 percent effect) would be considered a lower risk for environmental impacts.

Using the TST hypothesis approach in the NPDES WET Program has several benefits. By incorporating *b* in the hypothesis equation, using the TST approach, there is explicit acknowledgement of the fact that the organism response at the IWC can be less than the control organism response by a certain amount and still be considered acceptable (i.e., non-toxic). In that way, truly non-toxic samples (as defined in the TST approach) can be addressed in a clearer manner than is possible with the traditional hypothesis testing approach as practiced in the NPDES WET Program. A low false positive rate in the TST approach is further addressed by having a low β ($\beta \leq 0.05$), which means more statistical power to identify an acceptable effluent (as defined by EPA's regulatory management decisions [RMDs]) as non-toxic in the NPDES WET Program. In addition, because the null hypothesis in the TST approach is opposite to what is used in the traditional hypothesis testing approach, false negatives are explicitly addressed (α in the TST approach addresses the false negative rate). As mentioned previously, the current NPDES WET Program does not control for false negatives. Thus, the TST approach allows permitting authorities to minimize the occurrence of false negatives (i.e., declaring the IWC non-toxic when it is actually exhibiting unacceptable toxicity), while also minimizing the occurrence of false positives (i.e., declaring the IWC toxic when it is actually acceptable). The TST approach has the added advantage of providing permittees with a clear incentive to improve the precision of test results (e.g., decrease within-test variability and/or use more replicates within a WET test than the minimum required in the EPA WET test method) to reach a definitive conclusion as to whether unacceptable toxicity is observed in a test. Thus, using the TST approach, a permittee can in fact *prove a negative*, i.e., that their effluent is acceptable (non-toxic).

Table 2. Expression of null and alternative hypotheses using the TST approach and relationships between error rates and resulting decisions based on this approach. Entries correspond to the probability decision given in parentheses. The probability of a false positive (i.e., rejecting a null hypothesis that should not have been rejected) is represented by α and the probability a false negative (i.e., failing to reject the null hypothesis when it should have been rejected) is represented by β.

	True condition	
Decision	**Null hypothesis** Treatment mean ≤ b × Control mean **Sample is toxic**	**Alternative hypothesis** Treatment mean > b × Control mean **Sample is non-toxic**
Treatment mean ≤ b × Control mean **Sample is toxic**	Correct decision (1-α)	False positive (β)
Treatment mean > b × Control mean **Sample is non-toxic**	False negative (α)	Correct decision (1-β) (power)

2.0 TST METHODOLOGY

2.1 Regulatory Management Decisions for the TST Approach

Toxicity is not an absolute quantity but rather an effect that is determined relative to a control or reference sample using a given WET test method. In the TST approach, what is considered unacceptable or acceptable toxicity are explicit RMDs. For *chronic* testing in EPA's NPDES WET Program, the *b* value in the TST null hypothesis is set at 0.75, which means that a 25 percent effect (or more) is considered a demonstration of unacceptable toxicity in a given WET test. Using a 25 percent effect threshold as the *b* coefficient is consistent with EPA's use of a 25 percent inhibition concentration (IC25) as an acceptable WET endpoint for examining chronic WET data. Responses substantially less than a 25 percent effect would be interpreted as a lower risk potential. The unacceptable toxicity RMD threshold for acute WET methods is set higher than that for chronic WET methods because of the severe environmental implications of acute toxicity (lethality or organism death). Therefore, for *acute* WET tests, the *b* value in the TST approach is set at 0.80 (i.e., ≥ 20 percent effect in the effluent in acute WET tests is considered unacceptable).

For both acute and chronic WET test methods, the low-risk RMD threshold is set at a 10 percent mean effect at the IWC within a WET test. Thus, one can *prove the negative* (i.e., an effluent is *acceptable or considered non-toxic under NPDES*) if that condition is met in a WET test. For mean effect levels greater than 10 percent but less than the unacceptable toxicity RMD threshold (20 percent for acute and 25 percent for chronic WET tests), the TST approach will still declare the IWC non-toxic depending on within-test variability: the lower the variability in the WET test, the more likely the sample will be declared non-toxic on the basis of the mean responses observed under these test conditions.

EPA's RMDs using the TST approach are used to specify unacceptable toxicity in WET tests most of the time when it occurs (i.e., a low false negative rate). As mentioned previously, under the traditional hypothesis testing approach currently used in the NPDES WET Program, the false negative rate was not controlled. Using the TST approach, the false negative rate RMD is $0.05 \leq \alpha \leq 0.25$, which translates to at least 75 percent probability that an effluent causing unacceptable toxicity will be declared toxic. As noted in the previous paragraph, the unacceptable toxicity RMD threshold is defined as ≥ 20 percent effect of the IWC in acute WET tests and ≥ 25 percent effect of the IWC in chronic WET tests.

EPA also desires to minimize the probability that the IWC is declared toxic when in fact it is acceptable (i.e., low false positive rate). Under the traditional hypothesis testing approach currently used in the NPDES WET Program, the false positive rate is set at 0.05 or 5 percent. Therefore, in the TST approach, the desired false positive rate is also set at 0.05 or 5 percent ($\beta \leq 0.05$). A $\beta = 0.05$ in the TST approach means that 95 percent of the time, a truly acceptable effluent (≤ 10 percent mean effect at the IWC) will be declared non-toxic in the NPDES WET Program. Depending on the minimum WET test design required in the EPA methods (e.g., number of replicates and number of organisms per test concentration) and achievable laboratory control precision for a WET test method, α will be set between 0.05 and 0.25 while still

maintaining a $\beta \leq 0.05$. Extensive analyses were used to identify the lowest α for a given WET test method for which $\beta = 0.05$ and all other RMDs are met.

The RMD thresholds above represent boundaries in terms of desired α and β rates. An $\alpha = 0.20$ for a chronic test method, for example, means that the Type I error rate will be approximately 20 percent at a mean effect of 25 percent. At higher levels of effect in the IWC, actual Type I error rates would be lower; at lower mean effect levels in the IWC, Type I error rate would be somewhat higher, depending on the test method. Therefore, at mean effect levels between the 10 percent non-toxic RMD boundary and the unacceptable toxicity RMD boundary (20 percent for acute and 25 percent for chronic WET test methods), there are differing probabilities of an effluent being declared toxic depending on within-test variability and the difference in mean responses observed between control and IWC. As a result, there will be some instances in which TST will declare a test toxic, whereas the traditional hypothesis approach would declare that test non-toxic (particularly when within-test variability is high or the mean effect at the IWC is near 25 percent, as explained in Section 1.1). Similarly, there will be some instances in which TST will declare an effluent non-toxic but the traditional hypothesis approach would declare that test toxic (when within-test variability is low and the mean effect at the IWC is less than the 20 percent toxicity RMD threshold for acute test methods or 25 percent for chronic toxicity test methods, as explained in Section 1.1).

WET test design and the types of WET endpoints measured influence test sensitivity (e.g., control coefficient of variation or CV). Therefore, TST α error rates are identified for different types of test designs. For example, all fish chronic WET test methods that use a similar test design and have the same type of test endpoints (e.g., growth and survival) would have the same α value. Varying α by WET test design is appropriate for the TST approach. Given the way that the hypotheses are formulated in the TST approach (see Table 2), α represents what is considered β in the traditional hypothesis testing approach, and an acceptable β error was not identified in the current EPA TSD's approach to the EPA NPDES WET Program. Setting α as well as β in the TST approach addresses both false positives and false negatives.

2.2 Setting the Test Method-Specific Alpha Level

Several types of analyses were conducted to determine the appropriate α level for each WET test method. First, representative effluent and reference toxicant data meeting EPA WET test method's test acceptability criteria (TAC) were obtained from several state databases, which included multiple laboratories and wastewater effluents. Valid effluent WET data that met the following data selection requirements were considered to be a representative sample.

- Cover a range of NPDES permitted facility types, including both industrial and municipal permittees
- Represent many facilities for a given EPA WET test method (i.e., no one facility dominates the data for a given WET test method)
- Cover a range of target (design) effluent dilutions on which WET reasonable potential (RP) and NPDES permit compliance are based, ranging from 10 percent to 100 percent effluent concentrations
- Generated by several laboratories for a given EPA WET test method

- Cover a range of observed effluent toxicity for each EPA WET test method (e.g., NOECs range from < 10 percent to 100 percent effluent)

For each of the nine EPA WET test methods examined, control precision was calculated on the basis of valid WET data compiled in this project. A similar analysis was performed for the control response for each of the nine test methods (e.g., mean number of offspring per female in the chronic *Ceriodaphnia dubia* test method) to characterize typical achievable test performance in terms of control response.

A Monte Carlo simulation analysis (a statistical method) was used to estimate the percentage of WET tests that would be declared toxic using the TST approach as a function of different α levels, within-test variability (control and effluent variability), and different effect levels. That analysis identified probable false positive error rates (i.e., declaring an effluent toxic when in fact it is not) under all WET test scenarios encountered. Using the RMDs defined above, an appropriate α level was then identified for each WET test design given a desired β error of ≤ 5 percent (0.05) when there is a 10 percent mean effect at the IWC. By simulating thousands of WET tests for a given scenario (mean percent effect and control CV), the percentage of tests declared toxic under a given effluent assessment scenario could be calculated and compared with other scenarios.

3.0 USING THE TST APPROACH IN WET DATA ANALYSES

3.1 Summary of Test Method-Specific Alpha Values

On the basis of all the analyses conducted in this project, EPA recommends the following alpha levels when using the TST approach in a two concentration (i.e., two treatments) data analysis comparison (e.g., IWC and control) (see Table 3).

Table 3. Summary of alpha (α) levels or false negative rates recommended for different WET test methods using the TST approach

EPA WET test method	*b* value	Probability of declaring a toxic effluent non-toxic False negative (α) error[a]
Chronic Freshwater and East Coast Methods		
Ceriodaphnia dubia (water flea) survival and reproduction	0.75	0.20
Pimephales promelas (fathead minnow) survival and growth	0.75	0.25
Selenastrum capricornutum (green algae) growth	0.75	0.25
Americamysis bahia (mysid shrimp) survival and growth	0.75	0.15
Arbacia punctulata (Echinoderm) fertilization	0.75	0.05
Cyprinodon variegatus (Sheepshead minnow) and *Menidia beryllina* (inland silverside) survival and growth	0.75	0.25
Chronic West Coast Marine Methods		
Dendraster excentricus and *Strongylocentrotus purpuratus* (Echinoderm) fertilization	0.75	0.05
Atherinops affinis (topsmelt) survival and growth	0.75	0.25
Haliotis rufescens (red abalone), *Crassostrea gigas (oyster), Dendraster excentricus, Strongylocentrotus purpuratus* (Echinoderm) and *Mytilus sp* (mussel) larval development methods	0.75	0.05
Macrocystis pyrifera (giant kelp) germination and germ-tube length	0.75	0.05
Acute Methods		
Pimephales promelas (fathead minnow), *Cyprinodon variegatus* (Sheepshead minnow), *Atherinops affinis* (topsmelt), *Menidia beryllina* (inland silverside) acute survival[b]	0.80	0.10
Ceriodaphnia dubia, *Daphnia magna, Daphnia pulex*, *Americamysis bahia* acute survival[b]	0.80	0.10

Notes:

a. (1) declare a sample toxic at least 75 percent of the time (alpha $\leq$ 0.25) when there is unacceptable toxicity (20 percent effect for acute and 25 percent effect for chronic test methods) and (2) declare an effluent non-toxic no more than 5 percent of the time (beta $\leq$ 0.05) when the mean effect at the critical effluent concentration is 10 percent for both acute and chronic WET tests (including sublethal endpoints). For more discussion on the RMDs, see Section 2.1 of this document.

b. Based on four replicate test design

3.2 Calculating Statistics for Valid WET Data Using the TST Approach

Appendix A includes a step-by-step guide for using the TST approach to analyzing WET test data. The appendix also includes a statistical flowchart and several examples. Note that the WET test method should follow the test condition requirements as specified in EPA's approved WET methods (USEPA 1995, 2002a, 2002b, 2002c).

The TST approach is used to statistically compare organism responses from two concentrations (i.e., treatments) of the WET test, the IWC and the control. Percent data (quantal data), such as percent survival or percent germination from a WET test, is first transformed as required in the EPA WET test manuals. Other types of WET data (e.g., growth or reproduction data) are not transformed. Data are then analyzed using Welch's t-test, a well-known modification of the standard t-test (Zar 1996), which is appropriate for the TST approach (see Appendix A).

Appendix B lists the critical *t* values that apply to WET testing using the TST approach given the number of degrees of freedom and the α level that applies for a given WET test method from Table 3 of this document. If the calculated *t* value for the WET test is greater than the critical *t* value (see Table B-1), the null hypothesis is rejected, i.e., the test result is *Pass* and **the effluent is declared *non-toxic***. If the calculated *t* value is less than the critical *t* value in Appendix B, the null hypothesis is not rejected, i.e., the test result is *Fail* and **the effluent *is* declared toxic.** Appendix A contains examples that demonstrate the formulae used in the TST approach and are designed to illustrate how the outcome is influenced by within-test variability and the mean effect of the IWC using the TST approach. Four different case examples are presented, three of which have equal variances between control and IWC: (1) *Ceriodaphnia* reproduction data having relatively high within-test variability, (2) *Ceriodaphnia* reproduction data having relatively low within-test variability and the same effect as in Example 1, (3) growth data from two fathead minnow chronic WET tests, both with relatively high within-test variability but small mean effect at the IWC; one test was conducted with the minimum number of replicates required in the EPA WET test method (four replicates) and the other test was conducted a priori with six replicates per concentration; and (4) calculations using the TST approach for an acute fathead minnow WET test.

Case Example #1 in Appendix A: ***Demonstrates a benefit of the TST approach by addressing false negatives***. A WET test that has relatively high within-test variability for a given WET test method and has an effect at the IWC approaching the RMD threshold (25 percent in this case because it is a chronic WET test) *is declared toxic* using the TST approach. Using the traditional hypothesis testing approach as recommended in the TSD, such test data typically lead to a conclusion that the effluent is not toxic (i.e., a false negative).

Case Example #3 in Appendix A: ***Demonstrates the benefits of increased within-test replication using the TST approach***. Increasing the replication before conducting the test, which thereby improves the precision and power of the WET test, increases the chances of rejecting the null hypothesis and declaring a truly acceptable effluent as *non-toxic* using the TST approach. That increases the ability to *prove the negative*, i.e., that an effluent is declared not toxic.

The TST approach can also be used for ambient toxicity (i.e., receiving water) tests and stormwater toxicity testing programs because the TST approach compares two treatments (for application of the TST approach to ambient toxicity testing, see Appendix C).

4.0 IMPLEMENTING THE TST APPROACH IN WET NPDES PERMITS

The TST approach is an alternative approach for analyzing and interpreting valid WET data. Use of the TST approach does not result in any changes to EPA's WET test methods. WET limits are simpler to communicate and understand (for example permit language for acute and chronic WET monitoring using the TST statistical analysis approach, see Appendix D) than the TSD approach. EPA recommends that permitting authorities decide up front which approach (the 1991 TSD approach, the TST approach, or another scientifically defensible approach that is sufficient to meet the statutory and regulatory requirements) they will incorporate and consistently use in their state's NPDES implementation procedures, including their RP procedures. The permitting authority should use the selected WET statistical approach consistently in all of their state NPDES permits.

4.1 Reasonable Potential (RP) WET Analysis

NPDES permitting authorities conducting an RP analysis must follow Title 40 of the *Code of Federal Regulations* (CFR) section 122.44(d)(1) to determine whether a discharge will ~~"c~~ause, have the [RP] to cause, or contribute to" an excursion of a numeric criterion or a narrative WET criterion. Some states have state-specific WET RP approaches in their water quality control plan or other NPDES policy or guidance.

For RP calculations using the TST approach, EPA recommends that permitting authorities use all valid WET test data generated during the current permit term and any additional valid data that are submitted as part of the permit renewal application. The TST RP approach necessitates having at least a minimum of four valid WET tests to address effluent representativeness (see EPA's TSD, Chapter 3, p. 57, under Step 2 in the section *Steps in Whole Effluent Characterization Process*). EPA also recommends that states request that their permittees provide the actual test endpoint responses for the control (i.e., control mean) and IWC concentration (i.e., IWC mean) for each WET test conducted to make it easier for permit writers to find the necessary WET test results when determining WET RP. WET test data are then analyzed according to the TST approach using the IWC and control test concentrations for all the valid WET test data available. For data sets with fewer than four valid WET data points, RP should be assessed using EPA's TSD RP approach because it addresses small WET data sets by incorporating an RP multiplying factor (see Section 3.3.2 of the TSD, p. 54) to account for effluent variability in small WET data sets. If WET test data are available and the TST statistical approach indicates that the IWC is toxic in any WET test, RP has been demonstrated (40 CFR 122.44(d)(1)(i)). Similar to the TSD approach, the TST approach can establish the existence of RP for WET even when no tests have been declared toxic using the TST to address concerns regarding the ~~"p~~otential to cause or contribute to toxicity." Appendix E presents the approach used to determine RP using the TST approach.

Note that using the TST approach might be to the permittee's advantage. If the permittee decides to incorporate additional replicates for the control and the IWC within a WET test, beyond the minimum required in the WET test method, the test power is increased. More test replicates increases test power, which means a higher probability of declaring a sample as non-toxic using the TST approach *if the effluent is truly non-toxic*. A demonstration is provided in Appendix A (Case Example #3), which illustrates that as an intended consequence of the TST approach

methodology. Thus, using the TST approach, a permittee has a greater ability to *prove the negative* (i.e., their effluent does not have RP).

In those cases where the WET RP outcome is *yes*, a WET limit is expressed in the permit. In those situations where the RP outcome is *no*, WET monitoring requirements should still be incorporated in the permit. Also in the permit, a test result of *Fail* (i.e., sample declared toxic) during monitoring, would trigger additional steps in the permit. In either of those situations—either a WET limit or a WET monitoring requirement, if toxicity is demonstrated—states should specify an approach to address toxicity in the permit. Doing so often includes increased frequency of WET testing and additional permit requirements to perform a toxicity reduction evaluation.

4.2 NPDES WET Permit Limits

Using the TST approach, WET NPDES permit limits would be expressed as *no significant toxicity of the effluent at the IWC using the TST analysis approach*. A test result of *Pass* is when the calculated *t* value is greater than *the critical t value*. A test result of *Fail* is when the calculated *t* value is less than *the critical t value*.

Beyond assessing WET data for the NPDES Program, WET tests are used to assess toxicity of receiving water (watershed assessment for CWA section 303(d) determinations) and stormwater samples. Often as a first assessment of receiving or stormwater toxicity, researchers test a control and a single concentration (e.g., 100 percent receiving water or stormwater). In such cases, the TST approach can be used in the same way a t-test is used. Such analysis is used to determine whether organism response in a specified ambient concentration is significantly different than the control organism response (for further information, see Appendix C).

5.0 RECOMMENDATIONS FOR NPDES IMPLEMENTATION OF THE TST APPROACH

5.1 EPA Regions and NPDES States (Permitting Authorities)

Permitting authorities should consider adding the TST approach to their implementation procedures for analyzing valid WET data for their current NPDES WET Program. Permitting authorities should consider the practical programmatic shift from the traditional hypothesis testing approach to the TST approach by opening a dialogue with their regulated community. In addition, they might want to begin to identify what changes might be needed to assimilate the TST approach into any regulations, policy, guidance, and training within their respective NPDES WET Programs. EPA also recommends that permitting authorities decide up front which RP approach (the 1991 TSD approach, the TST approach, or another scientifically defensible approach that is sufficient to meet the statutory and regulatory requirements) the permitting authority will incorporate and consistently use in their state's NPDES implementation procedures. The permitting authority should then use the WET statistical approach (either the TSD approaches or the TST data analysis approach) selected throughout all its state NPDES permits. Again, the traditional hypothesis testing approach recommended in EPA's TSD is still considered valid as applied; however, that approach can now be advanced through the TST approach by providing new incentives to permittees to generate valid, high quality WET data.

The RMDs incorporated into the TST approach were selected on the basis of considerable research and analysis involving several of the EPA WET test methods. Lower *b* values (i.e., for chronic test methods using a 0.70 instead of 0.75 *b* is unacceptable) are not recommended because it would mean that a lower fraction of test control response (i.e., greater effect at the IWC) is considered acceptable. EPA chose the acute and chronic *b* values to minimize effects on aquatic ecosystems. Likewise, the alpha values identified by EPA using the TST approach were determined on the basis of the predetermined *b* values and therefore should *not* be altered.

The permitting authority should consider carefully how the TST approach will be implemented in NPDES permits. Example permit language is shown in Appendix D. In consideration of maintaining NPDES WET Program implementation consistency, the TST approach should be used in place of, *and not in addition to*, the traditional hypothesis testing (NOEC) approach for WET analysis.

5.2 NPDES Permittees

One of the intended benefits of the TST approach is that increasing the precision and power of the WET test increases the chances of declaring a truly *acceptable effluent as non-toxic*. The permittee has greater control over the interpretation of WET test results using the TST approach because the RMDs are transparent, and the level of WET data quality needed to obtain unequivocal results can be determined beforehand. For example, conducting tests with more test replicates improves the power of the WET test, which can then support and provide a defensible basis for a permittee's demonstration that its effluent is *acceptable (i.e., in compliance with the permit*) if the mean effect is truly within the RMDs as defined in the TST approach. Using the TST approach, there is a *lower* rate of WET tests declared toxic for tests that are truly acceptable because of the increased power of the WET test when the permittee increases its number of

replicates in a WET test or achieves better replication within a test through improved test method performance. Thus, the TST approach increases the ability of the permittee to *prove the negative*, that the effluent is *non-toxic* if it is truly *acceptable*. Where a permittee has concerns about WET data quality, EPA recommends increasing the number of replicates in tests, even if the permitting authority has not yet adopted the TST approach.

6.0 SUMMARY OF THE TST APPROACH

EPA's TSD approaches are valid and can still be used by EPA Regions and their NPDES states. The TST approach is another statistical option for analyzing valid WET test data. The TST approach can be applied to acute (survival) and chronic (sublethal) endpoints and is appropriate to use for both freshwater and marine EPA WET test methods. The TST approach requires no more time or expertise than is presently expended when using the TSD hypothesis testing statistical approach and can be used with a well-recognized statistical test. Below is a brief outline of both the TST and TSD hypothesis testing approaches relevant to the information in this document and a short list of the benefits derived when using the TST approach.

TST Approach

- Considered additional guidance only—TST is a statistical approach for analyzing WET test data as an alternative option to the traditional hypothesis testing approach provided in EPA's TSD
- Expresses NPDES WET permit limit ~~as~~ no significant toxicity of the effluent at the in-stream waste concentration" using the TST analysis approach
- Provides a positive incentive to NPDES permittees to generate valid, high quality WET data to the permitting authority by improving test performance or increasing the number of replicates within a WET test (which increases statistical power of WET test)
- Addresses both false negative (declared non-toxic when actually toxic) and false positive (declared toxic when actually non-toxic) error rates in a WET test

Traditional Hypothesis Test (EPA TSD)

- Existing approaches remain valid and can still be used by NPDES permitting authorities
- In existing guidance, WET permit limits are expressed as *no observed effect concentration (NOEC)* at the IWC
- Provides relatively less incentive to permittees to generate high quality valid, WET data or to increase the number of replicates within a WET test to increase statistical power of a WET test
- False negative error rate in a WET test is not addressed

Benefits When Using the TST Approach in WET Data Analysis

- ***The TST approach*** is similar to statistical concepts used in other EPA programs and at other federal agencies
- ***Transparent RMDs.*** RMDs are transparent because they are incorporated into the WET data analysis process, e.g., what effect level is considered toxic and what effect level is considered acceptable.
- ***WET test method-specific alpha and beta error rates.*** Both error rates are directly incorporated into the TST statistical approach, thereby increasing confidence in WET test interpretation.
- ***High quality WET test data incentive.*** Provides a positive incentive for the permittee to generate valid, high quality WET data; better test performance (lower within-test

variability) helps ensure appropriate WET decisions using the TST approach (e.g., a truly acceptable effluent will be declared non-toxic).

- ***Streamlined, simpler statistical analysis.*** Flowchart for analyzing valid WET data under the TST approach is much simpler because fewer statistical tests are needed.
- ***RP analysis is simpler.*** Because the calculation of the individual test result, using the TST statistical approach, incorporates both error rates in the analysis, the RP determinations can rely on a direct calculation of the percent effect at the IWC. Thus, the RP procedures are much simpler to use than the RP statistical procedures recommended in the TSD.

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APPENDIX A

STEP-BY-STEP PROCEDURES FOR ANALYZING VALID WHOLE EFFLUENT TOXICITY DATA USING THE TEST OF SIGNIFICANT TOXICITY APPROACH

APPENDIX A: STEP-BY-STEP PROCEDURES FOR ANALYZING VALID WET DATA USING THE TST APPROACH

The following is a step-by-step guide for using the TST approach to analyze valid WET data for the NPDES Program. This guide is applicable for a two-concentration valid WET data analysis of an in-stream waste concentration (IWC) or a receiving water concentration (RWC) compared to a control concentration. For further information regarding conducting WET tests and proper quality assurance/quality control needed, see the EPA WET test method manuals. Refer to the flowchart shown in Figure A-1 in this appendix as you proceed through this guide.

Step 1: Conduct WET test following procedures in the appropriate EPA WET test method manual. That includes following all test requirements specified in the method (USEPA 1995 for chronic west coast marine methods, USEPA 2002a for chronic freshwater test methods, USEPA 2002b for chronic east coast marine test methods, and USEPA 2002c for acute freshwater and marine WET test methods).

Step 2: For each test endpoint specified in the WET test method manual (e.g., survival and reproduction for the *Ceriodaphnia* chronic WET test method), follow Steps 3–7 below. Note that the guide refers to an effluent concentration tested, which is assumed to be the IWC as specified in the permit or a receiving water concentration for ambient testing. For example, if no mixing zone is allocated, the IWC is 100 percent effluent.

Note: If there is no variance (i.e., zero variance) in the endpoint in both concentrations being compared (i.e., all replicates in each concentration have the same exact response), then skip the remaining steps in the flowchart and do the following. Compute the percent difference between the control and the other concentration (e.g., IWC) and compare the percent difference against the RMD values of 25% for chronic and 20% for acute endpoints. Percent mean effect is calculated as:

$$\%\ \text{Effect at IWC} = \frac{\text{Mean Control Response} - \text{Mean Response at IWC}}{\text{Mean Control Response}} \times 100$$

If the percent mean response is $\geq$ the RMD, the sample is declared toxic and the test is "*Fail*". If the percent mean response is < the RMD, the sample is declared non-toxic and the test is "*Pass*".

Step 3: For data consisting of proportions from a binomial (response/no response; live/dead) response variable, the variance within the i^{th} treatment is proportional to $P_i (1 - P_i)$, where P_i is the expected proportion for the treatment. That clearly violates the homogeneity of variance assumption required by parametric procedures such as the TST procedure because the existence of a treatment effect implies different values of P_i for different treatments, i. Also, when the observed proportions are based on small samples, or when P_i is close to zero or one, the normality assumption might be invalid. The arcsine square root (arcsine $\sqrt{P}$) transformation is used for such data to stabilize the variance and satisfy the normality requirement. The square root of percent data (e.g., percent survival, percent fertilization), expressed as a decimal fraction (where 1.00 = 100 percent) for each treatment, is first calculated. The square root value is then

arcsine transformed before analysis in Step 4. Note: Excel and most statistical software packages can calculate arcsine values.

Step 4: Conduct Welch's t-test (Zar 1996) using Equation 1:

Equation 1

$$t = \frac{\overline{Y}_t - b \times \overline{Y}_c}{\sqrt{\frac{S_t^{\,2}}{n_t} + \frac{b^2 S_c^{\,2}}{n_c}}}$$

where

$\overline{Y}_c$ = Mean for the control

$\overline{Y}_t$ = Mean for the IWC

s_c^2 = Estimate of the variance for the control

s_t^2 = Estimate of the variance for the IWC

n_c = Number of replicates for the control

n_t = Number of replicates for the IWC

b = 0.75 for chronic test methods; 0.80 for acute test methods

Note on the use of Welch's t-test: Welch's t-test is appropriate to use when there are an unequal number of replicates between control and the IWC. When sample sizes of the control and treatment are the same (i.e., $n_t = n_{c)}$, Welch's t-test is equivalent to the usual Student's t-test (Zar 1996).

Step 5: Adjust the degrees of freedom (df) using Equation 2:

Equation 2

$$\upsilon = \frac{\left(\frac{S_t^{\,2}}{n_t} + \frac{b^2 S_c^{\,2}}{n_c}\right)^2}{\frac{\left(\frac{S_t^{\,2}}{n_t}\right)^2}{n_t - 1} + \frac{\left(\frac{b^2 S_c^{\,2}}{n_c}\right)^2}{n_c - 1}}$$

For tests using Welch's t-test, df is the value obtained for *v* in Equation 2 above. Because *v is* most likely a non-integer, round *v* to the next smallest integer, and that number is the df.

Step 6: Using the calculated *t* value from Step 4, compare that *t* value with the critical *t* value table in Appendix B using the test method-specific alpha values shown in Table A-1. To obtain the correct critical *t* value, look across the table for the alpha value that corresponds to the WET test method (for the alpha value, see Appendix A, Table A-1) and then look down the table for the appropriate df.

Step 7: If the calculated *t* value is less than the critical *t* value, the IWC is declared toxic and the test result is *Fail*. If the calculated *t* value is greater than the critical *t* value, the IWC is not declared toxic and the test result is *Pass*.

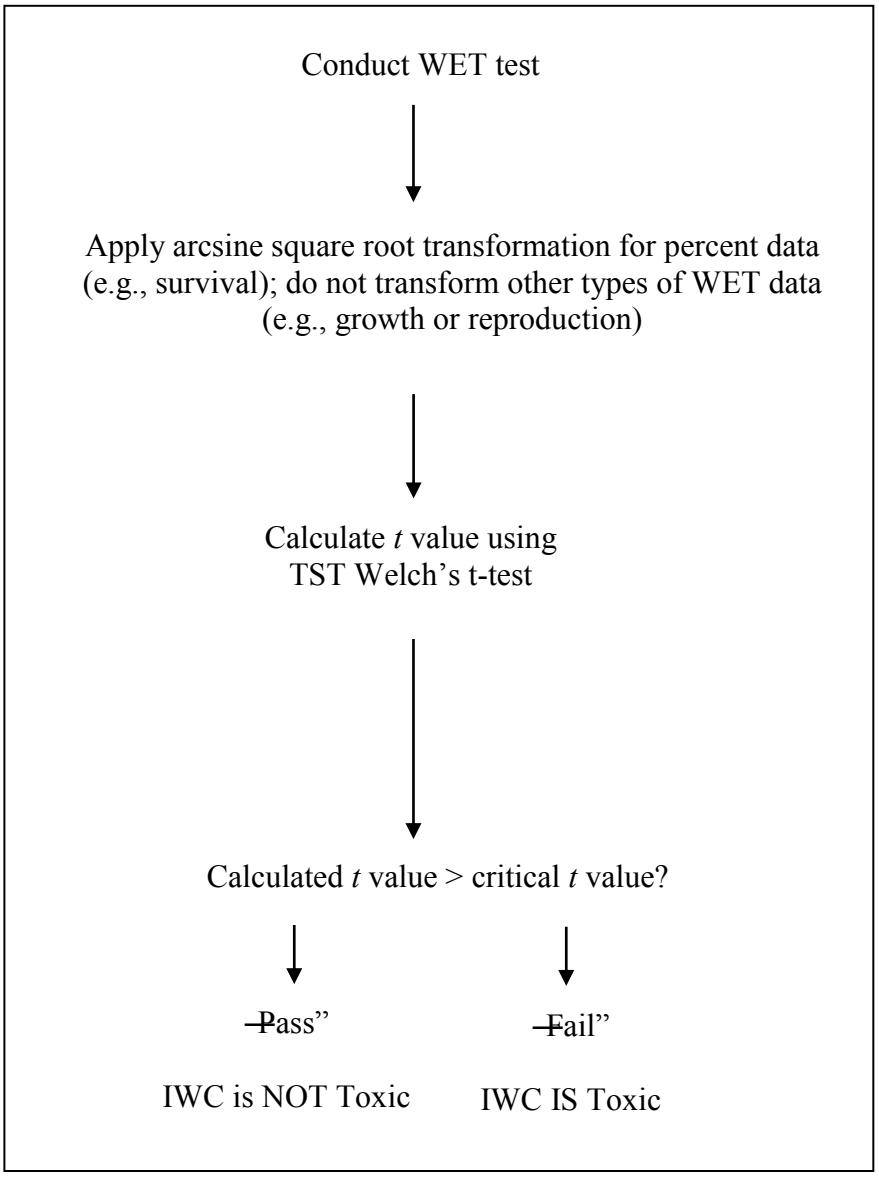

Figure A-1. Statistical flowchart for analyzing valid WET data using the TST approach for control and the IWC, receiving water, or stormwater.

Table A-1. Summary of alpha (α) levels or false negative rates recommended for different WET test methods using the TST approach

EPA WET test method	*b* value	Probability of declaring a toxic effluent non-toxic False negative (α) error[a]
Chronic Freshwater and East Coast Methods		
Ceriodaphnia dubia (water flea) survival and reproduction	0.75	0.20
Pimephales promelas (fathead minnow) survival and growth	0.75	0.25
Selenastrum capricornutum (green algae) growth	0.75	0.25
Americamysis bahia (mysid shrimp) survival and growth	0.75	0.15
Arbacia punctulata (Echinoderm) fertilization	0.75	0.05
Cyprinodon variegatus (Sheepshead minnow) and *Menidia beryllina* (inland silverside) survival and growth	0.75	0.25
Chronic West Coast Marine Methods		
Dendraster excentricus and *Strongylocentrotus purpuratus* (Echinoderm) fertilization	0.75	0.05
Atherinops affinis (topsmelt) survival and growth	0.75	0.25
Haliotis rufescens (red abalone), *Crassostrea gigas (oyster), Dendraster excentricus, Strongylocentrotus purpuratus* (Echinoderm) and *Mytilus sp* (mussel) larval development methods	0.75	0.05
Macrocystis pyrifera (giant kelp) germination and germ-tube length	0.75	0.05
Acute Methods		
Pimephales promelas (fathead minnow), *Cyprinodon variegatus* (Sheepshead minnow), *Atherinops affinis* (topsmelt), *Menidia beryllina* (inland silverside) acute survival[b]	0.80	0.10
Ceriodaphnia dubia, *Daphnia magna, Daphnia pulex*, *Americamysis bahia* acute survival[b]	0.80	0.10

Notes:

a. (1) declare a sample toxic at least 75 percent of the time (alpha $\leq$ 0.25) when there is unacceptable toxicity (20 percent effect for acute and 25 percent effect for chronic test methods) and (2) declare an effluent non-toxic no more than 5 percent of the time (beta $\leq$ 0.05) when the mean effect at the critical effluent concentration is 10 percent for both acute and chronic WET tests (including sublethal endpoints). For more discussion on the RMDs, see Section 2.1 of this document.

b. Based on four replicate test design

Case Example 1: Chronic *Ceriodaphnia* Reproduction Test with High Within-Test Variability

Step 1: Conduct WET test

Replicate/statistic	Control	Treatment
1	27	32
2	38	28
3	27	25
4	34	28
5	37	20
6	35	15
7	30	27
8	31	31
9	36	31
10	39	30
Mean	33.4	26.7
Std. deviation	4.402	5.417
N (# of replicates)	**10**	**10**

Step 2: Follow Steps 3–7 for each endpoint required in the test method

The following example is for chronic *Ceriodaphnia dubia* reproduction endpoint only.

Step 3: Transform data using an arcsine square root transformation, if necessary

Not necessary because reproduction is not percent data.

Step 4: Conduct Welch's t-test

$$t = \frac{\overline{Y}_t - b * \overline{Y}_c}{\sqrt{\frac{S_t^2}{n_t} + \frac{b^2 S_c^2}{n_c}}} = \frac{26.7 - (0.75 \times 33.4)}{\sqrt{\frac{29.34}{10} + \frac{(0.75)^2 (19.38)}{10}}} = 0.82$$

Step 5: Adjust the df

$$v = \frac{\left(\frac{S_t^2}{n_t} + \frac{b^2 S_c^2}{n_c}\right)^2}{\frac{\left(\frac{S_t^2}{n_t}\right)^2}{n_t - 1} + \frac{\left(\frac{b^2 S_c^2}{n_c}\right)^2}{n_c - 1}} = \frac{\left(\frac{29.34}{10} + \frac{(0.75)^2 (19.38)}{10}\right)^2}{\frac{\left(\frac{29.34}{10}\right)^2}{10 - 1} + \frac{\left(\frac{(0.75)^2 (19.38)}{10}\right)^2}{10 - 1}} = 15$$

Step 6: Calculated *t* value > critical *t* value? 15 df and test method alpha = 0.20 (Table A-1)

Critical *t* value = 0.87

0.82 < 0.87

Step 7: Declare effluent toxic or not

Calculated *t* < critical *t* value. Therefore, ***effluent is declared toxic; test result is FAIL***.

Case Example 2: Chronic *Ceriodaphnia* Reproduction Test with Low Within-Test Variability

Step 1: Conduct WET test

Replicate/statistic	Control	Treatment
1	29	31
2	38	28
3	31	25
4	34	28
5	36	22
6	35	21
7	30	27
8	31	26
9	36	29
10	34	30
Mean	33.4	26.7
Std. deviation	2.989	3.268
N (# of replicates)	10	10

Step 2: Follow Steps 3–7 for each endpoint required in the test method

The following example is for chronic *Ceriodaphnia dubia* reproduction endpoint only.

Step 3: Transform data using an arcsine square root transformation, if necessary

Not necessary because reproduction is not percent data.

Step 4: Conduct Welch's t-test

$$t = \frac{\overline{Y}_t - b \times \overline{Y}_c}{\sqrt{\frac{S_t^2}{n_t} + \frac{b^2 S_c^2}{n_c}}} = \frac{26.7 - (0.75 \times 33.4)}{\sqrt{\frac{10.68}{10} + \frac{(0.75)^2\ (8.93)}{10}}} = 1.32$$

Step 5: Adjust the df

$$v = \frac{\left(\frac{S_t^2}{n_t} + \frac{b^2 S_c^2}{n_c}\right)^2}{\frac{\left(\frac{S_t^2}{n_t}\right)^2}{n_t - 1} + \frac{\left(\frac{b^2 S_c^2}{n_c}\right)^2}{n_c - 1}} = \frac{\left(\frac{10.68}{10} + \frac{(0.75)^2 (8.93)}{10}\right)^2}{\frac{\left(\frac{10.68}{10}\right)^2}{10 - 1} + \frac{\left(\frac{(0.75)^2\ (8.93)}{10}\right)^2}{10 - 1}} = 16$$

Step 6: Calculated *t* value > critical *t* value? 16 df and test method alpha = 0.20 (Table A-1)

Critical *t* value = 0.86

1.32 > 0.86

Step 7: Declare effluent toxic or not

Calculated *t* > critical *t* value. Therefore, ***effluent is declared Non-Toxic; test result is PASS***.

Case Example 3: Benefit of Increased Replication in Chronic Fish Growth Test with Low Mean Effect and High Within-Test Variability

Step 1: Conduct WET test

Replicate/statistic	Control	Treatment
1	0.366	0.303
2	0.399	0.379
3	0.354	0.311
4	0.422	0.236
Mean	0.385	0.307
Std. deviation	0.031	0.058
N (# of replicates)	**4**	**4**

Step 2: Follow Steps 3–7 for each endpoint required in the test method

Step 3: Transform data using an arcsine square root transformation, if necessary

Not necessary because growth is not percent data.

Step 4: Conduct Welch's t-test

$$t = \frac{\overline{Y}_t - b \times \overline{Y}_c}{\sqrt{\frac{S_t^2}{n_t} + \frac{b^2 S_c^2}{n_c}}}$$

$$= \frac{0.307 - (0.75 \times 0.385)}{\sqrt{\left(\frac{0.00342}{4} + \frac{(0.75)^2\ (0.00096)}{4}\right)}} = 0.58$$

Step 5: Adjust the df

$$v = \frac{\left(\frac{S_t^2}{n_t} + \frac{b^2 S_c^2}{n_c}\right)^2}{\frac{\left(\frac{S_t^2}{n_t}\right)^2}{n_t - 1} + \frac{\left(\frac{b^2 S_c^2}{n_c}\right)^2}{n_c - 1}}$$

$$= \frac{\left(\frac{0.00342}{4} + \frac{(0.75)^2\ (0.00096)}{4}\right)^2}{\frac{\left(\frac{0.00342}{4}\right)^2}{4-1} + \frac{\left(\frac{(0.75)^2\ (0.00096)}{4}\right)^2}{4-1}} = 4$$

Step 6: Calculated *t* value > critical *t* value? 4 df, alpha = 0.25 (Table A-1); Critical *t* value = 0.74

0.58 < 0.74

Step 7: *Effluent is declared toxic, test result is FAIL.*

Step 1: Conduct WET test

Replicate/statistic	Control	Treatment
1	0.366	0.303
2	0.399	0.379
3	0.354	0.311
4	0.422	0.236
5	0.343	0.364
6	0.407	0.247
Mean	0.382	0.307
Std. deviation	0.032	0.058
N (# of replicates)	**6**	**6**

Step 2: Follow Steps 3–7 for each endpoint required in the test method

Step 3: Transform data using an arcsine square root transformation, if necessary

Not necessary because growth is not percent data.

Step 4: Conduct Welch's t-test

$$t = \frac{\overline{Y}_t - b \times \overline{Y}_c}{\sqrt{\frac{S_t^2}{n_t} + \frac{b^2 S_c^2}{n_c}}}$$

$$= \frac{0.307 - (0.75 \times 0.382)}{\sqrt{\frac{0.00342}{6} + \frac{(0.75)^2 (0.00101)}{6}}} = 0.79$$

Step 5: Adjust the df

$$v = \frac{\left(\frac{S_t^2}{n_t} + \frac{b^2 S_c^2}{n_c}\right)^2}{\frac{\left(\frac{S_t^2}{n_t}\right)^2}{n_t - 1} + \frac{\left(\frac{b^2 S_c^2}{n_c}\right)^2}{n_c - 1}}$$

$$= \frac{\left(\frac{0.00342}{6} + \frac{(0.75)^2\ (0.00101)}{6}\right)^2}{\frac{\left(\frac{0.00342}{6}\right)^2}{6-1} + \frac{\left(\frac{(0.75)^2\ (0.00101)}{6}\right)^2}{6-1}} = 7$$

Step 6: Calculated *t* value > critical *t* value? 7 df, alpha = 0.25 (Table A-1); Critical *t* value = 0.71

0.79 > 0.71

Step 7: *Effluent is declared Non-Toxic; test result is PASS.*

Case Example 4: Fish Acute Toxicity Test Example

Step 1: Conduct WET test

Replicate/statistic	Control	Treatment
1	10	10
2	10	8
3	10	9
4	10	8
Mean	10	8.75
Variance	0.000	0.917
N (# of replicates)	**4**	**4**

Step 2: Follow Steps 3–7 for each endpoint required in the test method

The following example is for acute *Pimephales promelas* survival endpoint only.

Step 3: Transform data using an arcsine square root transformation

Replicate/statistic	Control	Treatment
1	1.412	1.412
2	1.412	1.107
3	1.412	1.249
4	1.571	1.107
Mean	1.412	1.218
Variance	0.000	0.021
N (# of replicates)	**4**	**4**

Step 4: Conduct Welch's t-test

$$t = \frac{\overline{Y}_t - b \times \overline{Y}_c}{\sqrt{\frac{S_t^2}{n_t} + \frac{b^2 S_c^2}{n_c}}} = \frac{1.218 - (0.80 \times 1.412)}{\sqrt{\frac{0.021}{4} + \frac{(0.80)^2\ (0.000)}{4}}} = 1.2297$$

Step 5: Adjust the df

$$v = \frac{\left(\frac{S_t^2}{n_t} + \frac{b^2 S_c^2}{n_c}\right)^2}{\frac{\left(\frac{S_t^2}{n_t}\right)^2}{n_t - 1} + \frac{\left(\frac{b^2 S_c^2}{n_c}\right)^2}{n_c - 1}} = \frac{\left(\frac{0.021}{4} + \frac{(0.80)^2\ (0.000)}{4}\right)^2}{\frac{\left(\frac{0.021}{4}\right)^2}{4-1} + \frac{\left(\frac{(0.80)^2\ (0.000)}{4}\right)^2}{4-1}} = 3$$

Step 6: Calculated *t* value > critical *t* value? 3 df, alpha = 0.10 (Table A-1)

Critical *t* value = 1.64

1.229 < 1.64

Step 7: Declare effluent toxic or not

Therefore, ***effluent is declared toxic; test result is FAIL***.

Literature Cited

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APPENDIX B

CRITICAL *t* VALUES FOR THE TEST OF SIGNIFICANT TOXICITY APPROACH

Table B-1. Critical values of the *t* distribution. One tail probability is assumed.

Degrees of freedom	Alpha				
	0.25	**0.20**	**0.15**	**0.10**	**0.05**
1	1	1.3764	1.9626	3.0777	6.3138
2	0.8165	1.0607	1.3862	1.8856	2.92
3	0.7649	0.9785	1.2498	1.6377	2.3534
4	0.7407	0.941	1.1896	1.5332	2.1318
5	0.7267	0.9195	1.1558	1.4759	2.015
6	0.7176	0.9057	1.1342	1.4398	1.9432
7	0.7111	0.896	1.1192	1.4149	1.8946
8	0.7064	0.8889	1.1081	1.3968	1.8595
9	0.7027	0.8834	1.0997	1.383	1.8331
10	0.6998	0.8791	1.0931	1.3722	1.8125
11	0.6974	0.8755	1.0877	1.3634	1.7959
12	0.6955	0.8726	1.0832	1.3562	1.7823
13	0.6938	0.8702	1.0795	1.3502	1.7709
14	0.6924	0.8681	1.0763	1.345	1.7613
15	0.6912	0.8662	1.0735	1.3406	1.7531
16	0.6901	0.8647	1.0711	1.3368	1.7459
17	0.6892	0.8633	1.069	1.3334	1.7396
18	0.6884	0.862	1.0672	1.3304	1.7341
19	0.6876	0.861	1.0655	1.3277	1.7291
20	0.687	0.86	1.064	1.3253	1.7247
21	0.6864	0.8591	1.0627	1.3232	1.7207
22	0.6858	0.8583	1.0614	1.3212	1.7171
23	0.6853	0.8575	1.0603	1.3195	1.7139
24	0.6849	0.8569	1.0593	1.3178	1.7109
25	0.6844	0.8562	1.0584	1.3163	1.7081
26	0.684	0.8557	1.0575	1.315	1.7056
27	0.6837	0.8551	1.0567	1.3137	1.7033
28	0.6834	0.8546	1.056	1.3125	1.7011
29	0.683	0.8542	1.0553	1.3114	1.6991
30	0.6828	0.8538	1.0547	1.3104	1.6973
inf	0.6745	0.8416	1.0364	1.2816	1.6449

APPENDIX C

APPLICATION OF THE TEST OF SIGNIFICANT TOXICITY APPROACH TO AMBIENT TOXICITY PROGRAMS

APPENDIX C: APPLICATION OF THE TST APPROACH TO AMBIENT TOXICITY PROGRAMS

In ambient and stormwater toxicity testing, a laboratory control and a single concentration (i.e., 100 percent ambient water or stormwater) are often tested. In these two-concentration WET tests, the objective is to determine if a given sample or site water is toxic, as indicated by a significantly different organism response compared to the control. In the WET testing design, the determination of Pass or Fail (i.e., non-toxic or toxic) is ascertained using a traditional t-test (USEPA 2002c). EPA test methods recommend (USEPA 1995, 2002a, 2002b, 2002c) that the statistical significance (i.e., Pass/Fail) of a two-sample test design for ambient and stormwater toxicity testing be determined only using either a modified t-test (if homogeneity of variance is not achieved) or a traditional t-test (if homogeneity of variance is achieved).

To demonstrate the value of the TST approach in ambient toxicity programs, ambient toxicity test data from California's Surface Water Ambient Monitoring Program (SWAMP) was used for 409 chronic tests for *Ceriodaphnia dubia* and 256 chronic tests for *Pimephales promelas* using EPA's 2002 WET test methods (USEPA 2002a). Valid WET data for each EPA WET test method were subjected to the same statistical analyses as described in Section 2 of this document.

Chronic *Ceriodaphnia dubia* Ambient Toxicity Tests

Table C-1 summarizes results of the 409 *Ceriodaphnia dubia* ambient toxicity tests analyzed and an $\alpha = 0.20$ for this test method. Although the majority of the tests examined resulted in the same decision using either the TST or the traditional t-test approach, approximately 6 percent of the tests (24 tests) would have been declared non-toxic using the traditional t-test approach with mean effect levels $\geq$ 25 percent. In addition, 2 percent of the tests (7 tests) would have been declared toxic using the traditional t-test approach at mean effect levels < 15 percent and as low as 7 percent.

Table C-1. Comparison of results of chronic *Ceriodaphnia* ambient toxicity tests using the TST approach and the traditional t-test analysis. $\alpha = 0.20$ and *b* value = 0.75 for the TST approach. $\alpha = 0.05$ for the traditional hypothesis testing approach

Both approaches declare toxic	Only TST declares toxic	Only traditional approach declares toxic	Both approaches declare non-toxic
19.8%	5.9%	1.7%	72.6%

Figure C-1 shows ranges of CV values observed in *Ceriodaphnia dubia* ambient toxicity tests for those samples declared toxic using either the TST approach or the traditional t-test, but not both approaches. As expected, within-test variability was relatively high (higher CVs) for those tests found non-toxic using a t-test but toxic using the TST approach. The results demonstrate the lack of control of false negative rates using the traditional hypothesis testing approach when control variability is relatively high. Under those conditions, the traditional t-test did not have the power to detect toxicity when it was present. Figure C-1 also demonstrates that the TST approach recognizes a negligible effect as non-toxic when within-test variability is relatively low and the

mean percent effect is well below the risk management level of 25 percent. Under such conditions, the traditional t-test declared some samples toxic using this WET test method, even when the mean effect was as little as 7 percent. The TST approach, however, declared all such samples non-toxic using the recommended $\alpha = 0.20$. Thus, the TST approach reduces the number of tests declared as toxic when effects are actually well below the risk management decision.

Chronic *Ceriodaphnia* ambient WET tests that are identified as non-toxic (Pass) using the traditional hypothesis approach (NOEC) generally have high within-test variability (high control CVs) as compared to using the TST approach.

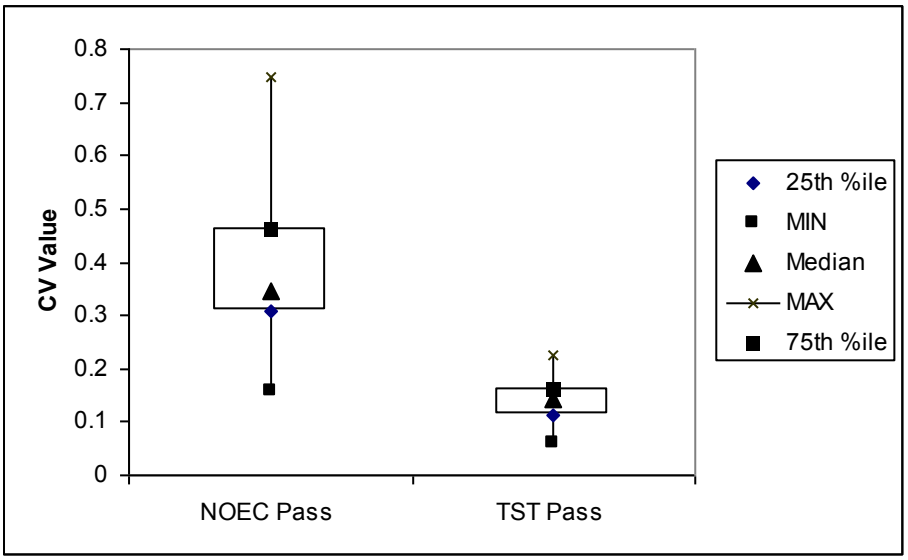

Figure C-1. Range of CV values observed in chronic *C. dubia* ambient toxicity tests for samples that were found to be non-toxic using the standard t-test but toxic using the TST approach (NOEC Pass) and for those samples declared toxic using t-test but not the TST approach (TST Pass). California's SWAMP WET test data.

Similar to the *Ceriodaphnia* ambient test data, within-test variability was higher in those chronic fathead minnow ambient tests found *non-toxic* using a t-test but toxic using the TST approach (Figure C-2). Similarly, those tests declared *non-toxic* by the TST approach but toxic using t-test had lower within-test variability and mean effect levels < 25 percent (Figure C-2). Thus, similar to the chronic *Ceriodaphnia* ambient tests, data from chronic fathead minnow ambient tests demonstrate that the TST approach can provide as much protection as the traditional t-test approach while also identifying those samples that are truly acceptable from a regulatory management decision.

Fish ambient WET tests that are identified as non-toxic using the traditional hypothesis approach (NOEC) generally have high within-test variability (high control CVs) as compared to using the TST approach.

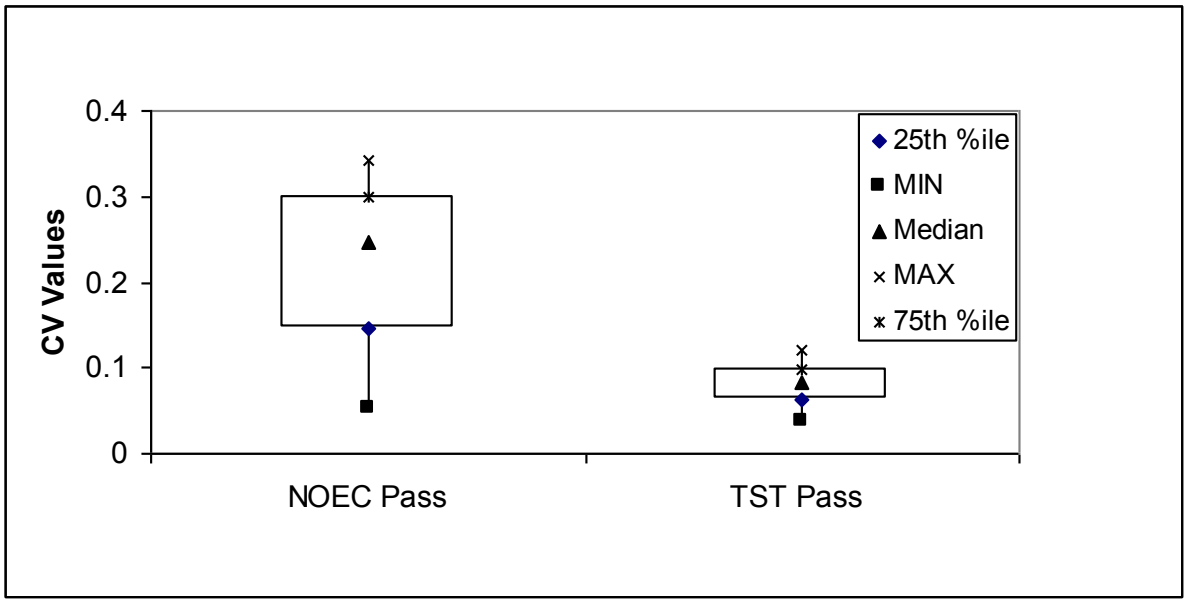

Figure C-2. Range of CV values observed in chronic *P. promelas* ambient toxicity tests for samples that were declared to be non-toxic using the standard t-test but toxic using the TST approach (NOEC Pass) and for those samples declared toxic using t-test but not the TST approach (TST Pass). California's SWAMP WET test data.

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APPENDIX D

EXAMPLE NATIONAL POLLUTANT DISCHARGE ELIMINATION SYSTEM PERMIT LANGUAGE USING THE TEST OF SIGNIFICANT TOXICITY APPROACH

APPENDIX D: EXAMPLE NPDES PERMIT LANGUAGE USING THE TST APPROACH

ACUTE WHOLE EFFLUENT TOXICITY (WET) NPDES PERMIT LANGUAGE

xx. <u>Acute Whole Effluent Toxicity (WET) Requirements</u>

1. Monitoring Frequency

The permittee must conduct ***monthly/quarterly/semiannual*** acute toxicity tests on 24-hour composite effluent samples. Once each calendar year, at a different time of year from the previous years, the permittee must split a 24-hour composite effluent sample and concurrently conduct two toxicity tests using a fish and an invertebrate species; the permittee must then continue to conduct routine ***monthly/quarterly/semiannual*** toxicity testing using the single, most sensitive species.

Acute toxicity test samples must be collected for each point of discharge at the designated NPDES sampling station for the effluent (i.e., downstream from the last treatment process and any in-plant return flows where a representative effluent sample can be obtained). During years ***1, 2, 3, 4, and 5*** of the permit, a split of each sample must be analyzed for all other monitored parameters at the minimum frequency of analysis specified by the effluent monitoring program.

2. Freshwater Species and WET Test Methods

Species and short-term WET test methods for estimating the acute toxicity of NPDES effluents are in the fifth edition of *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA/821/R-02/012, 2002; Table IA, 40 CFR Part 136). The permittee must conduct 96-hour static renewal toxicity tests with the following vertebrate and invertebrate species, respectively:

- ***Vertebrate:*** The fathead minnow, *Pimephales promelas* (Acute Toxicity Test Method 2000.0)
- ***Invertebrate:*** The daphnid, *Ceriodaphnia dubia* (Acute Toxicity Test Method 2002.0)

3. Acute WET Permit Triggers

a. There are no acute toxicity effluent limits for this discharge. For this permit, the determination of Pass or Fail from a multiple-effluent concentration acute toxicity test at the IWC is determined using the Test of Significant Toxicity (TST) approach that is described in the *National Pollutant Discharge Elimination System Test of Significant Toxicity Implementation Document* (EPA/833/R-10-003). The acute WET permit trigger is any one WET test where a test result is *Fail* (during the monthly reporting period) at the acute in-stream waste concentration (IWC). For this discharge, the IWC is **XXX** percent (e.g., either is 100 percent or an effluent at the mixing zone to be determined at the time of permit issuance) effluent. To calculate

either a Pass or Fail of a multiple-effluent concentration acute toxicity test at the IWC, follow the instructions in Appendix A in the *National Pollutant Discharge Elimination System Test of Significant Toxicity Implementation Document*. A *Pass* result indicates no toxicity of the multiple-effluent concentration test at the IWC, and a *Fail* result indicates toxicity of the multiple-effluent concentration test at the IWC. The permittee must report either a *Pass* or a *Fail* on the Discharge Monitoring Report (DMR) form. If a result is reported as Fail, the permittee must follow Section 6 (Accelerated Toxicity Testing and TRE/TIE Process) of this permit.

- OR -

3. Acute WET Permit Limit

b. There is an acute toxicity effluent limit for this discharge. For this permit, the determination of Pass or Fail from a multiple-effluent concentration acute toxicity test at the IWC is determined using the Test of Significant Toxicity (TST) approach which is described in the *National Pollutant Discharge Elimination System Test of Significant Toxicity Implementation Document* (EPA/833-R-10-003). The acute WET permit trigger is any one WET test where a test result is *Fail* (during the monthly reporting period) at the chronic in-stream waste concentration (IWC). For this discharge, the IWC is **XXX** percent (e.g., either is 100 percent or an effluent at the mixing zone to be determined at time of permit issuance) effluent. To calculate either a Pass or Fail of the multiple-effluent concentration acute toxicity test at the IWC, follow the instructions in Appendix A in the *National Pollutant Discharge Elimination System Test of Significant Toxicity Implementation Document*. A Pass result indicates no toxicity of the multiple-effluent concentration at the IWC and a Fail result indicates toxicity of the multiple-effluent concentration test at the IWC. The permittee must report either a *Pass* or a *Fail* on the DMR form. If a result is reported as Fail, the permittee must follow Section 6 (Accelerated Toxicity Testing and TRE/TIE Process) of this permit.

4. Quality Assurance – EPA WET Test Methods

a. Quality assurance measures, instructions, and other recommendations and requirements are in the EPA 2002 WET test methods manual previously referenced.

b. This permit is subject to a determination of Pass or Fail from a multiple-effluent concentration acute toxicity test at the IWC (for statistical flowchart and procedures, see Appendix A, Figure A-1 of the *National Pollutant Discharge Elimination System Test of Significant Toxicity Implementation Document*). The acute in-stream waste concentration (IWC) for this discharge is **XXX** percent effluent.

c. Effluent dilution water and control water should be prepared and used as specified in the EPA WET test methods manual *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA/821/R-02/012, 2002).

d. If organisms are not cultured in-house, concurrent testing with a reference toxicant must be conducted. If organisms are cultured in-house, monthly reference toxicant testing is sufficient. Reference toxicant tests and effluent toxicity tests must be conducted using the same test conditions (e.g., same test duration).

e. If either the reference toxicant or effluent toxicity tests do not meet all test acceptability criteria in the EPA WET test methods manual, the permittee must resample and retest within 14 days.

f. If the discharged effluent is chlorinated, chlorine must not be removed from the effluent sample before toxicity testing without written approval by the permitting authority.

5. Initial Investigation TRE Work Plan

Within 90 days of the permit effective date, the permittee must prepare and submit to the permitting authority a copy of its Initial Investigation Toxicity Reduction Evaluation (TRE) Work Plan (1–2 pages) for review. That plan must include steps the permittee intends to follow if toxicity is measured above an acute WET permit limit or trigger and should include the following, at minimum:

a. A description of the investigation and evaluation techniques that would be used to identify potential causes and sources of toxicity, effluent variability, and treatment system efficiency.

b. A description of methods for maximizing in-house treatment system efficiency, good housekeeping practices, and a list of all chemicals used in operations at the facility.

c. If a Toxicity Identification Evaluation (TIE) is necessary, an indication of who would conduct the TIEs (i.e., an in-house expert or outside contractor).

6. Accelerated Toxicity Testing and TRE/TIE Process

a. If an acute WET permit limit or trigger is exceeded and the source of toxicity is known (e.g., a temporary plant upset), the permittee must conduct one additional toxicity test using the same species and EPA WET test method. This WET test must begin within 14 days of receipt of WET test results exceeding an acute WET permit limit or trigger. If the additional toxicity test does not exceed an acute WET permit limit or trigger, the permittee may return to the regular testing frequency.

b. If an acute WET permit limit or trigger is exceeded and the source of toxicity is not known, the permittee must conduct six additional toxicity tests using the same species and EPA WET test method, approximately every two weeks, over a 12-week period. This testing must begin within 14 days of receipt of WET test results exceeding an acute WET permit limit or trigger. If none of the additional toxicity tests exceed an acute WET permit limit or trigger, the permittee may return to the regular testing frequency.

c. If one of the additional toxicity tests (in paragraphs 6.a or 6.b) exceeds an acute WET permit limit or trigger, within 14 days of receipt of this WET test result, the permittee must initiate a TRE using, according to the type of treatment facility, EPA WET TRE manual, *Toxicity Reduction Evaluation Guidance for Municipal Wastewater Treatment Plants* (EPA/833/B-99/002, 1999) or EPA WET TRE manual, *Generalized Methodology for Conducting Industrial Toxicity Reduction Evaluations* (EPA/600/2-88/070, 1989). In conjunction, the permittee must develop and implement a Detailed TRE Work Plan that must consist of the following: further actions undertaken by the permittee to investigate, identify, and correct the causes of toxicity; actions the permittee will take to mitigate the effects of the discharge and prevent the recurrence of toxicity; and a schedule for such actions.

d. The permittee may initiate a TIE as part of a TRE to identify the causes of toxicity using the same species and EPA WET test method and, as guidance, EPA WET TIE/TRE method manuals: *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures* (EPA/600/6-91/003, 1991); *Methods for Aquatic Toxicity Identification Evaluations, Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA/600/R-92/080, 1993); *Methods for Aquatic Toxicity Identification Evaluations, Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA/600/R-92/081, 1993).

7. Reporting of Acute Toxicity Monitoring Results

a. The permittee must submit a full laboratory report for all toxicity testing as an attachment to the Discharge Monitoring Report (DMR) for the month in which the toxicity test was conducted; the laboratory report must contain the following: the toxicity test results, the dates of sample collection and initiation of each toxicity test; all results for effluent parameters monitored concurrently with the toxicity test(s); and progress reports on TRE/TIE investigations.

b. The permittee must provide the actual test endpoint responses for the control (i.e., control mean) and IWC concentration (i.e., IWC mean) for each WET test conducted to make it easier for permit writers to find the necessary WET test results when determining WET RP.

c. The permittee must notify the permitting authority in writing within 14 days of exceedance of an acute WET permit limit or trigger. Such notification must describe actions the permittee has taken or will take to investigate, identify, and correct the causes of toxicity; the status of actions required by this permit; and schedule for actions not yet completed; or reason(s) that no action has been taken.

8. Permit Reopener for Acute Toxicity

In accordance with 40 CFR Parts 122 and 124, this permit may be modified to include effluent limitations or permit conditions to address acute toxicity in the effluent or receiving waterbody, as a result of the discharge; or to implement new, revised, or newly interpreted water quality standards applicable to acute toxicity.

CHRONIC WET NPDES PERMIT LANGUAGE

xx. Chronic Whole Effluent Toxicity (WET) Requirements

1. Monitoring Frequency

The permittee must conduct ***monthly/quarterly/semiannual*** chronic toxicity tests on 24-hour composite effluent samples. Once each calendar year, at a different time of year from the previous years, the permittee must split a 24-hour composite effluent sample and concurrently conduct three toxicity tests using a fish, an invertebrate, and an alga species; the permittee must continue to conduct routine ***monthly/quarterly/semiannual*** toxicity testing using the single, most sensitive species.

Chronic toxicity test samples must be collected for each point of discharge at the designated NPDES sampling station for the effluent (i.e., downstream from the last treatment process and any in-plant return flows where a representative effluent sample can be obtained). During years ***1, 2, 3, 4, and 5*** of the permit, a split of each sample must be analyzed for all other monitored parameters at the minimum frequency of analysis specified by the effluent monitoring program.

2. Freshwater Species and EPA WET Test Methods

Species and short-term EPA WET test methods for estimating the chronic toxicity of NPDES effluents are in the fourth edition of *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* (EPA/821/R-02/013, 2002; Table IA, 40 CFR Part 136). The permittee must conduct static renewal toxicity tests with the following:

- Fathead minnow, *Pimephales promelas* (Larval Survival and Growth Test Method 1000.0)
- Daphnid, *Ceriodaphnia dubia* (Survival and Reproduction Test Method 1002.0);
- Green alga, *Selenastrum capricornutum* (also named *Raphidocelis subcapitata*) (Growth Test Method 1003.0).

3. Chronic WET Permit Triggers

a. There are no chronic toxicity effluent limits for this discharge. The chronic WET permit trigger is any one WET test (either biological endpoint of survival or sublethal) where a test result is *Fail* (during the monthly reporting period) at the chronic in-stream waste concentration (IWC). For this discharge, the IWC is **XXX** percent (e.g., either is 100 percent or an effluent at the mixing zone to be determined at time of permit issuance) effluent. To calculate either a Pass or Fail of the multiple-effluent concentration chronic toxicity test at the IWC, follow the instructions in Appendix A in the *National Pollutant Discharge Elimination System Test of Significant Toxicity Implementation Document* (EPA/833-R-10-003). A Pass result indicates no toxicity at the IWC, and a Fail result indicates toxicity at the IWC. The

permittee must report either a Pass or a Fail on the DMR form. If a result is reported as Fail, the permittee must follow Section 7 (Reporting of Chronic Toxicity Monitoring Results) of this permit.

- OR -

3. Chronic WET Permit Limits

b. There is a chronic toxicity effluent limit for this discharge. The chronic WET permit trigger is any one WET test (either biological endpoint of survival or sublethal) where a test result is *Fail* (during the monthly reporting period) at the chronic in-stream waste concentration (IWC). For this discharge, the IWC is **XXX** percent (e.g., either is 100 percent or an effluent at the mixing zone to be determined at time of permit issuance) effluent. To calculate either a Pass or Fail of the multiple-effluent concentration chronic toxicity test at the IWC, follow the instructions in Appendix A in the *National Pollutant Discharge Elimination System Test of Significant Toxicity Implementation Document* (EPA/833-R-10-003). A Pass result indicates no toxicity at the IWC, and a Fail result indicates toxicity at the IWC. The permittee must report either a Pass or a Fail on the DMR form. If a result is reported as Fail, the permittee must follow Section 7 (Reporting of Chronic Toxicity Monitoring Results) of this permit.

4. Quality Assurance – EPA WET Test Methods

a. Quality assurance measures, instructions, and other recommendations and requirements are in the EPA WET test methods manual previously referenced in this permit.

b. This permit is subject to a determination of Pass or Fail from a multiple-effluent concentration chronic toxicity test at the IWC (for statistical flowchart and procedures, see *National Pollutant Discharge Elimination System Test of Significant Toxicity Implementation Document*, Appendix A, Figure A-1). The chronic in-stream waste concentration (IWC) for this discharge is **XXX** percent (e.g., either is 100 percent or an effluent at the mixing zone to be determined) effluent.

c. Effluent dilution water and control water should be standard synthetic dilution water as described in the EPA WET test methods manual, *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* (EPA/821/R-02/013, 2002). If the dilution water is different from test organism culture water, a second control using culture water must also be used.

d. If organisms are not cultured in-house, concurrent testing with a reference toxicant must be conducted. If organisms are cultured in-house, monthly reference toxicant testing is sufficient. Reference toxicant tests and effluent toxicity tests must be conducted using the same test conditions (e.g., same test duration).

e. If either the reference toxicant or effluent toxicity tests do not meet all test acceptability criteria in the EPA WET test methods manual, the permittee must resample and retest within 14 days.

f. Following Paragraph 10.2.6.2 of the freshwater EPA WET test methods manual, all chronic toxicity test results from the multi-concentration tests required by this permit must be reviewed and reported according to EPA guidance on the evaluation of concentration-response relationships in *Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing (40 CFR Part 136)* (EPA/821/B-00-004, 2000).

g. If the discharged effluent is chlorinated, chlorine must not be removed from the effluent sample before toxicity testing without written approval by the permitting authority.

5. Initial Investigation TRE Work Plan

Within 90 days of the permit effective date, the permittee must prepare and submit to the permitting authority a copy of its Initial Investigation Toxicity Reduction Evaluation (TRE) Work Plan (1–2 pages) for review. That plan must contain steps the permittee intends to follow if toxicity is measured above a chronic WET permit limit or trigger and should include the following, at minimum:

a. A description of the investigation and evaluation techniques that would be used to identify potential causes and sources of toxicity, effluent variability, and treatment system efficiency.

b. A description of methods for maximizing in-house treatment system efficiency, good housekeeping practices, and a list of all chemicals used in operations at the facility.

c. If a Toxicity Identification Evaluation (TIE) is necessary, an indication of who would conduct the TIEs (i.e., an in-house expert or outside contractor).

6. Accelerated Toxicity Testing and TRE/TIE Process

a. If a chronic WET permit limit or trigger is exceeded and the source of toxicity is known (e.g., a temporary plant upset), the permittee must conduct one additional toxicity test using the same species and EPA WET test method. This WET test must begin within 14 days of receipt of WET test results exceeding a chronic WET permit limit or trigger. If the additional toxicity test does not exceed a chronic WET permit limit or trigger, the permittee may return to their regular testing frequency.

b. If a chronic WET permit limit or trigger is exceeded and the source of toxicity is not known, the permittee must conduct six additional toxicity tests using the same species and EPA WET test method, approximately every two weeks, over a 12 week period. This testing must begin within 14 days of receipt of WET test results exceeding a chronic WET permit limit or trigger. If none of the additional toxicity tests exceed a chronic WET permit limit or trigger, the permittee may return to their regular testing frequency.

c. If one of the additional toxicity tests (in paragraphs 6.a or 6.b) exceeds a chronic WET permit limit or trigger, within 14 days of receipt of this WET test result, the permittee must initiate a TRE using as guidance, according to the type of treatment facility, the EPA TRE manual, *Toxicity Reduction Evaluation Guidance for Municipal Wastewater Treatment Plants* (EPA/ 833/B-99/002, 1999) or EPA TRE manual, *Generalized Methodology for Conducting Industrial Toxicity Reduction Evaluations* (EPA/600/2-88/070, 1989). In conjunction, the permittee must develop and implement a Detailed TRE Work Plan that must contain the following: further actions undertaken by the permittee to investigate, identify, and correct the causes of toxicity; actions the permittee will take to mitigate the effects of the discharge and prevent the recurrence of toxicity; and a schedule for such actions.

d. The permittee may initiate a TIE as part of a TRE to identify the causes of toxicity using the same species and EPA WET test method and, as guidance, EPA WET TIE/TRE method manuals: *Toxicity Identification Evaluation: Characterization of Chronically Toxic Effluents, Phase I* (EPA/600/6-91/005F, 1992); *Methods for Aquatic Toxicity Identification Evaluations, Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA/600/R-92/080, 1993); *Methods for Aquatic Toxicity Identification Evaluations, Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA/600/R-92/081, 1993).

7. Reporting of Chronic Toxicity Monitoring Results

a. The permittee must submit a full laboratory report as an attachment to the DMR for all toxicity testing for the month in which the toxicity test was conducted; the laboratory report must contain the following: the toxicity test results, the dates of sample collection and initiation of each toxicity test; all results for effluent parameters monitored concurrently with the toxicity test(s); and progress reports on TIE/TRE investigations.

b. The permittee must provide the actual test endpoint responses for the control (i.e., control mean) and IWC concentration (i.e., IWC mean) for each WET test conducted to make it easier for permit writers to find the necessary WET test results when determining WET RP.

c. The permittee must notify the permitting authority in writing within 14 days of exceedance of a chronic WET permit limit or trigger. The notification must describe actions the permittee has taken or will take to investigate, identify, and correct the causes of toxicity; the status of actions required by this permit; and schedule for actions not yet completed; or reason(s) that no action has been taken.

8. Permit Reopener for Chronic Toxicity

In accordance with 40 CFR Parts 122 and 124, this permit may be modified to include effluent limitations or permit conditions to address chronic toxicity in the effluent or receiving waterbody, as a result of the discharge; or to implement new, revised, or newly interpreted water quality standards applicable to chronic toxicity.

APPENDIX E

WHOLE EFFLUENT TOXICITY REASONABLE POTENTIAL ANALYSIS USING THE TEST OF SIGNIFICANT TOXICITY APPROACH

APPENDIX E: WET RP ANALYSIS USING THE TST APPROACH

For reasonable potential (RP) calculations using the TST approach, EPA recommends that permitting authorities use all the valid WET test data generated during the current permit term and any additional valid data that are submitted as part of the permit renewal application. The permitting authority should be using at least a minimum of four valid WET tests to address effluent representativeness using the TST RP approach. WET test data are then analyzed according to the TST approach using the IWC and control test concentrations for all valid WET test data available. For the RP approach, data sets with fewer than four valid WET data points should be assessed using EPA's Technical Support Document (TSD) RP approach because it addresses small WET data sets by incorporating an RP multiplying factor (see Section 3.2.2 of the TSD, p. 54) to account for effluent variability in small WET data sets.

EPA also recommends that states request that their permittees provide the actual test endpoint responses for the control (i.e., mean of control) and IWC concentration (i.e., mean of IWC) for each WET test conducted to make it easier for permit writers to find the necessary data with which to calculate WET RP with this approach. EPA recommends that permitting authorities decide up front which approach (the 1991 TSD approach, the TST approach, or another scientifically defensible approach that is sufficient to meet the statutory and regulatory requirements) they will incorporate and consistently use in their state's NPDES implementation procedures, including for their RP procedures. Permitting authorities should consistently use the selected WET statistical approach in all the state NPDES permits.

All valid WET test data are then analyzed according to the TST approach using the IWC and control test concentrations. If WET test data are available and the TST statistical approach indicates that the IWC is toxic in any WET test (~~"~~effluent **cause(s)** toxicity"), RP has been demonstrated (40 CFR 122.44(d)(1)(i)). For example, if results of five WET tests are available using the TST approach and the results are Pass, Pass, Fail, Pass, Pass, because at least one test was a Fail (i.e., TST declared the effluent toxic in at least one test), RP has been demonstrated.

To address concerns regarding the ~~"~~**potential** to cause or contribute to toxicity," a second assessment is applied to determine whether the effluent has RP even if all test results are *Pass* using the TST approach.

The current TST approach results in four outcomes with respect to RP at the IWC:

1. **Caused (effluent is toxic):** RP is demonstrated if any one test using the TST approach indicates a test result is *Fail* (i.e., using the statistical test (Appendix A) and *t* table (Appendix B), the test result is *Fail*; see Example A below in Table E-1);
2. **Potential to Cause:** Effluent has reasonable potential to cause (RP is demonstrated) if any test exhibits a mean effect at the IWC > 10 percent as compared to the mean control response, even if the test result is *Pass* using TST (see examples B-D, Table E-1); and
3. **No RP (effluent is non-toxic at the IWC):** Effluent does not cause or have reasonable potential to cause if the tests are each a *Pass* using the TST approach and the mean effect at the IWC is always ≤ 10 percent.

4. **Insufficient valid WET data (fewer than 4 tests or no data):** If fewer than four valid WET data are available, follow the TSD RP procedure for WET.

The second outcome is where the determination of RP is critical to demonstrate that the discharge has the reasonable *potential* to cause an excursion above the state toxicity water quality standards. In the TST approach, the regulatory management decision threshold for non-toxicity in WET tests under the NPDES WET Program is 10 percent mean effect at the IWC. At or below that mean effect level, the TST approach is designed to declare a WET test as non-toxic (i.e., *Pass*) most (at least 95 percent) of the time to help control for false positives. For purposes of RP assessment then, a 10 percent mean effect level at the IWC is used as a threshold, above which potential to cause is indicated, and the effluent has demonstrated RP. Any test with a mean effect at the IWC > 10 percent would demonstrate a potential for RP even if the TST test result is *Pass*. Equation E-1 below demonstrates how the effluent effect is calculated at the IWC.

$$\%\ \text{Effect at IWC} = \frac{\text{Mean Control Response} - \text{Mean Response at IWC}}{\text{Mean Control Response}} \times 100 \qquad \textbf{Equation E-1}$$

Table E-1. Examples illustrating the reasonable potential approach using TST and data from *Ceriodaphnia* chronic survival and reproduction WET tests

Example	Pass/Fail based on TST analysis	Mean control response	Mean response @ IWC	% effect at IWC	Reasonable potential?
A	Fail	26.3	17.0	35.4%	Yes
B	Pass	26.3	23.4	11.0%	Yes
C	Pass	28.6	22.0	23.1%	Yes
D	Pass	22.4	20.9	6.7%	No

United States
Environmental Protection
Agency

Office of Research and
Development
Washington, DC 20460

EPA/600/6-91/005F
May 1992

EPA

Toxicity Identification Evaluation:

Characterization of Chronically Toxic Effluents, Phase I

EPA/600/6-91/005F
May, 1992

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T.J. Norberg-King
Environmental Research Laboratory
Duluth, MN 55804

D.I. Mount,
J.R. Amato,
D.A. Jensen,
and
J.A. Thompson
ASci Corporation
Duluth, MN 55804

National Effluent Toxicity
Assessment Center
Technical Report 02-92

Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Duluth, MN 55804

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Foreword

This guidance document has been prepared to assist dischargers and/or their consultant laboratories in conducting chronic aquatic toxicity identification evaluations (TIEs). TIEs may be required by the state or federal agencies as a result of enforcement actions or as a condition of the discharger's National Pollutant Discharge Elimination System (NPDES) permit or may be conducted voluntarily by permittees. This document will assist the state and federal agencies and permittees in overseeing and determining the adequacy of the TIE in toxicity reduction evaluations (TREs).

This document discusses methods to characterize the chemical/physical nature of the constituents in effluents which cause their chronic toxicity. The general approach for toxicity identification evaluations is described in the document *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures* (EPA, 1988A; EPA, 1991A), hereafter referred to as the *"acute Phase I manual."* The acute Phase I manual provides much of the basis for the statements and guidance provided in this chronic Phase I characterization document. This chronic TIE manual and the acute Phase I manual should be used as companion documents, because all the guidance of the acute Phase I manual is not repeated here.

The general approach for the chronic characterization is divided into Tier 1 and Tier 2. Tier 1 consists of the EDTA and sodium thiosulfate additions, the graduated pH test, aeration and filtration manipulations, and the use of the C_{18} solid phase extraction (SPE) resin. For Tier 1, the tests are all done using the effluent sample without any pH adjustments (i.e., at the initial pH (pH *i*) of the effluent). Tier 2 manipulations are added when Tier 1 tests are not definitive in characterizing the toxicity. Tier 2 includes the aeration, filtration, and C_{18} SPE steps of Tier 1 performed at pH 3 and pH 10 and returned to pH *i* prior to testing.

The chronic Phase I procedures should provide information on whether the toxicants are volatile, chelatable, filterable, reducible, non-polar, or pH sensitive. These characteristics are indicated by comparing the results of toxicity tests conducted using unaltered and manipulated effluent samples. As with the acute TIE, the characterization results from the chronic TIE can be used for the treatability approach in a TRE (EPA, 1991A).

These chronic TIE methods are not written as rigid, required protocols, but rather as general guidance for conducting TIEs with effluents. These acute and chronic methods should also be applicable to samples from ambient waters, sediment pore and elutriate waters, and leachates. The methods to identify (Phase II; EPA, 1989A) and confirm (Phase III; EPA, 1989B) the cause of toxicity in effluent samples evaluated with the acute Phase I procedure are also applicable to effluent samples evaluated with this chronic Phase I procedure. The identification and confirmation documents are being revised (EPA, 1992A; EPA, 1992B) to reflect additional information from this manual and the revised acute Phase I manual (EPA, 1991A) to discuss the aspects of TIEs for both acute and chronic toxicity.

In September of 1991, we solicited peer-review comments until January 31, 1992 from all persons who obtained the document from any of the following locations: EPA's Office of Water, Washington, D.C., each EPA Regional Water Division Office, EPA's Environmental Research Laboratory-Duluth, MN, or EPA's Center for Environmental Research Information (CERI), Cincinnati, Ohio. Appropriate technical comments were incorporated into this manual.

Abstract

This manual is intended to provide guidance to aid dischargers in characterizing the type of toxicants that are causing chronic toxicity in industrial and municipal effluents. In a regulatory context, a toxicity identification evaluation (TIE) may be required as part of the National Pollutant Discharge Elimination System (NPDES) permit or as an enforcement action. TIEs may also be conducted by permittees on a volunteer basis to characterize their discharge toxicity.

The Phase I chronic toxicity methods are modified from those described in the acute Phase I TIE manual (EPA, 1988A; EPA, 1991A) and additional techniques are incorporated. This chronic Phase I manual describes procedures for characterizing the physical/chemical nature of toxicants in effluents that exhibit chronic toxicity to freshwater species, although many of the principles and procedures are similar for TIEs on marine species. Aliquots of effluent samples are manipulated and the resulting effect on toxicity measured. The objective is to characterize the toxicants so that appropriate analytical methods can be chosen to identify the toxicants.

The general approach to the chronic toxicity characterization is a two tiered approach, where usually Tier 1 is applied before proceeding to Tier 2. Tier 1 consists of filtration, aeration, use of additives to chelate or reduce the toxicants, minor pH adjustments, and use of a separation technique with the C_{18} solid phase extraction (SPE) resin. Each effluent is characterized in Tier 1 by performing the manipulations at the initial pH (pH *i*) of the effluent. Tier 2 consists of the Tier 1 manipulations combined with pH adjustments of additional aliquots of the effluent sample, and the Tier 2 characterization steps include aeration, filtration, and the C_{18} solid phase extraction of effluent samples adjusted to pH 3 and pH 10.

The Phase I characterization methods were developed for the short-term *"chronic"* test methods using two species, *Ceriodaphnia dubia* and the fathead minnow (*Pimephales promelas*) (EPA, 1989C). Chronic threshold levels for the various additives (sodium thiosulfate, EDTA, methanol) used in some of the characterization tests are provided for these species. Although developed for these species, the characterization techniques should be applicable to other species as well, provided threshold levels are established.

The guidance provided in this manual is intended to be supplemental to that given in the acute Phase I manual (EPA, 1991A). Sections of this chronic Phase I TIE manual discuss quality assurance, effluent handling, facilities and equipment, health and safety, dilution water, principles of the chronic TIE testing, and the Phase I characterization tests as a two tiered approach. The use of the whole effluent test as a *baseline test* (in manner similar to the acute Phase I characterization procedure), the appropriate treatment of dilution water for blanks and the toxic levels of the additives for two species are described. Use of short-cuts, reduced test volumes, reduced test duration, and a small number of replicates are discussed. The importance of sample type, frequency of sample collection and renewal, and descriptions of all manipulations are discussed, along with a section on the application of combining several of the characterization tests.

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Tables

Acknowledgments

Many people at the National Effluent Toxicity Assessment Center (NETAC) at the Environmental Research Laboratory-Duluth (ERL-Duluth) have provided assistance to produce this manual by performing the toxicity tests, chemical analyses, and data analyses as well as providing advice based on experience. This document is the result of the input by the NETAC group which has consisted of both federal and contract staff members, and includes Gary Ankley, Larry Burkhard, Liz Durhan, Don Mount, Shaneen Murphy, and Teresa Norberg-King (federal staff), and Joe Amato, Lara Andersen, Steve Baker, Tim Dawson, Nola Englehorn, Doug Jensen, Correne Jenson, Jim Jenson, Marta Lukasewycz, Liz Makynen, Greg Peterson, Mary Schubauer-Berigan, and Jo Thompson (contract staff). The toxicity test data generated for this document and the biological data upon which this report is based was produced by Doug Jensen, Jo Thompson, Tim Dawson, Greg Peterson, Nola Englehorn, Shaneen Murphy, Mary Schubauer-Berigan, Joe Amato, and Jim Jenson. The skillful assistance and dedication of Debra Williams and Jane Norlander (NETAC) in producing this document are gratefully acknowledged.

Comments were received from the following people and organizations: Charles Carry and LeAnne Hamilton for the County Sanitation Districts of Los Angeles County, Whittier, CA.; David Mount and J. Russell Hockett for ENSR Consulting and Engineering, Fort Collins, CO; Norman LeBlanc for Hampton Roads Sanitation District, Virginia Beach, VA; Charlie Webster, State of Ohio Environmental Protection Agency (EPA), Columbus, OH.; Michael Gallaway, State of Ohio EPA, Columbus, OH; Robert Berger for the Association of Metropolitan Sewerage Agencies (AMSA), Washington, D.C.; and Kerrie Schurr for EPA Region 10, Seattle, WA. We want to thank those individuals or organizations for reviewing the report, and in turn improving the document with their comments.

In the review comments, a suggestion was made to summarize all the effort on TIEs by government, state, academia, contract laboratories, and industries to date. While the TIEs at Duluth can be summarized, data from all the possible sources are difficult if not impossible to obtain. Contract laboratories and industrial data are protected for confidentiality and proprietary reasons, and information about the kinds of toxicants, the types of discharges, the time-frame for the TIE, and the costs are difficult to obtain. Numerous toxicity problems have been resolved as TIEs are initiated because of better plant operation. In fact, during a workshop (Aquatic Habitat Institute, 1992) held March 17 and 18, 1992 in Richmond, CA, these issues were discussed, and presenters of chronic TIE data indicated chronic TIEs have been much more successful than expected.

This work was supported in part by the Office of Water, Permit Division, Washington, D.C., through the backing of Rick Brandes and Jim Pendergast, who have provided strong support for the whole effluent water quality-based approach.

Section 1
Introduction

The United States Federal Water Pollution Control Act Amendments (commonly referred to as the Clean Water Act (CWA); (Public Law 92-500 of 1972) states that the discharge of toxic pollutants in toxic amounts is prohibited. In the CWA, the National Pollutant Discharge Elimination System (NPDES) was established; this system provides a mechanism whereby point source wastewater discharges are permitted. NPDES permits contain effluent limits that require baseline use of treatment technologies (best available technology). The technology-based limits are independent of receiving water impact, and additional water quality-based limits may be needed in order to meet the goal of the CWA of "*no toxics in toxic amounts.*" State narrative and state numerical water quality standards are used in conjunction with EPA's water quality criteria and other toxicity databases to determine the adequacy of the technology-based permit limits and the need for any additional water quality-based controls.

When limits were first written into the permits, they were based primarily on physical factors such as biological oxygen demand (BOD), suspended solids (SS), and color. Additional components were added in subsequent amendments to the CWA; for example, the list of 126 *"priority pollutants"* of which many or most were required to be monitored by the permittees. Water quality criteria were used to develop the water quality-based limits for these pollutants. However, water quality criteria or discharge limits exist for only a few of the thousands of chemicals in use.

An important objective of the NPDES program is the control of toxicity of discharges and to accomplish this objective, EPA uses an integrated water quality-based approach. Published water quality criteria are converted to standards that consist of both chemical-specific numeric criteria for individual toxics and narrative criteria. The states' narrative water quality criterion generally requires that the waters be free from oil, scum, floating debris, materials that will cause odors, materials that are unsightly or deleterious, materials that will cause a nuisance, or substances in concentrations that are toxic to aquatic life, wildlife or human health. Use of toxicity testing and whole effluent toxicity limitations is based on a state's narrative water quality criterion and in some cases, a state numeric criterion for toxicity.

EPA, in 1984, issued a policy statement (Federal Register, 1984) that recommends an *"integrated approach"* for controlling toxic pollutants. This *integrated approach* is referred to as the water quality-based approach and is described in detail in the Technical Support Document (hereafter referred to as the TSD; EPA, 1985A; EPA, 1991B). The control regulations for EPA (Federal Register 23868, 1989) establish specific requirements that the *integrated approach* be used for water quality-based toxics control. This *integrated approach* results in NPDES permit limits to control toxic pollutants through the use of both chemical-specific and whole effluent toxicity limitations as a means to protect both aquatic life and human health. This combination of chemical specific and whole effluent toxicity limitations is essential to the control of toxic pollutants. Once the permit limits are set, compliance is established through routine monitoring of effluent quality. In this manner, water quality-based limits (when following EPA, 1991B) will protect water quality and prevent the state water quality standards from being violated.

The whole effluent toxicity limitation aspect involves using acute and chronic toxicity tests to measure the toxicity of wastewaters. *Acute toxicity* refers to toxicity that occurs in a short period of time, operationally defined as 96 h or less. *Chronic toxicity* occurs as the result of long exposures in which sublethal effects (fertilization, growth, reproduction) are measured in addition to lethality. The chronic test is used to measure the effects of long-term exposure to chemicals, wastewaters, and leachates to aquatic organisms. True chronic toxicity tests include the life-cycle of the organism. For fish, the life-cycle test is infrequently conducted (Norberg-King, 1989A), and abbreviated test methods have been used to estimate chronic toxicity. These tests are the 7-d growth and survival test (EPA, 1989C), or the 32-d embryo-larval early life stage test (Norberg-King, 1989A). These tests rely on the most sensitive life-cycle stages (i.e., embryos and larval fish) to estimate chronic toxicity (McKim, 1977; Woltering 1983; Norberg-King, 1989A). Hereafter, chronic tests refer to the short-term tests that are described in the EPA manuals (EPA, 1992C; EPA, 1992D; EPA, 1989C; EPA, 1985C).

Toxicity is a useful parameter to protect receiving waters from potential impacts on water quality and designated uses caused by the mixture of toxic pollutants in wastewaters. EPA has published manuals which provide test methods for use of freshwater and marine organisms to determine acute and chronic toxicity of effluents. These manuals have been available since 1978 and 1985, respectively (EPA, 1978; EPA,

1985B; EPA, 1985C; EPA, 1988B; EPA, 1989C) and have been recently revised (EPA, 1991C; EPA, 1992C; EPA, 1992D). These methods are used by federal, state and local governments to assess toxicity and determine compliance of permitted point source discharges. Since the late 1970's, toxicity has been measured in wastewaters; permit writers began using toxicity limits in the early 1980's. With the increased use of toxicity testing, substantial numbers of unacceptably toxic effluents have been identified. Now, some permittees are required to perform *toxicity reduction evaluations* (TREs) as a condition of the NPDES permit. The TSD defines a TRE as "*a site specific study conducted in a stepwise process designed to identify the causative agents of effluent toxicity, isolate the sources of toxicity, evaluate the effectiveness of toxicity control options, and then confirm the reduction in effluent toxicity.*" *Toxicity identification evaluations* (TIEs), which are a part of the TRE, consist of methods to characterize (Phase I; EPA, 1988A; EPA, 1991A; EPA, 1991D), identify (Phase II; EPA, 1989A; EPA, 1992A), and confirm (Phase III; EPA, 1989B; EPA, 1992B) the cause of acute and chronic toxicity in effluents.

The TIE approach (EPA, 1988A; EPA, 1991A) relies on the use of organisms to detect the presence of toxicants in the effluent. Information about the physical/chemical characteristics of the effluent's toxicity is gained (by the various manipulations) and if possible the number of constituents in the effluent is reduced before any analyses begin. Using this approach, analytical problems can be simplified and the costs reduced. Toxicity throughout the TIE must be tracked to determine if the toxicity is consistently being caused by the same substance. Once the physical/chemical characteristics of toxicants are known, a better choice of analytical methods can be made. Knowledge of physical/chemical characteristics of any effluent is used for the treatability approach to TRE's (EPA, 1989D; EPA, 1989E).

As with the acute Phase I TIE approach, the chronic Phase I TIE is based on manipulations designed to alter a group of toxicants (such as oxidants, cationic metals, volatiles, or non-polar organics) so that toxicity is changed. Chronic toxicity tests are conducted after each manipulation to indicate the effect on the toxicity of the effluent. Based upon the manipulations that change toxicity, inferences about the chemical/physical characteristics of the toxicants can be made. Using several samples of the effluent for these characterization steps provides information on whether the nature of compounds causing the chronic toxicity remains consistent. The tests do not provide information on the variability of toxicants within a characterization group. From these data the toxicant characteristics can be identified as pH sensitive, filterable, volatile, soluble, degradable, reducible, or EDTA chelatable. Such information indicates how samples must be handled for analyses and which analytical methods should be used.

The recommended procedure is to concentrate on the characterization steps that are most clean-cut and have the major effect of reducing the toxicity in the effluent. If toxicity in every effluent sample is not caused by the same toxicant(s), the characterization tests should indicate if the type of toxicant(s) is the same or different. Once identification is initiated, and suspects identified, the varying causes of toxicity can be evaluated because the concentration of toxicants should be tracking with the toxicity. In the earlier version of this document (EPA, 1991D) we suggested that samples be subjected to Phase I techniques until no additional responses are found (which was suggested to be at least three samples). After conducting several Phase I evaluations for chronic toxicity, we have determined that if the effluents' toxicity is readily characterized after Phase I even with one sample it may be prudent to proceed with Phase II (EPA, 1992C) to measure the toxicant(s). Use of toxicity patterns as the TIE progresses can be helpful if patterns are tracked, beginning with the first samples. Following characterization, a decision is made to proceed with identification (Phase II; EPA, 1989A; EPA, 1992A) and confirmation (Phase III, EPA, 1989B; EPA, 1992B) or to conduct treatability studies where the identification of the specific toxicants (cf., acute treatability procedures (EPA, 1989D; EPA, 1989E)) is not made.

Chronic toxicity must be present frequently enough so that an adequate number of toxic samples can be obtained. Enough routine toxicity testing should be done on each effluent before a TIE is initiated (EPA, 1991B), to ensure that toxicity is consistently present. It is not important that the same amount of toxicity is present in each sample; in fact, variable levels of toxicity can assist in determining the cause of toxicity. If toxicity is not consistently present, when it occurs the toxicity can be pursued and if a toxicant(s) is suspected, the non-toxic samples may be used to eliminate suspects. One cannot assume that if the effluent showed acute toxicity and a TIE was completed, identifying the cause(s) of acute toxicity and action taken to remove the acute toxicant from the effluent, that the sublethal toxicity exhibited is due to the same compound.

Section 2
Quality Assurance, Health, and Safety, and Facilities and Equipment

2.1 Quality Assurance

The quality assurance plan (QAP), as described in *Standard Methods for the Examination of Water and Wastewater* (APHA, 1989) (describes standards to conduct performance evaluations) is primarily for analytical analyses. A QAP for toxicity testing can be developed, but determining the recovery of known additions for toxicity testing is not possible. For TIEs the combination of chemistry and biology requires a level of checks and balances not typically used under other situations. A step-by-step QAP for all steps of a TIE is not always possible due to the unknown toxicant(s) requiring various follow-up testing and analytical procedures; however as a TIE progresses, additional or different tests may be needed and many aspects of the TIE QAP can be addressed as the TIE proceeds. Adhering to the general guidelines of a strong QAP is important however, and should increase the probability of the TIE succeeding. As additional steps are recognized, the details should be added to the QAP.

Specific quality control (QC) procedures for aquatic toxicity tests are different than the specific QC procedures for chemical analytical methods. Both procedures have common goals that are to know that reliable data are generated, to recognize and eliminate unreliable data, and to have methods which assist investigations in resolving problems for future work. The quality assurance (QA/QC) guidance given by EPA (1989C) for the short-term tests lists numerous items of concern for toxicity testing. These are: (a) effluent sampling/handling, (b) test organisms, (c) facilities, equipment and test chambers, (d) analytical methods, (e) calibration and standardization, (f) dilution water, (g) test conditions, (h) test acceptability, (i) test precision, (j) replication and test sensitivity, (k) quality of organisms, (l) quality of food, (m) control charts, and (n) record keeping and data evaluation. Many of these should be closely followed, and the reader is encouraged to review the guidance in relation to QA/QC in both the short-term effluent test manual (EPA, 1989C; EPA, 1992C) and the acute Phase I manual (EPA, 1991A).

2.2 QA/QC Cost Considerations and Testing Requirements

For the chronic TIE, cost considerations are important and concessions in the requirements of the QC may have to be made. In some instances, the data will demand stringent control while in others, the QC can be lessened without impact to the overall endpoint of the TIE.

TIEs can require a great number of toxicity tests. The use of all aspects of the standard test protocols (EPA, 1989C; EPA, 1991C) is not necessary in Phase I. The factors of time requirements, number of tests and the test design (i.e., five replicates versus ten, four dilutions versus five) must be considered and weighed against the type of questions that are posed. For example, the need for water chemistry data are specific for each Phase I test. The testing requirement (EPA, 1989C) according to the permit requirement most likely included pH, daily measurements of DO, temperature, conductivity, alkalinity, and hardness measurements in the low, middle, and high concentrations for the five test dilutions of the effluent. However, hardness measurements are not pertinent for the methanol eluate collected from a solid phase extraction column. The post C_{18} SPE column effluent samples are more similar to the effluent and a concern for low dissolved oxygen (DO) exists, while the test solutions of the methanol eluate are more similar to the dilution water and the possibility of low DOs is not as great a concern. In contrast, frequent pH measurements on all test concentrations are needed to determine the impact of pH sensitive compounds.

As TIEs are reliant on a strong QAP, there are several aspects of a QA/QC program for chronic TIEs that should be delineated. In regard to test organism quality, there are steps for culturing organisms that should help provide the necessary QC verification that is needed to ensure the animals are representative in their sensitivity. These steps are simply routine items such as monitoring and recording the young production (for cladocerans) of the culture brood animals once a month, conducting monthly reference toxicant tests (including maintaining control charts), monitoring the preparation dates for the reconstituted waters used, and monitoring the types and age of the foods fed (Norberg-King, 1989B). For fathead minnows, it is useful to monitor the survival of the breeding stock, and the percent hatchability of the embryos, to verify that new genetic stock is introduced on a regular basis, and to conduct monthly reference toxicant tests (Norberg-King and Denny, 1989; Denny, 1988). Similar parameters for other species that are used are also desirable.

Since toxicity tests in the early part of the chronic Phase I do not generally follow all the effluent testing

requirements (EPA, 1989C), the QC measures are not as strict because the data are primarily informative rather than definitive. When Phases II (identification) and III (confirmation) are initiated, then QC aspects should be reconsidered and the tests modified. Phase I procedures frequently use one species and later stages of the TIE (Phase III) use more than one species to determine whether the cause of toxicity is the same for other species of the aquatic community.

Reference toxicant tests are not conducted with each set of Phase I manipulations because of the amount of labor and large numbers of animals required for testing. In general, the utility of the reference toxicant test is to know that the organisms are responding as expected. Since only relative differences are needed at this stage (Phase I), reference toxicant data are much less useful for the characterization interpretation but are important for the knowledge of the quality of the test organisms and general test procedures. For various manipulations of the TIE, organism responses are compared to either the *baseline test* (see Section 6) or the response of organisms in the dilution water treatments. Monthly reference toxicant tests should provide the necessary information about the quality of the organisms for the laboratory conducting the TIE. When a toxicant has been identified (Phase II) and tests for Phase III confirmation indicate it is the toxicant(s), that chemical should become the reference toxicant with the species used in the TIE.

Using receiving water as the dilution water in Phase III confirmation will help ensure that receiving water effects are properly considered (see Section 3, Dilution Water). The variability of the effluent, by nature of the TIE, is defined during the TIE, and this information will aid in determining the appropriate control option in order that the final effluent is safe upon discharge.

2.3 QA/QC and Chronic Testing Considerations

An inherent problem with effluents is that no effluent test can be repeated to assure that the toxicity is the same and that the toxicants are the same. However, repeated *baseline tests* (Section 6) can be done with the same effluent sample to determine how long that effluent sample can be used. The chemical and toxicological nature of the effluent shifts as an effluent is discharged or as an effluent sample is stored. Effluent constituents degrade (at unknown rates) and each constituent has its own rate of change. Analysis of each sample should be initiated as soon as the sample is received in the testing laboratory (generally <24 h). Until an effluent sample has been tested several times, there is no way to predict how long a sample can be stored before the toxicity changes. Testing of each sample can be done provided the toxicity remains and/or stabilizes; however this cannot be determined at the beginning of the Phase I battery of tests and will be known only through testing several samples a few times. Even though the toxicity remains, it is possible that the toxicant may change with time. The number of samples to evaluate and the number of tests to conduct must be weighed against the cost of the effort and how representative each effluent sample is of the effluent. Effluents that have low and non-persistent toxicity may need to be approached with the Tier 1 and Tier 2 characterization steps applied simultaneously (see Section 6).

In a chronic TIE, information obtained from a test should be maximized. This may mean paying particularly close attention to details such as small differences in the number of neonates the cladocerans are producing or the lack of food in the stomach of the larval fish. These parameters and any other observed characteristics during a test may be subtle indicators and quite informative about small changes in toxicity. For example, if all the animals exposed to the whole effluent die on day 4, and in some characterization test the animals don't reproduce or grow but are alive at day 7 of the exposure, that characterization manipulation reduced the toxicity, but did not remove it completely. Observations such as these may be just as useful as reductions in young production or growth.

While some abbreviations in the test design are made, the general principles for toxicity testing still apply. For example, all animals must be added to test solutions randomly. Animals must be placed in a test chamber one at a time. For the fathead minnows, use of an intermediate vessel to hold all 10 animals is preferable to ensure that animals are assigned randomly and that the volume of water added with the fish is minimized (1-2 ml). Also, transferring animals may require separate pipettes for each concentration or cleaning of the pipettes between concentrations to prevent cross contamination. However, we have observed that *C. dubia* do not have to be placed under the water; they can be added or transferred by dropping the water droplet containing the animal into the test solution. The problem frequently observed with *D. pulex* where animals are caught at the surface of the test solution (called "floaters") does not occur with *C. dubia*. Randomization, careful exposure time readings, use of animals of uniform narrow-age groups (i.e., *Ceriodaphnia* neonates 0-6 h old rather than 0-12 h old) should assist in quality data generation.

Standard operating procedures (SOPs) should be developed for each Phase I test, for preparing the reconstituted waters, preparing the foods for the test organisms, calibration and standardization for all measurements (temperature, DO, pH, conductivity, alkalinity, hardness, ammonia, chlorine), and other general routine practices.

An important aspect of TIEs is accurate and thorough data recording. All observations should be documented. Items that were not thought to be important at first may be useful in later stages of analysis and actually assist in the confirmation of the toxicant(s). These observations can be as simple as large bubbles produced during the aeration and filtration manipulations, large particles present in whole effluent, and low

pH upon arrival. It is best to record data so that any preconceived ideas of the toxicants are avoided. Data records should include records of test organisms (species, source, age, date of receipt, history and health), calibration records, test conditions, results of tests, and summaries of data. Once a control chart is developed using point estimates for reference toxicant tests, 1 out of 20 reference toxicity test results will be predicted to fall outside the acceptable limits if the 95% confidence intervals are used to develop the control chart (EPA, 1991C). If TIEs are conducted during such a period, the TIE data generated must be used with caution, and the investigator must carefully examine the TIE data to determine if the results are usable. The decision may be based on consistency of the concentration response data, *control blank* performance, and the consistency of the TIE results with those obtained with the same effluent sample.

2.4 QA/QC Blanks and Artifactual Toxicity

Throughout the TIE, dilution water samples are subjected to most of the procedures and analyses performed on the effluent sample (see Section 5.6). This is done to detect toxic artifacts (i.e., toxicity due to anything other than the effluent constituents causing toxicity) that are created during the effluent characterization manipulations (see Section 6). These manipulations can make QC/QA verifications difficult, as the use of such *blanks* for interpreting toxicity results is not standard toxicology. For example, typically organism responses from any toxicity test in standard aquatic toxicology are compared to the performance of *control* organisms which were in dilution water only. In the TIE, *controls* are used to judge organism performance (Section 5), and *toxicity controls* and *blanks* are used to evaluate whether a manipulation affected the toxicant(s), therefore the results of all characterization tests are not necessarily compared to the *baseline test*. For instance, post-column effluent samples that are collected and tested following concentration on a resin column have been filtered first. Therefore it is only logical to compare the post-column effluent toxicity (*post C_{18} SPE column test*; Section 6.6) to the toxicity observed in the filtered effluent sample (*filtration test*; Section 6.4) rather than to the unfiltered whole effluent (*baseline test*; Section 6.1) (see Section 5).

Artifactual toxicity can occur in several of the manipulations, particularly from the major pH adjustment manipulation (Tier 2). Toxicity results from tests relying on the addition of the reagents (EDTA, sodium thiosulfate, acids/bases) must be interpretable. Addition of both the acid (HCl) and the base (NaOH) can form a toxic product (e.g., NaCl). The addition of the acid and base may interfere with the growth and reproduction of the test organisms for the short-term chronic test, at lower levels than cause mortality in the acute test. Whether additives act in an additive, synergistic, or independent manner with the compounds in the effluent must be determined during the TIE but this is not likely to be clear during Phase I. Artifactual toxicity can occur in the aeration process, where contaminated air can be introduced. Also, contaminants can be leached from solid phase extraction (SPE) columns, and methanol leaching off the column can cause bacterial growth that will confound the results in the post-column *blank* and *post C_{18} SPE column tests*. Originality and judgement are needed to devise tests that will reveal artifactual toxicity (see Section 6) and some of these methods to deal with artifactual toxicity will be effluent specific.

2.5 Health and Safety Issues

For the toxicity identification work, hazards present in any effluent may not be known until Phase II identification steps have been started. Therefore, safety requirements for working with effluents (or other samples) of unknown composition must follow safety procedures for a wide spectrum of chemical and biological agents. Because all of the hazards in an effluent sample may not be known when a toxicant is identified, effluent samples should be treated as hazards of unknown composition throughout the TIE. Knowledge of the types of wastewater treatment applied to each effluent can provide some insight for the possible hazards. For example, unchlorinated primary treatment plant effluents containing domestic waste may contain pathogens. Chlorinated secondary effluents are less likely to contain such agents. Effluents from activated sludge treatment plants are less likely to contain volatile toxicants.

Because effluent characteristics are unknown, personnel should follow the guidelines for hazardous materials (EPA, 1991A; 1991C). Also, if any sample contains human waste, personnel should be immunized for diseases such as hepatitis B, tetanus, polio, and typhoid fever.

Each laboratory should provide a safe and healthy work place. All laboratories should develop and maintain effective health and safety programs (APHA, 1989; EPA, 1991C). Each program should consist of: (a) designated health and safety officers, (b) formal written health and safety plans, (c) on-going training programs, and (d) periodic inspections of emergency equipment and safety violations. Further guidance on safety practices is provided in other documents (APHA, 1989; EPA, 1991A; 1991C).

2.6 Facilities and Equipment

The laboratory facilities and equipment needed to conduct TIEs are discussed in the acute Phase I manual (EPA, 1988A; EPA, 1991A). Most of the equipment for conducting the short-term tests are delineated elsewhere (EPA, 1989C; EPA, 1992C). The reagents used for the chronic Phase I characterization are identical to those described in the acute Phase I manual (EPA, 1991A). Compressed air systems with oil-free compressors and air filters to provide high purity air are very important (EPA, 1991A). All glassware should be rigorously cleaned, and the glassware used for filtering must be rigorously cleaned to remove residual contaminants from the glass frit(s). Filtering equipment may

need to be made of plastic to avoid leaching of metals or other toxicants from glass when acid washes are used (see Section 6). Use of stainless steel frits can be used provided pH adjustments are not made since metals will rinse off the stainless steel at extreme pH's and cause toxicity. Ultra pure acids and bases (e.g., Suprapur®, E. Merck, Darmstadt, Germany) should be used to prevent impurities in the acids/bases from interfering in the toxicity results.

Section 3
Dilution Water

Dilution water used for chronic TIE's must meet several requirements. Obviously it must support adequate performance of the test animals in regard to growth, survival, and reproduction since these are the effects measured in the tests. Secondly, it must not substantially change the animals' response to the sample toxicants. Because the characteristics of the toxicants are not known, there is no way to be sure which dilution water characteristics are important. Hardness and alkalinity are most often used to select the dilution water but these parameters are generally of little importance for non-polar organics. Rarely is the organic matter content considered and yet for both non-polar organics and metals, organic matter has more effect on toxicity than hardness. Experience in the acute TIE work has shown pH to be the single most important water quality characteristic for characterizing the cause of toxicity.

The most important consideration, in addition to those mentioned above, is that the water be consistent in quality and not contain contaminants that could produce artificial toxicity. For example, if there was a nontoxic concentration of a non-polar organic present in the dilution water, when samples are concentrated, it might be toxic and this can confound the identification of the components causing toxicity in the effluent. The best policy is to use a high purity reconstituted water or a well water of known suitability. Receiving water should not be used until Phase III, when it is the water of choice to evaluate the toxicant in the receiving water system (see Section 2.2).

A reconstituted water of similar pH, hardness and alkalinity to that of the effluent is a first approximation of an appropriate water; however, organic matter is hard to duplicate. Experience has shown that for the *Ceriodaphnia* test, the addition of food[1] to the water has been helpful to provide some organic material. With food added, traces of contaminants can be less toxic. If higher concentrations of effluent are to be used, the choice of the dilution water is less important because the characteristics of the effluent dilution mixture will resemble those of the effluent. As information is gained about the toxicant characteristics, the choice of dilution water can be improved.

The impact of dilution water choice depends on the IC25 (see Section 5.8) of the effluent. If toxicity changes substantially from sample to sample, but the dilution water selected does not match the effluent in water characteristics yet is kept the same throughout several samples for Phase I, then the effect of the effluent in the dilution water can also vary across samples. As the TIE progresses into Phase II, attributing relative toxicity to various constituents must be more refined. For instance, suppose the suspect toxicant is a cationic metal whose toxicity is hardness dependent. Also, suppose that the whole effluent has a hardness of 300 mg/l as $CaCO_3$ (very hard water) but the dilution water has a hardness of 40 mg/l as $CaCO_3$. In this case, the hardness in each of the test dilutions will be different from that of either the whole effluent or the dilution water. Provided the cationic metal concentrations vary over the course of the TIE period, the amount of toxicity (as toxic units[2], TUs) due to a particular metal concentration will also vary depending upon the effect concentration in the effluent. If the first whole effluent sample contains 160 µg/l of zinc (for this example, 160 µg/l is 1.0 TU_c in very hard water) and the test is conducted using a dilution water of 40 mg/l as $CaCO_3$ (soft water), the no effect concentration would be 100% where hardness is 300 mg/l and the effluent would have <1 TU_c. The second whole effluent sample contains 480 µg/l of zinc. One would expect this sample to possess 3 TUs (480 µg/l ÷ 160 µg/l). The toxicity due to the second effluent sample would likely contain more than 3 TUs because the hardness at the effect level (<100%) would be much lower than at 100% effluent (where hardness is 300 mg/l as $CaCO_3$). The effect

[1] Food added for the *C. dubia* tests are the yeast-cerophyll-trout food (YCT) and the algae (*Selenastrum capricornutum*) at a rate of 0.1 ml/15 ml (EPA, 1989C). Although at ERL-Duluth the algae has been added at the rate of 0.05 ml/15ml until May of 1991 when we increased the level (EPA, 1989C).

[2] TUs is a means of normalizing the concentration term (i.e., LC50, NOEC, IC25 as percent effluent; see Section 5.8) to a unit of toxicity. The use of the TUs approach allows effluent toxicity to be compared (provided test species and test duration are the same) to a suspect toxicant's toxicity. The toxicity of an effluent and a chemical are different and different concentrations of each equal one LC50 (1 TU). TUs of an effluent can be calculated for either acute or chronic toxicity endpoints. The number of acute TUs in the effluent is 100% ÷ LC50 = TU_a and the chronic TUs in the effluent is 100% ÷ NOEC = TU_c or 100% ÷ IC25 = TU_c (EPA, 1991B). For specific chemicals the TU_c is equal to the concentration of the compound present in the effluent divided by the acute test LC50 for TU_a or the chronic test NOEC or IC25 for the TU_c. The assignment of TU_c is necessary for the correlation step (Phase III) when effluent toxicity TUs are compared to suspect toxicant(s) TUs.

level would be near 20-25% effluent where hardness would be <100 mg/l as $CaCO_3$ and 1 TU of zinc would be <160 µg/l. In addition, if one were to use receiving water for the diluent, the hardness might change dramatically and confound calculation of TU's in a like manner if the effect concentration was <100% effluent.

Section 4
Effluent Samples

To determine whether an effluent sample is typical of the wastewater discharge may require a number of samples to be tested. Experience has shown that the use of several samples spanning two to three months has been successful in characterizing many effluents. TIE work on atypical samples may be problematic and these TIE procedures were not developed for one-time episodic events. However, the very nature of atypical samples may provide valuable assistance in the TIE effort by identifying the type of toxicant(s) that previously was not suspect. This is probably more likely when an atypical sample has greater toxicity than the other samples. In addition, the atypical toxic sample may aid a discharger in recognizing wastewater treatment plant upsets and assist the discharger in implementing prevention procedures or generally improve and maintain better wastewater plant housekeeping efforts, which in turn may eliminate the episodic toxicity problems.

The acute Phase I manual discusses the quantitative and qualitative changes in effluents (EPA, 1988A; EPA, 1991A) that may affect toxicity. Varying concentrations of toxicants, different toxicants, water quality characteristics, and analytical and toxicological error are all factors in determining the toxicity of an effluent. Although the toxicity of an effluent over time appears unchanged, there may be more than one toxicant involved in each sample, and not necessarily the same ones.

At the same time a sample is collected, information on the facilities treatment system (normal operation; aberrant processes) may be useful. When dealing with industrial discharges, details of the process being used may be helpful. These details and others should be recorded and provided to the laboratory conducting the TIE at the time of sample shipment. When samples are received, temperature, pH, chronic toxicity, hardness, conductivity, total residual chlorine (TRC), total ammonia, alkalinity and DO should be measured. Figure 4-1 provides a typical format to record such information.

Since most TIEs are not performed on-site, the effluent samples must be shipped on ice to the testing location. The samples should be cooled to 4°C or less prior to shipment and they should be shipped in sturdy ice chests to prevent either temperature increases or container breakage during shipment. Primary requirements of the TIE are that toxicity occurs frequently in the effluent samples and that the toxicity of each sample (held at 4°C) remains in the efiluent sample for a sufficient period of time. If samples repeatedly lose their toxicity after shipment, steps should be taken to preserve toxic fractions (Section 6.7) for later testing and analysis. For example, if the initial characterization tests indicate the presence of non-polar organics, one tool to use is to concentrate large volumes (5-10 L) of effluent when the sample arrives (see Section 6). Use of the Phase II (EPA, 1992A) non-polar fractionation procedure is the preferred way to concentrate the non-polar toxicants for subsequent analysis and testing. While efforts must be expended on this procedure, it can be a crucial step to aid in identifying potential toxicants (in instances where toxicity is present and lost in the effluent). The information on when toxicity degrades or is lost may become useful as the toxicant(s) is identified (see Section 9; EPA, 1991A). Filterable toxicants which degrade quickly in the effluent may be recovered from the filters with solvent and stored for future use (cf., *filtration test*; Section 6.4).

For one chronic Phase I TIE, a typical volume of effluent needed to ship is 19 L (5 gal) but of course this will depend on the options chosen for the TIE (Section 6) and 38 L (10 gal) may be more helpful once identification and confirmation begin on any sample. The second edition of the acute Phase I TIE manual (EPA, 1991A) recommends that samples be initially collected and stored in both glass and plastic to determine whether effluent stored in either container affects the toxicity. Some compounds (such as surfactants) are less toxic if water samples containing them are stored in plastic containers. Prior to initiating the characterization it may be useful to collect and test several preliminary samples to determine which containers to use during the TIE to provide samples that are the most representative of the effluent (see Phase I, Section 6 (EPA, 1991A) for more details). Less volume (≤2 L) is needed for these tests.

Composite samples should be used for Phase I. Later, in Phases II and III, where variability is desired, grab samples should be used. Samples that are consistent (i.e., composite samples) give results that are easier to interpret and lead more rapidly to identification (Phase II) and confirmation (Phase III) of the cause of toxicity. Grab samples can provide the maximum effluent toxicity; however, it is more difficult to catch

Figure 4-1. Example data sheet for logging in samples.

Sample Log No.: _____

Date of Arrival: _____

Date and Time
of Sample Collection: _____

Facility: _____

Location: _____

NPDES No.: _____

Contact: _____

Phone No. _____

Sampler: _____

Sample Type: ☐ Grab ☐ Composite
☐ Glass ☐ Plastic
☐ Prechlorinated
☐ Chlorinated
☐ Dechlorinated

Sample Conditions Upon Arrival:

Temperature _____

pH _____

Total Alkalinity _____

Total Hardness _____

Conductivity/Salinity _____

Total Residual Chlorine _____

Total Ammonia _____

Condition of treatment system at time of sampling:

Status of process operations/production (if applicable):

Comments:

intermittent peaks of toxicity (such episodic events may not be caused by the same toxicant that causes routine toxicity).

Multiple effluent samples in each test should *not* be used in Phase I as is done for permit testing (EPA, 1989C). We have found that using only *one* composite sample for each set of Phase I characterization tests is adequate. If several effluent samples are used for renewals during the chronic Phase I TIE and the toxicants are different or change in their ratios one to another, the interpretation of Phase I will be nearly impossible. Indeed such variability must be identified but it should be done after at least one or preferably most of the toxicants are known. The use of *one* sample is more important in Phase III, (EPA, 1992B) where toxicity data are correlated to the measured concentrations in the effluent. If multiple samples are used, this correlation can not be readily done because the same toxicant may not be present in each sample, or it is present in varying concentrations and other toxicants may appear.

Existing routine toxicity test data should be examined. If one notes a sudden response such as death in the middle to the end of the test period and especially if it is associated with a new sample, the effect being measured may actually be acute rather than chronic and if so the approach may be switched to an acute TIE approach. The investigative approach should be adjusted to respond to such situations. When the permit test is conducted and the test fails, it may be desirable to try to identify the toxicants in those permit compliance samples. This can be done by collecting the appropriate volume needed for a chronic TIE of either the daily samples or the three samples used for the short-term toxicity test (EPA, 1992C). Additional short-term toxicity tests can be conducted on each

sample prior to any TIE tests on each sample or preferably additional short-term tests would be initiated on each new sample during the 7-d test to evaluate whether it is the cumulative toxicity from all samples or whether one or two samples are driving the toxicity. We have observed in several effluent tests that the toxicity during the short-term chronic test can be caused by one or two samples and these samples cause the chronic test to demonstrate that the effluent is toxic in less than the full 7-d of the *C. dubia* or fathead minnow tests. When the toxicity that occurs in ≤48 h (*C. dubia*) or ≤96 h (fathead minnow) with any one of the samples from the permit compliance samples or any sample collected for the TIE, is observed as >50% mortality, acute TIE procedures can be applied to more quickly characterize the toxicant(s).

Section 5
Toxicity Testing

5.1 Principles

The test organism is used as the detector of chemicals causing chronic toxicity in effluents and other aqueous media. The response to toxic levels of chemicals is a general one; however the organism is the only tool that can be used specifically to measure toxicity. Only when the cause of toxicity is characterized can chemical analytical methods be applied to identify and quantify the toxicants.

Chronic TIE's will usually be triggered by the use of the toxicity test methods as found in the short-term chronic toxicity test manuals (EPA, 1989C; EPA, 1992C). These methods rely on sublethal endpoints as the indicator of chronic toxicity for the Phase I manipulations, therefore conducting the tests strictly as detailed in those manuals is not always necessary and sometimes not possible. Modifications have been developed and these include: (a) reduced test volumes, (b) shorter test duration, (c) smaller number of replicates, (d) reduced number of test concentrations, and (e) reduction in the frequency of the test solution renewal. In addition, the frequency of preparation of manipulated samples for test solution renewal must be established and this issue is discussed in the following section. Any loss of test precision due to these modifications is not as critical during Phase I characterization as it is in Phase II and Phase III (EPA, 1992A; EPA, 1992B). During Phase I the analyst is searching for an obvious alteration in effluent toxicity, which may be obtained using modified chronic test methods. Confirmation testing (Phase III) conducted according to the standard methodologies will confirm whether the toxicant(s) detected in the characterization and identification steps (Phases I and II) is the true toxicant.

5.2 Test Species

In most cases, freshwater effluents will be subjected to this evaluation because they have been found to be chronically toxic to the cladoceran, *C. dubia*, or to the fish, fathead minnow (*P. promelas*), or possibly to the cladocerans, *D. magna* or *D. pulex*. Freshwater effluents discharged into marine environments are evaluated for toxicity using marine species or may be assessed with freshwater species (EPA, 1991D). TIE guidance for the marine species will be forthcoming in the fall of 1992 (George Morrison, personal communication, ERL-Narragansett, RI).

The species which detected the toxicity which in turn triggered the TIE, is the first choice for the TIE species. When an alternative species is chosen one must prove that it is being impacted by the same toxicant(s) as the species which initially detected the toxicity. The species need not have the same sensitivity to the toxicant(s), but each species' threshold must be at or below the toxicant concentration(s) present in the effluent. One method of proving that the species are being affected by the same compound(s) is to test several samples of the effluent over time to both species. If the effluent possesses sufficient variability, and the two species IC25s (see Section 5.8 below for a description of the IC25) change in proportion to one another, the analyst may assume that the organisms are reacting to changing concentrations of the same compound. Further proof that the two species are responding to the same toxicant should surface during Phase III. If the toxicant is the same for both species, then characterization manipulations which alter toxicity to one species should also alter toxicity to the second species. The extent to which toxicity is altered for each will depend upon the efficiency of the manipulation and the organism's sensitivity to the toxicant. Steps applied in Phase III will confirm whether the two species are indeed sensitive to the same toxicant in the effluent. Extensive time and resources may be wasted if one discovers during Phase III that the organism of choice is not responding to the same toxicant as the species which triggered the TIE.

For the above mentioned reasons, we recommend when at all possible to use the organism which prompted the TIE. Our chronic TIE experience has been based on tests with *C. dubia* and/or larval fathead minnows. Obvious constraints on the use of other species are availability, size, age, and adaptability to test conditions. Also, the threshold levels for additives and reagents must be determined for other species.

5.3 Toxicity Test Procedures

Measures to conserve time and resources required to conduct a chronic Phase I must be used in order to make the procedures cost-effective. The application of all aspects of the standard short-term chronic tests to Phase I in terms of replicates, routine water chemistries, test duration, and volume is not practical due to time constraints and expense. Variations of the procedures need to be implemented whenever possible.

As mentioned above, smaller test volumes can be used in all tests with *C. dubia* and in most instances with fathead minnows. For example, 10 ml in a 1 oz

plastic cup (or 30 ml glass beaker) has been adequate for *C. dubia* and 50 ml in a 4 oz plastic cup (10 fish per cup) has been used successfully to test the fathead minnows (or 100 ml in a 400 ml glass beaker). There are two precautions to watch for in the chronic TIE tests—1) evaporation of test solutions and 2) transfer of toxicants while moving the animals. If evaporation reduces test volumes, efforts to reduce the evaporation must be made or larger volumes must be used. The volume of water added with each transfer should be minimized, because the volume used in the test is small, and the resultant test concentration could be diluted, thereby reducing toxicity. Using the same size test chambers and consistent volumes should be maintained in Phase I; when Phases II and III are initiated, tests should be conducted following the test protocol that was used to trigger the TIE. This may be important in Phase I to be as sure that the oxygen requirements for the test species are met and that toxicity is not due to physical restrictions of the test procedure.

If a reduction in the number of replicates per test concentration is used, one must assume that precision is sufficient enough to decipher changes in toxicity that must be measured. For the *C. dubia* test, five animals per concentration (one per cup) and for the fathead minnow test, two replicates per concentration and 10 fish per replicate have been found to be adequate for interpreting the changes in toxicity. However this smaller data set is not amenable to all statistical requirements as described for the short-term tests (EPA, 1989C; see Section 5.8). Use of more organisms and more replicates may be preferable if Phase I data are likely to be used in Phase III confirmation (see Sections 2.2 and 2.3).

A shortened version of the 7-d *C. dubia* test, referred to as the 4-d test, may be useful in the TIE. The 4-d test does not have to be as sensitive as the 7-d test, just sensitive enough that the toxicity changes occurring in Phases I and II of the TIE (using 4-d tests) would be the same as the 7-d tests. The 4-d day test was found to produce similar results for single chemicals (Oris et al., 1991), but in tests in our laboratory with effluents, the 4-d test has not been as sensitive for all effluents tested as the 7-d test in determining the effects on young production and survival. Masters et al. (1991) tested *C. dubia* to one effluent (three times), three surfactants, three metals, and three organic compounds with the 4-d and 7-d exposures. They found that for the most part the effluent toxicity was similar for the 4-d and 7-d test results but for the surfactants the 7-d test was more sensitive. For the metals (cadmium, lead, and zinc), ethylene glycol, and pentachlorophenol, the chronic toxicity values for both tests were very similar while the 4-d test was more sensitive for phenol.

In the 4-d test, when animals are initially exposed at 72 h they are ready to produce their first brood. Therefore, toxicity can be underestimated because these animals are predisposed to produce their first brood, unlike the animals exposed as neonates (24 h old). The exposure during a 4-d test may miss their most sensitive life stage. However for the Phase I where the purpose is to detect differences following various manipulations, this issue is not as important as the ability to rapidly conduct the characterization. Use of the shorter term test will decrease the cost of Phase I TIE's. In the confirmation of toxicity (Phase III), the 7-d test is required because the toxicity as measured in the 7-d test (with more replicates, more dilutions, more volume) was used to detect toxicity for the permit, and should be used to confirm the cause of toxicity.

To conduct a 4-d test with *C. dubia*, neonates (0-12 h old) are placed in the dilution water that will be used to conduct the TIE. At present these animals are held in groups of three, two or individually in test containers (with 15 ml of culture water) and fed daily until they are 72 h (±6 h) old in a similar test fashion (Oris et al., 1991). The animals are then transferred to the *baseline test* solutions or the various characterization test solutions. The test is then continued for 4-d using the endpoint of three broods.

The use of known parentage (EPA, 1989C) for the *C. dubia* test is important when the number of replicates is reduced, and helpful for Phase I, II or III tests and in routine tests as well (EPA, 1992C). For Phase I, this known parentage approach allows the young of one female to be used across one replicate of all dilutions and the control (i.e., 5 animals), the young from another female for the next replicate set of dilutions and control, and so on until all test cups contain one young animal. By this technique, animals from a given female that later appear atypical in appearance or movement or produce no young when others in the same test concentration are producing normally can legitimately be dropped from the data set without statistical bias (Norberg-King et al., 1989). The ability to discard such data without bias improves precision. Precision will be better when $n \geq 7$ per treatment for *C. dubia* or $n \geq 4$ for the fathead minnow test.

5.4 Concentrations to Test

The level of toxicity for any given discharger most likely will have been established with some degree of certainty from previous tests that were conducted on the effluent that triggered the TIE.

Therefore during Phase I of the TIE, we have found that four effluent dilutions and a control are adequate to define the toxicity of the sample while reducing the cost of the tests. Now for the TIE, the key to choosing the concentrations to test is to select those that will assist in the detection of small changes in toxicity, which is essential in the chronic TIE. For example, if the NOEC (from a previous data set) is 12% (or IC25 is 10%), then a concentration series such as 6.3%, 12.5%, 25%, and 50% would be logical; or perhaps closer concentration intervals may be desired. Using 20% as the high concentration and a dilution factor of 0.7, would mean the concentrations to test would be 7%, 10%, 14%, and 20%. If the NOEC (from historical data) is 40-50% (or above 50%), then the concentrations to test should be, for example, 25%, 50%, 75%, and 100% or

40%, 60%, 80%, and 100%. Choice of dilution factor and test concentration range is a matter of judgement and depends on needed precision and practicality.

In nearly all examples in this document, the concentrations of 12.5%, 25%, 50%, and 100% are used. We are assuming that if effluents have ICp (or NOEC) values below 10%, the effluent is likely to show acute toxicity and if so, an acute TIE approach should be used. If chronic work is to be done on a highly toxic effluent, the same recommendations given in the acute manual should be used; that is, use concentrations of 4x, 2x, 1x and 0.5x the IC25 or IC50 value (see Section 5.8 for which value to select). For example, if the IC25 is 5% effluent, we would suggest using a range such as 20%, 10%, 5% and 2.5% for the various tests. It is best to use the same dilution sequence within a series of tests (Tier 1) when tests are to be compared to each other for differences in toxicity.

5.5 Renewals

For *C. dubia*, daily renewals of the test media (as required in the chronic manual, EPA, 1989C) are not necessary in Phase I as long as the toxicity of the effluent can be measured with one or two renewals. Because available sample volume is limiting in some manipulations, fewer renewals are desirable. As with the test duration (4-d vs. 7-d) the acceptability of less frequent renewals must be established by comparison with whichever test duration is selected. However in Phase III, tests must be conducted similarly to the routine biomonitoring test. For the fathead minnow test the frequency of sample replacement must be daily to maintain adequate water quality because the live food organisms (brine shrimp, *Artemia salina*) die 2-8 h after being added to the freshwater test solutions. A *baseline test* (see Section 6) is always conducted when the sample is received. The suitability of reduced renewal frequency can efficiently be evaluated at this time by conducting comparative *baseline tests* simultaneously with different renewal frequencies.

The number and types of chemical measurements taken initially and at the renewal intervals (referred to as finals) should be based on the need for these measurements and their usefulness (see Section 2). Initially, little judgement about the value of these can be made, but as toxicant characteristics are identified, the usefulness of various measurements can be judged. Initially, the usual measurements (hardness, alkalinity, conductivity; EPA, 1989C) should be made but some of these can be dropped as the TIE progresses. For example, if non-polar toxicity is found, then hardness and alkalinity need not be closely monitored. However if a metal is suspected, then these measurements are important. Low levels of dissolved oxygen in the fathead minnow test are a greater concern than in the *C. dubia* test, and the pH between the two tests will be dissimilar after 24 h of exposure. The pH measurement is frequently needed and for toxicants such as ammonia it is extremely important (EPA, 1992A). If an effluent contains greater than 5.0 mg/l of ammonia, the pH should be carefully measured at least daily (or more often) in *all* test concentrations. Since ammonia is a highly pH dependent toxicant, one must be aware of variable pH drift in the Phase I treatments which may lead to erroneous conclusions. One generalization, however, can be made. For characteristics that are unlikely to change, such as conductivity and hardness, both initial and final measurements need not be made-once is enough.

5.6 Toxicity Blanks

A risk of the reliance on a toxicity response in the characterization step of TIEs is the probability that artifactual toxicity is created during sample manipulations (see Section 2.4). While a particular manipulation may cause some degree of artifactual toxicity, if the toxicity is predictable the test may still retain its validity. Since chronic tests are more sensitive to artifactual toxicity, lower concentrations of additives or less severe conditions must be used as compared to the acute test.

The presence of artifactual toxicity caused by contaminated acids, bases, air, filters and columns and by intentional additives are detected by treatment *blanks* and *toxicity controls*. A *blank* is dilution water manipulated the same as the effluent, and then it is toxicity tested to determine if the manipulation added any toxicity. The *toxicity control* is the reference used to judge the impact of a manipulation. Sometimes the *toxicity control* is the *baseline test*, at other times it will be a characterization test. For example, the *toxicity control* for the *EDTA addition test* is the *baseline test* while the *toxicity control* for the *post C_{18} SPE column test* is the *filtration test* (filtered whole effluent). Treatment *blanks* for either the *EDTA addition test* or the *sodium thiosulfate addition test* are not appropriate as the testing of these additives in clean dilution water is not representative of the effluents' characteristics. The *toxicity control* must be distinguished from the *control* treatment (animals in standard culture or dilution water; also described as "performance controls") which is always used. *Controls* provide information on the health of the test organism and the test conditions while the *blanks* provide information on the cleanliness of the acids and bases, the aeration system, the filter apparatus, the C_{18} SPE column, and other apparatus used.

Although artifactual toxicity may appear in the dilution water *blanks*, artifactual toxicity in the effluent matrix may not be observed. One must decide whether the test results from that manipulated sample are meaningful. For example, if the aeration manipulation caused toxicity in the dilution water *blank* but aeration removed the effluents' toxicity then the conclusion that aeration was an effective treatment is valid. However, if the dilution water *blank* was toxic and it appeared aeration did not remove the effluent's toxicity then one cannot conclude that aeration was not effective without further investigation.

5.7 Renewal of Manipulated Samples

One must decide whether a manipulated sample to be used for renewal during the test should be prepared (e.g., aerated or passed over a C_{18} SPE column) as a batch sample for the entire test or prepared separately for each renewal. This choice may be dependent on the persistence of the effluent toxicity, but whether daily samples are prepared or batch samples are prepared and used for renewals of the tests should be decided by the investigator, and the same methods should be performed consistently throughout the TIE. As a general guideline, we have chosen to discuss these Phase I steps as though one aliquot of effluent samples prepared for the characterization tests is used for all renewals. However for either daily or batch samples, the same techniques should be used for all the manipulations. For example, a sample for the *filtration test* (Section 6) may be batch prepared on day 1. Then on day 2, a batch sample for the *aeration test* should be prepared. Yet for the *EDTA and sodium thiosulfate addition tests*, these additives should be added to the effluent dilutions on the day of each renewal as batch solutions for each dilution (e.g., add EDTA to 50 ml of 100% effluent, let sample sit and dispense to test cups). This is true for the methanol addition and the graduated pH manipulations as well. To test the post C_{18} SPE column samples for some effluents, daily samples may need to be prepared because of bacterial growth problems in samples stored for several days.

Since Phase I TIE work is often concerned with the qualitative evaluation of toxicity, rather than quantitative, there is no reason why a test could not be terminated sooner than 7 d, if the answer to the particular question posed has been found. For example, if the *baseline test* with a sample indicates a complete inhibition of *C.dubia* reproduction by day 5 of a 7-d test, and one of the manipulated samples (e.g., aeration) shows normal reproduction, there may be little point in continuing that test, because toxicity was altered. This type of judgmental decision is harder to make in a chronic fathead minnow test based on growth; however, by careful observation of factors such as survival or behavior, the trend of the toxicity response may be discerned earlier than 7 d. Sufficient measurable growth of the fathead minnows may have been achieved by 5-d. Experiments with fish exposed to zinc and selenium for 5-d and 7-d indicated that sufficient growth differences could distinguish the toxic effect even at 5-d (Norberg-King, 1989). However, if this information is needed in Phase III, it is important to correlate the same type of data and terminating the test early may require additional tests later on.

Because the chronic test is longer and requires more laboratory work than the acute test, loss of toxicity of any effluent sample is more troublesome when it occurs. If the presence of toxicity is not measured in the whole effluent before Phase I tests begin, much work will be wasted if the sample is non-toxic initially. On the other hand, to delay by waiting for the test may also result in the loss of toxicity. The best approach is to examine existing data sets for evidence of toxicity loss due to storage of samples. If there are none then start a *baseline test*, and upon the onset of chronic toxicity (e.g., 60% mortality, no reproduction by day 5 in high test concentrations of a 7-d test, absence of food in the gut of the fishes), additional follow-up manipulations of Phase I tests should be started. Toxicity degradation can be a useful tool in identification and confirmation (cf., Section 2). Once it has been determined that the sample toxicity degrades quickly, Tier 1 and Tier 2 steps should be started on the day of arrival. Removal of headspace in effluent storage containers may help minimize the loss of toxicity.

5.8 Test Endpoints and Data Analysis

For evaluating whether any manipulation changed toxicity, the investigator should not rely on statistical evaluations only. Some treatments may have a significant biological effect that was not detected by the statistical analysis. Judgement and experience in toxicology should guide the interpretation.

Endpoints for the most commonly used freshwater short-term chronic tests are growth, reproduction, and survival. Historically, the effect and no effect concentrations have been determined using the statistical approach of hypothesis testing to determine a statistically significant response difference between a control group and a treatment group. The no effect level, called the no observed effect concentration (NOEC), and the effect concentration, called the lowest observed effect concentration (LOEC), are then statistically defined endpoints. The NOEC/LOEC are heavily affected by choice of test concentrations and test design. For example, these effect levels are dependent not only on the concentration intervals (dilution sequence) chosen, but the number of organisms, the number of replicates used, and the choice of the statistical analysis for the data (i.e., parametric or non-parametric). The minimum significant difference detected in hypothesis tests can be quite variable (e.g., 10% or 50%; Stephan and Rogers, 1985) and yet this difference is used to determine the NOEC. In the chronic testing manual (EPA, 1989C), the minimum number of replicates (a relatively large number), organisms, and dilutions for the *C. dubia* and fathead minnow short-term tests are needed to meet the hypothesis testing requirements. When less replicates, fewer numbers of dilutions and fewer test organisms are used (as in the chronic TIE) the hypothesis tests will not be able to detect smaller differences that are needed for chronic TIEs. Therefore, hypothesis testing is not suitable for Phase I purposes and a point estimation method must be used.

The linear interpolation method described in the supplement to the freshwater chronic manual (EPA, 1989C) calculates a point estimate of the effluent concentration that causes a given percent reduction based

on the organisms response. The inhibition concentration (ICp[3]) program (Norberg-King, 1989; DeGraeve et al., 1988; EPA, 1989C) was developed for the purpose of analyzing data from the short-term tests. This method of analysis is not as dependent on the test design as hypothesis analysis and is particularly useful for analyzing the type of data obtained from Phase I testing. When analyzing data for the ICp estimates, only one test endpoint is determined. For *C. dubia* all the data are used. If all animals have died, the data are entered as zeros and if some animals have some young but the adult dies, the partial brood values are used. We have found with some effluents that when the 4-d test is routinely applied during a chronic TIE, often the first brood is produced and then the adult dies. In other cases we have observed no adult mortality in the 4-d or 7-d test, but at the same effluent exposure concentrations the 7-d test animals will not produce any young while the 4-d test animals produce their first brood. The dose response from this 4-d test is not typical in the 7-d test, and the production of young can be problematic in data interpretation and analysis since mortality also occurred. For example, when analyzing the data using the ICp program, the effects of survival and young production are incorporated into one estimate for the IC50 and IC25. Yet there is no doubt that 0-40% survival is a significant reduction in survival that indicates toxicity, and would cause a routine test to fail (EPA, 1989C). Therefore when this occurs, to track toxicity in the TIE, it may require calculating the IC25/IC50 for young production and survival and then recalculating the IC25/IC50 for survival alone. For the fathead minnow test in the routine monitoring test and the TIE tests, the weights are calculated as mean weight per original fish rather than mean weight per surviving fish (EPA, 1992C). Also the program allows direct comparison of results from tests conducted using different concentration intervals. The level of inhibition (p) used as an endpoint (e.g., 25 or 50%) is not critical, although the IC25 is generally suggested as an equivalent for the NOEC (EPA, 1991B). Confidence intervals are calculated using a bootstrap technique, and these confidence intervals can be used to determine the significance of toxicity alterations observed in Phase I. A *"significant reduction"* in toxicity must be determined by each laboratory for each effluent and in combination with the precision of reference toxicant tests that the performing laboratory achieves. The use of the IC50 for Phase I TIEs may be more useful when trying to correlate the characterization test results to the effluent toxicity. However, an IC50 may not be able to be estimated while the IC25 can; use of a consistent endpoint effect level is important for subsequent TIE work (EPA, 1992A: EPA, 1992B). We have observed substantial toxicity reductions in characterization tests, yet it does not always appear to be a significant reduction when only the IC25s are compared. When this happens the sample size should be increased with subsequent testing in order to more clearly differentiate the toxicity and the dose response curve should be studied. Once the toxicant is identified, the number of replicates is increased and more dilutions are used (Phase III; EPA, 1992B), which increases the confidence in the IC25.

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[3] The ICp program (Release 1.1) calculates confidence intervals which are limiting when the sample size is ≤5 and these confidence intervals are less than 95% in version 1.1 (R. Regal, personal communication, University of Minnesota, Duluth, MN). This is being corrected in the revision of the program now underway (for more information, contact Teresa Norberg-King). The ICp program is available by sending a formatted disk to Teresa Norberg-King, EPA, 6201 Congdon Boulevard, Duluth, MN 55804.

Section 6
Characterization Tests

The chronic Phase I manipulations follow the same approach and employ the same type of manipulations used in the acute TIE (EPA, 1991A). These include aeration, filtration, C_{18} SPE extraction and chromatography, chelation with EDTA, oxidant reduction and/or complexation with sodium thiosulfate, and toxicity testing at different pH values (Figure 6-1). The main differences between the acute and chronic techniques are that the concentrations of additives must be lower and the test conditions must be less severe in a chronic TIE because the chronic test organisms are more sensitive to these conditions. The pH adjustment procedures in Tier 2 are changed from the acute Phase I because we found that consistent, representative blanks with reconstituted water could not be obtained at higher pH's.

The following characterization steps are all based on the use of *Ceriodaphnia* or fathead minnows. Obviously, use of other species will require consideration of appropriate test volumes and additive concentrations. As discussed in the acute manual, if the TIE is done with species different from the species used in the permit, one must demonstrate that both species are sensitive to the same toxicant(s) (see Section 5).

More than one effect is measured in chronic tests (reproduction or growth and survival) and because partial effects are more frequent in short-term chronic tests than in acute tests, a graded response with concentration is often seen. A graded response allows one to better judge small changes in toxicity—an advantage not often available in acute tests. Also, effects (initial mortality, delayed mortality, aborted young, reduced young, poor growth) can be observed and used in interpreting the results as can the time to onset of effect be used. Such effects can be useful in distinguishing the response to different toxicants.

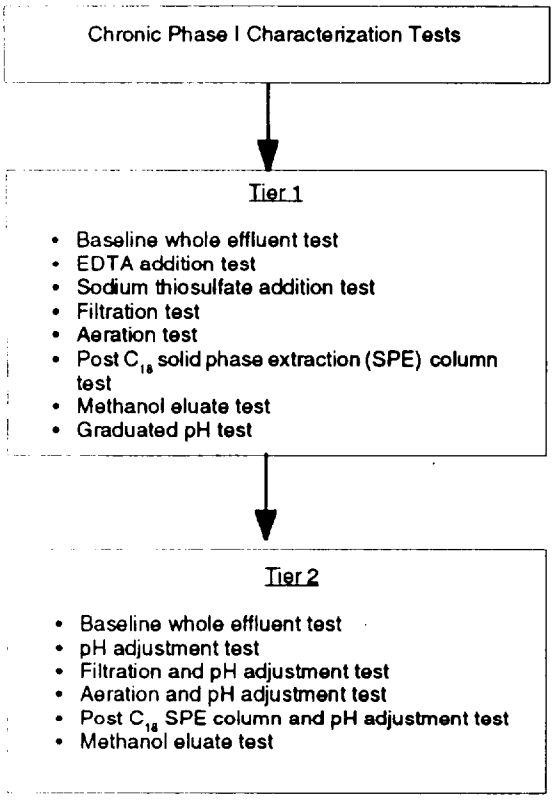
For acute TIEs, tests are quick and relatively inexpensive, so the need to maximize their usefulness is lessened. The chronic test is more work not only because the test is longer and more complex, but also because more sample volume is needed. For example, for tests such as the sublation test (a subsequent step in the *aeration test* (Section 6.4)) sample size can be very restricting. In addition, if an effluent is not always toxic, a decision has to be made as to whether to test for the presence of toxicity first, before manipulations are started. If the effluent is not toxic and all the manipulations are set up, the results may be of no value. On the other hand, if the presence of toxicity is first established, often a week will have passed and by the time manipulations are tested, the toxicity may have degraded. Unfortunately, there is no clear answer to which way to proceed. When there are data for effluent toxicity for preceding months, examination of these data may assist in the decision.

In the acute TIE, the *initial test* (EPA, 1991C) is used to set the range of concentrations to test. However in the chronic TIE, an equivalent of the *initial test* is not practical, therefore historical data must be used to make such judgements. Lacking historical data, a judgement will have to be made to set the test range and guidance for this is given in Section 5.4.

For chronic Phase I characterization, the use of two tiers of characterization tests is suggested (Figure 6-1). Tier 1 is done without major pH adjustments. Experience with acute TIEs has shown that major pH adjustments are usually not needed. Tier 2 is performed only when Tier 1 does not provide sufficient information, and consists of filtration, aeration and the C_{18} separation technique of Tier 1 with an effluent sample adjusted to both pH 3 and pH 10. Therefore when the characterization tests indicate Tier 2 is not required, resources needed to conduct the TIE are significantly reduced.[4] Each characterization test used in the Tier 1 or Tier 2 has as its foundation the information in the acute Phase I manual (EPA, 1988A; EPA, 1991A). The principles, methods, and interpretation of results are based on the acute manual, and the tests for Tier 1 (Figure 6-2) are discussed in Sections 6.1-6.8. All tests within a Tier (1 or 2) should be started on the same day. Starting chronic tests involves more effort than acute tests, and logistics must be planned (for instance, available animals of the appropriate age for the chronic test, sufficient food supply for more chronic tests, adequate supply of dilution water for all test renewals). Tests need to be started on the same day in order to compare results of each manipulation test to others and to the *baseline test* (Section 6.3) results (Table 6-1). Once the Tier 1 data are generated, they are compared, and interpretations are made to see which inferences can be drawn concerning the nature of the toxicants. Usually, multiple manipulations and a retest of selected manipulations will be effective in

[4] A recent estimate of the cost of the Tier 1, Phase I for chronic toxicity was equivalent to the full Phase I acute TIE (Aquatic Habitat Institute, 1992).

Figure 6-1. Overview of characterization tests.



yielding information concerning the nature of toxicants before additional effluent samples are tested (see Sections 6.15, 6.16 and acute Phase I manual, EPA 1991A).

Sample Preparation for the Characterization Tests

As for acute TIE tests, we suggest doing certain chemical measurements and the manipulations on one day and then starting the tests the next day (Table 6-1). This schedule balances the work load more evenly. When the sample is received (day 1), various measurements (Section 4) are taken and some preparatory manipulations for the Tier 1, Phase I are done.

First, the routine chemical measurements are taken as discussed in Section 4. DO, conductivity, and pH should be measured on the 100% effluent to ensure that the values are in the physiologically tolerable range for the test species. If these are at levels that could be toxic (EPA, 1989C), there is little point to test the effluent sample without some sample manipulation. In addition, the water hardness and alkalinity should be measured so that the appropriate dilution water can be selected (see Section 3, Dilution Water). As the TIEs have progressed, we have begun to match both the hardness and the alkalinity of the dilution water to similar values for the effluent.

Figure 6-2. Tier 1 sample preparation and testing overview.

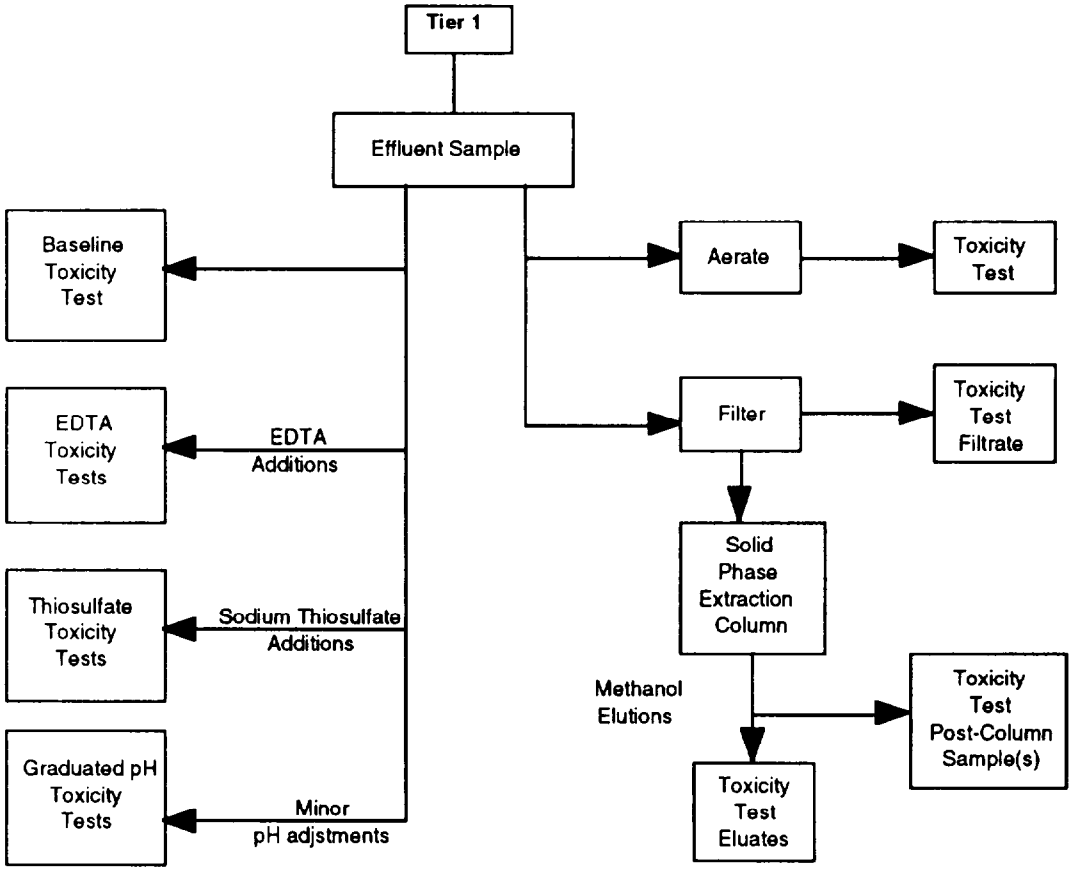


Table 6-1. Outline of Phase I effluent manipulations Tier 1 and Tier 2.

Description	Section
DAY 1 SAMPLE ARRIVAL:	
Measure	4.0
• temperature	
• conductivity	
• pH	
• DO	
• alkalinity	
• hardness	
• total ammonia	
• total residual chlorine	
Perform Sample Manipulations	6.0
• filter effluent	6.4
• perform solid phase extraction (SPE)	
• collect effluent	6.6
• collect methanol eluate	6.7
DAY 2 TOXICITY TESTING:	
Warm aliquot of whole effluent and aliquots of filtered effluent, post C_{18} SPE column effluent, and methanol eluates.	
Initiate Tier 1 Tests	
• *baseline toxicity test*	6.1
• *EDTA addition test*	6.2
• *sodium thiosulfate addition test*	6.3
• *aeration test*	6.4
• *filtration test*	6.5
• *post C_{18} SPE column test*	6.6
• *methanol eluate test*	6.7
• *graduated pH test*[1]	6.8
ADDITIONAL TESTING ON SUBSEQUENT DAYS[2]*:*	
Tier 2 Tests	
• *pH adjustment test*	6.10
• *aeration and pH adjustment test*	6.11
• *filtration and pH adjustment test*	6.12
• *post C_{18} SPE column and pH adjustment test*	6.13
• *methanol eluate test for pH adjusted samples*	6.14

[1] Experimentation may be needed for this test (see text for details).

[2] Tier 2 is primarily for those effluents where the results from Tier 1 did not indicate any clear pattern of toxicity change following manipulation (see text for details).

The initial pH of effluent upon arrival at the testing laboratory is referred to as pH *i*, which is not necessarily the pH of the effluent at air equilibrium[5]. The pH of the sample after being warmed, may be selected as pH *i* rather than the pH upon arrival. The important point is to use the same pH *i* for all subsequent tests. As an effluent warms to 25°C in an open container, CO_2 escapes and the pH may rise from 7.2-7.6 to 8-8.5. In some tests, once the food is added the pH may rise faster or in some cases (e.g., the fathead minnow growth test), once the food has been in the test solution for a period of time, the pH may be lower (e.g., 7.5-7.6). These changes may be important for interpreting the data in a chronic TIE, and pH should be measured in the test dilutions that determine the test endpoint. Of course, since the endpoint may be unknown, pH is typically measured in all test concentrations.

Since samples are cooled for shipping and storage, upon warming to 25°C, some of the samples are apt to be supersaturated. Supersaturation can usually be monitored by measuring DO. If DO is too high, it should be reduced to acceptable levels as described by EPA (1989C) for the routine monitoring test or by maximizing surface-to-volume ratio of the container to facilitate more rapid exchange of equilibrium of the sample and atmospheric oxygen. *Ceriodaphnia* are less sensitive to supersaturation than newly hatched fathead minnows. For chronic Phase I tests, routine water chemistry measurements (such as DO, pH, temperature) are more important than in acute Phase I tests.

The manipulations performed the day the sample arrives are filtering, extraction on the C_{18} SPE column, and collection of the methanol eluates (see Sections 6.5 and 6.7 below). The aliquots of filtered effluent and post-column effluent will be held until the next day (day 2) to start the tests. Of course these samples should be stored in the refrigerator at 4 (± 2°C). This sample preparation schedule is particularly convenient for laboratories who rely on courier services to deliver samples, which typically occurs late in the morning.

On day 2, the EDTA addition test should be prepared first so that compounds that are EDTA chelatable, yet may require an equilibration time for complexation, can be chelated (see Section 6.4). Then the rest of the manipulations (aeration, sodium thiosulfate additions, graduated pH adjustments) should be started. For the laboratory that is experienced in chronic toxicity testing, the amount of time required to conduct the Tier 1 sample manipulations and set up the toxicity tests is about 6-10 h.

6.1 Baseline Test

General Approach: To determine the effects of Phase I manipulations on the toxicity of the effluent, its inherent toxicity must be determined. The toxicity measured in this test is used to gauge toxicity changes caused by some manipulations and to detect changes in the sample's toxicity during storage. *Baseline tests* must be repeated each time additional manipulation tests are started.

Methods: The *baseline test* will be initiated using concentrations based on the historical data for each particular discharger. For the TIE, use of four (and

[5] EPA suggests that toxicity must be prevented under worst case scenarios (EPA, 1991B) which may mean the routine monitoring tests were conducted at high pH's.

three) dilutions have been sufficient for defining toxicity (Section 5.4). If the toxicity is low, in order to draw distinctions between the concentrations used in the test for the various characterization tests, the dilutions may need to be set closer, for example, 40%, 60%, 80%, 100%. In this test, and all subsequent characterization tests, the test concentrations, test volumes and number of replicates should be kept the same as described in Section 5, Toxicity Testing.

On day 2, an aliquot of the effluent is warmed slowly in a warm water bath to test temperature (25°C). The various test concentrations are prepared using the appropriate hardness reconstituted water. Next, routine chemistries are measured (initial pH, temperature, DO). The use of *dilution water controls* is not required for every manipulation but at least two sets of *controls* should be included to estimate reproducibility. In addition, the tests are conducted using one *C. dubia* per one 10 ml test volume in a 1 oz plastic cup (or glass beaker) and five animals per treatment. For the fathead minnow tests, two replicates per treatment, 10 fish in 50 ml in a 4 oz plastic cup, or 100 ml in a 400 ml beaker, are assumed.

Interpretation of Results/Subsequent Tests: The *baseline tests* serve as the basis for determining the effects produced by various characterization tests. This test serves as the *toxicity control* for some of the other tests. If *baseline tests* done on subsequent days with additional manipulations indicate that the toxicity of the effluent is decreasing, either every effort should be expended to characterize the toxicity more quickly (i.e., Phase II identification or Tier 2 tests) or another sample should be obtained. The *"shelf life"* of the toxicity can be determined after a few samples have been evaluated.

Special Considerations/Cautions: The *controls* in this test will provide information on the health of the test organisms, the dilution water, the test glassware and equipment used to prepare the test solutions and the cleanliness of the test chambers. This *baseline test* serves as the *toxicity control* for some subsequent Tier 1 or Tier 2 tests.

6.2 EDTA Addition Test

General Approach: This test is designed to detect effluent toxicity caused by certain cationic metals. The addition of EDTA to water and effluent solutions can produce non-toxic complexes with many cationic metals. Loss of toxicity with EDTA addition(s) suggests that cationic metals are causing toxicity.

EDTA is a strong chelating agent and because of its complexing strength, it will often displace other soluble forms (such as chlorides and oxides) of many metals. The ability of EDTA to chelate any metal is a function of pH, the type and speciation of the metal, other ligands in the solution, and the binding affinity of EDTA for the metal. And the complexation of metals by EDTA may vary according to the sample matrix. The specific form of metal that causes toxicity in the water matrix may be more important than the total concentration of the metal.

Cations strongly chelated by EDTA include aluminum ($^{3+}$), cadmium, copper, iron, lead, manganese ($^{2+}$), nickel, and zinc (Stumm and Morgan, 1981). EDTA weakly chelates barium, calcium, cobalt, magnesium, strontium, and thallium (Flaschka and Barnard, 1967). EDTA can form relatively weak chelates with arsenic and mercury and anionic forms of metals (selenides, chromates and hydrochromates) will not be chelated.

For some cationic metals for which EDTA forms relatively strong complexes, the acute toxicity to *C. dubia* is reduced (Mount, 1991; Hockett and Mount, In Preparation). EDTA was shown to chelate the metal causing the acute toxicity (at 4x the LC50) for copper, cadmium, lead, manganese ($^{2+}$), nickel, and zinc to *C. dubia* in both dilution water and effluents. However, they also found that EDTA did not remove/reduce the acute toxicity of silver, selenium (either as sodium selenite or sodium selenate), aluminum ($Al(OH)_4^-$), chromium (either as chromium chloride or potassium dichromate), or arsenic (either sodium m-arsenite or sodium arsenate) when tested using moderately hard water and *C. dubia* (Hockett and Mount, In Preparation).

In the acute Phase I manual (EPA, 1988A), the recommended amount of EDTA to be added was high because the authors thought calcium and magnesium had to be complexed in order to complex toxic metals (D. Mount, personal communication, NETAC, Duluth, MN). The mass of EDTA required was approximated by the amount needed for the titration of hardness or the measurement of calcium and magnesium when titration was not possible due to interferences. A third choice was to use 0.5x the EDTA LC50 for the test species (EPA, 1991A). Ideally the amount of EDTA to add would be just enough to chelate the toxicant(s) without causing toxicity or otherwise changing the matrix of the effluent. Without knowing how much toxicant must be chelated, the amount of EDTA to add must be estimated. Recently, the role of calcium and magnesium was tested in our laboratory. Acute toxicity tests with *C.dubia* were conducted in moderately hard and very hard reconstituted water using copper, cadmium, and zinc at 4x, 2x, and 1x the LC50 of each. When one metal and EDTA were present at approximately a 1:1 molar basis, all the toxicity was removed regardless of water hardness (J. Thompson, personal communication, NETAC, Duluth, MN). These results indicate that calcium and magnesium concentrations do not affect the levels of EDTA needed to remove the acute cationic metal toxicity. Whether toxicity reduction using the 1:1 molar ratio is true for chronic toxicity has not yet been evaluated in a likewise manner (cf., *Interpretation of Results/Subsequent Tests* below). However, EDTA and nitrotriacetic acid (NTA) were effective in chelating the toxicity of one concentration of either cadmium or copper to *C. dubia* at molar ratios of less than 1:1 (Zuiderveen and Birge, 1991). However, NTA pos-

sesses the characteristic of increasing the toxicity of some metals therefore NTA is limited in its usefulness for the TIE.

The threshold levels for *C. dubia* and fathead minnows to EDTA were determined using 7-d tests in different hardness waters and the results are given in Table 6-2. For *C. dubia*, the chronic toxicity of EDTA is not water hardness dependent, but for fathead minnows the sublethal toxicity appears to be greater in softer waters. This is in contrast to the acute toxicity of EDTA to *Ceriodaphnia* which indicated that EDTA toxicity decreased with increased water hardness (Phase I; EPA, 1991A). Natural waters and effluents have many constituents in addition to those added to reconstituted waters, and the behavior of EDTA in effluents (or receiving waters) could be different than in simple reconstituted water.

Methods: The goal is to add enough EDTA to reduce metal toxicity, without causing EDTA toxicity or substantially changing the water quality. The toxicity of EDTA as determined in clean reconstituted water is likely to be higher than the toxicity of EDTA added to an effluent. Therefore, the EDTA toxicity values contained in Table 6-2 represent maximum toxicity in any effluent. The toxic concentration of EDTA in one effluent will probably not be the same as the concentration causing toxicity in a different effluent or even a different sample of the same effluent. To be safe, the concentrations of EDTA added to any effluent should be less than the expected effect concentration of EDTA in clean water. For either species, two EDTA concentrations are added to two sets of two effluent dilutions. EDTA stock solution is added after the effluent dilutions are prepared so that the EDTA concentrations for each addition are constant across each set of effluent dilutions. A stock solution of EDTA (ethylene–diaminetetraacetic acid, disodium salt dihydrate) is prepared in distilled water. This EDTA stock solution should be prepared so that only microliter amounts of the stock are needed to minimize effluent dilution. No more than 5% dilution of the effluent aliquot by EDTA stock should occur.

To perform the effluent dilution test, two sets of effluent dilution concentrations are prepared (e.g, 100%, 50%, 25%,) and each set receives one of two addition levels of EDTA (Table 6-3). By using non-toxic concentrations of EDTA, there is less chance for artifactual toxicity; since the total amount of metal to be chelated is probably low for most chronically toxic effluents, there is no reason to add high levels of EDTA. The additive levels are based on the assumption that the calcium and magnesium need not be chelated in order to chelate the toxic metals, although the amount of EDTA added is most likely still an excess.

An EDTA stock solution of 2500 mg/l can be prepared. For the *C. dubia* tests, 0.06 ml is added to three separate 50 ml aliquots in the first effluent dilution set (i.e., 25%, 50%, 100%) to obtain a 3.0 mg/l final EDTA concentration. In the second dilution set, 0.16 ml is added to the other set of 50 ml effluent aliquots for a final concentration of 8.0 mg/l. For the fathead minnow tests, the same concentration of an EDTA stock solution can be used but the volume of stock additions must be doubled for the 100 ml test volume/concentration.

Table 6-2. Chronic toxicity of EDTA (mg/l) to *C. dubia* and *P. promelas* in various hardness waters using the 7-d tests.

Species	Water Type	IC50 95% C.I.	IC25 95% C.I.	NOEC	LOEC
C. dubia	VSRW	4.5 3.6-6.0	3.0 2.1-3.9	2.5	5.0
	SRW	7.5 6.2-8.3	4.9 3.7-5.7	3.1	6.3
	MHRW	8.8 4.7-13	5.9 3.4-10	5.0	10
	HRW	7.5 6.2-9.8	5.5 0.98-6.9	5.0	10
	VHRW	7.8 6.7-8.6	6.1 4.0-6.8	5.0	10
	VHRW	12 10-14	8.3 4.2-10	7.5	15
P. promelas	SRW	136 130-139	103 94-110	100	200
	MHRW	163 150-188	132 123-144	100	200
	HRW	236 227-248	—[1]	200	400
	VHRW	287 269-300	230 203-247	200	400

[1] Value could not be determined, value would be less than lowest test concentration.

Note: C.I. = confidence interval; VSRW = very soft reconstituted water; SRW = soft reconstituted water; MHRW = moderately hard reconstituted water; HRW = hard reconstituted water; VHRW = very hard reconstituted water.

Table 6-3. Concentrations of EDTA to add for chronic TIEs. Values given are the final exposure concentration in mg/l.

Species	Water Type[1]	Concentrations (mg/l)	
C. dubia and *P. promelas*	SRW, MHRW, HRW, VHRW	3.0	8.0

[1] In very soft water, the final concentrations of EDTA must be lower in order to not have EDTA induced toxicity, for example 1.0 mg/l and 5.0 mg/l.

Note: SRW = soft reconstituted water; MHRW = moderately hard reconstituted water; HRW = hard reconstituted water; VHRW = very hard reconstituted water.

To allow the EDTA time to complex the metals, solutions should be set up on day 2 and all solutions containing EDTA are allowed to equilibrate while other manipulations are being prepared before test organisms are introduced. A minimum of 2 h equilibration time should elapse before organisms are added.

Since EDTA is an acid, the pH of the effluent after addition of EDTA should be checked, although additions at these low levels should not lower the pH of the effluent. The amount of change in solution pH will depend upon the buffering capacity of the effluent and the amount of reagent added. If the pH of the effluent has changed, readjustment of the test solution pH to pH *i* should be performed.

The EDTA is not added to one batch of effluent on day 2; rather at each renewal EDTA is added to the renewal test solutions prior to dispensing into the test chambers in the identical way that the test solution was first made (allowing equilibration time).

Interpretation of Results/Subsequent Tests: Toxicity may be removed at all exposures provided the addition of EDTA does not cause toxicity. If the effluent is less toxic (i.e., EDTA addition IC50 (or IC25) shows less toxicity than *baseline test* IC50 (or IC25)) in either of the EDTA addition dilution tests, then EDTA removed or reduced the toxicity and cationic metal toxicity is probably present. If, in either test, the effluent is more toxic than in the *baseline test*, EDTA itself may be causing toxicity and the test should be repeated using lower EDTA concentrations. If toxicity is not reduced below the *baseline test*, the probability of cationic metals causing toxicity in the effluent is low and higher concentrations of EDTA can be tried, although this may or may not be useful.

Table 6-4 shows the results of a chronic zinc test and the reduction of the toxicity by the addition of EDTA. When *C. dubia* were tested in very hard reconstituted water, zinc was chronically toxic at 55 µg/l and EDTA was chronically toxic at 15 mg/l. When EDTA was added to solutions of 55 µg/l zinc at 2.5, 5.0, and 7.5 mg/l EDTA respectively, the toxicity of the zinc was removed but at 15 mg/l EDTA, EDTA itself was toxic. Such trends may be similar to the toxicity reduction observed in effluents. If toxicity is reduced in a systematic manner, such as in the example, proceed to Phase II methods for identification of those metal(s) which are chelated by EDTA. Additions of EDTA at 3 mg/l and 8 mg/l removed the toxicity of copper to *C. dubia* in a 7-d two-renewal test with hard reconstituted water at levels of 210 µg/l and 105 µg/l of copper. In addition to removing toxicity due to metals, EDTA reduces the acute toxicity of some cationic surfactants. This reduction of toxicity may also occur in chronically toxic effluents, and the toxicity reduced by EDTA should not be assumed to be due only to cationic metals. See Section 6.4 *Aeration Test* for subsequent tests to conduct if cationic metals are not present in the effluent at chronically toxic levels but EDTA reduced toxicity.

Table 6-4. The chronic toxicity of zinc (µg/l) to *C. dubia* in very hard reconstituted water and the toxicity of zinc when EDTA is added.

Zinc[1] Conc.	Mean Young per Female				
	EDTA Additions (mg/l)				
µg/l	0	2.5	5.0	7.5	15
0	19.2	18.6	17.5	17.6	6.8
3.4	19.4	—[2]	—	—	—
14	17.8	22.0	23.2	20.8	1.8
55	8.2	20.8	19.0	16.6	5.3

[1] Measured values.
[2] EDTA not added to this zinc concentration.

Special Considerations/Cautions: If pH in the EDTA tests is greatly different from that in the *baseline test*, the test might need to be redone. There is no way to distinguish the effect of pH change on the toxicity of a pH sensitive toxicant (e.g., ammonia) from toxicity changes caused by EDTA. A change of 0.1 pH unit can cause substantial errors if ammonia is involved. Before the test is reinitiated, data from the *graduated pH test* should be examined to evaluate whether the toxicity is pH dependent. This test data may be useful in deciding whether the *EDTA addition test* should be redone. EDTA additions to dilution water are not relevant *controls* for the EDTA additions to effluent; therefore, the *toxicity control* is the *baseline test*. The *control* of the *baseline test* serves as the QC for the health of the test organisms, the quality of the dilution water, and general test conditions.

If all dilutions where EDTA is added should cause mortality, one possibility is that the stock solution of EDTA is contaminated and the stock solution should be checked by conducting another test with a new EDTA stock.

6.3 Sodium Thiosulfate Addition Test

General Approach: Oxidative compounds (such as chlorine) and other compounds (such as copper and manganese) can be made less toxic or non-toxic by additions of sodium thiosulfate ($Na_2S_2O_3$). Toxicity from bromine, iodine, ozone, and chlorine dioxide is also reduced. Sodium thiosulfate has been routinely used to reduce the toxicity of substances such as chlorine (EPA, 1989C).

Reductions in effluent toxicity observed with sodium thiosulfate additions may also be due to the formation of metal complexes with the thiosulfate anion (Giles and Danell, 1983). The ability of sodium thiosulfate to form a metal complex is rate dependent and metal dependent (Smith and Martell, 1981) and sodium thiosulfate is not a particularly strong ligand for metal complexation. Cationic metals that appear to have this potential for complexation, based upon their equilibrium

stability constants, include cadmium, copper, silver, and mercury ($^{2+}$) (Smith and Martell, 1981). The rate of complexation is specific for various metals and some cationic metals may remain toxic in the 24-h or 48-h renewal period of the chronic toxicity test due to the slow rate of complexation or the stability of the complex. The thiosulfate anion is not very stable, and the ability of sodium thiosulfate to complex the compound(s) causing chronic toxicity without daily renewals has not been tested completely.

Recent findings have shown that the acute toxicity of certain cationic metals may be reduced by levels of sodium thiosulfate added in the acute Phase I tests (EPA, 1988A; EPA, 1991A). The acute toxicity of several cationic metals was shown to be removed by sodium thiosulfate in standard laboratory water. The acute toxicity at 4x the LC50s of copper, cadmium, mercury, silver, and selenium (as selenate) to *C.dubia* was removed by sodium thiosulfate additions at levels suggested in the acute Phase I manual. However, for zinc, manganese, lead, and nickel, the acute toxicity was not removed by the sodium thiosulfate additions (Mount, 1991; Hockett and Mount, In Preparation). The toxicity of mercury with the addition of sodium thiosulfate was reduced for 24 h but not 48 h which indicates it may not have been completely complexed by the thiosulfate. If the acute toxicity of metals can be reduced or complexed by sodium thiosulfate, the same may be true for chronic toxicity. However, for *C. dubia* 7-d tests with hard reconstituted water, sodium thiosulfate levels of 5 mg/l and 10 mg/l did not remove or reduce the chronic toxicity of copper at the same concentrations where EDTA complexed the toxicity (cf., Section 6.2).

The test animals will probably tolerate more sodium thiosulfate than would ever be needed to render oxidants or metals non-toxic in effluent samples, especially the fathead minnows in comparison to the *C. dubia* (Table 6-5). The presence of oxidants or complexable metals will reduce the concentrations of sodium thiosulfate below the nominal concentrations added.

Table 6-5 gives the toxicity values in various reconstituted waters. The effect concentrations for *C. dubia* and fathead minnows were measured in waters of different hardnesses (soft, moderately hard, hard, and very hard water (EPA, 1989C)). For *Ceriodaphnia*, the results indicate that the sublethal toxicity is unchanged regardless of the water type (Table 6-5). The toxicity tests with sodium thiosulfate and fathead minnows (7-d growth test) indicate that the toxicity due to sodium thiosulfate is greater in softer waters.

Methods: Two sets of effluent dilutions (such as 25%, 50%, 100%) each set with a different level of thiosulfate concentration (Table 6-6) are prepared regardless of whether *C. dubia* or fathead minnows are used as the TIE test organism. The concentration of thiosulfate remains constant across one set of effluent concentrations within a series (identical to *EDTA addition test*). Small volumes (microliter) of the sodium thiosulfate stock solution should be added to minimize the dilution (5% of total volume). Non-toxic concentrations of sodium thiosulfate are used to reduce the pro–

Table 6-5. Chronic toxicity of sodium thiosulfate (mg/l) to *C. dubia* and *P. promelas* in various hardness waters using the 7-d tests.

Species	Water Type	IC50 95% C.I.	IC25 95% C.I.	NOEC	LOEC
C. dubia	SRW	39 30-42	26 15-33	30	60
	HRW	38 26-44	27 20-36	30	60
	VHRW	43 37-44	34 21-37	30	60
P. promelas	SRW	1,070 1,041-1,1005	820 785-859	750	1,500
	MHRW	2,001 1,891-2,161	720 550-1,528	750	1,500
	HRW	4,871 4,633-5,051	3,590 3,226-3,800	3,000	6,000
	VHRW	8,522 8,053-8,704	6,780 6,065-7,073	6,000	12,000

Note: C.I. = confidence interval; SRW = soft reconstituted water; MHRW = moderately hard reconstituted water; HRW = hard reconstituted water; VHRW = very hard reconstituted water.

Table 6-6. Concentrations of sodium thiosulfate to add for chronic TIEs. Values given are the final exposure concentration in mg/l.

Species	Water Type[1]	Concentrations (mg/l)	
C. dubia and *P. promelas*	SRW, MHRW, HRW, VHRW	10	25

[1] In very soft water, the final concentrations of sodium thiosulfate must be lower in order to not have sodium thiosulfate induced toxicity, for example 1.0 mg/l and 5.0 mg/l.

Note: SRW = soft reconstituted water; MHRW = moderately hard reconstituted water; HRW = hard reconstituted water; VHRW = very hard reconstituted water.

bability of artifactual toxicity, yet sufficient concentrations are needed to remove/reduce oxidants.

For a *C. dubia* test, to the first effluent dilution set (i.e., 25%, 50%, 100%), 0.20 ml of sodium thiosulfate stock (2500 mg/l) is added to each 50 ml dilution to obtain final concentrations of sodium thiosulfate of 10 mg/l. To the second effluent dilution set, 0.50 ml of the same stock solution is added to 50 ml of each test dilution to obtain final concentrations of 25 mg/l (Table 6-6).

The fathead minnow test is similar except that twice the volume of the same thiosulfate stock is needed (because of 100 ml test volumes) to achieve the same final concentrations (Table 6-6).

The sodium thiosulfate is not added to a batch of the effluent on day 2; rather, at each renewal, sodium thiosulfate is added to the renewal test solutions in a manner identical to the way they were first prepared.

Interpretation of Results/Subsequent Tests: The results of the *sodium thiosulfate addition tests* are compared to one another and to the *baseline test* results to determine whether or not toxicity reduction occurred. Toxicity may be completely reduced, partially reduced, or not reduced. If toxicity appears to be reduced and/or removed, then more tests to determine whether the toxicity is due to an oxidant or to some metal should be performed. When chlorine concentrations are ± 0.1 mg/l total residual chlorine (TRC), there may be a toxicity problem for *C. dubia*. A significant drop in the chlorine level in the whole effluent may occur in the first 24-h period after sample collection and testing. Therefore, tests repeated on an aged sample may give different results if an oxidant is involved but may give the *same* results if a metal is involved.

For cases where oxidants account for only part of the toxicity, sodium thiosulfate may only reduce, not eliminate, the toxicity. Yet the *sodium thiosulfate addition test* is useful even when chlorine appears to be absent in the effluent. Oxidants other than chlorine occur in effluents, and even if the effluent is not chlorinated this test should not be omitted. Both thiosulfate and EDTA reduce the toxicity of some metals and this information can be helpful in identifying the toxicant. (However, this effect of thiosulfate/metal complexation has not been demonstrated for chronic toxicity.) In cases where both the *sodium thiosulfate addition test* and the *EDTA addition test* reduce the toxicity in the effluent sample, there is a possibility that the toxicant(s) may be a cationic metal(s). Many oxidants are reduced by aeration but if aeration does not reduce toxicity, Phase II methods for identification of cationic metal(s) toxicants should be investigated. No change in toxicity suggests either no oxidants or certain metals.

Special Considerations/Cautions: The general test conditions, quality of the dilution water, and health of the test organisms are tracked by the *controls* in the *baseline test*. Additions of sodium thiosulfate to dilution water are not relevant *controls* for thiosulfate additions to effluent to determine if the thiosulfate was toxic. Therefore the *toxicity control* is the *baseline test*.

If all dilutions where sodium thiosulfate is added should exhibit mortality, one possibility is that the stock solution of sodium thiosulfate is contaminated and this phenomena should be checked by conducting another test.

6.4 Aeration Test

General Approach: Changes in toxicity due to aeration at pH *i* may be caused by substances that are oxidizable, spargeable, or sublatable. The chemical/physical conditions of the aeration process will also affect whether or not the toxicity is reduced or removed.

Sparging of samples is done using air which includes oxidation as a means of toxicity removal. In our experience, typically volatile compounds that are highly water soluble (such as ammonia) will not be air-stripped at pH *i* by this method. If aeration is one of the mechanisms that removes the toxicity, then additional tests must be performed to identify which mechanism is removing the toxicity. Subsequent tests with nitrogen can be used to determine if toxicity reduction was due to oxidation. Also, air or nitrogen sparging can cause surface active agents to sublate. As bubbles break at the surface, sublatable compounds will be deposited on the sides of the aeration vessel. Sublatable toxicity identification requires special sample removal and rinsing (see below). A visible deposit does not indicate the presence or absence of such toxicants.

Methods: For the aeration process, the volume of effluent and dilution water aerated is kept the same even though all of the dilution water volume is not needed for the *aeration blank*. The flow rate, bubble size, geometry of apparatus and time of aeration should be consistent among treatments. Taller water columns and smaller bubbles should ensure better stripping; therefore, the aeration vessel should be half-full or greater for this process. Each aliquot (effluent and dilution water) should be moderately aerated for a standard length of time (60 min). Use of gas washing bottles (Kontes Glass Co., Vineland, NJ) fitted with

glass frit diffusers located at the bottom of the vessel for aeration is suggested because they sparge the sample effectively. During aeration, the pH of the effluent is not maintained at "pH *i*."

The volume of effluent aerated should be the same for either a 4-d *C. dubia* test or a 7-d *C. dubia* two renewal test (four dilutions, five replicates for each dilution; see Section 5), although there is excess of solutions for the 4-d test. Use of 300 ml of effluent (or dilution water) in a 500 ml gas washing bottle or 500 ml in a 1 L bottle and a flow-rate of 500ml/min is suggested. Any loss of volume and any formation of precipitates should also be recorded.

Interpretation of Results/Subsequent Tests: If the aerated effluent has less toxicity than the *baseline test*, and the *aeration blank* is not toxic, aeration was effective in reducing toxicity. If the toxicity of the aerated effluent is less than the *baseline test*, even though the *aeration blank* is toxic, the results indicate that aeration is an effective removal technique. If the effluent toxicity is not reduced or it is more toxic after aeration than in the *baseline test* (and the *aeration blank* was non-toxic, then either toxicity was concentrated during the aeration process or toxicity was added or created during the aeration process (see *Special Considerations/Cautions* below).

Typically, using this aeration technique, ammonia is not air-stripped from the sample at pH *i*. However, if total ammonia is at least 10 mg/l or higher and the pH is above 8.0, ammonia measurements in the aerated sample may be useful if the aeration manipulation resulted in a toxicity reduction.

If a substantial reduction in toxicity is observed, then the mechanism for the toxicity removal must be determined. To determine if the reduction is due to oxidation, sparging, or sublation, the air should be replaced by nitrogen. The flow of nitrogen through the sample must be the same as for air. If nitrogen sparging as well as air sparging removes or reduces the toxicity, then oxidation as the removal process is eliminated. If aeration only succeeds in reducing toxicity, then oxidation may be involved. It is possible that a toxicant can be removed through sparging *and* oxidation in which case air should reduce toxicity more than nitrogen.

The presence of sublatable substances can be determined (whether air or nitrogen is used) by removing the aerated sample from the aeration vessel by siphoning or pipetting without contact with the sides of the aeration vessel. The geometry of the aeration vessel (i.e., at least a half-full cylinder) must remain the same as in the initial aeration experiment but the recovery of sublated compounds can be difficult. Dilution water added to the aeration vessel is used as a rinse to remove the sublate residue on the walls. To attempt this recovery, use of graduated cylinders with ground glass stoppers has been successful for acute testing (EPA, 1991A) because the water can be shaken vigorously to contact all surface areas to recover the sublatables. This sublation procedure is effective for dissolved surfactants, and while sewage particles adsorb surface active particles tightly, the actual sublation process may take some time (i.e., >1 h) (AHPA, 1989). If toxicity is not recovered from the vessel walls, the presence of such compounds cannot be ruled out. Specific procedures, for the larger volumes needed in the chronic tests, have not yet been developed.

In some instances, sublatable toxicants may not be removed by dilution water, and the use of solvents (e.g., methanol) may be needed for better recovery. However, the solvent will have to be reduced in volume (aired down) in order to have an adequate concentration factor in the test solution and a sufficiently low concentration of solvent for the subsequent toxicity tests (see Sections 6.7 and 6.8 for methanol toxicity information). Of course, dilution water *blanks* must also be subjected to all steps to check for artifactual toxicity.

Special Considerations/Cautions: Removal of compounds by precipitation can occur through oxidation. However, the *filtration test* should not change toxicity of the effluent if oxidation is involved but filtration might also remove the toxicity of some sublatable compounds absorbed to particles and therefore the results of the *aeration test* can be compared to the *filtration test*.

Use of nitrogen to sparge the sample is likely to drastically reduce the DO. For instance, 1 h of nitrogen sparging has caused the DO to drop below 4 mg/l. To increase the DO before initiating the test after a sample has been sparged with nitrogen, transfer the sample to a container with a large surface area to water volume ratio. The DO should rise to >5 mg/l without additional aeration.

The *baseline test* serves as the *toxicity control* and the aeration of the dilution water (*aeration blank*) provides information on the system apparatus. The general test conditions, quality of the dilution water, and health of the test organisms are tracked by the *controls* in the *baseline test*. No significant toxicity should occur in the *aeration blank*. Toxicity in the *aeration blank* implies toxic artifacts from the aeration process, the glassware, or a dilution water problem. If the *aeration blank* is toxic, check the results of the test of the *filtration blank*. If both *blanks* are toxic, then most likely there is a problem with the dilution water but if only the *aeration blank* is toxic, artifactual toxicity arose during that manipulation.

6.5 Filtration Test

General Approach: Filtration of the effluent sample provides information on whether the toxicity is filterable yet provides relatively little specific information about which class of toxicant may be causing the toxicity. Reductions in the toxicity caused by filtering alone may imply toxicity associated with suspended solids or removal of particle-bound toxicants. Whether compounds in the effluent are in solution or sorbed to particles is

dependent on particle surface charge, surface area, compound polarity and charge, solubility, and the matrix of the effluent. If particles are removed, other compounds may be bound to them and are not available to cause toxicity. The way the toxicant is bound to the particulates is probably more important when using filter feeders as the toxicity test organism in short-term chronic tests. This is primarily a route of exposure for filter feeders as compared to the fathead minnow. Toxicity can also be reduced by filtering if a toxicant(s) is not particle-associated; we have observed that some chemicals in a dilution water stock are removed by filtering (e.g., DDT).

The filtration step also serves an important purpose for another Phase I manipulation, the solid phase extraction (SPE) (Section 6.6), where aliquots of the effluent typically must be filtered before application to the SPE sorbent (see *Interpretation of Results/Subsequent Tests* below). If many particles are present in the sample, the sorbent may act as a filter itself or the column will become plugged.

Methods: The use of a positive pressure filtration system is superior to the use of a vacuum filter because volatile compounds may be removed by vacuum filtering and hence confuse the effect of filtering (see *Interpretation of Results/Subsequent Tests*).

As in the acute Phase I, prepare the filters (typically 1 µm glass fiber filters without organic binder) by passing an appropriate volume (approximately one-fourth of effluent volume to be filtered) of high purity water over the filter(s) in the filter housing. This water is discarded, a small aliquot of the dilution water is filtered (prepare excess, at least 500 ml for the *C. dubia* 7-d test and 800 ml for the fathead minnow 7-d test) and discarded (100 ml) and the rest collected. A portion of the filtered dilution water is collected and used for testing and a portion reserved for the *post C_{18} SPE column test blank* (Section 6.6). For example, the last 400 ml of the filtrate is collected for the *C. dubia* 7-d *filtration blank* and *post C_{18} SPE column blank* tests.

Next the effluent sample is filtered using the same filter, and a portion of the filtrate is collected for toxicity testing and a portion set aside that will be concentrated on the C_{18} column. When filtering the effluent, filter enough sample for this test and enough sample (>1 L) to use for the SPE step described below. For some effluents, one filter will not suffice. A technique we use is to prepare several filters at once by stacking 5-8 filters together followed by rinses of high purity water and dilution water using the same rinse volumes as above. Then the filters are separated, and set aside, using one at a time for the effluent sample. If the samples measure quite high in total suspended solids, pre-filtering using a larger pore size filter may help. Again, appropriate *blanks* must be obtained for any pre-filtering. Low levels of metals on the glassware or the filters could cause interferences in toxicity interpretation. Pre-rinsing the filters and glassware with high purity water adjusted to pH 3 may provide consistently clean *blanks* and possibly less contamination in effluent samples. If the sample cannot be effectively/easily filtered due to many fine particles, centrifuging may be better (again *blanks* must be prepared).

The filter housing should be thoroughly cleaned between effluent samples to prevent any particle build-up or toxicity carryover. We have found large filter apparatus (1 L), removable glass frits, or plastic filtering apparatus (Millipore®) to be useful. The glassware cleaning procedure that is described in the acute Phase I TIE manual should be sufficient for chronic TIE work (EPA, 1991A). The glass frits may require rigorous cleaning (i.e., soak in strong acid (10% v/v) for 20-40 min) to remove residuals that may remain after filtering, since the glass frit may itself act as a filter.

Interpretation of Results/Subsequent Tests: If toxicity in the whole effluent is reduced by filtration, a method for separating the toxicants from other constituents in the effluent has been achieved. This should advance the characterization considerably because any subsequent analysis will be less confused by non-toxic constituents. If appropriate, one should determine if toxicity loss was due to volatilization. Comparisons of pressure filtering and vacuum filtering should indicate if volatilization is involved. For further characterization, the mechanism of removal should be determined (precipitation, sorption, changes in equilibrium or volatilization).

Identification efforts should be focused on the residue on the filter after testing indicates that the toxicant(s) is not volatile. To recover the toxicity from the filter(s), use of acidic and basic water as well as various organic solvents can be tried. The recovery achieved by these various methods provides information about pK_a and water solubility of the toxicants. Filtration has reduced the quantity of total cationic metals present in some effluents. The recovery of the metal and acute toxicity was successful when dilution water adjusted to pH 3 was used to extract the filter (EPA, 1991A). Filter extraction into smaller volumes than that of the effluent sample filtered will give a higher concentration of toxicant, perhaps allowing the use of acute test endpoints. However, evidence then must be gathered to be sure the toxicants causing acute toxicity are the same as those causing chronic toxicity. Use of solvents will require solvent reduction or solvent removal (exchange) before testing (see Phase II; EPA, 1992A). Sonication of filters is another approach but the manipulation must be accompanied by proper *blanks* in similar fashion to those needed for the pH 3 extraction of the filter extraction step described above.

If large volumes of an effluent (~2 L over one 1 µm filter) can be readily filtered, the effluent should be filtered for the *filtration test* and unfiltered effluent can be passed over the C_{18} SPE column (see Section 6.6; *Post C_{18} SPE column test*). Once it has been demonstrated that filtration does not reduce toxicity in the effluent, and the toxicity is recovered in the *methanol eluate test* the routine filtering can be eliminated. By

this approach the amount of testing to be done is decreased, yet the tracking of toxicity is possible. We have infrequently experienced any effluents that have low amounts of filterable solids where the effluent could be concentrated without filtering. If any effluent sample has reduced toxicity in the *filtration test* and toxicity is not observed in the *methanol eluate test*, characteristics of the toxicant(s) will be described as filterable and not C_{18} recoverable.

If the toxicity cannot be recovered from the filter, was not volatile (see Section 6.4 *aeration test*) and no other manipulations changed toxicity, use of Tier 2 is a good subsequent step. Toxicity could have been removed by the glass frit, and use of a plastic filter apparatus or stainless steel frits may assist in identifying that the toxicant(s) removed is on the frit or filter. Filter-removable toxicity in Tier 2 is more difficult to identify (because of the radical pH adjustments) because of irreversible reactions and potential for artifactual toxicity (see Section 6.12 below).

Special Considerations/Cautions: The filtered dilution water and filtered effluent sample also serve as the *toxicity blank* and *toxicity control* respectively for the *post C_{18} SPE column test* (see Section 6.6). The results of the effluent *filtration test* should be compared with the *filtration blanks* and no major change in the trend of young production, growth or survival should occur in the *filtration blanks* in comparison to the *controls* in the *baseline test*. If the *filtration blanks* are acceptable, then the results of the *filtration test* and the *baseline test* should be compared.

As a *toxicity blank* for the SPE tests, if the *filtration blank* is either slightly or completely toxic, but the post C_{18} SPE column effluent is not toxic (and effluent toxicity was unchanged after filtration), the *filtration blank* toxicity can be ignored since the effluent toxicity was removed. However, as work proceeds to identification, the *blank* toxicity will have to be eliminated or else it could introduce an artifact and lead to a misidentification of the cause of toxicity.

6.6 Post C_{18} Solid Phase Extraction Column Test

General Approach: The C_{18} SPE column is used to determine the extent of the effluent's toxicity that is due to compounds that are removed or sorbed onto the column at pH *i* (cf., *post C_{18} SPE column and pH adjustment test*; Section 6.13 below). By passing effluent through a SPE column, non-polar organics, some metals, and some surfactants are removed from the sample. In addition, these columns may also behave as a filter (see *filtration test* above).

Compounds in effluent samples interact with the C_{18} and depending upon the polarity and solubility of the compounds, the sorbent may extract the chemicals from the water solution/effluent onto the column. Extraction occurs when the compounds have a higher affinity for sorbent than for the aqueous phase. Non-polar organic chemicals are extracted because the C_{18} sorbent is very non-polar in comparison to the polar water phase; this extraction process is referred to as reverse phase chromatography.

The effluent that passes over the column is collected and the post-column effluent is toxicity tested in order to determine if the column removed toxicity. If the toxicity of the post-column sample is decreased, removal of toxicant(s) by the column is probable but if it is not, artifactual toxicity may be obscuring the removal. Steps to deal with this are given below in *Interpretation of Results/Subsequent Tests*. If the post-column sample is highly toxic, the capacity of the column to extract the toxicants may be exceeded or the column may have been inadequately conditioned.

Because toxicity may be retained by the C_{18} column, efforts to recover the toxicity are necessary. After a sample is passed over the C_{18} column, many of the compounds extracted by the sorbent at a neutral pH should be soluble in less polar solvents than water (i.e., hexane, methylene chloride, methanol, chloroform). However, most of the non-polar solvents are highly toxic to aquatic organisms. Sorbed non-polar organics are eluted from the column because they have higher affinity for the non-polar solvent than the C_{18} sorbent. The *methanol eluate test* (Section 6.7) is designed to determine if toxicants are non-polar.

Methods: The toxicity of the effluent, the type of test to be conducted, and the frequency of the solution renewal affect how much effluent must be filtered and passed over the C_{18} SPE column. First, the concentrations and the volume of the eluate needed for the *methanol eluate test* (Section 6.7) to test at 2x or 4x the whole effluent concentrations should be determined (keeping in mind that the methanol test level must be below the chronic threshold level for the species used; Section 6.7). However, limiting factors of the maximum volume to apply to a column, the minimum elution volume required, and the concentration that can be obtained within these confines must be calculated (Tables 6-7 and 6-8).

For example, our procedure has been to pass 1000 ml of 100% effluent over a 1 g (6 ml) column and elute with 3 ml of methanol which results in a theoretical 333x concentrate. The 1000 ml is the limit of sample volume over a 1 g (6 ml) column and the 3 ml methanol elution is slightly more than the minimum elution volume required (Table 6-7). However to test *C. dubia* at 4x, and to have the methanol concentration at a non-toxic chronic level (Table 6-9), the 3 ml must be further concentrated to 1.5 ml (now 666x whole effluent concentration). At present 3 ml of the eluate is concentrated in graduated centrifuge tubes to 666x by using a gentle stream of nitrogen gas over the surface of the methanol eluate in a warm water bath (25-30°C) to concentrate the 333x eluate to a final volume of 1.5 ml. For five replicates of 10 ml each, 0.30 ml of the eluate can be added to 50 ml of dilution water and the resultant effluent concentration is 4x and the methanol concentration is 0.6%. However the 1.5 ml eluate from the

Table 6-7. Factors to consider for the size of available pre-packed C_{18} SPE columns. Appropriate volumes of sample to apply to each column with respect to maximum volumes of sample and minimum elution volumes, and elution volumes frequently used in the TIE process.

Columns Available[1]		Conditioning Volume (ml)	Maximum Volume (ml) of Effluent	Minimum Elution Volume[2]	Methanol Elution Used (ml)[3]	No. Methanol Fractions[4]	Eluate Concentration
Size (ml)	g of Sorbent						
6	1	10	1,000	2.0, 2.4	3[5], 2.4	3	333x[5], 417x
12	2	24	2,000	4.8	3	3	417x
20	5	40	5,000	12	6	3	417x
60	10	120	10,000	24	12	3	417x

[1] 1 g columns are available from J.T. Baker Chemical Company, Phillipsburg N.J. (1 g, 6 ml columns have been extensively used at ERL-Duluth). 1 g, 2 g, 5 g, and 10 g columns are available from Analytichem International, Mega Bond Elut™, Harbor City, CA. Pumping rates for each column are proportional to volume based on 1 L at 5 ml/min; therefore 2 L at 10 ml/min, 5 L at 25 ml/min, and 10 L at 50 ml/min. We are currently evaluating the minimum elution volumes to determine if less eluting solvent can be used. Pumping rates for 5 L and 10 L may need to be slower when eluting each column. Yet how much the pump should be slowed will be a function of the toxicants. The contact time of the elution solvent with C_{18} sorbent may need to be increased if toxicity is not recovered in the methanol eluates.

[2] Minimum elution volume as recommended by the manufacturers. For the 1 g column, J.T. Baker recommends 2.0 ml and Mega Bond Elut™ recommends 2.4 ml, but 2.0 ml is probably adequate.

[3] Elution of two one-half volume aliquots is better for optimizing the elution efficacy

[4] For each fractionation of any size column, collect three separate 100% methanol fractions to use in *methanol eluate test* to attempt recovery of the non-polar toxicants (see text for more details).

[5] This procedure has been routinely used for acute TIEs. To maximize concentration and minimize methanol levels in concentration and minimize methanol levels in toxicity tests it is best to use the minimum elution volumes recommended by the manufacturer.

1 L fractionation will allow testing of 4x, 2x, 1x only if two solution renewals are used (Table 6-8). Daily renewals for a 7-d *C. dubia* test require a total of 3.7 ml at a water concentration of 0.6% methanol (which means 3 L of effluent must be fractionated to obtain 9 ml of 333x eluate which is concentrated to 4.5 ml to test at 4x) (Table 6-8).

To test at 2x using a 417x eluate from a 2.4 ml elution, 0.048 ml in 10 ml will result in the 2x test concentration. For a 7-d, daily renewal test at 2x, 1x, 0.5x, 3.0 ml is needed (5 replicates of 10 ml each) which will require 1 L of effluent to be concentrated (Table 6-8). By this procedure the final methanol concentration is 0.48%. The 417x concentrate can also be concentrated to 834x and use 0.048 ml/10 ml to test the eluate at 4x.

For the 7-d fathead minnow test using 50 ml per replicate and two replicates, a total of 7.4 ml of a methanol eluate is needed for test initiation and six renewals, which requires fractionation of 3 L of effluent. This assumes the methanol test concentration between species are kept the same. Actually the fathead minnows could probably be tested at methanol concentrations of ~1%, and using 0.96 ml of the 417x eluate per 100 ml will result in 4x effluent test concentration and a 1% methanol concentration (Table 6-9).

The methods below assume one effluent volume (usually the 100%) is concentrated and the post column effluent sample collected and used for all solution renewals during the test (Table 6-8). The procedure described below is an overview of the steps needed to prepare the column, collect methanol *blanks*, recondition the column, collect post-column effluent, and collect methanol eluate (steps needed for this test and the next test—Section 6.7). All steps are detailed in the acute Phase I manual (EPA, 1991A), and the major difference for the chronic Phase I is that fewer post-column samples (one or two versus three) are collected.

The general technique for conditioning and using the prepackaged SPE columns is as follows. Using a pump system with a reservoir for the effluent sample and teflon tubing, first pump 10-120 ml of HPLC grade methanol over the column to condition the sorbent (Table 6-7). This methanol is discarded. Without letting the column go to dryness, 10-120 ml of high purity water is passed over the column and discarded. Next, before the methanol *blank* is collected, the column is allowed to go to dryness. For 1 L of sample and a 1 g (6 ml) column, two 1.5 ml aliquots of 100% methanol are collected, combined, and tested as the *blank*. The elution is more efficient when two aliquots of 1.5 ml are collected in contrast to one elution of 3 ml. The collection of three 100% methanol eluates (2.4 or 3 ml each) has been more helpful for tracking toxicity than only one 100% methanol eluate sample. The use of three 100% methanol elutions is replaced when the Phase II fractionation procedures are applied. These 100% methanol eluates may need to be concentrated prior to testing (see Section 6.7). The containers to collect the methanol should be acid leached, hexane and acetone rinsed, and allowed to dry before use. After the methanol *blank* is collected, the column must

Table 6-8. Test volume of eluate needed for *methanol eluate test* with *C. dubia* or *P. promelas*. Volumes described are based on minimum elution volumes recommended (Table 6-7) and the highest test concentration possible with the methanol level at an acceptable concentration.

Test Species	Test Duration	No. Renewals & Original Sample	High Test Conc.	No. Rep.	Volume of Eluate Needed for Testing at: 333x[1]	417x[2]	Test Concentrations	Minimum Volume (L) of Effluent[3]
C. dubia	4-d	2	2x	5	1.05	0.84	2x, 1x, 0.5x	1
C. dubia	4-d	4	2x	5	2.10	1.68	2x, 1x, 0.5x	1
C. dubia	7-d	3	2x	5	1.58	1.26	2x, 1x, 0.5x	1
C. dubia	7-d	7	2x	5	3.68	2.94	2x, 1x, 0.5x	2
C. dubia	4-d	2	2x	10	2.10	1.68	2x, 1x, 0.5x	1
C. dubia	4-d	4	2x	10	4.10	3.36	2x, 1x, 0.5x	2
C. dubia	7-d	3	2x	10	3.16	2.52	2x, 1x, 0.5x	2
C. dubia	7-d	7	2x	10	7.35	5.88	2x, 1x, 0.5x	3
P. promelas	7-d	7	2x	2	7.35	5.88	2x, 1x, 0.5x	3
P. promelas	7-d	7	2x	4	14.70	11.76	2x, 1x, 0.5x	5
P. promelas	7-d	7	4x	2	14.70	11.76	4x, 2x, 0.5x	5
P. promelas	7-d	7	4x	4	29.40	23.52	4x, 2x, 0.5x	10

[1] For the 333x eluate concentration, this volume is based on the assumption that the *C. dubia* test solutions are prepared as 300 µl of 333x into 50 ml for 2x, 150 µl into 50ml for 1x, and 75 µl into 50 ml for 0.5x. More volume will be needed if serial dilutions are prepared (600 µl vs 525 µl). For the fathead minnow tests this assumes test solutions are prepared as 600 µl into 100 mL for 2x, 300 µl into 100 mL for 1x, and 150 µl into 100 mL for 0.5x. More volume will be needed if serial dilutions are prepared (1200 µl vs 1050 µl).

[2] For the 417x eluate concentration, this volume is based on the assumption that the *C. dubia* test solutions are prepared as 240 µl of 333x into 50 ml for 2x, 120 µl into 50ml for 1x, and 60 µl into 50 ml for 0.5x. More volume will be needed if serial dilutions are prepared. For the fathead minnow tests this assumes test solutions are prepared as 480 µl into 100 ml for 2x, 240 µl into 100 mL for 1x, and 120 µl into 100 ml for 0.5x. More volume will be needed if serial dilutions are prepared. For the 4x fathead minnow test, 960 µl per 100 ml must be prepared for the 4x solution.

[3] Volume is based on high test concentration (2x or 4x) tested without concentration to obtain eluate twice as concentrated. If further concentration is needed, twice as much effluent will be needed.

Table 6-9. Chronic toxicity of methanol (%) to *C. dubia* and *P. promelas* using the 7-d tests.

Species	Water Type	Test Renewal	IC50 95% C.I.	IC25 95% C.I.	NOEC	LOEC
C. dubia	SRW	daily	1.2 1.1-1.2	0.45[1] 0.35-1.0	<0.5	—
	SRW[2]	twice	1.4 —	0.45[1] 0.36-0.70	<0.5	—
	SRW[2]	twice	1.2 0.69-1.7	0.59 0.29-0.95	0.75	1.5
	SRW[2]	twice	1.3 —	0.83 0.34-1.0	0.75	1.5
P. promelas	SRW	daily	2.1 2.0-2.2	1.34 0.27-1.5	1.3	2.5

[1] Value is extrapolated.
[2] Tests all conducted independently.

Note: C.I. = confidence interval; SRW = soft water

be reconditioned with 10-120 ml of methanol (which is discarded). Without allowing the column to go to dryness, follow the methanol with an aliquot (10-120 ml) of high purity water, immediately followed by an aliquot of filtered dilution water. The amount of filtered dilution water needed will be dependent on the species and type of test to be conducted. The initial aliquot of the post-column water should be discarded (~200 ml) and the remainder of the post column dilution water should be collected. This post-column dilution water sample will serve as the dilution water *blank* for the *post C_{18} SPE column test.*

In order to optimize concentration of an effluent sample and not exceed the specifications of the sorbent capacity, when the maximum volume (Table 6-7) of a sample is passed over a column, the sorbent must be reconditioned following the collection of the post column dilution water. For example if 1.2 L of dilution water is needed and 5 L of effluent is to be concentrated on a 5 g column, without reconditioning the column between the dilution water and the effluent, the sorbent's capacity is likely to be exceeded. Toxicity might be observed in the *post C_{18} SPE column test* because of the excessive volume of dilution water and 5 L of effluent. The procedures for conditioning the column are similar to those above. The appropriate amount of methanol (Table 6-7) is used to condition the sorbent and the methanol is discarded. Before the column goes to dryness, follow the methanol with an aliquot (10-120 ml) of high purity water, immediately followed by the volume of filtered effluent to be concentrated. Again, collect about 200 ml of the post-column effluent and discard it. This is discarded to reduce the possibility of higher background concentrations of methanol in the post-column sample which might contribute to artifactual toxicity. Collect remainder of post-column effluent as a batch or in aliquots. If small quantities (<500 ml) of post-column effluent are needed for toxicity testing, separate post-column effluent samples may help determine if toxicity breakthrough occurred, and concentration factors will be different for the lower volumes.

Interpretation of Results/Subsequent Tests: The extraction efficiency of the column is evaluated by comparing the toxicity in the *post C_{18} SPE column test* to the *filtration test* data. This *post C_{18} SPE column test* is most useful when there is no post-column toxicity, and filtration did not reduce toxicity.

When toxicity in the post-column effluent is reduced or removed, then the next step is to compare the results with the *methanol eluate test.* If toxicity was recovered in the methanol eluates (see Section 6.7 below), then efforts to identify the toxicants (Phase II) should be initiated immediately.

If the post-column effluent toxicity was removed or reduced, but toxicity was not recovered in the methanol eluates (see below), it is possible that the toxicant is not eluted into 100% methanol and the C_{18} SPE column contains the toxicant. Use of the gradient of methanol and water fractions should be tried as well as testing the eluate at higher concentrations than 2x (i.e., 4x or 8x). If those tests do not indicate toxicity present in the eluates (see below) alternate elution schemes (EPA, 1992A) must be tried to recover the toxicant. It is important to recognize that the toxicity removed by the C_{18} SPE column is not necessarily due to non-polar compounds. Metals can be removed from some effluents via the C_{18} SPE sorbent. However, metals are not efficiently eluted in methanol or other organic solvents. Acid adjusted (pH 3) dilution water may be needed to elute toxicant(s) from the column. If this is done, the pumping rate of the pH-adjusted water should be slowed (perhaps by one-fourth of original pumping rate) to allow adequate contact time to elute the compound from the sorbent. In addition, compounds such as polymers or surfactants may be sorbed onto the column and some will elute with methanol while others do not.

The column can act as a filter itself and the various solvents used do not elute the toxicant. To check whether the C_{18} column is acting as a filter, unfiltered effluent can be passed over the C_{18} column and toxicity test results compared to those from the filtered effluent sample simultaneously. When effluent samples are readily filtered (e.g., ≥1.5 L for one 1 µm filter) filter the effluent to conduct the *filtration test* and use unfiltered effluent for the *post C_{18} SPE column test* and *the methanol eluate test.* When toxicity can be recovered in the methanol eluate, the toxicant(s) is most likely to be non-polar and since filtration can be eliminated for subsequent identification steps the amount of testing is subsequently reduced.

If the post-column toxicity was reduced and/or removed but not recovered in the *methanol eluate test*, the possibility exists that the toxicant has degraded or decomposed during the manipulation and the toxicant(s) was not concentratable.

As mentioned above, when no toxicity occurs in the post-column effluent (or the toxicity is reduced), and yet the *methanol eluate test* did not exhibit toxicity, metals may be involved or a non-polar that was not recovered in the solvent may be involved (discussed above). To check for cationic metal toxicity, the *post C_{18} SPE column test* should be combined with the *EDTA addition test* and the *sodium thiosulfate addition test* to characterize the post-column toxicity (see Section 6.16, *multiple characterization tests*).

For effluents that have shown that the toxicant is C_{18} recoverable, but the degradation of toxicity occurs fairly rapidly (i.e., the effluent sample is non-toxic in 1-2 weeks), it may be prudent to concentrate additional volumes of effluent immediately after the effluent arrives at the testing laboratory. Non-polar toxicants may not degrade in the methanol fractions as quickly in the effluent samples. Collect the methanol fractions (three 100% fractions) or the various methanol/water fractions as described in Phase II (EPA, 1992A) and hold them at 4°C for analysis as the TIE proceeds. Similarly,

once the cause of toxicity has been determined to be non-polar (C_{18} extractable) it might be more appropriate to immediately concentrate 10 to 20 L of effluent and for the elution step, replace the three 100% methanol elutions with the methanol/water procedures (EPA, 1992A). For chronic work, we have been using seven water/methanol fractions (50%, 75%, 80%, 85%, 90%, 95%, and 100%) rather than the eight used in acute TIEs because the toxicity has never recovered in the 25% fraction and by eliminating it the testing workload is reduced. It may be prudent to try two additional 100% methanol fractions following the seven fractions as well or follow it with alternate elution schemes (cf., Phase II; EPA, 1992A). By immediately concentrating the effluent, it is possible to optimize the amount of methanol available for testing and subsequent concentration for analysis and the post-column samples can be tested at one time. This eliminates duplication of effort that is required when additional methanol eluate is needed for subsequent work in Phase II.

Artifactual toxicity in the test containers may appear as a biological growth in the 100% post-column effluent and the effluent dilutions during the test. Effluents from biological treatment plants may develop this characteristic more readily than physical-chemical treatment plant effluents. This growth can negate actual toxicant removal by the column. While this growth does not occur in all effluents, when it does occur with one post-column effluent sample, the growth often occurs in each subsequent post-column effluent sample. The growth appears as a filamentous growth and gives a milky appearance in the test vessel. This growth has been linked to methanol stimulation of bacterial growth. Methanol is present in the post-column samples because methanol is constantly released from the sorbent during the sample extraction. Additional filtering of the post-column effluent sample through a 0.2 µm filter before testing to remove bacteria and eliminate the growth, has not been particularly successful. Artifactual toxicity from the post-column effluent may be avoided if the tests with the post-column samples are initiated on the same day the effluent is concentrated. To date, when we have collected the post-column samples and tested them on the same day, we have not experienced less artifactual toxicity than we found in those effluents where artifactual toxicity consistently has been a problem. However, less time elapses before animals are exposed to the test solution, therefore less time is available for bacteria to cause problems in the post-column sample matrix. Another option is to perform daily concentration of the effluent and extraction of the column during the 7-d test, as fresh post-column samples may minimize the artifactual toxicity.

When post-column artifactual growth is not readily eliminated, then a different solvent (acetonitrile) to prepare the column (but not for eluting) may be useful in reducing the post-column artifactual bacterial growth. Acetonitrile causes narcotic effects in toxicity tests, and is recommended only to condition the columns to avoid toxic concentrations. This technique has been successful on a limited number of effluents.

Special Considerations/Cautions: Careful observations and judgement must be exercised in detecting problems in the *post C_{18} SPE column test.* Low DO levels can occur in these samples. Through testing experience, the investigator will know whether toxicity appears as artifactual (i.e., growth, low DO) as opposed to the presence of the sample toxicity. If artifactual toxicity is not recognized, then a conclusion that the C_{18} SPE column did not remove toxicity can erroneously be made. For this reason if the post-column effluent is toxic, the methanol eluate must be tested (Section 6.7). This avoids the artifactual toxicity issue and the error can be avoided by determining the toxicity of the eluate.

The methanol elution process does not always produce predictable results with the same effluent sample. When toxicity is removed by the column but no toxicity occurs in the 100% methanol eluates, it does not indicate that the toxicity is *not* due to a non-polar toxicant(s). To check this possibility, immediately test the series of methanol/water fractions at concentrations of 4x or 8x. Not all non-polar organic compounds elute into 100% methanol as well as they do into lower methanol/water concentrations. Also toxicants may smear across the fractions and when <100% recovery of toxicity from the column is not 100%, toxicity may not be observed at 2x or 1x.

General test conditions will be tracked (dilution water, health of test animals) by the *controls* in the *baseline test.* The post-column dilution water *blanks* should be compared to those *controls* to determine if the column imparted toxicity. If the post-column dilution water *blank* was toxic, but no toxicity or artifactual toxicity occurred in the post-column effluent sample, the toxic *blank* can be ignored.

Results of the *post C_{18} SPE column effluent test(s)* must be compared to the results of the *filtration test* to determine if the manipulations effectively reduced toxicity. When the *post C_{18} SPE column test* is plagued by artifactual toxicity, the importance of the *methanol eluate test* increases. The results of the *post C_{18} SPE column test* must also be compared to the *baseline test* to determine if toxicity was removed by the C_{18} SPE column.

6.7 Methanol Eluate Test

General Approach: In order to elute toxicants from the C_{18} SPE sorbent, a relatively non-polar solvent is used. Hexane, one of the most non-polar solvents, can be used to remove highly non-polar compounds from the C_{18} SPE column. Yet hexane is one of the most toxic solvents to aquatic organisms and has a low miscibility with water. Methanol is more polar than hexane, but is much less toxic and will elute many

compounds. The use of methanol has been adopted as the eluant for the acute TIE (EPA, 1991A; EPA, 1989A) and the chronic TIE because of its low toxicity (Table 6-9) and its usually adequate ability to elute chemicals from the C_{18} SPE column.

Methods: The conditioning and elution steps are described in detail in the *post C_{18} SPE column test* above (see Section 6.6). For this test, we assume that the column extraction efficiency and elution efficiency are 100%.

If a 1 g (6 ml) SPE column was used with 1 L of 100% effluent, and a 3 ml methanol eluate was collected, the methanol eluate is a 333x concentrate of the original effluent (Table 6-7). Depending on the amount of effluent toxicity, this eluate may have to be concentrated further in order to test at a sufficient concentration (i.e., 4x) and have methanol concentrations in the test lower than the methanol effect concentration. In Table 6-9 the toxicity data for methanol toxicity to *C. dubia* and fathead minnows are given. The toxicity of methanol is slightly greater for *C. dubia* when the test solutions were renewed daily but not significantly for this characterization stage of the TIE. From these data, one can decide how much methanol can be added and how concentrated the eluant must be to achieve 2x or 4x the original effluent concentration. The choice of test concentration depends on the toxicity of the effluent; for example, if the effluent is toxic at ~25%, one may not need to achieve a 4x concentration. Some methanol toxicity can be present, as long as sufficient toxicity from the effluent is present to be measurable. As discussed in the *post C_{18} SPE column test*, the fathead minnows can be tested at 4x using only 0.96 ml of a 417x methanol eluate but the methanol concentration is about 1%, which cannot be tolerated by *C. dubia*.

Interpretation of Results/Subsequent Tests: If toxicity occurs in the *methanol eluate test* at any concentration tested, Phase II should be initiated. This step would include the use of a gradient of methanol/water eluant solutions to elute additional columns and conduct the toxicity tests on each fraction (Phase II; EPA 1989A; EPA, 1992A). Toxicants other than non-polar compounds may be retained by the SPE column but they are less likely to be eluted sharply or eluted at all (see Section 6.6). Non-polar toxicity can in some instances be distinguished from post-column artifactual toxicity if the eluate is checked for toxicity. Some toxicants (such as some surfactants) may not elute from the SPE column with methanol, but if toxicity is not recovered in the eluate, it does not exclude the possibility of a non-polar toxicant or metal (see Section 6.6 for additional discussion). Dilution water adjusted to pH 3 or pH 9 may be useful in eluting a toxicant(s) from the column. Some experimentation will be needed to determine the volumes of water to pump over the column. The pumping rate should be slowed considerably to allow sufficient contact time on the column (see details in Section 6.6 and Table 6-8).

At this time, we have not been successful in tracking chronic non-polar toxicity using the acute test endpoint with the methanol eluates, rather chronic tests have been needed to track the chronic toxicity.

A subsequent test that may be useful is to assess whether the toxicant must be metabolically-activated by the test organism before exhibiting toxicity. These activation reactions consist of oxidative metabolism by a family of enzymes collectively known as cytochrome P-450. Some toxicants require cytochrome P-450 activation before expressing toxicity. Piperonyl butoxide (PBO) is a synthetic methylenedioxyphenyl compound that effectively binds to, and blocks the catalytic activity of cytochrome P-450. When a non-toxic amount of PBO is added to an effluent test solution which contains a toxicant(s) that requires metabolic activation, the toxicity of the effluent can be reduced or completely blocked (EPA, 1991A). The relative specificity of PBO for blocking the toxicity of metabolically-activated organic compounds makes this test a useful part of the subsequent testing in the TIE. For example in the acute Phase I (EPA, 1991A) as a subsequent test, we suggest that PBO may be added directly to the effluent before adding the organisms. The 48 h LC50 of PBO is 1 mg/l for *C. dubia* and we have used 0.250 to 0.500 mg/l to effectively block the acute toxicity of metabolically-activated compounds for *C. dubia* in the effluent and the methanol eluate. The NOEC and the IC25 for PBO and *C. dubia* was determined as 63 µg/l and 89 µg/l, respectively. Low concentrations of PBO have reduced the chronic toxicity in the methanol eluate test and levels of 100 or 50 µg/l have been useful in chronic tests with *C. dubia*. The PBO should be added using a minimal amount of methanol as a carrier solvent since the level of methanol present in conjunction with the methanol eluate is present. Since PBO is not readily soluble in water, a superstock of 20 g/l is prepared by dissolving PBO in reagent grade methanol. An aliquot of the superstock is mixed in the standard laboratory dilution water to produce a stock solution at a concentration of 25 mg/l and aliquots of this stock solution are added to the test cups after addition of the methanol eluate, and the solution thoroughly mixed. This test should be conducted in similar fashion to the *EDTA addition test*. Appropriate *blanks* must be used, for example both the *methanol blank* and the *methanol eluate* must be tested with and without PBO. If toxicity occurs in the *methanol blank* fraction with the PBO additions, either PBO was present at toxic concentrations or the methanol concentration in the test was too high. If toxicity is observed in the *methanol eluate* with the PBO addition, but not in the *methanol eluate* without the PBO or either of the *blank eluates* (with PBO and without PBO), this result is not very informative. It is possible that the PBO has interacted in a synergistic fashion with another compound present in the test effluent that normally would not be toxic.

Compounds that are sparingly soluble in water may not be eluted from the column with methanol. If this

occurs, less polar solvents will have to be tried, but this technique will require solvent exchanges to avoid toxic solvent concentrations and other solvents may recover chemicals not toxic in the effluent due to solubility problems. At this time, we have not used solvent exchanges for chronic toxicity tests, but are exploring the use of methylene chloride. The 48 h LC50 of methylene chloride to *C. dubia* is 0.13% and the chronic toxicity to *C. dubia* is ≤0.03%. Therefore it cannot readily be used as the primary solvent, but rather as the exchange solvent and may be of limited use for this effort. Additional work on the appropriate solvent exchange for chronic TIEs is on-going (EPA, 1992A).

Special Considerations/Cautions: The *baseline test* serves as the *toxicity control*, and the *methanol blank* serves as a comparison of the effects of methanol alone in water. The health of the test animals, the viability of the dilution water and general test conditions are evaluated by the *baseline controls*. If the effluent *methanol eluate* is *non-toxic* at 2x or 4x but the *methanol blank* is toxic, the *blank* toxicity can be ignored since no non-polar toxicity is recovered.

If effluent dilutions are set at 100%, 80%, 60%, and 40%, it might be useful to test the eluate at a multiple of these concentrations, i.e., 2x, 1.6x, 1.2x, 0.8x or concentrate them to 4x, 3.2x, 2.4x, or 1.6x to compare the *baseline toxicity* with the toxicity in the *methanol eluate tests*. The artifactual growth observed in the *post* C_{18} *SPE column test* from the methanol has not occurred in our *methanol eluate tests*. This is most likely due to the differences in how the methanol degrades/behaves in dilution waters which are low in methanol-oxidizing bacteria and other organic matter in contrast to effluent samples (even post-column effluents).

6.8 Graduated pH Test

General Approach: This test will determine whether effluent toxicity can be attributed to compounds whose toxicity is pH dependent. The pH dependent compounds of concern are those with a pK_a that allows sufficient differences in dissociation to occur in a physiologically tolerable pH range (pH 6-9). The toxicity depends on the form that is toxic (ionized versus un-ionized). Metal toxicity can be affected by pH differences through changes in solubility and speciation. pH dependent toxicity is likely to be affected by temperature, DO and CO_2 concentrations, and total dissolved solids (TDS). The graduated pH test is most effective in differentiating substantial toxicity related to ammonia from other causes of toxicity.

Ammonia is an example of a chemical that exhibits different ionization states and subsequently pH dependent toxicity. Ammonia is also frequently present in effluents at concentrations of 5 mg/l to 200 mg/l (or higher). Measuring the total ammonia in the sample upon its arrival will be helpful to assess the potential for ammonia toxicity. pH has a great effect on ammonia toxicity. For many effluents (especially with municipal effluents) the pH of a sample rises upon contact with air, typically the pH of effluents at air equilibrium ranges from 8.0 to 8.5. Literature data on ammonia toxicity (EPA, 1985D) can be used only as a general guide because the pH values for most ammonia toxicity tests as reported in the literature are usually not measured or reported fully enough to be useful in TIE tests. Additional data on ammonia toxicity for *C. dubia* and *P. promelas* is provided in the revised Phase II (EPA, 1992A). The acute Phase I manual has a lengthy description of the toxicity behavior of ammonia (EPA, 1991A) and Phase II provides additional information (EPA, 1992A).

One might expect ammonia to be removed during the Tier 2 *aeration and pH adjustment test* at basic pH (described in Section 6.11). Based on our experience, however, ammonia is not substantially removed by the methods used to aerate the sample described in this manual. (If a larger surface to volume ratio is used, this manipulation can reduce ammonia levels; see *Interpretation of Results/Subsequent Tests* below and Phase II; EPA, 1992A.) Other techniques which can be used to remove ammonia may also displace metals or other toxicants with completely different physical and chemical characteristics. For example, ion exchange resins (e.g., zeolite) remove ammonia, cationic metals, and possibly organic compounds through adsorption.

Toxicity related to metals may also be detected by the *graduated pH test*, although these effects are less well documented in effluents (and for chronic toxicity) than those associated with ammonia toxicity. The toxicity may change for both pH increases and decreases from neutral pH (pH 7). Such behavior is characteristic of aluminum and cadmium. Acute toxicity test experiments with *C. dubia* in clean dilution waters indicate lead and copper were more acutely toxic at pH 6.5 than at pH 8.0 or 8.5 (in very hard reconstituted water), while nickel and zinc were more toxic at pH 8.5 than at 6.5 (EPA, 1991A). In recent experiments during a chronic TIE, we have found that chromium is pH dependent on an acute basis for *C. dubia*, but not water hardness dependent. The pH dependence was not observed in acute tests unless food (YCT) (EPA, 1992C) was added during the 48 h acute test at test initiation. Therefore, caution must be exercised in interpreting the chronic toxicity results with effluents, because the toxicant(s) may behave in certain ways that are not documented in the literature.

By conducting tests at different pHs, the effluent toxicity may be enhanced, reduced or eliminated. For example (at 25°C) where ammonia is the primary toxicant, when the pH is 6.5, 0.180% of the total ammonia in solution is present in the toxic form (NH_3). At pH 7.5, 1.77% of the total ammonia is present as NH_3 and at pH 8.5, 15.2% is present as NH_3. This difference in the percentages of un-ionized ammonia is enough to make the same amount of total ammonia about three times more toxic at pH 8.5 as at pH 6.5. Whether or not toxicity will be eliminated at pH 6.5 and the extent to which toxicity will increase at pH 8.5 will depend on the total ammonia concentration. If the graduated pH test is done at two pHs using the same dilutions, one

should see toxicity differences between pH 6.5 and 8.5. The effluent effect level (expressed as percent effluent) should be lower at pH 8.5 than pH 6.5 if ammonia is the dominant toxicant.

The most desirable pH values to choose to test for the *graduated pH test* will depend upon the characteristics of the effluent being tested. The graduation scheme that includes the air equilibrium (the pH the effluent naturally drifts to) will allow a comparison of treatments to unaltered effluent (i.e., *baseline test*). For example, if the air equilibrium pH of the effluent is pH 8.0, it may be more appropriate to use pHs 6.5, 7.3 and 8.0. The pHs of many municipal effluents rise to 8.2 to 8.5 (or higher), so pHs such as 6.5, 7.5 and 8.5 may be more appropriate. In any case, it will be necessary to conduct the test at more than one effluent concentration (e.g., 100%, 50%, 25%) to determine what role, if any, the pH dependent compounds play in toxicity.

The challenge of the graduated pH test is to maintain a constant pH in the test solution. This is a necessity if the ratio of ionized to the un-ionized form of a pH sensitive toxicant is to remain constant and the test results are to be valid. However, in conducting either acute or chronic toxicity tests on effluents, it is not unusual to see the pH of the test solutions change 1 to 2 pH units over a 24-h period.

Methods: To lower the pH of the samples, either CO_2/air mixtures or HCl additions (or the combination of both) are used. The pH should be maintained throughout the 4-d or 7-d test with little variation (±0.2 pH units).

When CO_2/air (without any acid addition) is used to control the pH, the pH of the effluent samples is adjusted by varying the CO_2/air content of the gas phase over the water or effluent samples. By using closed headspace test chambers, the CO_2 content of the gas phase can be controlled. The amount of CO_2/air needed to adjust the pH of the solution is dependent upon sample volume, the test container volume, the desired pH, the temperature, and the effluent characteristics (e.g., dissolved solids). The exact amount of CO_2/air to inject for a desired pH must be determined through experimentation (on day 1) with each effluent sample *before* the *graduated pH test* begins. Therefore, the test may have to be set up later than the other Phase I tests (e.g., day 3) unless experimentation was initiated on day 1. The amount of CO_2 added to the chamber assumes that the liquid volume to gas volume ratio remains the same. Generally, as the alkalinity increases, the concentration of CO_2 that is needed to maintain the pH also increases. For adjusting pH's downward from pH 8.5 to 6, 0.5-5% CO_2 has been used. If more than 5% CO_2 is needed, adjust the solutions with acids (HCl) and then flush the headspace with no more than 5% CO_2/air. With appropriate volumes of effluent, experiments with variable amounts of CO_2/air and equilibrated for about 2 h, are used to select the needed CO_2 concentration. More than 5% CO_2 is not recommended as CO_2 toxicity is likely to be observed. When dilutions of an effluent have the same hardness (or alkalinity) and initial pH as the effluent, the same amount of CO_2 is usually needed for each dilution, but sometimes different amounts are needed in the higher effluent concentrations. Use of a dilution water of similar hardness (or alkalinity) as the effluent makes the CO_2 volume adjustments easier. When tests are conducted in these CO_2 controlled environments, dilution water *controls* for each pH should be included.

Acid is used first to adjust pH's when the amount of CO_2/air needed to adjust to the desired pH is greater than 5% CO_2/air. Again experimentation is needed to determine how much CO_2/air is needed. Techniques for acid adjustment are described in Section 6.10 below and also in the acute Phase I manual (EPA, 1991A).

For adding a mixture of CO_2/air to the headspace of the test compartments, a 1 L gas syringe (Hamilton Model S-1000, Reno, NV) is used. In most instances, the amount of CO_2 produced by the invertebrates has not caused further pH shifts, but with larval fathead minnows, the pH may drop from the additional amount of CO_2 respired by the fish bacterial metabolic CO_2 released.

For the pH controlled tests, the pH should be measured at least two to three times for each 24 h period when readings of survival and/or young production are made. If samples are not renewed daily (as may be the case for the *C. dubia* tests), then the headspace should be re-flushed with CO_2/air after the animals are fed. Again, some experimentation may be needed to determine the amount of CO_2/air needed for this step. In all *graduated pH tests*, the pH should be measured in all the chambers. If the pH drifts as much as 0.2 pH units, the results may not be usable and better pH control must be achieved. However, if pH fluctuates more than 0.2 pH units and toxicity is gone at one pH and not another, the toxicity results may be useful (see *Interpretation of Results/Subsequent Tests* below).

Measurements of pH must be made rapidly to minimize the CO_2 exchange between the sample and the atmosphere. Avoid vigorous stirring of unsealed samples because at lower pH values, the CO_2 loss during the measurement can cause a substantial pH rise. In addition, measure the DO because toxicants such as ammonia have different toxicities when DO is decreased (EPA, 1985D). Keep in mind that if the test animals have been dead for awhile, the pH and/or DO of the test water most likely will have changed. Therefore, pH measurements should be made as soon as possible if animals die rapidly.

Methods that use continuous flow of a CO_2/air mixture, such as tissue cell incubators, may be preferable and give better pH control. A pH feedback system can be used to control the CO_2-mix to the incubators. At this time we have not attempted to use a continuous flow of CO_2 and cannot recommend a system to use.

Maintaining pH above the air equilibrium pH (generally above pH 8.3) is difficult to achieve because the

concentration of CO_2 must be very low, and microbial respiration can increase the CO_2 levels in the test chamber. Frequently we use a dilution water that has a higher pH (i.e., very hard reconstituted water) to prevent pH drift downward.

Interpretation of Results/Subsequent Tests: For the *graduated pH test*, the pHs selected must be within the physiological tolerance range for the test species used (which generally is a pH range of 6 to 9). In this pH range, the amount of acid or base added is negligible, and therefore the likelihood of toxicity due to increased salinity levels is low.

When ammonia is the dominant toxicant, the toxicity at pH 6.5, should be less than in the pH 8 test. However, ammonia is not the only possible cause of toxicity. Using the pH of the *baseline test*, the relative toxicity of each pH adjusted solution can be predicted if ammonia is the sole cause of toxicity (EPA, 1989A; EPA, 1992A).

However, if ammonia is only one of several toxicants in an effluent, this procedure will be hard to interpret. For this reason, if total ammonia concentrations in the 100% effluent are greater than 20 mg/l, include a pH 6 (rather than 6.5) and pH 7.3 (±0.2) effluent treatment interfaced with other Phase I tests. Complicating effects of metal toxicity may be reduced by adding EDTA to the test solutions. However, the ability of EDTA to detoxify metals may also change with pH, although we have not experienced this effect yet.

Other metals may exhibit some degree of pH dependence, but these are not as well defined. Whether the metal toxicity can be discerned will depend in large part on the concentration of other toxicants in the sample. In order to detect metal toxicity, one must be cautious when selecting a dilution water if the test solutions are low effluent concentrations. Artifactual toxicity due to metals may be created if the hardness of the dilution water is much different from that of the effluent (see Section 3). This effect may be magnified for metals when coupled with the pH change. A dilution water similar in hardness to that of the effluent must be used for this test to reveal metal-caused toxicity. If more than one pH dependent toxicant is present, the pH effects may either cancel or enhance one another.

In the acute TIEs, we have suggested the use of hydrogen ion buffers to maintain the pH of effluent test solutions and to compare these test results to those from CO_2 adjusted samples. Three hydrogen ion buffers were used by Neilson et al. (1990) to control pH in toxicity tests in concentrations ranging from 2.5 to 4.0 mM. These buffers were chosen based on the work done by Ferguson et al. (1980). These buffers are: 2-(N-morpholino) ethane-sulfonic acid (Mes) (pK_a = 6.15), 3-(N-morpholino) propane-sulfonic acid (Mops) (pK_a=7.15), and piperazine-N,N'-bis (2-hydroxypropane) sulfonic acid (Popso) (pK_a=7.8). We have replaced the Popso buffer with another buffer which is more readily soluble in order to achieve better pH control around the pH 8.0 range. This buffer is N-tris(hydroxymethyl) methyl-3-amino propanesulfonic acid (Taps) (pK_a = 8.4) and has been used primarily for the chronic *C. dubia* tests at this time.

The acute toxicity of these Mes, Mops, and Popso buffers is low to both *C. dubia* and fathead minnows (Phase I; EPA, 1991A) (48-h and 96-h LC50s for all buffers are ≤25 mM for both species). Sublethal levels of the buffer are added to hold the pH of test solutions for the acute Phase I tests (see EPA, 1991A). Chronic toxicity results using these three buffers indicated that 16 mM did not cause reduced survival or growth for the fathead minnow 7-d test. For *C. dubia*, 4mM of all four buffers has not caused reduced survival or reproduction in either the 4-d or 7-d tests. Use of the buffers is preliminary and the effects due to interferences from the buffers themselves have not been studied. It is possible that the buffers may reduce the toxicity of some toxicants.

The buffers must be weighed and then added to aliquots of the effluent dilutions and control water as batches. Then adjust to desired pH with acid and base to the selected values and add the test organisms. Solutions should be left for several hours to equilibrate, especially for the Popso buffer which has low solubility in water (in contrast to other buffers). While our experience with the buffers is limited, we have found the amount of any buffer needed to hold a pH is effluent specific. Once the pH is adjusted to the desired pH, the test solutions need not be covered tightly to maintain pH; however pH should be measured at each survival reading at all dilutions. The test results with the buffers should mimic those of the earlier *graduated pH test* if ammonia is the suspect toxicant.

The methods described in Phase II can be used to add identify ammonia as the pH sensitive toxicant. Use of the air-stripping method to remove ammonia from the sample at high pH's should help evaluate whether toxicant(s) other than ammonia are present (Phase II, 1992A). The results of this air-stripping test should be compared with the *aeration test* results of Phase I, the *baseline effluent test* and the *graduated pH test*. If the ammonia concentration is decreased and the toxicity is reduced or absent, more evidence that ammonia is playing a role in the toxicity of the effluent has been generated. Other compounds could precipitate with the pH adjustment and concentration during air-stripping and when water is added back into the solution, they may not be available.

Special Considerations/Cautions: The *controls* in the CO_2 controlled chambers for each pH and the *baseline test* act as checks on the general health of the test organisms, the dilution water and most test conditions. If the effluent pH in the *baseline test* is close to that of the pH adjusted test solutions, the toxicity expressed in the two tests should be similar. Significantly greater toxicity may suggest interference from other factors such as the ionic strength related toxicity (if the

pH was adjusted with HCl) or CO_2 toxicity. Dilution water tested at the various pH's does not serve as *blanks*, as the effluent matrix may differ from that of the dilution water. However, if acids and bases are added (with or without CO_2 additions) then *toxicity blanks* with the same amounts of acid/base added need to be tested to determine the cleanliness and effects of the acids and bases. Other compounds with toxicities that increase directly with pH may lead to confounding results or may give results similar to ammonia. Monitoring the conductivity of the effluent solutions after the addition of the acids and bases may also be helpful in determining artifactual toxicity.

6.9 Tier 2 Characterization Tests

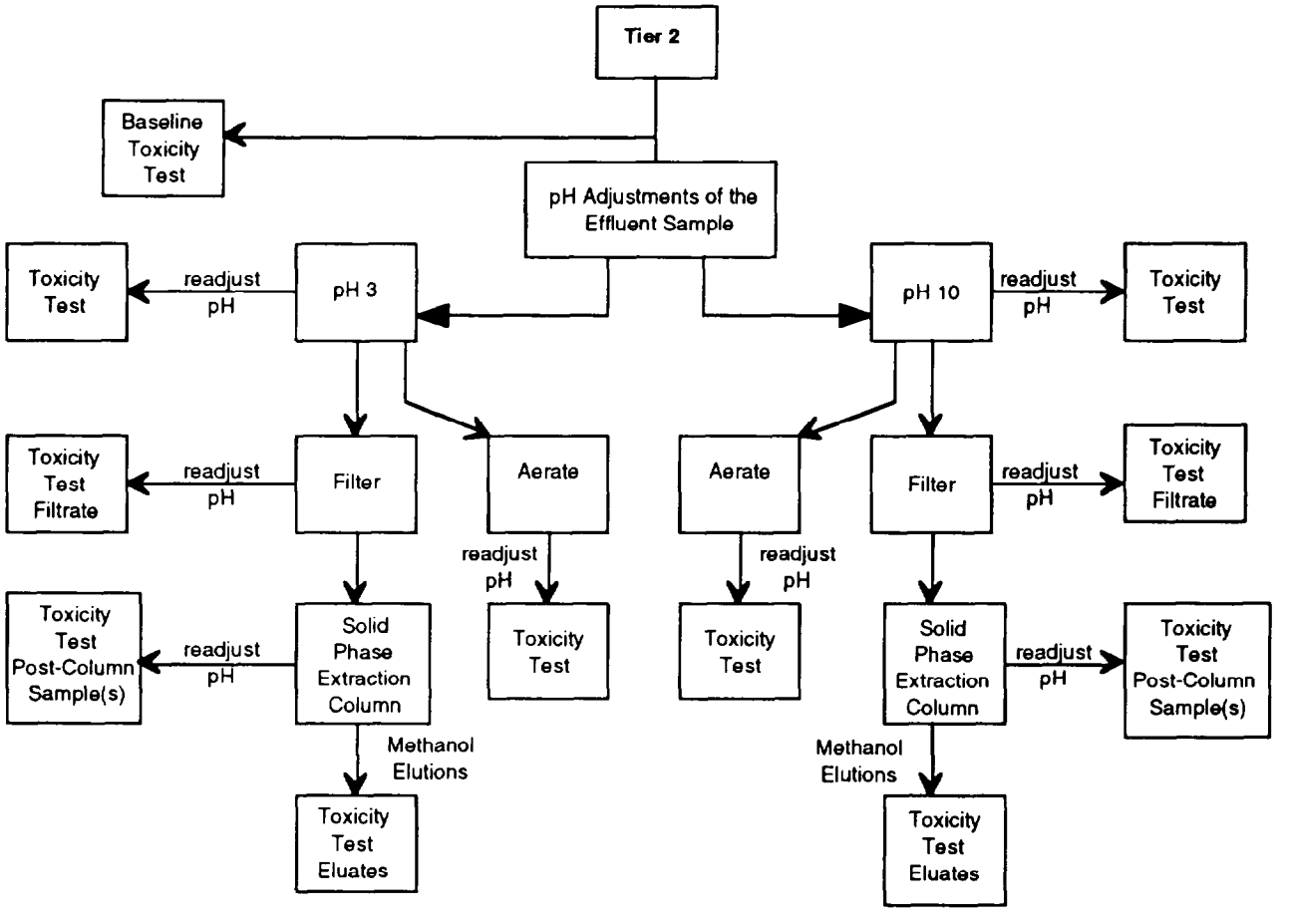
Two tiers are used in the chronic TIE approach primarily because in our experience, radical pH adjustment often is not needed. Only when the manipulations in Tier 1 do not indicate clear patterns is Tier 2 conducted. Tier 1 manipulations do not involve the use of drastic pH manipulations to characterize the toxicity of the sample. The pH adjustments are used to affect toxicity when the Tier 1 tests are not adequate or to assist in providing more information on the nature of the toxicants (Figure 6-3).

Changes in pH can affect the solubility, polarity, volatility, stability, and speciation of a compound. These can change the bioavailability of the compounds, and also their toxicity. The Phase I acute manual (EPA, 1991A; EPA, 1988A) discusses the effect of pH on groups of compounds at length, therefore only an abbreviated discussion of pH effects will be covered in this document.

Un-ionized forms of chemicals are generally less polar than the ionized form, and the ionized forms interact with water molecules to a greater extent. Compounds may be more toxic in the un-ionized form, as was discussed above in Section 6.8 *graduated pH test*. Un-ionized forms may be easily stripped from water using aeration, or extracted with SPE techniques and subsequent elution with non-polar solvents. Also, changes in solubility with pH change may cause compounds to be removed by filtration. The form of metals can be altered by pH and organic compounds can be degraded at extreme pH values.

Even if the chemical species are unchanged, changes in the pH of the solution may affect the toxicity of a given compound. The cell membrane permeability and the chemistry of the toxicant may be affected.

Figure 6-3. Tier 2 sample preparation and testing overview.



Changing pH and returning it to pH *i* after a short time (~1 h) will not always change the toxicity. However, this adjustment may result in a reduction, loss or increase in the toxicity. Sometimes only the pH adjustment in combination with a manipulation (e.g., filtering, solid phase extraction) changes toxicity when the same pH unadjusted manipulation test did not.

6.10 pH Adjustment Test

General Approach: For this Tier 2 test, the effluent is adjusted to either pH 3 or pH 10, and left at those pHs until other manipulations (aeration, filtration, and C_{18} SPE post-column effluent samples) are ready to be readjusted to pH *i*. The pH adjustment alone may not change toxicity, if equilibrium is slow. Satisfactory *blanks* in chronic tests with various reconstituted waters adjusted to pH 11 have not been consistently produced, but acceptable *blanks* have been obtained at pH 10 (and pH 3), while pH 11 adjustments have not been problematic in some effluent matrices. Since pH 11 was subjectively chosen, we recommend adjustment to pH 10 for chronic TIE's. The *pH adjustment test* serves as a *toxicity control* for the pH adjustments combined with aeration, filtration and the C_{18} SPE column manipulation. As described in Tier 1 and the acute Phase I manual, pH may drift very differently during the toxicity tests following these more severe pH manipulations. Therefore, monitoring and control of test pH is necessary.

Methods: An aliquot of effluent is pH adjusted to pH 3 and another aliquot is adjusted to pH 10, along with dilution water samples which will serve as *blanks*. Enough sample and dilution water are pH adjusted to provide the necessary volumes for the *aeration and pH adjustment test*, the *filtration and pH adjustment test*, and the *post C_{18} SPE column and pH adjustment test*. Minimal dilution of the effluent should occur, and the use of 0.01 N, 0.1 N, and/or 1.0 N solutions of acids/bases (Suprapur®, E. Merck, Darmstadt, Germany) to adjust pH are suggested. The volumes and strengths of the acid/base additions should be recorded as this information may be useful in determining if artifactual toxicity should be expected. This information can be helpful when subsequent testing is conducted and knowledge of the volumes of acid/base added to the previous samples assists in making the pH adjustments more rapidly.

Interpretation of Results/Subsequent Tests: A decrease in toxicity compared to the *baseline test* should be pursued to detect the mechanism of toxicity reduction. Often precipitation occurs after drastic pH change. If precipitation does occur, then the *filtration and pH adjustment test* will likely remove the toxicant and efforts should be focused on recovery and identification from the filter. Similarly, if the C_{18} SPE column or aeration changes toxicity, these manipulations should be pursued. If toxicity is only reduced by pH change, (which is not common) not much can be made of the information, and clustering of several manipulations as well as adding additional techniques such as ion exchange should be explored. Dilution from the acid and base additions should also be checked. Degradation of toxicity is a possibility also, but is nearly impossible to detect at this stage.

The adjustment of pH (to pH 3 or pH 10 and back to pH *i*) may cause toxicity problems. Just the addition of the NaOH or HCl may be the cause of the toxicity and may also occur in the dilution water *blanks* or only in the effluent sample. The effect on effluent toxicity of the Na^+ and Cl^- additions, depends on the TDS concentration of the effluent. The acid/base additions are typically more toxic in dilution water than in effluent, unless the effluent TDS concentration is high, and the additional concentrations of acid/base result in toxic TDS concentrations. These effects are of more concern in chronic TIE's. The effect of NaCl additions on TDS can be tracked by measuring conductivity. Appreciable increases in conductivity should be a warning to evaluate TDS toxicity caused by acid and base addition.

Increases in toxicity compared to the *baseline test* may be a result of either an increase in TDS or toxicant changes. TDS as a toxicant may be eliminated by calculating the TDS at the ICp value. Effluents that have high toxicity require high dilution to determine the ICp, and at such great dilution the TDS is subsequently diluted sufficiently to remove TDS as a candidate. If this is not the case, NaCl can be added to an aliquot of effluent to see if the acid/base additions could have caused the increased toxicity. Table 6-10 provides chronic toxicity information for NaCl in various hardness waters for *C. dubia* and fathead minnows.

Precipitates can remove toxicity through sorption of such chemicals as non-polar organics. In this case the precipitate is only the mechanism of removal, not the toxicant itself. The C_{18} SPE column is likely to remove the toxicity in such cases; however, in Tier 2 a pH change can also desorb toxicants from particles and make them bioavailable and therefore toxic.

Different pH drift during the *baseline toxicity test* and those after manipulations has been discussed (EPA, 1991A). For a valid test, the pH during the test must be known and maintained the same as in the pH *i* test. If the drift of the pH varies considerably, confusion in interpreting the results can arise if a compound whose toxicity is pH dependent is present in the sample. If good pH control is not maintained incorrect conclusions are likely to be made and mislead the TIE process.

Special Considerations/Cautions: The addition of acids and bases to the effluent does not give comparable results of acids and bases added to the dilution water. The amount of acid and base added to each sample will more than likely be dissimilar. However, dilution water toxicity *blanks* to assess the additions of the acid and base are needed to determine whether toxic concentrations of ions have been reached and to determine the cleanliness of the acid and base solutions that are used in this manipulation and subsequent pH manipulation tests. The *controls* from the *baseline test* provide information on the health of the test organ-

Table 6-10. Chronic toxicity of sodium chloride (g/l) to *C. dubia* and *P. promelas* in various hardness waters using the 7-d tests.

Species	Water Type	IC50 95% C.I.	IC25 95% C.I.	NOEC	LOEC
C. dubia	SRW	1.3 1.2-1.5	0.93 0.76-0.96	0.63	1.3
	MHRW	1.6 1.4-1.7	1.3 0.24-1.3	1.0	2.0
	HRW	1.5 1.3-1.6	1.2 1.0-1.3	1.0	2.0
	VHRW	1.4 1.1-1.6	1.0 0.58-1.2	1.0	2.0
P. promelas	SRW	0.84 0.76-1.1	0.67 0.63-0.77	0.50	1.0
	SRW	1.3 1.2-1.5	0.93 0.76-0.96	0.63	1.3
	MHRW	1.5 1.4-1.6	1.2 1.1-1.2	1.0	2.0
	HRW	3.2 2.9-3.3	2.3 2.0-2.5	2.0	4.0
	VHRW	4.5 3.9-4.9	3.2 2.4-3.5	2.0	4.0

Note: C.I. = confidence interval; SRW = soft reconstituted water; MHRW = moderately hard reconstituted water; HRW = hard reconstituted water; VHRW = very hard reconstituted water. laboratory test conditions. The *pH adjustment test* serves as the *toxicity control* (or perhaps the "*worst case*" *toxicity control*) for the subsequent pH adjustment/characterization tests.

isms, dilution water, and laboratory test conditions. The pH adjustment test serves as the toxicity control (or perhaps the "worst case" toxicity control) for the subsequent pH adjustment/characterization tests.

6.11 Aeration and pH Adjustment Test

General Approach: Aeration at pH 3 or pH 10 may make toxicants oxidizable, spargeable or sublatable, that are not so at pH *i*. If this does occur, avenues are then available to characterize and identify, similar to the procedures described for aeration at pH *i* in Tier 1. For this test, two effluent aliquots which were adjusted to pH 3 and pH 10 in the *pH adjustment test* are each aerated for a period of time, for example, 1 h. The aeration process can concentrate compounds due to loss of volume, and caution should be exercised in this aeration process and lost water may need to be replaced with dilution water.

Methods: The steps for this procedure should be identical to those used in the non-pH adjusted sample aeration (Section 6.4). The pH of the effluent may drift during the aeration, and it should be checked at 30 min intervals and readjusted to the original pH (pH 3 or 10) if it has drifted more than 1 pH unit. The amount of NaCl added from the acid/base additions may be different in aerated samples than for *pH adjustment test* and proper compensation for this difference must be made as described above. The volume of effluent aerated should be compared to the amount of original sample volume prepared.

After aeration is completed, adjustments back to pH *i* should be made on all samples at the same time. The formation of any precipitates should be noted, but the importance of precipitates (if any) will not be known at this point in the characterization.

Interpretation of Results/Subsequent Tests: If aeration with either pH adjustment removes or reduces the toxicity, additional tests must be performed to identify whether sparging, sublation, or oxidation removed the toxicity, as described in Tier 1 (Section 6.4). If toxicity is reduced because of precipitation, the results for this test and the *filtration and pH adjustment test* should be similar, but if oxidation is a problem, pH adjustment and filtration will not affect the toxicity of the effluent. At pH 10 the total ammonia levels can be reduced by aeration. However, the geometry of the aeration technique (i.e., small surface area) for this *pH adjustment and aeration test* described here is not particularly conducive to ammonia removal. However, if aeration at pH (10) reduces toxicity compared to the toxicity in the *aeration test* at pH *i* and the *baseline test*, measure the total ammonia level in the sample to determine if it was stripped from the effluent.

Special Considerations/Cautions: The results of this test should be compared to the *toxicity control* (*pH*

adjustment test) and the *baseline test*. The *aeration and pH adjustment blank* should be compared to the pH adjustment *blank*. If the effluent toxicity is reduced in the effluent following pH adjustment/aeration, and the *blank* is toxic, the *blank* can be ignored and the results indicate toxicity removal. However, if toxicity is the same or greater, artifactual toxicity cannot be ruled out and further tests must be conducted. Compare the results of the *aeration and pH adjustment blank* to the *filtration and pH adjustment blank* and the *pH adjustment blank* (Sections 6.10 and 6.12). If all have toxicity, then artifactual toxicity occurred from the pH adjustment, while if only the *aeration and pH adjustment blank* has toxicity, then the artifactual toxicity crept in during the aeration manipulation and the test should be repeated.

6.12 Filtration and pH Adjustment Test

General Approach: Since a pH change can cause toxicants to precipitate or cause solubilized toxicants to sorb on particles, filtration at altered pH values can be used as a tool in characterizing the effluent. Therefore, by filtering pH adjusted effluent, compounds that were in solution without a pH adjustment may no longer be in solution or any toxicants associated with particles may be removed by the filtration process. Differences in the toxicity caused by filtering (at pH *i*) compared to the *pH adjustment test* (Section 6.10) may imply toxicity associated with suspended solids. If pH affects the filterability of the toxicants, solubility changes are implied at those pH values. Once the toxicants are filtered, the particles may be recoverable from the filter if toxicity has not degraded.

Methods: Details of preparing filters are generally the same as described in Tier 1 (Section 6.5), except the high purity water used to rinse the filters must be pH adjusted to the appropriate pH, as should the dilution water for the *blank*.

Effluent samples adjusted to pH 3 or pH 10 (Section 6.10) are filtered, readjusted to pH *i*, and the filtrate toxicity tested. Stainless steel filter housings are not to be used for this step, because stainless steel will frequently bleed metals when a pH 3 solution being filtered is in contact with the stainless steel. An inert plastic or properly cleaned glass housing should be used.

Interpretation of Results/Subsequent Tests: The results of the *filtration and pH adjustment test* are compared to the *toxicity controls*—the *baseline test* and the *pH adjustment test*. If the effluent is more toxic after filtration and contamination is not the cause, the breaking of an emulsion might be involved. If the toxicity is removed or reduced by the filtration step and dilution is not the cause, then toxicants have been separated from the whole effluent and efforts should focus on identifying the compounds filtered out. The next step is to recover the toxicity as described in Tier 1 *filtration test*. This may be accomplished using a pH adjusted sample of water, perhaps using the pH opposite of that used in the filtration process.

Special Considerations/Cautions: The pH adjusted and filtered dilution water serves as a *blank* and the pH adjusted and filtered effluent sample serves as a *toxicity control* for the solid phase extraction step (Section 6.13). The results of the *filtration and pH adjustment test* should be compared to the effluent *pH adjustment test* and the *baseline test*. The *filtration blank* should be compared to the *baseline control*, the *aeration blank*, and *pH adjustment blank*. Toxicity in the *filtration blank* implies toxic artifacts from the filtration process, the glassware, the pH adjustment or a dilution water problem. If the *baseline control* performance is acceptable, the *blank* toxicity was most likely created during the pH adjustment or filtration. If the *aeration and pH adjustment blank* is non-toxic, and if the *filtration blank* is toxic, and the filtered effluent sample is still toxic or more toxic, artifactual toxicity cannot be ruled out. To check if it occurred during the manipulation, the experiment must be repeated. If the *filtration blank* is toxic, yet the filtered pH adjusted effluent indicates that toxicity is reduced/eliminated, the toxicity in the *blank* can be ignored.

6.13 Post C_{18} Solid Phase Extraction (SPE) Column and pH Adjustment Test (pH 3 and pH 9)

General Approach: Shifting the ionization equilibria at high and low pHs may cause the C_{18} SPE column to extract different compounds than at pH *i*. pH adjusted and filtered effluent is passed over a prepared C_{18} SPE column to remove non-polar organic compounds (cf., *post C_{18} SPE column test*, Section 6.6 above). Organic acids and bases may be made less polar by shifting their equilibrium to the un-ionized species. By adjusting the effluent samples to a low pH and a high pH, some compounds that are in the un-ionized form should sorb onto the column. However, the C_{18} packing degrades at high pH, so pH 9 (rather than pH 10 or pH 11) is used in this manipulation. Specific manufacturer's data should be checked for acceptable pH range. We have had no experience in eluting toxicants off the C_{18} SPE column that would be sorbed only at an altered pH, and therefore we can only provide general rules to follow in these cases except those inferred from how ionizable compounds behave in regard to pH change.

Methods: All of the procedures for this manipulation and the use of the C_{18} SPE column are the same as is described in Tier 1 for the SPE extraction at pH *i* (Section 6.6) with one exception. All water passed through the column (rinse, *blank* and effluent) should be acidified or rendered basic depending on which pH is under investigation (see Section 6.12). The potential for bacterial growth and artifactual toxicity in the post-column samples remain the same as for pH *i*.

Interpretation of Results/Subsequent Tests: The extraction efficiency of the column is assessed by comparing the results of the *post C_{18} SPE column and pH adjustment test* (pH 3 and pH 10) to the *filtration and pH adjustment test*, and the *pH adjustment test*. Again post-column test results are the most interpretable when there is no artifactual toxicity and toxicity was removed.

When the toxicity is removed, compare the results of the test with the *methanol eluate test* below (Section 6.14). If toxicity is removed that was not removed under pH *i* and recovered in the methanol eluate, efforts to identify the toxicants should be started. If methanol does not recover toxicity, a pH adjusted water should be tried. For further discussions of the interpretation of the results, see Section 6.6 above.

Special Considerations/Cautions: Careful observations and judgement must be exercised in detecting problems in the *post C_{18} SPE column and pH adjustment test*. Low DO levels can occur in these samples (cf., Section 6.6). Through testing experience, the investigator will know whether toxicity appears as artifactual (i.e., growth, low DO) or as lack of toxicity removal. If artifactual toxicity is not recognized, then an erroneous conclusion that the C_{18} SPE column did not remove toxicity can be made.

General test conditions (dilution water, health of test animals) will be tracked by the *controls* in the *baseline test*. The post-column dilution water *blanks* should be compared to those *controls* to determine if the column imparted toxicity. If the post-column dilution water *blank* was toxic, but no toxicity or artifactual toxicity occurred in the post-column effluent sample the toxic *blank* can be ignored.

Results of the *post-column effluent test(s)* must be compared to the results of the *filtration and pH adjustment test* to determine if the manipulations effectively reduced toxicity. When the *post C_{18} SPE column test* data is plagued by artifactual toxicity, the importance of the *methanol eluate test* increases.

6.14 Methanol Eluate Test for pH Adjusted Samples

General Approach: This test is essentially the same as the *methanol eluate test* in Section 6.7, except that the columns were prepared with pH adjusted waters/effluents (see Section 6.13).

Methods: These are identical to those in Section 6.7, except the pH of the rinse water, blank and effluent sample has to be adjusted to pH 3 or pH 9 (lowered from pH 10).

Interpretation of Results/Subsequent Tests: If the toxicity is recovered in the eluate, identification should be initiated. Refer to Sections 6.6, 6.7, and 6.13 for more information.

Special Considerations/Cautions: The *baseline test* serves as the *toxicity control*, and the *methanol blank* (for *pH adjusted samples*) serves as the *toxicity control* for the effects of methanol in water. The health of the test animals, the viability of the dilution water and general test conditions are evaluated by the *controls*.

The artifactual growth observed in the *post C_{18} SPE column test* (with and without pH adjustments) from the methanol has not occurred in *methanol eluate tests*. This is most likely due to the differences in how the methanol degrades/behaves in dilution waters which are low in bacteria and other organic matter in contrast to effluent samples (even post-column effluents).

6.15 Toxicity Characterization Summary

Phase I will not usually provide information on the specific toxicants. If effluent toxicity is consistently reduced, for example, through the use of the C_{18} SPE column, this does not prove the existence of a single toxicant because several non-polar organic compounds may be causing the toxicity in the effluent over time, but use of the C_{18} SPE technique in Phase I detects the presence of these compounds as a group. This lack of specificity is very important to understand for subsequent Phase II toxicant identification. Efforts should concentrate on those manipulations affecting toxicity in which the toxicant is isolated from other effluent constituents, such as the SPE column, filtration and aeration.

After the Tier 1 group of Phase I tests has been completed, the results will usually show that some manipulations increased toxicity, some decreased it, and others effected no change. In some instances, Tier 1 results allow the researcher to proceed immediately into the Phase II identification, and sometimes Phase I (Tier 1 and/or Tier 2) and Phase II combinations are needed to determine the cause of toxicity (cf., EPA, 1992A). Of course, new approaches are frequently devised as more Phase I TIEs are completed.

Toxicity may be changed by two or more tests, and if so, then more conclusive inferences might be possible than when only one manipulation changed the toxicity.

If all of the toxicity is not removed, it is possible that other toxicants could be present in the effluent so that only partial removal was obtained. Frequently more than one manipulation affects toxicity but only infrequently is there no effect from any manipulation. Even if toxicity is affected by only one manipulation, one still does not know whether or not there are multiple toxicants. When several manipulations affect toxicity, it still does not ensure that there are multiple toxicants. There is also no way to tell at this stage if there are multiple toxicants, whether or not they are additive, partially additive or independent. In our experience with acutely toxic effluents, we have not found synergism, but independent action has commonly been found. Some toxicants identified in effluents have been additive, but more often these have been only partially additive.

The two objectives which usually move the TIE along more rapidly are to separate and concentrate the toxicant(s). Therefore, the first step in Phase II (EPA, 1989A) will often be to reduce the number of constituents accompanying the toxicants. These efforts may reveal more toxicants than are suggested by Phase I testing. In Phase II one may discover that toxicants of quite a different nature are also present but were not in evidence in Phase I and if this is the case, different Phase I characterizations may then be needed. Once the analytical methods to identify one or more of the toxicants is found, efforts to confirm the cause should be initiated immediately (EPA, 1989B; EPA, 1992B).

As discussed earlier, the amount of time necessary to adequately characterize the physical/chemical nature and variability of the toxicity will be discharge specific. For a given discharge, the factors that will affect the length of time it takes to move through Phase I is the appropriateness of Phase I tests to the toxicants, the existence of long- or short-term periodicity in individual toxicants and the variability in the magnitude of toxicity. An effluent which consistently contains toxic levels of a single compound that can be neutralized by more than one characterization test should be identified and moved into Phase II more quickly than an ephemerally toxic effluent with highly variable constituents, few of which or none of which are impacted by any of the Phase I tests. Several samples should be subjected to the Phase I characterization tests but not all manipulations have to be done on all subsequent samples. The decision to do subsequent tests on these samples to confirm or further delineate initial results is a judgement call and will depend on whether or not the results of Phase I are clear-cut. Sometimes it may be reasonable to start Phase II and Phase III on the first sample.

If the Phase I characterization tests that remove or neutralize effluent toxicity vary by the sample, the number of tested samples must be increased and the frequency of testing should be sufficient to include all major variability. The differences seen among samples can be used to decide when further differences are not being found. Phase I characterization testing should continue until there is reasonable certainty that new types of toxicants are not appearing. No guidance can be given as to how long this may take—each problem for every discharger is unique. While the toxicity of samples can be very different, the same characterization tests must be successful in removing and/or neutralizing effluent toxicity.

Often the next step of the TIE is obvious; at other times the outcome of Phase I will be confusing and the next step will not be obvious. In our experience with acutely and chronically toxic effluents, once one toxicant is identified, identification of subsequent toxicants becomes easier because: (a) the toxicity contribution of the identified toxicant can be established for each sample; (b) the number of Phase I manipulations that will affect the toxicity of the known toxicant can be determined; (c) one can determine whether the identified and the unidentified toxicant(s) are additive; (d) if some manipulations affect the toxicity due only to the unidentified toxicants, some of their characteristics can be inferred; and (e) one can determine if the relative toxicity contributions of identified and unidentified toxicants varies by sample. Such information can be used to design tests to elucidate additional physical/chemical characteristics of the toxicants that cause chronic toxicity.

6.16 Use of Multiple Characterization Tests

Type and amount of testing is dependent on the toxicity persistence in the effluent, the nature of the toxicity, and reassessment of previous Phase I results (observed trends in the characteristics can be very important). Several tests could each partially remove the effluent toxicity because several compounds are causing the toxicity, or that one toxicant can be removed by several Phase I steps. For example, if several toxicants are acting to cause the toxicity, then the *graduated pH test* and the *post C_{18} SPE column test* both might result in a partial toxicity reduction. If sodium thiosulfate and EDTA both reduce toxicity, cationic metals might be suspect.

In the acute Phase I (EPA, 1991A), the use of multiple manipulations (combining two of the Phase I tests) was advocated and this same concept is also useful for the chronic TIE as well. For effluents with multiple toxicants, especially if they are not additive, multiple manipulations are helpful. Especially when no single manipulation removes all the toxicity, multiple manipulations should be tried.

When the C_{18} SPE column only partially removes toxicity, Phase I manipulations with the post-column sample should be tried. For this multiple manipulation, the post C_{18} SPE column effluent can be treated as whole effluent, and several of the Phase I steps can be conducted on the post-column effluent such as the *EDTA addition test*, the *thiosulfate addition test*, and the *graduated pH test*. However, these combinations are useful only with the post-column effluent provided that no artifactual toxicity is present.

If the C_{18} SPE column partially removes toxicity, pass an aliquot of the post-column effluent over an ion exchange column to determine the characteristics of the remaining toxicity. If a non-polar toxicant and ammonia are suspected, then passing the sample over the C_{18} SPE column and then over zeolite may assist in accounting for all of the toxicity. Likewise, passing the effluent over zeolite and then over the C_{18} SPE column may provide additional insight. To gain this knowledge toxicity tests must be performed after each manipulation and not just on the multiple manipulated sample.

Effluent characterization must be approached without any preconceived notion or bias about the cause of

toxicity because many constituents are present in effluents and their chemistry is often unknown, resulting in circumstantial evidence that is frequently misleading. Certainly all available information and experience should be used to guide the investigative effort but temptations to reach conclusions too soon must be resisted. Sometimes the answer being sought is only whether or not a specific substance is causing toxicity. Obviously in such cases testing is specifically selected to answer that question and therefore not all manipulations need to be performed.

Section 7
Interpreting Phase I Results

After Phase I on one sample or several samples is completed, the investigator must carefully evaluate the data, draw conclusions, and make decisions about the next steps that are needed. Sometimes the next step is obvious, at other times the outcome will be confusing and the next step will not be obvious. Several general suggestions, based on our experience to date, may provide some help.

In this section, various examples of Phase I results are given with interpretation suggestions. This discussion is repeated from the acute Phase I characterization manual (EPA, 1991A), and not all aspects have been evaluated for chronic TIEs yet. These examples should be used only as guides to thinking and not as *definitive* diagnostic characteristics. Since almost any toxicant can be present in effluents, clear-cut logic is not totally dependable in interpreting results. Rather, one must use the weight of evidence to proceed, and be aware that artifacts cannot at this point always be identified.

One should avoid making categorical assumptions to every extent possible. For example, to assume that the toxicity is due to a non-polar toxicant(s) because the toxicity in the post C_{18} SPE column effluent was removed, often is an error. Metals may also be the toxicant adsorbed by the SPE column; we have observed zinc, nickel, and aluminum concentrations remaining on the C_{18} SPE column. However, if the toxicity can be recovered in the methanol fraction, then the theory that a non-polar toxicant(s) is causing the toxicity is better substantiated, because metals do not elute with methanol and therefore do not produce toxicity in the methanol fraction toxicity test (cf., Phase II).

Example 1. Non-polar toxicant(s). The Phase I results implicating non-polar toxicants are:

- Toxicity in the *post C_{18} SPE column test* was absent or reduced.
- Toxicity was recovered in the *methanol eluate test.*

Toxicants other than non-polar compounds may be retained by the SPE column but they are less likely to be eluted sharply. Also, artifactual post-column toxicity can occur, but non-polar toxicity is typically distinguished from the artifactual toxicity when the eluate is checked for toxicity. Some toxicants (metals, some surfactants) may not elute from the SPE column with methanol and so failure to recover the toxicity in the eluate does not exclude the possibility of a non-polar toxicant. Recovery of toxicity in the eluate at pH *i* is less likely to be an artifact than recovery only at pH 3 or pH 9. For those instances where methanol does not recover C_{18}-removable toxicity, other solvents may be needed to elute the toxicants (see Phase II; EPA, 1992A).

Example 2. Cationic Metals. This group of metals has varied chemical/physical behaviors which result in less definitive Phase I results. The following characteristics can be used only in a general way to point to metals as the cause of the toxicity:

- The toxicity is removed or reduced in the *EDTA addition test.*
- The toxicity is removed or reduced in the *post C_{18} SPE column test.*
- The toxicity is removed or reduced in the *filtration test*, especially when pH adjustments and filtration are combined.
- The toxicity is removed or reduced in the *sodium thiosulfate addition test.*
- Erratic dose response curve observed.

No single characteristic is definitive, with the possible exception of EDTA. In addition, toxicity may be pH sensitive in the range at which the graduated pH test is performed but may become more or less toxic at low or high pH depending on the particular metals involved. This characteristic for chronic toxicity has not yet been demonstrated to the extent it was for the acute toxicity of several metals (EPA, 1991A).

Example 3. Total dissolved solids (TDS). TDS consists of a group of common cations and anions (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, SO_4^{-}, NO_3^{-}, Cl^{-}, CO_3^{-}) and in parts of the United States, this group is called "salinity." TDS is usually measured by conductivity, density or refraction, none of which measure specific compounds or ions. The toxicity of any given amount of TDS will depend on the specific composition. TDS behaves as a mixture of toxicants, which do not cause toxicity through osmotic stress. Evidence of this is that the LC50s of the individual salts expressed in moles, are quite different. If osmotic stress were the mode of action, the concentration in moles at the LC50s would be similar (EPA, 1991A). One cannot use marine organisms to circumvent TDS unless NaCl is by far the

dominant TDS. Marine organisms regulate Na^+ and Cl^- but like freshwater organisms, they too are sensitive to non-NaCl TDS.

For these reasons, only very general relationships exist between toxicity and TDS. Because of their varied nature, they do not sort out clearly in Phase I. Rather, unless conductivity is very high (e.g., 10,000 µmhos/cm), one might suspect TDS when nothing else is indicated. For example, if high TDS were present and caused by calcium sulfate ($CaSO_4$), toxicity is likely to be removed in the *pH adjustment test* at pH 10 or in the *filtration and pH adjustment test* at pH 10, whereas if the TDS were due to NaCl, toxicity would likely not be affected.

As a general guide, when conductivity exceeds 1,000 and 3,000 µmhos/cm at the effect concentration for *Ceriodaphnia* and fathead minnows, respectively, TDS toxicity might be suspect. The conductivity of 100% effluent is not the relevant reading, but rather the conductivity at the concentrations bracketing the effluent no effect and effect concentrations.

Following are some Phase I general indicators that TDS might be a suspect:

- No pH adjustments changed the toxicity, unless a visible precipitate occurs upon pH adjustment, pH adjustment and filtration, and pH adjustment and aeration.
- No loss of toxicity in the *post C_{18} SPE column test*, or a partial loss of toxicity but no change in conductivity measurements.
- No change in toxicity with the *EDTA addition test*, *sodium thiosulfate addition test* or in the *graduated pH test*.

In addition, there are two tests that can be used that are not included in Phase I but may help to characterize the toxicity:

- Use acid/base ion exchange resins (EPA, 1992A). When toxicity is removed or reduced, the toxicity could be due to TDS.
- Use of activated carbon to remove toxicity (EPA, 1992A). When no toxicity is removed by passing the effluent over carbon, TDS could be responsible for toxicity.

An additional caution is that where TDS is marginally high, the addition of NaCl from pH manipulations can increase TDS enough to produce artifactual TDS toxicity. The conductivity of the solutions before and after the pH adjustments should be monitored closely to avoid this.

Example 4. Surfactants. There are three main groups of surfactants and/or flocculants (anionic, cationic and nonionic) that may occur in effluents. The Phase I behavior of these types of compounds may vary depending on which particular groups are present.

The general Phase I results implicating a surfactant(s) as the toxicant(s) are:

- Toxicity is reduced or removed in the *filtration test*.
- Toxicity is reduced or removed by the *aeration test*. In some cases, the toxicity is recoverable from the walls of the aeration vessel after removing the aerated effluent sample.
- Toxicity is reduced or removed in the *post C_{18} SPE column test*. The toxicity may or may not be recovered in the *methanol eluate test*.
- Toxicity is reduced or removed in the *post C_{18} SPE column test* using unfiltered effluent. Toxicity reduction/removal is similar to that observed in the *filtration test* and toxicity may or may not be recovered in *methanol eluate test* or the extraction of the glass fiber filter.
- Toxicity degrades over time as the effluent sample is kept in cold storage (4°C). Degradation is slower when effluent is stored in glass containers rather than plastic containers.

Example 5. Ammonia. Ammonia concentrations can be measured easily, and because it is such a common effluent constituent, determining the total ammonia concentration in the whole effluent is a good first step (see Section 4). If more than 5 mg/L of total ammonia is present, additional evaluations should be done. Sole dependence on analyses is not advisable because the chronic effects of ammonia and some other toxicants (e.g., such as surfactants) is not well known. Even though the ammonia concentration is sufficient to cause toxicity, other chemicals may be present to cause toxicity if the ammonia is removed. Three indicators of ammonia toxicity are:

- The concentration of total ammonia is 5 mg/L or greater.
- In the *graduated pH test* the toxicity increases as the pH increases.
- The effluent is more toxic to fathead minnows than to *Ceriodaphnia*.

Example 6. Oxidants. In effluents, oxidants other than chlorine may be present. Measurement of a chlorine residual (TRC) is not enough to conclude that the toxicity is due to an oxidant. In general, oxidants are indicated by the following:

- The toxicity is reduced or removed in the *sodium thiosulfate addition test*.
- Toxicity is removed or reduced in the *aeration test*.

- The sample is less toxic over time when held at 4°C (and the type of container does not affect toxicity).
- *Ceriodaphnia* are more sensitive than fathead minnows.

Of course, TRC greater than 0.1 mg/L in 100% effluent might indicate chlorine as the oxidant causing the toxicity. In addition, the dechlorination with SO_2 provides evidence of chlorine toxicity in the same manner as the sodium thiosulfate addition test.

Section 8
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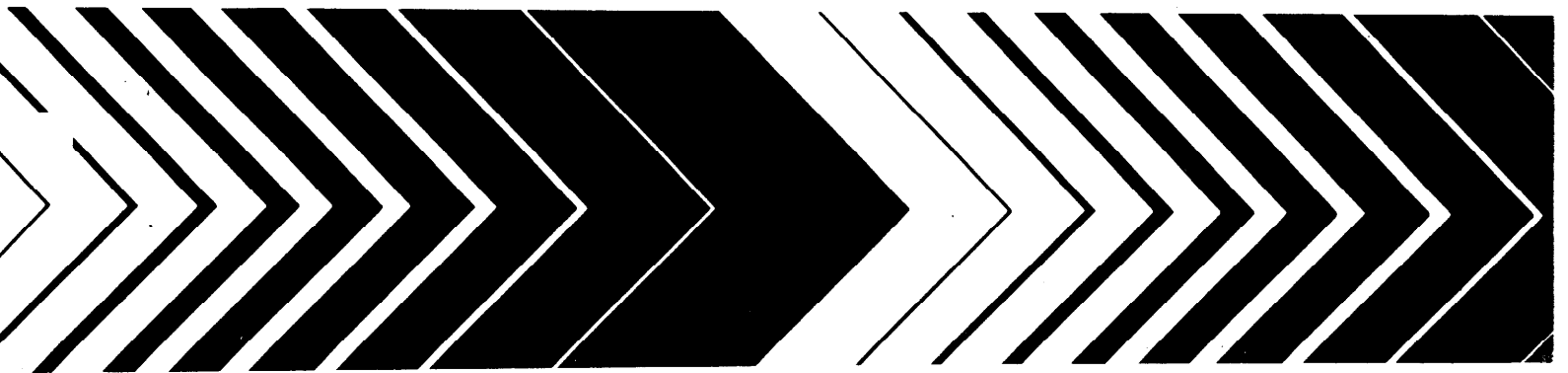
Methods for Aquatic Toxicity Identification Evaluations

Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity



EPA/600/R-92/080
September 1993

Methods for Aquatic Toxicity Identification Evaluations

Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity

by

E. J. Durhan[1]
T. J. Norberg-King[1]
L. P. Burkhard[1]

With Contributions from:

G. T. Ankley[1]
M. T. Lukasewycz[2]
M. K. Schubauer-Berigan[2]
J. A. Thompson[2]

[1]U.S. Environmental Protection Agency
[2]ASci Corporation - Contract No. 68-C0-0058

Previous Phase II Methods
by D. I. Mount and L. Anderson-Carnahan
EPA-600/3-88/035

National Effluent Toxicity Assessment Center
Technical Report 01-93

Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Duluth, MN 55804

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Foreword

This document is one in a series of guidance documents intended to assist dischargers and their consultants in conducting acute or chronic aquatic toxicity identification evaluations (TIEs). TIEs might be required by state or federal agencies resulting from an enforcement action or as a condition of a National Pollutant Discharge Elimination System (NPDES) permit. The methods described in this document will also help to determine the adequacy of effluent TIEs when they are conducted as part of a toxicity reduction evaluation (TRE).

This Phase II document is the second of a three phase series of documents that provide methods to characterize and identify the cause of toxicity in effluents. The first phase of the series, Phase I (EPA, 1991A; EPA, 1992), characterizes the physical/chemical nature of the acute and chronic toxicant(thereby simplifying the analytical work needed to identify the toxicant(Phase II provides guidance to identify the suspect toxicants, and the last phase, Phase III (EPA, 1993A) provides methods to confirm that the suspect toxicants are indeed the cause of toxicity. The recent TIE documents (EPA, 1991 A; EPA, 1992; EPA, 1993A; and this document) have been produced or revised to include chronic toxicity recommendations and additional information or experiences we have gained since the original methods were printed (EPA, 1988A; EPA, 1989A; EPA, 19898).

This Phase II document provides identification schemes for non-polar organic chemicals, ammonia, metals, chlorine, and surfactants that cause either acute or chronic toxicity. The document is still incomplete in that it does not provide methods to identify all toxicants, such as polar organic compounds. This Phase II manual also incorporates chronic and acute toxicity identification techniques into one document.

While the TIE approach was originally developed for effluents, the methods and techniques directly apply to other types of aqueous samples, such as ambient waters, sediment pore waters, sediment elutriates, and hazardous waste leachates. These methods are not mandatory protocols but should be used as general guidance for conducting TIEs.

The sections of both Phase I documents (EPA, 1991A; EPA, 1992) which address health and safety, quality assurance/quality control (QA/QC), facilities and equipment, dilution water, testing, sampling, and parts of the introduction are applicable to Phase II. These sections, however, are not repeated in their entirety in this document.

Abstract

This manual and its companion guidance documents describe a three phase approach for dischargers to identify the causes of toxicity in municipal and industrial effluents (Phase I, EPA, 1991A; EPA, 1992; and Phase III, EPA, 1993A). In 1989, the document titled Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures was published as a guidance document for identifying the cause of toxicity in acutely toxic effluents (EPA, 1989A). This new Phase II document provides details for more types of samples, tests and test procedures that can be used to identify the specific chemical(s) responsible for acute or chronic effluent toxicity when the cause of toxicity is related to non-polar organic compounds, ammonia, surfactants, chlorine, or metals. Phase I characterization and Phase III confirmation manuals, the other guidance documents in the three phase TIE approach, have also been produced or updated to include both chronic toxicity information and new developments made since the first set of documents were printed. The TIE approach is applicable to effluents, ambient waters, sediment pore waters or elutriates, and hazardous waste leachates.

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Abbreviations

AA	Atomic Absorption
C_{18}	Octadecylcarbon Chain
C_8	Octylcarbon Chain
CTAS	Cobalt Thiocyanate Active Substances
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
EDTA	Ethylenediamine Tetraacetic Acid
ERL-D	Environmental Research Laboratory-Duluth
GC	Gas Chromatography .
GC/MS	Gas Chromatography/Mass Spectrometry
Heppso	N-(2-Hydroxyethyl) Piperazine-N'-2-Hydroxypropane Sulfonic Acid
HPLC	High Performance Liquid Chromatography
ICp	Inhibition Concentration Percentage
ICP-AES	Inductively Coupled Plasma-Atomic Emission Spectroscopy
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
K_{ow}	Octanol-Water Partition Coefficient
LAS	Linear Alkylbenzene Sulfonate
LC/MS	Liquid Chromatography/Mass Spectrometry
LC	Lethal Concentration
MBAS	Methylene Blue Active Substances
Mes	2-(N-Morpholino) Ethane-Sulfonic Acid
Mops	3-(N-Morpholino) Propane-Sulfonic Acid
NETAC	National Effluent Toxicity Assessment Center
NIST	National Institute of Standards and Technology
NOEC	No Observed Effect Concentration
NPDES	National Pollutant Discharge Elimination System
PBO	Piperonyl Butoxide
Pops0	Piperazine-N,N'-bis (2-Hydroxypropane) Sulfonic Acid
QA/QC	Quality Assurance/Quality Control
SPE	Solid Phase Extraction
s s	Suspended Solids
Taps	N-tris-(Hydroxymethyl) Methyl-3-Aminopropane Sulfonic Acid
TDS	Total Dissolved Solids
TIE	Toxicity Identification Evaluation
TOC	Total Organic Carbon
TRE	Toxicity Reduction Evaluation
TU	Toxic Unit
YCT	Yeast-CerophyP-Trout food

Acknowledgments

This document presents additional methods and improvements made to the procedures of Methods *for* Aquatic Toxicity *Identification Evaluations: Phase II Toxicity Identification Procedures* (EPA-600/3-88/035) by Donald Mount and Linda Anderson-Carnahan. This manual reflects new information, techniques, and test procedures developed by the National Effluent Toxicity Assessment Center (NETAC) since the previous Phase II document was printed in 1989. This Phase II document is based on the efforts of both federal and contract staff of the NETAC group. We gratefully acknowledge the following individuals' contributions to the research and development of the methods for this document: Penny Juenemann and Shaneen Schmitt (federal staff); Joe Amato, Lara Andersen, Steve Baker, Tim Dawson, Joe Dierkes, Nola Englehorn, Doug Jensen, Correne Jenson, Jim Jenson, Liz Makynen, Phil Monson, Don Mount, and Greg Peterson (contract staff).

Through the support of Rick Brandes and Jim Pendergast (EPA, Permits Division) and Nelson Thomas (ERL-D), the NETAC staff members developed and revised the TIE series of documents.

Section 1
Introduction

1.1 General Overview

The major objective of Phase II is to identify the suspected toxicant(s) in effluent samples using toxicity identification evaluation (TIE) procedures. Some general guidance to achieve this goal might be furnished by the results of acute or chronic Phase I tests (EPA, 1991 A; EPA, 1992), but for many effluents, such as those that contain non-polar organic toxicants, both separation and concentration steps will be needed to achieve the stated objective. If metals are the suspect toxicants, atomic absorption (AA) spectrometry should be sensitive enough to measure toxic concentrations directly in the sample, and the number of metals is small enough that toxicity can be attributed without separating one from another. The same principle applies to toxicants such as ammonia and chlorine; measurements can be made without separating or concentrating the effluent. However, if non-polar organic chemicals are suspected, separation is usually necessary for analytical and toxicological reasons.

Because there are often many constituents within the classes of chemicals (e.g., non-polar organics) identified in Phase I, initial efforts are most productively directed towards separating the toxic from the non-toxic constituents. With the need to identify the toxicant(s) quickly, comes the temptation to analyze too soon. Using methods such as gas chromatography/mass spectrometry (GC/MS) one can identify many non-polar organics that are present in the whole effluent mixture, but the association of toxicity with compound identification is very difficult to make for several reasons:

- There can be hundreds of compounds present in the mixture, and to investigate all of them would be very time consuming.
- Toxicity data for many of the chemicals identified are usually not available; chronic data are especially scarce.
- Separate constituents are often not commercially available; therefore, their toxicities cannot be measured and compared to effluent toxicity.
- Interactions (additivity, synergism, antagonism) are not known for the given mixtures and one must know interactions to apportion toxicity.

Therefore, it is suggested that the search for a separation technique to simplify the mixture into toxic and non-toxic 'subsamples be the first priority, rather than spending time investigating non-toxic components. If there is a single suspect toxicant such as ammonia, then separation needs are limited largely by the analytical requirements. If the toxicity is caused by one constituent, the number of other non-toxic constituents is irrelevant when attributing toxicity. However, Phase I results do not usually lead to a single suspect toxicant and, therefore, separation may be necessary.

When a method for separating the toxicant(s) is found, concentration might be an inherent part of the procedure (as in solvent extraction) which will simplify the problem of finding a method to concentrate the toxicity. At each stage of the separation and concentration process, measurement of toxicity is the best way to evaluate the success or failure of the manipulations.

The interpretation of TIE results can be different than in the classical research approach, where experiments are designed to either accept or reject a hypothesis. In TIE work, an experiment usually permits acceptance but not rejection of the hypothesis. For example., if ammonia is the suspect toxicant, it can be removed using zeolite resin. If the post-zeolite effluent is still toxic, you can conclude that there are additional toxicants present. If the post-zeolite effluent is not toxic, you cannot conclude that there are no additional toxicants because the zeolite might have removed other toxicants in addition to the ammonia.

The always present question of whether or not there is more than one toxicant immensely complicates data interpretation. Phase I results might not give an indication of multiple toxicants unless the toxicant classes change over time or from sample to sample. Phase II results are often such that one cannot tell whether the situation is one of partial removal of a single toxicant or

toxicity resulting from multiple toxicants. The issue might be resolved when one toxicant is identified and measured analytically. Experience shows that the best choice is to try to focus on the toxicant that appears easiest to identify. Usually that will be a toxicant that can be separated from the sample (e.g., extracted or recovered from a sorbent that reduces the toxicity) and for which there is a broad spectrum analytical identification method. Above all, data should always be interpreted under all probable scenarios, i.e., one toxicant, multiple toxicants, and even different toxicants from sample to sample.

Experience gained since the first Phase II (EPA, 1989A) document was printed has shown that effluent toxicants are not always strictly additive. When they are not additive, the toxicant present in the largest number of toxic units (TUs)[1] will determine the toxic units of the effluent. Non-additive toxicity will not be reduced by manipulations that remove toxicants present in fewer TUs than the major toxicant. Two or more toxicants might be present in approximately equal TUs, however, the ratio of TUs might change over different sampling times so that different chemicals determine the toxicity of the effluent. These important problems can be dealt with in Phase III (EPA, 1993A). In Phase II, the objective is to find which toxicants are present in toxic concentrations. However, failure of additivity may confuse Phase II results. Minor toxicants might not be noticed until the major one has been removed. In addition, additivity cannot be determined until at least one toxicant has been identified. Usually Phase II and Phase III merge and overlap, therefore such concerns regarding non-additivity must be incorporated in Phase II, at least in the latter stages.

As effluent constituents are identified, a sorting process begins in which a decision must be made as to whether or not each one identified contributes to the toxicity of the effluent. Usually, this is based on the estimated concentration and the constituents' toxicities. Analytical error in quantitation might be large (1 O-fold or more) because recoveries and instrument response factors probably will not yet have been determined on a particular chemical. Uncertainty about toxicological data is caused by differences in species sensitivity and water quality effects, when literature values are available. Confidence in an acute toxicity value (LC50) will vary depending on the quality of the test, the number of times it was repeated, and the completeness by which the results and conditions were described. Data on chronic effect levels are often scarce and rarely have tests been repeated. Species sensitivity frequently varies from 1 OO-fold to 1,000-fold; an error will likely be introduced when the published toxicity data for species other than the test species are used. When the uncertainty of the toxicity data is high, a maximum of 100-fold difference between measured concentrations and literature effect would be acceptable to classify a chemical as a suspect. If one has good data for the test species being used, then this difference might be reduced (e.g., to IO-fold). Since these decisions are always subjective, they will sometimes be wrong no matter how carefully they are made. Perhaps most important is use of an iterative process to make these decisions. First evaluate candidates that have concentrations higher than or closest to their chronic or acute effect levels and if these prove to be negative, then examine those that have concentrations below their effect levels. Remember that the suspected toxicant concentrations at the dilution equal to the effect level concentration are the important concentrations to compare. At some point, a decision must be made whether the true toxicants have not yet been identified or measured and that different sample preparation or analyses must be used.

For some effluents, Phase I results might not have provided any guidance for selecting the appropriate Phase II procedures to follow. Other characterization steps that might be helpful are solvent extraction (acidic or basic), sample evaporation, size exclusion chromatography, lyophilization, and vacuum or steam distillation (Jop et al., 1991; Walsh et al., 1983). We have little experience upon which to recommend procedures in these cases. It is most important to realize that the more severe the effluent treatment, the more likely it is that toxic artifacts will be created. These toxic artifacts could then be confused with effluent toxicity; therefore, artifactual toxicity must be monitored for each technique by using blanks.

Phase II efforts should develop into Phase III confirmation (EPA, 1989B; EPA, 1993A) as soon as good evidence is obtained that one or more candidates are probable toxicants. The primary product of Phase II is the chemical identification of the suspected toxicants to furnish the basis for Phase III testing. The techniques described in this document are useful for TIE work with effluents as well as ambient waters (Norberg-King et al., 1991) and sediment pore water or elutriates (EPA, 1991 B).

1.2 Biological Testing Considerations

The Phase I characterization documents (EPA, 1991 A; EPA, 1992) provide detailed discussions of various issues that are important in decision making throughout the TIE. The guidance covers use of various species, test concentrations, effluent sample types, testing requirements for quality assurance (QA), test endpoints, frequency of changing the test solutions, and more. All of these issues will not be discussed at length here and the user is encouraged to refer to the acute Phase I or the chronic Phase I as companion documents for the TIE process. As the Phase II identification and Phase III confirmation steps are initiated, QA requirements should

[1] TU calculations are described in EPA, 1992. The TUs of whole effluent equals 100% divided by the LC50, NOEC, or ICp (IC25, IC50) of the effluent. The TU of a specific chemical equals the concentration of the compounded divided by the effect level of the compound.

be revisited and the types of tests modified as needed. Several of these testing concerns are addressed below.

During Phase I, the analyst is searching for an obvious alteration in effluent toxicity, which might be obtained by using modified acute or chronic test methods. Confirmation testing (Phase III) conducted according to the standard methodologies will confirm whether the suspect toxicant(s) detected in the characterization and identification steps (Phases I and II) is the true toxicant.

In characterizing the toxicity in Phase I, factors such as time requirements, number of tests and the test design had to be considered and weighed against the type of questions that are posed. EPA has published manuals that describe the acute or chronic test methods to determine the toxicity of effluent or receiving waters to freshwater and marine organisms (EPA, 1991 C; EPA, 1993B; EPA, 1993C), and these tests are typically those that indicated the presence of toxicity which the TIE initiated. Deviations from these standard effluent testing protocols were discussed in both the acute Phase I (EPA, 1991A) and the chronic Phase I (EPA, 1992) manuals. For either the acute or the chronic Phase I procedures, the test volumes, number of test concentrations, and number of replicates were all reduced from the standard test methods (EPA, 1991 C; EPA, 19938). Additional modifications for the short-term chronic tests (EPA, 19938) including shorter test duration and a reduction in the frequency of the test solution renewal are suggested.

Throughout this document the TIE procedures for acutely toxic samples are based on the following species: *Ceriodaphnia dubia, Pimephales promelas, Daphnia magna, Daphnia pulex, Hyalella azfeca,* and *Chironomus tentans.* Almost all acute tests have been conducted using 10 ml of test solution in a 1oz plastic cup (or 30 ml glass beaker). TIE procedures with chronically toxic effluents are based on tests using either C. *dubia* or larval fathead minnows *(P. promelas).* In our laboratory, the chronic tests with C. *dubia* generally are conducted using 10 ml of test solution in 1 oz cups and the chronic tests with fathead minnows are conducted using 50 ml of test solution in a 4 oz plastic cup (10 fish per cup). Use of other species is constrained only by availability, size, age, and adaptability to test conditions, and the threshold levels for additives and reagents for the other organisms must be determined.

As soon as good evidence is obtained to implicate a suspect toxicant(the procedures for performing the toxicity tests can be changed. Therefore in Phase II, the time to modify the tests from the way they were conducted in Phase I may depend on when the toxicant is identified, and generally there is more flexibility for this in Phase II than in Phase III. The quality control (QC) measures in Phase I were not very strict because the data are primarily informative rather than definitive. The identity of the suspect toxicant(s) furnishes the basis upon which Phase III testing will be conducted, which will require stricter QC measures.

Initially, the use of modified protocols in Phase II may continue; however, once specific toxicant(s) identification has been made, Phase II (and Phase III) testing conditions should be similar to the methods described in the protocol that was used to trigger the TIE. Although a shortened version of the 7-d C. *dubia* test (which is referred to as the 4d test) may have been used in Phase I, the use of this test changes in Phase II (and Phase III). In order to use the 4d test in Phases I and II of the TIE, the 4d test must detect similar trends of toxicity as the 7-d test does. However, in Phase III the 7-d test is required because the toxicity as measured in the 7-d test (with additional replicates, more test concentrations, additional volume) was used to detect toxicity for the permit, and should be used to confirm the cause of toxicity. In the early Phase II chronic toxicity evaluation steps, the qualitative evaluation of toxicii might be useful and there is no reason why a toxicity test could not be terminated sooner than day seven, if the answer to a particular question has been found.

Information obtained from all toxicity tests should be maximized. For instance, in acute toxicity tests, monitoring time to mortality might be useful. In chronic toxicity tests, time to young production of the cladocerans or the lack of food in the stomach of the larval fish might be useful parameters. Observations such as these made during a test might be subtle indications and quite informative of small changes in toxicity. For example, if there is complete mortality on day four of the baseline effluent test, and in the EDTA addition test (Section 4) the animals either do not reproduce or grow yet they are alive at day seven of the exposure, the indication is that the toxicity was reduced. These results suggest that either an additional toxicant is present or the EDTA concentration was not sufficient to remove all cationic metal toxicity. These types of observations in the short-term tests might be just as useful as reductions in young production or growth. For evaluating whether any manipulation changed toxicity, the investigator should not rely only on statistical evaluations of test endpoints (see below and Phase I; EPA, 1992). Some treatments may have a significant biological effect that was not detected by the statistical analysis. Judgement and experience in toxicology should guide the interpretation.

In addition, for acute or chronic toxicity tests, randomization, careful exposure time readings, use of animals of uniform narrow-age groups (i.e., *C. dubia* neonates O-6 h old rather than O-12 h old) might assist in detecting smaller differences in tests. For example, in the chronic *C. dubia* tests, it is important to use organisms of known parentage (EPA, 19938) when the number of, replicates is reduced from ten to five. For C. *dubia,* daily renewals of the test media (as required in the chronic manual; EPA, 1993B) might not be necessary in Phase I or early Phase II testing as long as the toxicity of the

using 20% effluent as the high concentration and a dilution factor of 0.7, the concentrations to test would be 7%, 10%, 14%, and 20%. If the NOEC (from historical data) is 4050% (or above 50%), then the concentration series to test might be either 25%, 50%, 75%, and 100% or 40%, 60%, 80%, and 100%. Choice of dilution factor and test concentration range is a matter of judgement and depends on precision required and practicality.

After conducting Phase I procedures on an effluent sample, the amount of effluent available for subsequent identification work can be sufficiently reduced so that it may be impractical to try to conduct each step as described in this manual. This is most likely to be a concern for the non-polar organic identification techniques and other methods that require large volumes of effluent to identify the toxicant. Therefore, when the volume of an effluent sample is limited, it might be possible to track toxicity through the non-polar identification steps without quantifying the amount of toxicity that is being tracked. Essentially, this means that the toxicity tests are done without dilutions and the results would indicate only that toxicity was present or absent; the degree of toxicity present would not be measured. Once a suspect toxicant is identified, it is important that the amount of toxicity removal is known (through the use of dilutions) because this information can be used to correlate a suspect toxicant to the effluent toxicity in the Phase III confirmation.

If the number of replicates per test concentration is reduced, one must assume that precision is sufficient to decipher changes in toxicity that must be measured. One problem in using reduced replicates and low numbers of test concentrations in chronic tests is that this smaller data set is not amenable to all statistical requirements as recommended for the short-term tests (EPA, 1989C; see Section 5.8). Use of more organisms and more replicates than in the Phase I modified tests might be preferable if Phase I and/or Phase II data are likely to be used in Phase III confirmation (See Sections 2.2 and 2.3).

For acute toxicity tests, usually the LC50 or EC50 is reported for the toxicity data (calculated as recommended in EPA, 1991C). Endpoints for the most commonly used freshwater short-term chronic tests are growth, reproduction, and survival. The no effect level (the NOEC), and the effect concentration (the lowest observed effect concentration (LOEC)) are determined using the statistical approach of hypothesis testing to determine a statistically significant response difference between a control group and a treatment group. The NOEC/LOEC are heavily affected by choice of test concentrations and test design (see Phase I; EPA, 1992). The linear interpolation method (EPA, 1993B) provides a point estimate of the effluent concentration that causes a given percent reduction based on organism response. To calculate the inhibition concentration percentage (ICp), a computer program (Norberg-King, 1993; DeGraeve et al., 1988; EPA, 1989C) is available and the assumptions for the method are not the same as the test design requirements for hypothesis-based analyses. This point estimation method is particularly useful for analyzing the type of data obtained from chronic TIE tests using dilutions (see Phase I; EPA, 1992). Confidence intervals are calculated using a bootstrap technique and might be useful in determining if significant toxicity alterations have been observed. A significant reduction in toxicity and the precision of reference toxicant tests must be determined by each laboratory for each effluent. The use of the IC50 for Phase I TIEs might be more useful in correlating the characterization test results to the effluent toxicity than an IC25. However, there are situations when an IC50 may not be able to be estimated while the IC25 can. Above all, it is most important to use a consistent effect level for TIE toxicity testing (EPA, 1992A; EPA, 1992B). When substantial toxicity reductions occur in the toxicity tests, it may not always appear to be a significant reduction when the IC25s are compared. In order to further evaluate whether toxicity reductions occurred, the sample size (number of replicates, number of concentrations) should be increased in subsequent testing in an effort to differentiate toxicity responses from the sample size limitations. The dose response curves should then be compared to see if responses are similar. Once the toxicant is identified, the number of replicates should be increased, the dilution sequence might be modified and more dilutions used (see Phase III; EPA, 1993A). This should increase the confidence in the IC25 (or any other ICp value chosen) estimate.

Section 2
Non-Polar Organic Compounds

2.1 General Overview

The procedures described in this section presume that the results of Phase I tests have implicated non-polar organic compounds as the cause of acute or chronic toxicity. Results of Phase I tests that clearly implicate a non-polar organic toxicant typically are (1) all toxicity is removed by the C_{18} Solid Phase Extraction (SPE) column and (2) toxicity was observed in the methanol eluate test (see Section 8.6, EPA, 1991 A; and Section 6.7, EPA, 1992). In some instances, toxicity might not be removed completely by the SPE column, but sufficient toxicity is recovered in the methanol eluate to suggest a non-polar organic toxicant is present. While toxicants other than non-polar organic compounds might be removed by the column (e.g., metals), the elution for such toxicants is unlikely to be similar to that of the non-polar organic compounds. However, there is also the possibility that non-polar toxicants such as surfactants will be removed by the column and not recovered in the methanol eluate. The goal in this section is to separate the non-polar organic toxicants from the many non-toxic components of the sample to simplify the analytical work needed to identify the toxicant.

This section provides the general background information on non-polar organic compounds along with methods for concentrating and separating the toxicants for samples with acute and/or chronic toxicity. While the method provides a stepwise procedure, there are instances where the investigator may have to modify the approach to achieve the best results.

Also provided in this section are procedures that might prove helpful in less common situations. Metabolic blockers can be used to reduce or eliminate certain organophosphate compounds from exhibiting their toxicity (Section 2.5.1). In the instance when toxicity is not recovered in the methanol eluate and toxicity is not evident in the post-column effluent, alternate SPE elution procedures might be needed (Section 2.6).

A flow diagram of the general procedures followed in identifying non-polar organic toxicants is shown in Figure 2-I. In this procedure, the C_{18} SPE column is used to extract non-polar organic compounds from effluent samples. These compounds are then selectively

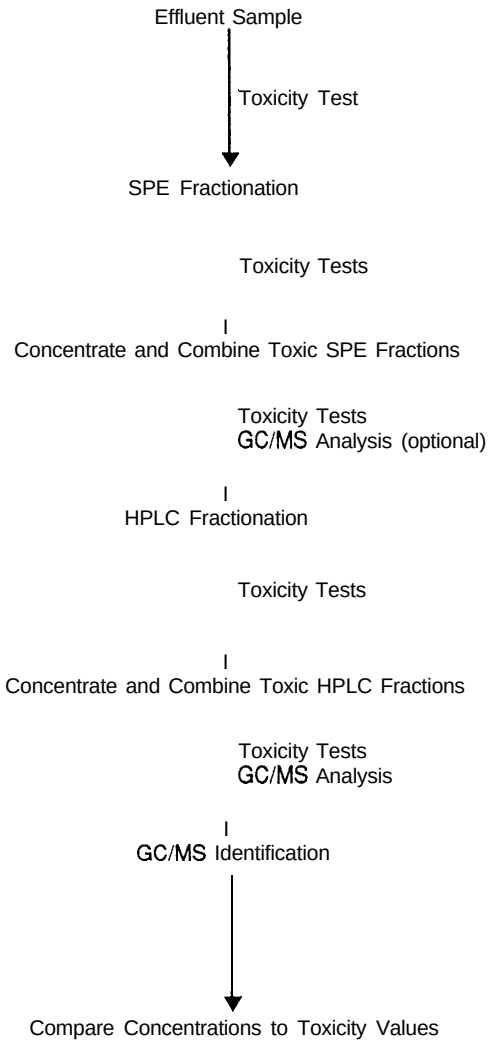

Figure 2-1. Phase II schematic for the identification of non-polar organic toxicants.

stripped off the column by eluting the C_{18} sorbent with solvent/water mixtures that are increasingly less polar. As a series, the 'fractions" resulting from column elutidn contain analytes that are decreasingly polar and decreasingly water soluble. Each fraction is then tested for toxicity. The fractions that exhibit toxicity are concentrated, and chromatographed using reversed phase High Performance Liquid Chromatography (HPLC). The resulting HPLC fractions are collected and tested for toxicity. The toxic HPLC fractions are concentrated into methanol by using another C_{18} SPE column. The concentrates are toxicity tested as before and analyzed using gas chromatography/mass spectrometry (GC/MS). Those constituents that are identified by GC/MS are roughly quantitated, by assuming that the identified constituents and the internal standard have the same response factor, and the estimated concentrations are compared to available toxicity values for each chemical. If this process reveals strong suspect toxicants, mass balance testing (Phase III; EPA, 1993A) could be started to determine whether additional toxicants are present. If no suspect toxicants are identified by GC/MS, a longer analysis time on the HPLC might help the identification by increasing the separation between toxic and non-toxic components, especially if there are many constituents present. Also, additional constituents might be identified by increasing the concentration factor by using larger effluent samples. At some point, the probability that the toxicants are not chromatographing on the gaschromatograph or the mass spectrometer is not detecting the toxicants must be considered if no suspect toxicants are identified. Use of other types of mass spectrometry, such as liquid chromatography/mass spectrometry (LC/MS) or direct probe mass spectrometry may be useful. Some effluents might require the SPE fractionation of several different samples befoie good suspect toxicants are found (Burkhard et al., 1991: Lukasewycz and Durhan, 1992).

The sorbents that we recommend for use in SPE and HPLC columns are chemically identical. The column packing is composed of silica gel which has been reacted with octadecyl silane to produce a covalent bonded phase one layer thick. The mechanism of extraction with C_{18} sorbents is relatively simple. Extraction of effluent compounds occurs because the C_{18} sorbent competes for the non-polar compounds more strongly than the surrounding water molecules of the effluent. Sorption of non-polar organics is also influenced by ionic strength, pH, and total organic carbon (TOC) levels. The same compounds will partition on both SPE and HPLC columns and the order of elution of chemicals will be approximately the same. The major difference between the SPE and HPLC columns is the amount of resolution achieved. The particle size employed in HPLC columns is smaller, providing a greater surface area and better component resolution. Despite less resolution, SPE columns have the advantage of possessing a higher loading capacity in general than HPLC columns. The SPE column could be considered as a preparatory column for sample cleanup while the HPLC column gives far more refined and controlled separation of sample constituents.

To elute non-polar organic toxicants extracted by the C_{18} SPE column, the sorbed compounds must have a higher affinity for the eluting solvent than for the octadecyl functional group (C_{18}). Choosing a solvent for elution is complicated because the toxicants' identities are not known. In general, the solvent should be less polar than water and more polar than the C_{18} functional group. The degree of solvent strength required to elute the toxicants is also unknown. Since methanol is less polar than water, has a very low toxicity (EPA, 1991A; EPA, 1992) and elutes chemicals from C_{18} sorbents, it has been a good solvent choice for most TIE purposes to date.

During sequential column elutions with successively increasing methanol in water concentrations, the relatively hydrophilic, polar compounds are eluted first, and the more hydrophobic non-polar compounds are eluted last. Given the strength of methanol as a solvent for non-polar compounds, it is possible that very hydrophobic (octanol water partition coefficient (log K_{ow}) ≥ 4) effluent compounds will not be eluted from the C_{18} sorbent. If toxicity caused by a very hydrophobic compound is extracted by the SPE column but not eluted by methanol, less polar solvents might be used to elute the SPE column (Section 2.6.1).

Once toxicity is found in one or more C_{18} SPE effluent fractions, the toxic fractions can be concentrated, then fractionated using HPLC. HPLC separation is used to reduce the number of non-polar organic chemicals associated with the toxicant(s) and to simplify analytical identification. The toxic HPLC fractions are concentrated and then analyzed by GC/MS. The estimated concentrations of constituents in the final concentrate (based on an internal standard) are then compared to their toxicity values to decide which may be sufficiently high in concentration to cause toxicity. If none are found, higher concentration factors, other analytical methods (e.g., LC/MS), and better separation are recommended.

Fractionation and toxicity testing procedures for non-polar organic toxicants causing either acute or chronic toxicity are presented in different sections of this chapter. The acute toxicity (Section 2.2) and chronic toxicity (Section 2.3) sections, have similar outlines and were written so either section could be used independently. As a result, some details that apply to both acute and chronic toxicity are repeated. After using Section 2.2 or Section 2.3 the investigator should then follow the identification techniques described in Sections 2.4 and 2.5. Additional identification techniques are included in Sections 2.5.1 and 2.5.2, and alternate fractionation methods are found in Section 2.6.

2.2 Acute Toxicity: Fractionation and Toxicity Testing Procedures

In the initial stages of Phase II, toxicity tests may be conducted on C_{18} SPE effluent fractions and blank fractions to detect the presence of toxicants and not to quantify the magnitude of the toxicity in each. As in the **toxicity** tests conducted during Phase I, careful measure-

ment of test solution water chemistry parameters is not required, and duplicate exposures are not needed during initial stages of Phase II. The major purpose of this step is to assess whether or not acute toxicity is present in the effluent fractions and the blank fractions. However, as suspect toxicants are identified, quantitative toxicity measurements will be needed to compare with the analytical measurements. If Phase II data will be used to correlate effluent toxicity to toxicant concentrations (Phase III), then more replicates per concentration, randomization of test concentrations, careful observation of organism exposure times, and organisms of approximately the same age should be used (Section 1.2). Also, the amount of eluate that is collected from the SPE fractionation, SPE concentration, and the amount of eluate used for testing and GC/MS analysis should be measured at all steps. The volume of eluate must be measured to determine the actual toxicity concentration in each step of the procedure. If it is expected that the Phase II data will be needed later, it is prudent to measure the degree of toxicity in the eight SPE effluent fractions at the onset of testing. We rarely see blank fraction toxicity; therefore, there is little need to evaluate the toxicity of the blank fractions with dilutions.

2.2.1 *Sample Volume*

The volume of effluent needed depends on its toxicity, the toxicity of the chemicals causing effluent toxicity, and the sensitivity of the analytical method. Since only the first of these will usually be known when Phase II begins, trial and error will dictate volume size. For acutely toxic effluents with LC50 values in the range of 25-100%, 2,000 ml have usually been adequate to perform one complete Phase II procedure, i.e., C_{18} SPE and HPLC fractionations, and GC/MS identification. Examples of the variables that should be considered when deciding what volume of effluent to fractionate are provided in Appendix A, Tables A-l and A-2.

2.2.2 *Filtration*

For acute tests, glass fiber filter(s) (1 µm nominal pore size) should be prepared as described in Section 8 of Phase I (EPA, 1991A). Both 45 mm and 90 mm diameter filters have been used routinely, the 90 mm filter allows about four times more effluent to be passed over the filter than one 45 mm filter. All filters and glassware should first be pre-rinsed with pH 3 high purity water (e.g., Milli-Q® Water System, Millipore Co., Bedford, MA) to remove any metal residues followed by a high purity water rinse which is discarded. The filter should then be rinsed with 200 ml of dilution water (rather than with high purity water) and a sample collected after most of the volume has been filtered, to provide the filter toxicity blank. In subsequent steps, a dilution water column blank will be collected after passing the filtered dilution water through the SPE column. The same type of dilution water should be used for the filter blank as for the column blank (Section 2.2.4). Usually, a reconstituted water is used for these procedures (EPA, 1991C).

The volume of effluent that can be passed through a single filter is sample specific. If more than one filter is needed (as is often the case) a single filter blank can be prepared by stacking three to eight pre-rinsed filters in one filter holder, followed by a dilution water rinse. The filters are then separated and used one at a time to filter the effluent sample. If samples are high in suspended solids additional pre-filtration may be needed. Centrifugation may also be useful for reducing solids in the sample. The decision to use a vacuum or a pressure system for filtering should have been made during the filtration tests of Phase I. If a volatile chemical is indicated in Phase I, pressure filtration should be used.

Filtration equipment should be thoroughly cleaned before use to prevent any toxicity carry-over or particle buildup from previous samples. We have found that glass vacuum filtering apparatus with stainless steel filter supports (for samples without pH adjustments), or plastic pressure filtering devices are the most useful. We have also found that if removable glass frits are used, they can be rigorously cleaned with aqua regia for 20-40 min followed by rinsing with copious amounts of water to remove residual effluent particles, since glass frits may act as a filter. The removable stainless steel filter supports do not require as rigorous cleaning as fritted glassware, and therefore are a good substitute.

A portion of the filtered sample must be reserved for toxicity testing while the rest is used for C_{18} extraction. If the filtration toxicity blank exhibits slight or complete toxicity, but the post C_{18} SPE column effluent is not toxic (and effluent toxicity was unchanged after filtration), the blank toxicity can be ignored since the effluent toxicity was removed (see Phase I). However, as the identification process continues, the blank toxicity will have to be eliminated, or it could lead to a misidentification of the cause of toxicity.

When effluent samples are readily filtered (-2,000 ml for one 90 mm 1µm filter) it may be possible to filter the effluent for the filtration test of Phase I but then use unfiltered effluent with the C_{18} SPE column test and the methanol eluate test (Phase I). Once it has been demonstrated that filtration does not reduce toxicity, routine filtering of these effluents (before passing the effluent through the SPE column) can be eliminated. This will reduce the amount of toxicity testing required.

2.2.3 *Column Size*

Various sizes of C_{18} SPE columns are available ranging from 100 mg to 10,000 mg packing material. We routinely have used Baker® 1,000 mg columns for 1,000 ml of effluent (J.T. Baker Chemical Co, Phillipsburg, NJ). Volumes for a 1,000 mg C_{18} SPE column are used in the following description, since this is the size most often used for acutely toxic effluents. Other available column sizes and the appropriate volumes to be used in their preparation are listed in Table 2-I. Positive pressure pumps (EPA, 1991 A) are convenient for the large volume

effluent samples because flow rate can be controlled. Vacuum manifolds can be used for drawing the small samples and solvents through the column. Whichever system is used, it must be made of materials that dilute acid and solvents do not destroy, or from which chemicals are not leached that are toxic or that interfere with analytical measurements. Teflon, glass, and stainless steel are all acceptable choices.

2.2.4 C,, SPE Column Conditioning

The 1,000 mg C_{18} SPE columns are conditioned by pumping 10 ml of 100% methanol through the column at a rate of 5 ml/min. The pumping rate can be increased to 40-50 ml/min when using the larger C_{18} SPE columns (e.g., 5 g or 10 g). The volumes of conditioning solvent recommended for other size columns are shown in Table 2-1. We most commonly use methanol as the conditioning solvent but other water miscible solvents such as acetonitrile, ethanol, or isopropanol may be substituted. Before *the packing goes dry,* 10 ml of high purity water must be added. As the last of the high purity water is passing through the column, 25 ml of filtered dilution water is added. The last 10 ml of dilution water is collected for a dilution water column blank. After the dilution water has been collected, pumping is continued until no dilution water emerges from the column.

2.2.5 *Elution Blanks*

To generate elution blanks from a 1,000 mg column, two successive 1.5 ml volumes of 25% methanol/water (%v/v) are pumped sequentially through the conditioned column and collected in one analytically clean, labeled glass vial to produce a 3 ml sample. This procedure is repeated with two successive 1.5 ml volumes of 50%, 75%, 80%, 85%, 90%, 95% and 100% methanol/water. The column should be allowed to dry for a few seconds between each elution with the different 3 ml volumes of methanol/water solutions. This will result in eight 3 ml SPE fraction blanks (Figure 2-2). The volume of methanol solutions used for elution will vary depending on column size as shown in Table 2-1.

2.2.6 Column Loading with Effluent

The same column is then reconditioned with 10 ml of 100% methanol and 10 ml of high purity water, as described in Section 2.2.4. *Without allowing the column to dry,* 1,000 ml of filtered effluent is pumped through the column at a rate of 5 ml/min (Figure 2-3). The pumping rate can be increased to 40-50 ml/min when using the larger C_{18} SPE columns (e.g., 5 g or 10 g). Three samples (-25 ml) of the post-C,, SPE column effluent are collected after 25 ml, 500 ml and 950 ml of the sample has passed through the column. Each post-column aliquot is toxicity tested to determine the presence of acute toxicity in the post-column effluent. This information can be used to determine whether the toxicant is removed from the eff luent by the column. As Phase II progresses, the recommendation is to increase the volume of post-column effluent collected to 50-60 ml so that dilutions can be made and LC50 values obtained. Pumping is continued until no effluent emerges from the column.

The efficiency of the C_{18} SPE column is determined by the extraction efficiency (i.e., how well the column sorbent removes the effluent components) and the elution efficiency (i.e., how well sorbed effluent compounds are removed from the column by the solvent elution). For purposes of the TIE, "efficiency" applies only to recovery of those compounds causing or affecting effluent toxicity. Since most acute effluent tests do not require large volumes of post-column effluent, the question of extraction efficiency can be determined by measuring the toxicity of the post-C,, column effluent sample. The toxicity of each aliquot collected after different vol-

Table 2-1. Solid Phase Extraction (SPE) Column Fractionation Information'

C_{18} SPE Sorbent Amount[2] (mg)	Volume Conditioning Solvent (ml)	High Purity Water Volume (ml)	Maximum Volume Effluent (ml)	Minimum (500x) Elution Volume[3] (ml)	Suggested (333x) Elution[4] Volume[3] (ml)
100	2	2	100	2 x0.1	2 x 0.15
500	5	5	500	2 x 0.5	2 x 0.75
1,000	10	10	1,000	2 x 1.0	2 x 1.5
5,000	50	50	5,000	2 x 5.0	2 x 7.5
10,000	100	100	10,000	2x 10	2 x 15

[1]The information is based on manufacturer's guidance and experimental data from ERL-D.
[2]The smaller columns (100, 500, and 1,000 mg sorbent) are available pre-packed from J.T. Baker Chemical Co., the larger columns (5,000 and 10.000 mg sorbent) are available pre-packed from Analytichem International.
[3]Elution with two successive aliquots of the volume listed.
[4]The 333x concentration factor is most often used for acute work.

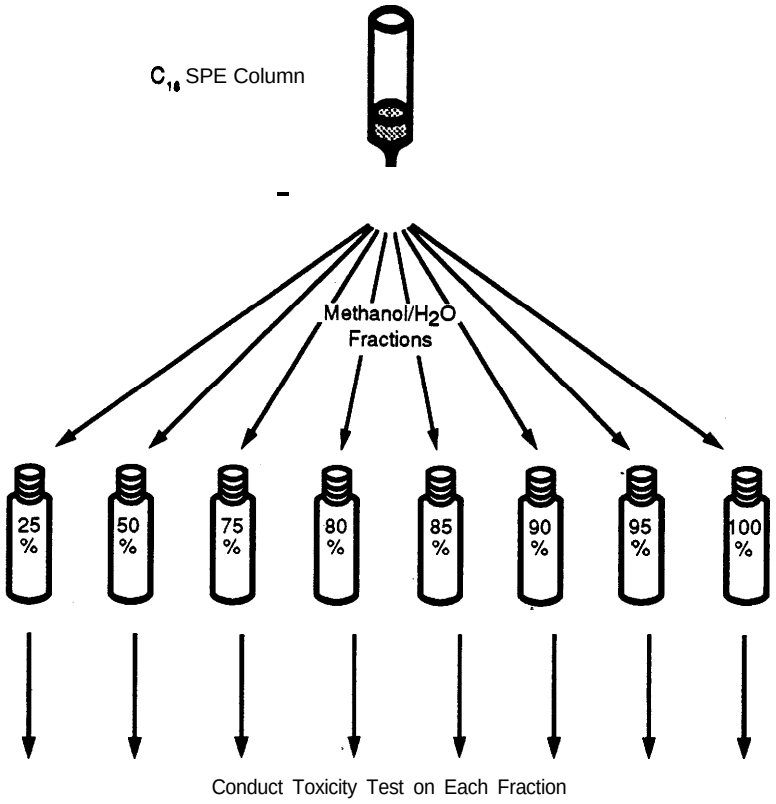

Figure 2-2. Procedures for eluting the SPE column with a gradient of methanol/water solutions.

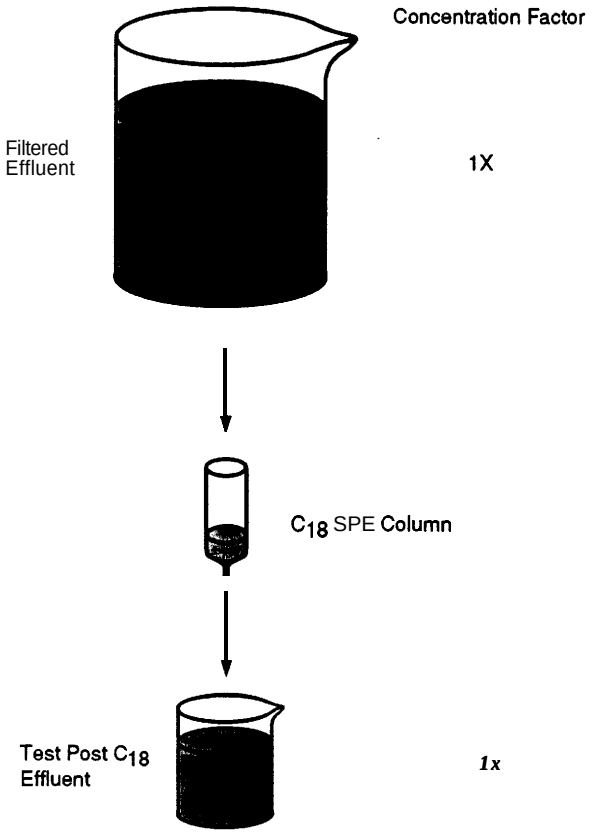

Figure 2-3. Concentrating effluent on the C_{18} SPE column.

umes of effluent have passed through the column can be compared. If there is toxicity in these aliquots, but it is independent of the volume of effluent previously passed through the column, then the post-column effluent toxicity is probably caused by toxicants that are not extracted by the column. If toxicity increases as the volume of post-column effluent passed through the column increases, the capacity of the column to sorb the toxicants was probably exceeded.

In some post-column effluent, a biological growth may occur during toxicity testing which may result in artifactual toxicity. Such growth can make it appear as if the toxicant is not removed by the column. While this growth does not occur in all effluents, when it does occur with one post-column effluent sample, the growth often occurs in each subsequent post-column effluent sample from the same preparation. The growth may appear to be filamentous and give a milky appearance in the test vessel. This effect has been linked to methanol stimulation of bacterial growth. Methanol is present in the post-column samples because a small amount of methanol is constantly released from the column during the sample extraction. Effluents from biological treatment plants may develop this characteristic more readily than industrial effluents.

Additional filtering of the post-column effluent sample through a 0.2 µm filter before testing to remove bacteria and eliminate the growth has been helpful. To avoid artifactual toxicity as much as possible in the post-column effluent, initiate the tests with the post-column samples on the same day the effluent is extracted even if fractions are not tested simultaneously. For those few effluents where we have not eliminated this type of artifactual toxicity, holding the post-column effluent is problematic in that more time is available for bacteria to cause problems in the post-column sample matrix. When post-column artifactual growth is not readily eliminated, a different solvent (e.g., acetonitrile) to condition the column (but not for eluting) may be useful in reducing the post-column artifactual bacterial growth. This artifactual growth has not occurred in the toxicity tests with methanol SPE fractions (Section 2.2.8).

2.2.7 C_{18} SPE Column Elution

Once the effluent sample has been loaded onto the column, elution can begin. To elute a 1,000 mg column, two successive 1.5 ml volumes of the 25% methanol/water mixture are pumped through the column and collected in one labelled, analytically clean vial to make a 3 ml sample. Subsequently, two successive 1.5 ml volumes of each of the 50%, 75%, 80%, 85%, 90%, 95% and 100% methanol/water are pumped through the column and collected in separate vials (Figure 2-2). The next elution volume should be added when no more of the preceding one is emerging from the column.

This entire procedure (conditioning through elution) is repeated using a second 1,000 mg C_{18} SPE column for the second 1,000 ml of filtered effluent. The dilution water column blank samples should be kept separate. The corresponding fractions from the blank and the sample from each 1,000 ml fractionation can be combined. For example, the 3 ml 100% methanol sample fraction from the first column and the 3 ml 100% methanol sample fraction from the second column are combined to produce a total of 6 ml. There will be eight 6 ml blank fractions and eight 6 ml effluent fractions.

The vials containing the methanol/water fractions are tested immediately or sealed with perfluorocarbon or foil-lined caps and stored under refrigeration. These fractions represent a 'first cut" separation of effluent components. Elution volumes will vary if columns of different sizes are used or if the particular effluent under study or the research question being posed dictates method modification.

2.2.8 *Blank and Effluent Fraction Toxicity Tests*

The next step is to determine the toxicity of the blank and effluent fractions. While the choice of test concentration depends on the toxicity of the effluent in most instances, we have used a high test concentration of 2x or 4x (the LC50 or 100% effluent) for acutely toxic effluents. The methanol content in the fractions limits the concentration that can be tested, and at this point the amount of methanol is assumed to be 100% in all the fractions for dilution calculations; however, this is not assumed for add-back tests (described below). Usually 120 µl of each blank and sample fraction (333x) is injected into separate 10 ml aliquots of dilution water to test at 4x the 100% effluent[2]. This will give a 1.2% methanol concentration which is below the methanol LC50 for both C. *dubia* and fathead minnows in the 100% methanol fraction. The resulting methanol concentration must be adjusted for the species tested (see Section 8 of EPA, 1991 A). During the initial stages, five animals in each 10 ml aliquot are used without duplicates. Using the above volumes, the tested solution is more concentrated (i.e., 4x) than 100% effluent, assuming 100% extraction and 100% elution in one fraction. These test solutions can be diluted to provide an LC50 for each sample fraction. Blank fractions need not be diluted, since hopefully they are nontoxic.

Individual chemicals in the fractions could be toxic even when they are not toxic in the whole effluent, since the concentration tested may be as high as 4x whole effluent. Therefore, to be toxic at whole effluent concentrations, an individual fraction must have an LC50 of 25% or less. Since there is no way to know whether the toxicant(s) eluted over more than one fraction or what the percent extraction and elution efficiency are, fraction tox-

[2] In the Phase II document published in 1989, testing at 5x was recommended, the methanol level was 1.5% at this concentration. In order to lower the methanol level, this was changed to 4x in this document.

icity up to 100% (4x whole effluent) should not be disregarded.

If toxicity occurs in any of the fraction blank tests and it is small relative to the toxicity in the corresponding sample fraction (e.g., 20% mortality versus 80%), the sample fraction results should not be dismissed. If all organisms die in the blanks and the effluent fractions, dilutions should be tested to make sure the sample fraction is substantially more toxic than the blank. In general, blanks should not have measurable toxicity.

If the SPE fractions are toxic at effluent concentrations of lx or 2x and toxicity is reduced in two of the three post-column effluent samples, the toxicant could still be a non-polar organic compound. If the effluent fractions are not toxic individually and the post-column samples are non-toxic, it is possible that the toxicity has been spread across several fractions or has not been recovered from the column. Combining and concentrating fractions may be useful or other elution procedures may be necessary. If toxicity is observed in the fractions at lx, 2x, or 4x and in the post-column effluent samples, it is possible that not all the toxicity is caused by non-polar compounds, that break-through of the toxicant has occurred, or that the toxicity is artifactual.

In addition to concentrating column artifacts to toxic levels, effluent constituents present at nonlethal levels may be concentrated to toxic levels in this test if they have a relatively high recovery value. Actual effluent toxicants with poor recovery may not be present in these test solutions at toxic levels. Spurious results of this nature will be identified in the later stages of Phase II and/or in Phase III.

Elution efficiency may be approximated by summing the amount of toxicity (i.e., TUs) in the toxic fractions (provided dilutions are tested) and comparing this value to whole effluent toxicity expressed as TUs. When summing acute toxicity, it is important that all values are for comparable endpoints (i.e., LC50s). Adding of TUs may be somewhat imprecise for several reasons. A single toxicant may occur in more than one adjacent fraction, in which case a small amount of the toxicant in one fraction may not be detectable because it is present below the effect concentration. For acute toxicity, this problem may be solved by combining a portion of each effluent fraction (and separately testing the corresponding blank fractions) and measuring total toxicity at ix. If more than one toxicant is present, the effluent fraction toxicity may not be strictly additive in their toxicities, and when separated into different fractions the sum of the fraction toxicities may be low even if extraction and elution efficiencies were 100%. Table 2-2 illustrates a hypothetical example. The toxicity test results from the test with a portion of all fractions or a few of the fractions may show somewhat greater toxicities than those of the whole effluent. This may be caused by enhanced toxicity due to matrix effects. When this occurs, it may be possible to compensate for toxicity enhancement by methanol, by adding methanol to the whole effluent and evaluating the toxicity. This methanol addition may in turn stimulate

Table 2-2. Comparison of Toxic Units (TUs) in Each Toxic Fraction to TUs of All Fractions Combined and Whole Effluent

Toxic Fraction (% Methanol)	TUs
75	0.5
80	1.2
85	0.6
SUM	2.3
Combined Fractions	2.7
Whole Effluent	2.5

biological growth, and if this happens, the test is negated. We have rarely used this approach since the fractions have seldom caused more toxicity than the effluent itself. At this point in Phase II, the effluent fractions should also be tested in water with TOC and suspended solids which mimics the effluent to lessen matrix effects on toxicity. As the identification step moves into Phase III, it is better to use dilution water that mimics effluent or receiving water characteristics.

*2.2.9 SPE Fractions: Concentration and Subsequent Toxicity **Testing***

The SPE fractionation provides a general separation of non-polar organics and except in relatively uncomplicated effluents, GC/MS analysis of the concentrates of toxic C_{18} SPE fractions will result in very complicated chromatograms from which the toxicant(s) cannot be distinguished from other effluent components. A secondary fractionation using HPLC is often needed to further simplify toxic effluent fractions prior to component identification by GC/MS analysis.

In order to maximize the chromatographic separation capability of the HPLC, the volume of the sample injected onto an analytical size HPLC column should be as small as possible (i.e., <0.5 ml); therefore, the toxic SPE fractions (usually >1 ml) must be concentrated prior to injection onto the HPLC column. This concentration step will provide the added benefit of an increase in concentrations of constituents in the HPLC fractions as well as rid the SPE fractions of water. The latter issue is important if GC/MS analysis will be performed on the concentrated SPE fraction prior to injection on the HPLC (Durhan et al., 1990).

The volume of the fractions from the initial SPE fractionation procedure and the number of fractions to be combined will determine the size of the SPE column to use for the concentration procedure. Table 2-3 contains information on the column sizes we have found to be most useful. In the procedure outlined below (Figure 2-4), we have used a 100 mg column which is the most commonly used size for concentrating SPE fractions of acutely toxic effluents. Most often the toxic effluent SPE

Table 2-3. Information for Concentrating SPE and HPLC Fractions'

C_{18} SPE Sorbent Amount (mg)	Volume Conditioning Solvent (ml)	High Purity Water Volume (ml)	Maximum Toxic Fraction Volume (ml)	Maximum Diluted Fraction Volume (ml)	Minimum Elution Volume[2] (ml)	Approximate Eluate Volume (ml)
100	1	1	20	100	3 x0.1	0.22
200	2	2	40	200	3 x 0.2	0.44
500	5	5	100	500	3 x 0.5	1.10
1,000	10	10	200	1,000	3x 1.0	2.20

'Concentration information is based on manufacturers guidance and experimental data from ERL-D
[2]Elution with three successive aliquots of the volume listed.

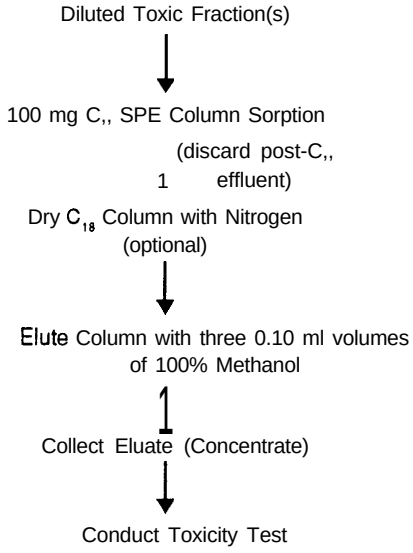

Figure 2-4. Procedure to concentrate toxic SPE fractions.

fractions are combined and diluted with high purity water and the corresponding blank fractions are treated similarly. In cases where there are multiple non-polar toxicants, and when toxicity occurs in several fractions, it may be more useful to concentrate each fraction separately for subsequent HPLC separation. The percent methanol in the diluted fraction sample should be ≤20% and the volume to which the fractions can be diluted is dependent on the amount of column packing. For example, the total volume of the diluted fractions should not exceed 100 ml for the 100 mg C_{18} SPE column (Table 2-3). No more than three toxic fractions of 6 ml each can be combined and concentrated on the 100 mg column. When the total volume of combined fractions or the individual fraction volume is above 20 ml, larger columns should be used; consult Table 2-3 for column size and elution volume information. The effluent and blank concentrates and the column blank are tested for toxicity to ensure that the toxicant is still in the concentrate and that artifactual toxicity was not introduced by the procedure. If there is not measurable toxicity in the concentrate, it is possible that the percentage of methanol in the diluted fraction was too high. The concentration procedure should then be repeated with a new set of toxicity tested fractions diluted to a lower methanol concentration, e.g., 10%.

Below is an example of how to prepare effluent and blank fraction concentrates. First, a 100 mg C_{18} SPE column is conditioned with 1 ml of methanol and 1 ml of high purity water similar to the procedures described in the SPE Column Conditioning Section (2.2.4). Column blanks for toxicity testing are obtained by rinsing the column with at least 20 ml of dilution water. After collecting the column blank, recondition the column with 1 ml of methanol and rinse with 1 ml of high purity water. The diluted blank fractions (for dilution guidance see Table 2-3) are then drawn through the 100 mg C_{18} SPE column under a pressure of 380 mm Hg using a vacuum manifold. Unlike the first fractionation step (Section 2.2.6) the post-column sample cannot be tested for toxicity because of its high methanol concentration (i.e., IO-20%). The column is then dried for 10 min using a gentle flow of nitrogen (1 0-20 ml/sec). Drying the column usually increases the recovery of toxicity, but sometimes toxicity is not recovered from the column, possibly as a result of volatilization. If this occurs the concentration procedure can be repeated without the nitrogen drying step.

After drying the sorbent, the luer tip of the column is fitted with a luer-lock needle and 100 µl of 100% methanol is placed into the column using a microliter syringe. Nitrogen is then applied to the column at a rate of -4 ml/sec to force the methanol through the sorbent. The luer-lock needle is needed to ensure the collection of small volumes; when using larger column sizes (e.g., ≥500 mg) this is not necessary. The first 100 µl aliquot of methanol applied to the column will yield approximately 25 µl of eluate. Two more 100 µl aliquots of 100% methanol are also forced through the column. The final volume of eluate collected will be approximately 220 µl. If desired, measure the exact volume collected (using a µl syringe) to calculate concentration factors (Table 2-3). As in most chromatographic separations and extractions, three separate smaller elutions of methanol are more efficient than one large one.

The 100 mg C_{18} SPE column is reconditioned with 1 ml of methanol and rinsed with 1 ml of high purity water. It is then used to concentrate the diluted toxic SPE column fractions, using the same procedure used for the blank fractions (Figure 2-4). In lieu of reconditioning the same column, two columns can be conditioned, one used for the diluted blank fractions and the other used for concentrating the diluted toxic SPE fractions. The resulting column blanks should be toxicity tested separately.

The original effluent volume of 2,000 ml is now concentrated into a 220 µl sample or a nominal concentration of 9,091x (ignoring the amount used for testing). As work progresses and more quantitative results are needed, the eluate volume must be measured to provide the correct concentration factor. If 9 µl of concentrate is diluted to 10 ml in dilution water, the resulting test concentration will be 8x whole effluent. Additional test concentrations (e.g., 4x, 2x, lx) can be prepared to determine an LC50 of the concentrate, and toxicity recovery can be calculated by comparing this LC50 to the LC50 of the effluent. The concentrate toxicity might be higher than the sum of the individual toxic fractions because some of the toxicant may have been in adjacent fractions that were concentrated in the first step (Section 2.2.7) but not detectable by the toxicity test of the single fraction. The concentrate toxicity may also be lower than expected because of low extraction and elution efficiencies. Where greater concentration factors are desirable, SPE fractionation should be repeated with additional volumes of effluent, followed by combining the toxic fractions before concentration. The size of the column used for concentrating may have to be increased, along with the appropriate changes in dilution and elution volumes (Table 2-3).

The important concern here is not 100% recovery of toxicants but enough recovery for GC/MS analyses and to obtain measurable toxicity in the HPLC fractions. If recovery is too low, changing or eliminating the column drying time may help. Sometimes recovery appears to increase with drying time while other compounds are volatilized from the column during the drying process. For concentrates analyzed using GC/MS, column drying to remove water is critical to GC column performance.

2.2.10 HPLC Separation

The same column packing functionality should be used in the HPLC column as the SPE column. At later stages, when more is known about the toxicants, other sorbent types may be used.

The HPLC conditions presented in this section are general. As more information on the effluent is gathered, HPLC conditions should be modified to achieve better separation and higher concentration factors. We use a flow rate of 1 ml/min on an instrument equipped with a 5 µm C_{18} reverse phase column (250 mm x 4.6 mm i.d.). The HPLC elution conditions will change depending on which SPE fractions have been concentrated. The HPLC conditions for the four most commonly toxic SPE fractions are listed in Table 2-4. Depending on the size of the HPLC injector and column, more than one HPLC fractionation run may be required to fractionate the entire blank concentrate. When multiple HPLC fractionations are conducted, collect all the corresponding HPLC fractions in the same set of vials. For example, if two HPLC fractionations were performed for the blank concentrate, 25-2 ml HPLC fractions would be obtained.

Table 2-4. Example HPLC Elution Gradients for Four Commonly Toxic SPE Fractions

75% or 85% SPE Fractions		85% or 90% SPE Fractions	
Time (min)	% Methanol/Water	Time (min)	% Methanol/Water
0	50	0	60
1	60	1	70
13	90	13	90
20	100	20	100
25	100	25	100

Using the HPLC equipment described above, all of the blank concentrate remaining after toxicity testing is injected (≤500 µl) and 25-1 ml fractions are collected in analytically clean glass vials (Figure 2-5). The same procedure is followed using the effluent sample concentrate. The vials should be sealed (e.g., with foil lined caps) and stored at 4°C after use. As soon as toxicant identification is obtained by GC/MS, then HPLC conditions (gradient, fraction size, and number of fractions) can be optimized for further fractionations.

2.2.11 HPLC Fraction Toxicity Tests

Before specific toxicants are identified, toxicity tests on each HPLC blank fraction and sample fraction are conducted using non-replicated exposures of five animals each. The amount of methanol in the HPLC fractions limits the concentration that can be tested. Assume that each fraction is 100% methanol to calculate the necessary dilution. A methanol concentration of 1.2% should not be exceeded for *C. dubia* and fathead minnow acute toxicity tests.

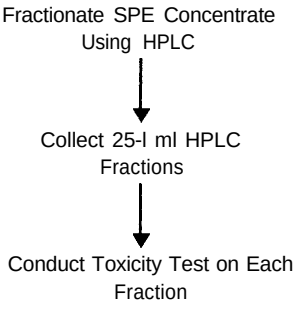

Figure 2-5. Procedure to fractionate acutely toxic SPE concentrates using HPLC.

For acute studies, when all of the SPE fraction concentrate remaining after toxicity testing is injected (one injection) on the HPLC (Figure 2-5), each resulting 1 ml HPLC fraction equals 2,000 ml of effluent (assuming no loss and toxicant elution in only one fraction) or a 2,000-fold concentration. If each HPLC fraction is then diluted for testing (80 µl to 10 ml) the resultant concentration is 16x the original effluent concentration. In preliminary Phase II testing the HPLC fractions are tested without dilutions. Only the toxic HPLC fractions are tested again with dilutions to generate an LC50. Some loss of toxicant tends to occur in each concentration step and the resulting toxicity may be decreased relative to the original effluent.

The blank fractions should not be toxic. If they are, then additional tests with dilutions must be conducted on both blanks and toxic fractions to find out whether there is enough additional toxicity in the sample fractions to warrant analysis

The toxicity of the HPLC fractions should be tested at twice (at least) the concentration at which the original SPE column fractions were tested because recovery of toxicity and analytical measurements indicate that up to 50% of the initial concentration of toxic compounds may be lost in this step (Durhan et al., 1990). The amount of methanol should not exceed the amount used in the SPE fraction tests described above (Section 2.2.8).

*2.2.12 HPLC **Fractions:** Concentration and Subsequent Toxicity Testing*

The HPLC fractions that exhibit toxicity and their corresponding blank fractions must be concentrated in a solvent suitable for GC/MS or other analytical techniques. The procedure is identical to that described in Section 2.2.9 and is depicted in Figure 2-6. Judgement must be used to decide whether to concentrate each toxic fraction separately or to combine various toxic and adjacent fractions prior to concentration. If, for example, three successive fractions exhibit toxicity, there is a good chance that the same toxicant is in all three. If there are other fractions that show toxicity but they are separated from the first set by several non-toxic fractions, there is high probability that the second set contains a toxicant different from the first three. There is also a good chance that at least one non-toxic fraction on either side of the toxic fractions contains some of the toxicant(The advantage of combining fractions is to reduce the work load and increase concentration in the final concentrate. The disadvantage is that more constituents that are not the toxicant(s) will also be concentrated. This decision is not always straightforward and must be based on trial and error, and experience. Blank fractions corresponding to the toxic fractions are concentrated the same way.

The HPLC fraction and blank concentrates should be finally checked for toxicity before GC/MS analysis. This concentrate is now nominally 9,091x more concentrated than the effluent. If 18 µl is diluted to 10 ml, the resultant test concentration will be 16x the original sample

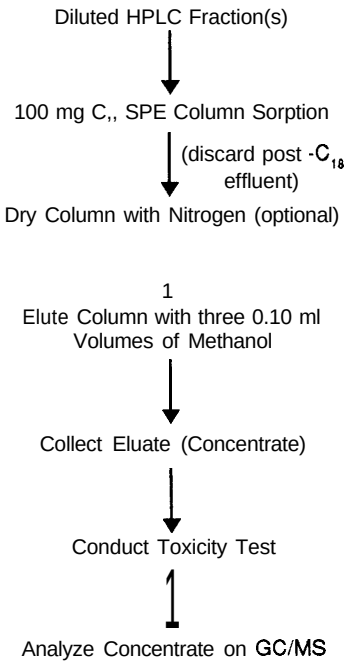

Figure 2-6. Procedure to concentrate toxic HPLC factions.

concentration. To quantitate toxicity and use the Phase II data later, additional lower concentrations should be tested (e.g., 8x, 4x, 2x); TUs of this concentrate can then be compared to previous toxicity test results. The HPLC concentrate should be tested at one to two times the test concentration of the HPLC fraction tests (i.e., 16x or 32x). This is the last opportunity to assure that the toxicant is still present in the concentrate before it is subjected to GC/MS analysis. Whether the toxicant is detected by the analytical detector (mass spectrometry in our laboratory) is always a question. Since GC/MS detects only about 20% of organic chemicals (EPA, 1989B), even such a broad spectrum method is not certain to identify the toxicant. As work progresses with more samples of the effluent and quantitative results are needed, the amount of eluate collected should be carefully measured and recorded to accurately calculate the concentration factors. In addition, the volume of concentrate removed for toxicity testing and analytical analyses should also be recorded.

2.3 Chronic Toxicity: Fractionation and Toxicity Testing Procedures

The chronic Phase II non-polar organic toxicity identification follows the same general approach and employs manipulations similar to those described for the acutely toxic non-polar organic compounds (Section 2.2). One major difference is that the concentration of the eluting solvent (e.g., methanol) must be lower in the chronic toxicity tests than in acute tests. In the initial stages of Phase II, toxicity tests may be conducted on C_{18} SPE effluent fractions and blank fractions to detect the presence of toxicants, and not to quantify the magnitude

of the toxicity in each. However, as suspect toxicants are identified, quantitative toxicity measurements will be needed to compare with the analytical measurements. If Phase II data will be used to correlate effluent toxicity to toxicant concentrations (Phase III), then more replicates per concentration, randomization of test concentrations, careful observation of organism exposure times, and organisms of approximately the same age should be used (Section 1.2). Also the amount of eluate that is collected from the SPE fractionation, SPE concentration, and the amount of eluate used for testing and GC/MS analysis should be measured at all steps. If it is expected that the Phase II data will be needed later, it is prudent to measure the degree of toxicity in the SPE effluent fractions (Section 23.5) at the onset of testing. We rarely see blank fraction toxicity, therefore, there is little need to evaluate the blank fraction toxicity with dilutions. Also, the volume of eluate must be measured to determine the actual toxicity concentration in each step of the procedure.

The following discussion is based on our experiences with C. dubia and fathead minnows (see Section 1.2). The use of other species will require reconsideration of the appropriate test volumes and methanol concentration for each step. Chronic testing is more labor intensive and generally requires more effluent sample volume than acute testing. For the most part, in the descriptions below, for *C. dubia* there are five replicates containing 10 ml of test solution and one animal per cup. For the fathead minnow tests, two replicates of 10 animals per 50 ml and the control are usually used (Section 1.2). Typically we use four concentrations and a control.

As soon as the cause of toxicity has been determined to be a non-polar organic compound (e.g., methanol eluate test; EPA, 1992) it is prudent to concentrate large volumes of effluent for the subsequent analyses. By concentrating large amounts of the effluent it is possible to plan the optimal usage of the amount of column eluate available for toxicity testing.

2.3.1 Sample Volume

The volume of effluent needed depends on its toxicity, the toxicity of the chemicals causing effluent toxicity, and the sensitivity of the analytical method. Since only the first of these will usually be known when Phase II begins, the volume of effluent to process should be considered at the beginning of the identification process to minimize the amount of re-fractionating and re-testing of effluent and fractions. Ideally, fractionation should provide enough volume of post-column effluent (Section 2.3.6), C_{18} SPE fractions (Section 2.3.8), SPE fraction concentrates (Section 2.3.9), HPLC fractions (Section 2.3.1 I), and HPLC fraction concentrates (Section 2.3.12) to conduct all chronic toxicity testing and chemical analyses, Because of the many factors affecting the amount of effluent needed, a significant amount of thought should be put into the volume of effluent to obtain and process at one time. It is prudent for the investigator to anticipate how many identification procedures will be done, and then calculate the volume of effluent needed using the particular test parameters desired, before extracting any effluent to ensure that sufficient volume of fractions, concentrates, and post-column effluent is available for the planned procedures. It may be best to perform these calculations with several different effluent volumes and test conditions to ascertain the optimal volume of effluent to fractionate. A worksheet to assist with these calculations and an example are provided in Appendix A.

The volumes of eluate needed for chronic toxicity testing at 2x, lx, and 0.5x are provided in Table 2-5 for the C. *dubia* and fathead minnow short-term tests based on the methanol concentration that can safely be used for the chronic tests. The amount of SPE fractionation eluate needed for toxicity testing is presented for the range of tests that are commonly performed with C. *dubia* or fathead minnows, these volumes can be used in the calculation worksheets found in Appendix A (Table A-l). The approximate volume of effluent that will be needed for testing with *C.* dubia and fathead minnows is listed in Table 2-6 for various fractionation schemes and toxicity testing parameters. When only a portion of the TIE procedures will be used, obviously less effluent volume will be needed. In Table A-2, the example calculations are based upon the use of minimum elution volume for the SPE columns (Table 2-1), concentrating only one SPE fraction (Section 2.3.9), and taking into account the toxicity testing (Sections 2.3.8, 2.3.9, 2.3.11, and 2.3.12) and GC/MS analysis volumes (Section 2.5). These parameters are discussed in detail below. If additional eluate is needed, the chronic tests must be repeated for each fractionation. In Phase II and Phase III more confidence in the toxicity estimates is needed than in Phase I, therefore tests may require more replicates. The volumes needed for those tests are also presented in Table 2-5. When only limited amounts of effluent are available, one must be creative and plan its usage very carefully to obtain meaningful results.

2.3.2 *Filtra tion*

For filtration of chronically toxic effluents, the use of glass fiber filters (1 µm nominal pore size) is recommended. Both 45 mm and 90 mm diameter filters have been used routinely, but the 90 mm diameter filter allows about four times more effluent to be passed over one filter than the 45 mm filter. All filters and glassware should first be pre-rinsed with pH 3 high purity water to remove any residual metals followed by a high purity water (e.g., Milli-Q® Water System) rinse which is discarded. Low levels of metals (e.g., µg/l) from the filters may cause toxicity interferences and pre-rinsing the filters may provide cleaner blanks and less contamination in effluent samples. To collect the dilution water filter blank, first pass a volume (-200 ml) of dilution water over the filter and discard it. Next, collect the volume of dilution water needed to conduct the filtration blank test. It is a good idea to prepare excess volume, at least 500 ml for the *C. dubia* 7-d test and 800 ml for the fathead minnow 7d test. A portion of the filtered dilution water is collected for testing and a portion is reserved for the solid phase extraction test blank (Section 6.6; EPA, 1992).

Table 2-5. Eluate Volumes Needed for Chronic SPE Fraction Toxicity Tests with *Ceriodaphnia* dubia and *Pimephales promelas*

Test Species	Test Duration	Original Sample& No. Renewals	High Test Conc. of SPE Fraction	No. Rep	Volume (ml) of 500x Eluate Needed for Testing'	Test Concentrations
C. dubia	4-d	2	2x	5	0.70	2x, lx, 0.5x
C. dubia	4-d	4	2x	5	1.40	2x, lx, 0.5x
C. dubia	7-d	3	2x	5	1.05	2x, lx, 0.5x
C. dubia	7-d	7	2x	5	2.45	2x, lx, 0.5x
C. dubia	4-d	2	2x	10	1.40	2x, lx, 0.5x
C. dubia	4-d	4	2x	10	2.80	2x, lx, 0.5x
C. dubia	7-d	3	2x	10	2.10	2x, lx, 0.5x
C. dubia	7-d	7	2x	10	4.90	2x, lx, 0.5x
P. promelas	7-d	7	2x	2	4.90	2x, lx, 0.5x
P. promelas	7-d	7	2x	4	9.80	2x, lx, 0.5x
P. promelas	7-d	7	4x	2	9.80	4x, 2x, lx
P. promelas	7-d	7	4x	4	19.60	4x, 2x, lx

[1]Test volumes per replicate are 10 ml/cup for C. *dubia* and 50 ml/cup for *P. promelas. The* fraction test solutions are prepared as one solution and divided into aliquots for the replicates. For the 500x eluate concentration, this volume is based on the assumption that the C. *dubia* test solutions are prepared as 200 µl of 500x into 50 ml for 2x, 100 µl into 50 ml for lx, and 50 µl into 50 ml for 0.5x. More volume will be needed if serial dilutions are prepared (400 µl vs 350 µl). For the fathead minnow tests this assumes test solutions are prepared as 400 µl into 100 ml for 2x, 200 ml into 100 ml for lx, and 100 µl into 100 ml for 0.5x. More volume will be needed if serial dilutions are prepared (800 µl vs 700 µl). For the 4x fathead minnow test, 800 µl per 100 ml can be prepared in a similar manner.

Table 2-6. Approximate Effluent Volumes Needed for the Chronic Non-Polar Organic Identification Procedures'

Test Species	Test Duration	Original Sample & Number of Renewals	High Test Conc. in SPE Fraction Test	No. Rep.	Are Dilutions Used ?	Volume Effluent (ml) Needed to Conduct SPE & GC/MS[2] Analyses	Volume Effluent (ml) Needed to Conduct SPE & HPLC & GC/MS[3] Analyses
C. dubia	4-d	4	2x	5	Yes	3,000	15,000
C. dubia	7-d	3	2x	5	Yes	2,000	15,000
C. dubia	7-d	7	2x	5	Yes	5,000	20,000
P. promelas	7-d	7	2x	2	Yes	10,000	50,000
P. promelas	7-d	7	2x	4	No	5,000	40,000

'Calculation of toxicity testing volumes assumes that: 4x high concentration for SPE concentrate test (Section 2.3.9), 8x high concentration for HPLC fraction test (Section 2.3.1 1), 16x high concentration for HPLC concentrate test (Section 2.3.12), concentration of only one toxic fraction (SPE and HPLC), the maximum amount of sample is concentrated on the SPE columns and all SPE columns are eluted with the minimum elution volume.

[2]TIE procedures used: SPE fractionation and GC/MS of SPE concentrate,

[3]TIE procedures used: SPE fractionation, GC/MS of SPE concentrate, HPLC fractionation, and GC/MS of HPLC concentrates.

After the filtration blank has been obtained, the effluent sample is filtered using the same filter, a portion of the filtrate is collected for toxicity testing, and a portion is set aside for concentrating on the C_{18} SPE column. For some effluents, one filter will often not suffice. A technique we use to prepare several filters at once is stacking three to eight filters together in one filter holder, followed by sequential rinses with pH 3 high purity water, high purity water and dilution water (using the same rinse volumes as above). Finally, the filters are separated and set aside, using one at a time for the effluent sample. If the samples have high suspended solids concentration, pre-filtering using a larger pore size filter may help, and the appropriate blanks should be used. If the sample cannot be effectively filtered due to the presence of many fine particles, centrifugation may be used (of course, blanks must be prepared).

The filter housing should be thoroughly cleaned before use to prevent any particle build-up or toxicity carry-over from previous samples. We have found large filtration apparatus (1,000 ml), removable glass frits, or plastic filtering apparatus (e.g., Millipore®) to be useful. The glassware cleaning procedure that is described in the acute Phase I TIE manual (EPA, 1991A) is sufficient for chronic TIE work. The glass frits may require rigorous cleaning (i.e., soak in aqua regia for 20-40 min) to remove residuals that may remain after filtering, since the glass frit may itself act as a filter. Also available are removable stainless steel filter supports in a glass vacuum filter apparatus (available from Millipore®). These filter supports do not require as rigorous cleaning as fritted glassware, and therefore are a good substitute.

When effluent samples are readily filtered (-2,000 ml for one 90 mm 1 µm filter) it may be possible to filter the effluent for the filtration test of Phase I but then use unfiltered effluent with the C_{18} SPE column test and the methanol eluate test (Phase I). Once it has been demonstrated that filtration does not reduce toxicity, routine filtering of these effluents (before passing the effluent through the SPE column) can be eliminated, which will reduce the amount of toxicity testing required.

2.3.3 Column Size

Available C_{18} SPE column sizes and the appropriate water and solvent volumes used in their preparation are listed in Table 2-1. Positive pressure pumps are the most convenient to use for the large volume effluent samples because the flow rate can be controlled. Pumps and vacuum manifolds can both be used for eluting C_{18} SPE columns. Whichever system is used, it should be made of materials that dilute acid and solvents do not destroy, or from which chemicals are not leached that are toxic or interfere with analytical measurements. Teflon, glass, and stainless steel are all acceptable.

When SPE is used for isolating non-polar organic toxicants, use the maximum volume of effluent and the minimum elution volume for the column size selected to optimize the concentration of toxicants in the methanol eluates. For example, if 6,000 ml is processed, it is best to use one 5,000 mg column with 5,000 ml and one ***1,000*** mg column with 1000 ml of effluent and elute both columns with the minimum elution volumes (Table 2-1) and combine eluates. It is always best to process the maximum volume of effluent on each column to achieve the highest concentration of toxicants in the eluate.

2.3.4 C_{18} SPE Column Conditioning

The 10,000 mg C, SPE columns (Analytichem International, Harbor City, CA) are conditioned by pumping 100 ml of methanol through the column at a rate of 40-50 ml/min. This size column can process 10,000 ml of effluent and is the largest commercially pre-packed SPE column available at this time. The example presented in this section will be for 10,000 ml effluent using a 10,000 mg SPE column. The volumes of conditioning solvent will change when other size columns are used, as shown in Table 2-1. We most commonly use' methanol as the conditioning solvent but other water miscible solvents such as acetonitrile, ethanol, or isopropanol may also be used to condition columns. ***Before*** *the* ***packing goes*** *dry*, 100 ml of high purity distilled water must be added. As the last of that water is passing through, filtered dilution water is added. The volume of dilution water needed may vary from 250 ml to 1,200 ml depending on the species tested. The first ***100*** ml is discarded and the remainder is collected for the dilution water column blank. After the dilution water has been collected, pumping is continued until no water emerges from the column.

Low dissolved oxygen (DO) in the post-column dilution water blanks (even in reconstituted waters) has occurred during some chronic tests; therefore, we discard the first 100-200 ml and collect the remainder of the post-column dilution water. Low DO has been a problem, particularly in the fathead minnow growth test, and is attributed to the small amount of methanol that bleeds into the post-column sample. This may be alleviated by discarding the first post-column aliquots.

2.3.5 ***Elution*** Blanks

For chronic work, we have been using seven methanol/water fractions (50%, 75%, 80%, 85%, 90%, 95%, and 100%) rather than the eight used in acute TIEs. By eliminating 25% methanol/water fraction used in acute work the toxicity testing workload is reduced, in turn a reduction in separation of toxic and non-toxic components can occur.

To collect the fraction blanks from the 10,000 mg column, two successive 10 ml volumes of 50% methanol in water are pumped through the conditioned column and collected in one analytically clean labeled vial, to make a 20 ml sample. This procedure is repeated six more times with two successive 10 ml volumes of 75%, 80%, 85%, 90%, 95% and 100% methanol/water solutions. The column should be allowed to dry for a **few seconds between** each elution with the different 20 ml volumes of methanol/water mixtures. This will result in seven 20 ml blank SPE

fractions. The volume of methanol solutions used for elution will vary depending on column size as shown in Table 2-1.

2.3.6 Column Loading with Effluent

The same 10,000 mg column is reconditioned with 100 ml of 100% methanol and 100 ml of high purity water, as described in Section 2.3.4. The sorbent must be reconditioned when the maximum volume of dilution water has been passed over the column, otherwise the sorbents' capacity will be exceeded. After the high purity water rinse and *without a//owing the column to dry,* 10,000 ml of filtered effluent sample is pumped through the column at a rate of about 40 - 50 ml/min.

Discard the first 100-200 ml of post-column effluent, to reduce the possibility of higher concentrations of methanol in post-column samples, which may contribute to artifactual toxicity. To evaluate the post-C_{18} SPE column effluent for toxicity, collect at least two aliquots (e.g., beginning and end) separately. If only small quantities (<500 ml) of post-column effluent are needed for toxicity testing (e.g., C. *dubia* test), several separate post-column effluent samples may be more helpful in determining if the toxicants are retained by the column. About 800 ml of post-column effluent is needed for the fathead minnow test if only one concentration (100%) of post-column effluent is tested for toxicity. If two concentrations (100% and 50%) are used, then the required volume for that species increases to 1,200 ml for each post-column aliquot. As Phase II progresses, the recommendation is to collect enough post-column effluent to conduct toxicity tests with dilutions.

2.3.7 C,, SPE Column Elution

To elute the C_{18} SPE column, two successive 10 ml volumes of the 50% methanol/water mixture are pumped through the column and collected in one labelled, analytically clean vial. Subsequently, two successive 10 ml total volumes of each of the 75%, 80%, 85%, 90%, 95% and 100% methanol/water solutions are pumped through the column and collected in separate vials. The next elution volume should not be added until no more of the preceding one is emerging from the column. This results in seven 20 ml SPE fractions. If one 5 g column and two 1 g columns are used to concentrate 7,000 ml of effluent, the corresponding fractions can be combined. For example, the 10 ml eluate of the 80% fraction from the 5 g column can be combined with the two 2 ml 80% fractions from the two 1 g columns. This applies to both sample and blank fractions for a total of 14 ml.

This entire procedure (conditioning through elution) is repeated using a second 10,000 mg C_{18} SPE column for a second 10,000 ml of filtered effluent. The dilution water column blank samples should be kept separate. The corresponding fractions from both the blanks and the sample from each 10,000 ml fractionation can be combined as described above. There will be seven 40 ml blank fractions and seven 40 ml effluent fractions, representing 20,000 ml effluent.

The vials containing the methanol/water fractions are sealed with perfluorocarbon or foil-lined caps, and stored at 4°C if not tested immediately. These fractions represent a 'first cut" separation of effluent components. Volumes will vary if columns of different sizes are used or if the particular effluent under study or the research question posed dictates method modification.

2.3.8 Blank and Effluent Fraction Toxicity Tests

While the choice of test concentration depends on the toxicity of the effluent (Section 1.2), in most instances we have used a concentration of 4x or 2x as the high test concentration for testing SPE fractions. The high test concentration of the SPE fraction is in part controlled by the tolerance of the organisms to methanol. For chronic testing the concentration of methanol should be less than 0.6% for *C. dubia,* and less than or equal to1% for fathead minnows (see Phase I; EPA, 1992).

If the minimum elution volumes are used, typically SPE eluates are 500x effluent concentration. For fathead minnow testing, eluates can be toxicity tested at 4x effluent concentration by diluting 80 µl to 10 ml, which results in a 0.8% methanol concentration. For *C. dubia,* eluates can be toxicity tested at 2x the 100% effluent concentration by diluting 40 µl to 10 ml which results in a methanol concentration of 0.4%. If there is the need to toxicity test the 500x eluate with C. *dubia* at 4x then the SPE eluates can be concentrated by gently airing the eluate down (using nitrogen) to half its original volume. However, by using this procedure you risk losing the toxicant because of evaporation or insolubility. Also realize that when a water and methanol mixture is aired down, the percent methanol composition changes, because methanol will evaporate faster than water.

If toxicity occurs in any of the fraction blank tests and it is small relative to the toxicity in the corresponding sample fraction, the sample fraction results should not be dismissed. If all organisms die in the blanks and effluent fractions, dilutions of each should be tested to make sure the sample fraction is substantially more toxic than the blank. In general, blanks should not have measurable toxicity.

When the post-column effluent sample is toxic and the fractions are toxic at effluent concentrations of lx or 2x, the toxicant could still be a non-polar organic compound. If the fractions are not toxic individually and the post-column sample is non-toxic, it is possible that the toxicity is spread out among the fractions. Combining and concentrating these fractions may be useful or other elution procedures may be necessary (Section 2.6). If the fractions are toxic and the post-column effluent is toxic, it is possible that the toxicant(s) is in the fractions, and that either an additional toxicant(s) is present in the post-

column effluent, that break-through of the toxicant(s) occurred, or that the toxicity is artifactual. If toxicity is recovered at lx, 2x, or 4x and in one of the post-column effluent samples, it is possible that not all the toxicity is caused by non-polar organic compounds or the possibility exists of break-through in the post-column sample.

For the chronic TIE, the question of extraction efficiency cannot be as readily addressed as it is for the acute TIE (Section 2.2.8). Measuring the chronic toxicity of the post-column effluent will be limited by the species tested, the test volumes required for the test and the frequency of sample replacement. Without a measure of the toxicity in the post-column effluent, conclusions regarding extraction efficiency are difficult to make. The limitations created by this concern are addressed in Phase III (EPA, 1993A). Artifactual toxicity in the post-column effluent has been a problem in chronically toxic effluents as it was in some acutely toxic effluents. For a detailed discussion of this artifactual toxicity that appears as a biological growth and suggestions to avoid it please refer to Sections 2.2.6 and 2.3.4., and EPA, 1992.

At this point in Phase II, the effluent fractions should also be tested in water with TOC and suspended solids that mimic the effluent to lessen matrix effects on toxicity. As the identification step moves into Phase Ill, it is better to use dilution water that mimics effluent or receiving water characteristics.

2.3.9 SPE Fractions: Concentration and Subsequent Toxicity Testing

The SPE fractionation provides a general separation of non-polar organics and except in relatively uncomplicated effluents, GC/MS analysis of the concentrates of toxic C_{18} SPE fractions will result in very complicated chromatograms from which the toxicant(s) cannot be distinguished from other effluent components. A secondary fractionation using HPLC is often needed to further simplify toxic effluent fractions prior to component identification by GC/MS analysis.

In order to maximize the chromatographic separation capability of the HPLC, the volume of the sample injected onto an analytical size HPLC column should be as small as possible (i.e., ≤0.5 ml); therefore the toxic SPE fractions (usually >1 ml) must be concentrated prior to injection onto the HPLC column. This concentration step will provide the added benefit of an increase in concentrations of constituents in the HPLC fractions as well as rid the SPE fractions of water. The latter issue is important if GUMS analysis will be performed on the concentrated SPE fraction prior to injection on the HPLC.

The volume of the SPE fraction and the number of toxic fractions to be combined will determine which size SPE column will be used for the concentration procedure. Table 2-3 contains information on column sizes and the appropriate volume of conditioning and eluting solvents we have found to be most useful. In the procedures outlined below we have used a 200 mg SPE column to concentrate one 40 ml toxic fraction from two 10,000 mg SPE columns. Often the toxic effluent SPE fractions are combined and diluted with high purity water. If enough toxicity occurs in each fraction it may be more useful to concentrate each fraction separately for subsequent HPLC separation. The corresponding blank fractions are similarly treated. The percent methanol in the diluted fraction sample should be ≤20% and the volume to which the fractions can be diluted is dependent on the amount of column packing. For example, the total volume of the diluted fraction(s) should not exceed 200 ml for the 200 mg C_{18} column (Table 2-3).

A 200 mg C_{18} column is conditioned with 2 ml of methanol and rinsed with 2 ml of water similar to the procedures described in the SPE Column Conditioning Section (2.3.4). The diluted blank fractions are then drawn through the 200 mg C_{18} SPE column under a pressure of 380 mm Hg using a vacuum manifold. When processing larger volumes, or using larger columns, positive pressure can be used. The solution passing through the column cannot be tested for toxicity because of its high methanol concentration (e.g., 1 0-20% methanol). The column is then dried for 10 min using a gentle flow of nitrogen (1 O-20 ml/sec). Drying ?he column usually increases the recovery of toxicity, but sometimes toxicity is not recovered from the column possibly because of volatilization. If this occurs, the concentration procedure can be repeated without the nitrogen drying step.

After drying the sorbent, the luer tip of the column is fitted with a luer-lock needle (to ease collection of small volumes) and 200 µl of 100% methanol is placed into the column using a microliter syringe. Nitrogen is then applied to the column at a rate of -4 ml/sec to force the methanol through the sorbent, which is then collected in a small glass vial. The first 200 µl aliquot of methanol applied to the column will yield approximately 125 µl of eluate. Two more 200 µl aliquots (applied separately) of 100% methanol are also forced through the column. The final volume of eluate collected will be approximately 440 µL. Measure the exact volume collected using a µl syringe or pipet. As in most chromatographic separations and extractions, three separate smaller elutions of methanol are more efficient than one large one.

The 200 mg C_{18} SPE column is reconditioned following the directions given above in Section 2.3.4. It is then used to concentrate the diluted toxic SPE fractions, using the same sequence used for the blank fractions (Figure 2-4). The concentrated blank fractions will serve as the dilution water column blank because it cannot be obtained for chronic toxicity testing as it can for acute testing.

When the total volume of fractions is above 40 ml, larger columns should be used; consult Table 2-3 for column size and elution volume information. The size of the column used for concentrating should be chosen to maximize concentration in the eluate. Therefore, choose the smallest column appropriate for the diluted fraction volume.

If there is a large toxicity loss after the concentration step, it is possible that the percentage of methanol in the diluted fraction was too high. The concentration procedure should then be repeated with a new set of toxicity tested SPE fractions diluted to a lower methanol concentration (e.g., 10%). Both the effluent and blank concentrates are toxicity tested at each step to track toxicity. Generally we suggest that this toxicity test be at least at two times higher than the concentration used in the first SPE fraction test. The tests are conducted exactly as the SPE fraction tests.

If an original effluent volume of 20,000 ml (using two 10,000 mg SPE columns) is now represented by a 440 µl concentrate, then the sample is 42,670x more concentrated than the effluent (accounting for volume removed for toxicity testing, see Table A-2 example). If 1 µl of concentrate is diluted to 10 ml in dilution water, the resulting test concentration will be about 4x whole effluent. However, the 4x test solution should be prepared as one sample before solutions are split among replicates. For example, 5 µl is diluted to 50 ml for five replicates with the C. *dubia* test described above (Table A-2). Additional test concentrations (e.g., 2x, Ix, 0.5x) can then be prepared to determine an IC25 or IC50 of the concentrate, and toxicity recovery can be calculated by comparing this value to the toxicity of the effluent. The concentrate toxicity might be higher than the sum of the individual toxic fractions because some of the toxicant may have been in adjacent fractions that were concentrated in the first step (Section 2.3.7) but not detectable by the toxicity test of the single fraction. The concentrate toxicity may also be lower than expected because of low extraction and elution efficiencies.

The important concern here is not 100% recovery of toxicants but enough recovery for GC/MS analyses to be successful and to obtain measurable toxicity in the HPLC fractions. If recovery is too low, changing or eliminating the column drying time may help. Sometimes recovery appears to increase with drying time while other compounds are volatilized from the column during the drying process. For concentrates analyzed using GC/MS, column drying to remove water is critical to GC column performance.

2.3. IO *HPLC Separation*

The same column packing functionality should be used in the HPLC column as is used in the SPE column, such as C,,. At later stages, when more is known about the toxicants, other sorbents might be more appropriate.

The HPLC conditions presented in this section are general. An important consideration of HPLC fractionation is the number of HPLC fractions to collect. Since chronic toxicity testing is very time consuming, deciding the appropriate number of fractions to collect is an important step. **However,** when choosing which collection scheme to use, keep in mind the trade-off between separation and toxicity testing load. When the fraction volume is increased (toxicity testing load decreases) the separation of the toxicants from the non-toxic components decreases. We have used a 20 min separation gradient with the collection of 20-1 ml fractions. There are many other collection options that could be used, such as 10-2 ml fractions or 4-5 ml fractions using the same separation gradient. As information on the effluent is gained, HPLC conditions should be modified from the general conditions described below, to achieve better separation and higher concentration factors.

We use a flow rate of 1ml/min on an instrument equipped with a 5 µm C_{18} reverse phase column (250 mm x 4.6 mm i.d.). The HPLC elution conditions will change depending on which SPE fractions have been concentrated. An example of HPLC conditions for commonly toxic SPE fractions is listed in Table 2-f. First, the blank concentrate is injected (≤500 µl) and 20-1 ml fractions are collected in analytically clean glass vials. Depending on the size of the HPLC injector and column, more than one HPLC fractionation run may be required to fractionate the entire blank concentrate. When multiple HPLC fractionations are conducted, collect and combine all the corresponding HPLC fractions in the same set of vials. For example, if two HPLC fractionations were performed for the blank concentrate, 20-2 ml HPLC blank fractions would be obtained. The same procedure is followed using the effluent sample concentrate. The vials should be sealed (e.g., with foil lined caps) and stored at 4°C if not tested immediately. As soon as toxicant identification is obtained by GC/MS (Section 2.5), then HPLC conditions (gradient, fraction size, and number of fractions) can be optimized.

Table 2-7. Example HPLC Elution Gradient for SPE Fractions from Chronically Toxic Effluent Samples

80 or 85% SPE Fractions	
Time (min)	% Methanol/Water
0	80
10	90
12	100
20	100

2.3.11 HPLC Fraction Toxicity Tests

In the HPLC fraction toxicity tests for chronically toxic effluents, the methanol in the HPLC fractions is one of the limiting factors of the concentration of the fractions that can be tested. Each fraction is assumed to be 100% methanol to calculate the necessary dilution. A 0.6% methanol concentration or less can be tested with C. *dubia*, while a 1% or less methanol concentration can be tested with fathead minnows.

In a chronic TIE with *C. dubia*, when all of the SPE concentrate remaining after toxicity testing from 20,000 ml effluent is injected on the HPLC (one injection),

each resulting 1 ml HPLC fraction equals 15,575 ml of effluent (assuming the toxicant elutes in only one fraction, see Table A-2). If 11 µl of each HPLC fraction is then diluted to 10 ml, the test concentration is 16x the original effluent concentration. However, the 16x solution should be prepared as one sample before aliquots are split to provide replicates. For instance, in the example used above, 55 µl should be diluted to 50 ml, which is then equally distributed into five test cups. Additional concentrations are prepared in a similar fashion to estimate the IC25 or IC50 and to compare toxicity recovery to the toxicity of the sample. Of course, some loss of toxicant will occur in each step and the toxicity may be less.

The blank fractions should not be toxic. If they are, then additional tests with dilutions must be conducted on both blanks and toxic fractions to find out whether there is enough additional toxicity in the sample fractions to warrant analysis.

The toxicity of the HPLC fractions should be tested at twice (at least) the concentration at which the SPE fraction concentrates were tested because recovery of toxicity and analytical measurements indicates that up to 50% of the initial concentration of toxic compounds may be lost in this step (Durhan et al., 1990). The concentration of methanol should not exceed the amount used in the SPE fraction tests described above (Section 2.3.8).

2.3.12 *HPLC Fractions: Concentration and Subseguen t Toxicity Testing*

The toxic HPLC fractions and their corresponding blanks must be concentrated in a solvent suitable for GC/MS or other analytical techniques. Use the procedure described in Section 2.3.9, Concentration of Fractions (Figure 2-6). Judgement must be used to decide whether to concentrate each toxic fraction separately or to combine various toxic fractions prior to concentration. if, for example, two successive fractions are toxic, there is a good probability that the same toxicant is present in both. If one toxic fraction is separated from the other by several nontoxic fractions, there is a high probability that they contain different toxicants. There is also a good probability that at least one nontoxic fraction on either side of the toxic fractions contains some of the toxicant. The advantage of combining fractions is to reduce the workload and to increase the amount of toxicant in the concentrate. The disadvantage is that more constituents that are not the toxicant will be included. The decision has to be based on trial and error and experience. Blanks corresponding to the toxic fractions are concentrated the same way.

The HPLC fraction and blank concentrates should also be checked for toxicity before analysis on the GC/MS. Generally, we suggest that these toxicity tests be done at concentrations at least 2x higher than the concentration used in the previous HPLC fraction tests. Hopefully, the amount of concentrate available will be enough to conduct the toxicity test and perform a GC/MS analysis. Dilutions of the concentrate may be useful to compare toxicity of this concentrate to each previous toxicity test result. The HPLC concentrate (of 20,000 ml effluent) is now 48,495x more concentrated than the effluent (see Table A-2). If 3 µl is diluted to 10 ml the resultant test concentration will be about 16x the original sample concentration. This 16x solution should be prepared as one solution before aliquots are removed for the replicates. For instance, 15 µl is diluted to 50 ml for use in the example given above, then split into five 10 ml test cups. It is prudent to verify toxicity in the HPLC concentrate before it is subjected to GC/MS analysis. Whether the toxicant is detected by the analytical detector is always a question. Since GC/MS detects only about 20% of organic chemicals (EPA, 1989B), even such a broad spectrum method is no guarantee that the toxicant will be identified.

2.4 GC/MS Analyses

Procedures and methods provided in this section are based upon our experience in performing GC/MS analyses on fractions from numerous effluents and are applicable to both acute and chronic toxicity identification. In general, these procedures should be used.

A GC/MS system equipped to perform standard chemical residue analyses is suggested; i.e., a 30 m capillary column, electron impact ionization, scan range of 50-500 amu, scan rate of 1 or 2 scans/sec, a GC temperature program of 50 to 300°C at 5°C/min, and a data system with library searching capability.

Prior to GC/MS analysis, the prepared blank and toxic fraction concentrates should be tested for toxicity (Figure 2-6). After verification of the toxicity in the methanol concentrate, inject 1 or 2 µl of the concentrate (to which an internal standard has been added) and collect the mass spectral data. Note, methanol is not a typical solvent for GC analysis and the injection of methanol on a capillary column will shorten the column's life. Therefore, routine GC/MS QA/QC procedures should be followed closely to monitor the performance of the column.

The mass spectral data should be collected, the chromatogram integrated, and all detected peaks library searched. Reverse search is preferred. Concentration estimates for all chromatographic peaks can be obtained by using the response factor of the internal standard. Usually the internal standard is added to a small aliquot (10-20 µl) of the concentrate prior to GC/MS analysis. The selection of internal standard to use is an individual choice, and many different standards are available. An external standard method could also be used for deriving concentration estimates.

The NIST (National Institute of Standards and Technology, Gaithersburg, MD) mass spectral library has been used in ERL-D for performing library searches. Other mass spectral libraries are available, but some of the larger libraries contain multiple spectra for some of the compounds in the database. Library searching results that contain multiple identifications of the same com-

pound are not as useful as those obtained using the NIST library.

Once the library search results are available, the search report for each peak must be examined to decide whether the identification by the search is valid and reasonable. The help of a trained GC/MS chemist is required to do this evaluation. Questions we consider in our laboratory when performing this process include: A) are all major ions present in the correct proportions?, B) is this identification consistent with other information about the fraction?, C) do forward and reverse searching provide similar fits? and 0) are the library searching fits greater than 70%? Factor A *must* be met! Consistency, factor B, considers circumstances such as "has the identified chemical been found in vastly different fractions," or "has the same identification been given to numerous peaks in the same chromatogram?" Both factors C and 0 are somewhat relative and depend a great deal on the sample and its matrix. In addition, the toxicants are often very minor components in the GC/MS total ion chromatogram and thus, the quality of the mass spectral data even after background subtraction can lead to poor results for factors C and 0.

After examination of the library search results, a list of identified chemicals is assembled and evaluated using the methods in the following section. For the confirmation analyses we suggest EPA method 625 (EPA, 1982).

2.5 Identifying Suspect Toxicants

If one toxicant is identified, then the goal of the rest of Phase II is to determine if there are any other toxicants contributing to effluent toxicity. Two parallel lines of investigation should be pursued to achieve that goal. The first is to determine whether or not the concentration of the suspect toxicant is sufficient to cause toxicity (EPA, 1993A). The second is to estimate the proportion of the effluent toxicity that is caused by the suspected toxicants, so that a decision can be made as to whether other toxicants are present in the effluent.

The first line of investigation should begin by comparing the estimated concentrations of identified chemicals in the SPE or HPLC concentrate to their known toxicity values. Recovery of 100% of each effluent toxicant in the C_{18} SPE fractions may not be crucial, because at this stage, only the estimated concentration of compounds in the fraction and the toxicity of the fraction are compared. Assumptions about the concentration of toxicant(s) in the whole effluent are not made at this point, nor is any statement made regarding recovery of whole effluent toxicity in C_{18} SPE column fractions. In later stages of Phase II, inferences regarding the relationship between the concentration of the suspected toxicant(s) in whole effluent and the observed toxicity in the SPE fractions are made. At this step, the compound quantification will have been performed using an internal or external standard response and since the compound's recovery is unknown, considerable error may be involved in the concentration estimate. Secondly, the toxicity data, if available, may be for a different species than that used in the TIE. Species differences are usually as large as 100-fold and often 1,OOO-fold. Given these two sources of uncertainty and the chance that they may reinforce one another, certainly if the estimated concentration of a chemical accounts for the toxicity within a factor of 100, the chemical should remain a suspect. To the extent that data for either quantitation or toxicity values of the compound are known to be better, concentration differences of smaller magnitude may be used to eliminate suspects.

Once a list of suspects is available, the measurements for both concentration and toxicity should be refined. This will usually require obtaining pure compound to make better analytical measurements and to establish acute or chronic toxicity estimates for the species of concern. This step requires as much separation as practical before analysis so that the list of suspects is small.

At this stage, only the concentration of the suspected toxicant(s) in the concentrate is known; until recovery through all the fractionation and concentration steps is complete, suspect compound concentrations in whole effluent are not known. Since the concentrate is virtually devoid of suspended solids and much of the effluent TOC, both of which may dramatically affect toxicity of non-polar organics, the toxicity of non-polar chemicals may be quite different in the fraction tests than in the effluent test. Therefore, the toxicity of suspects in the fraction test should be compared to the suspect's toxicity in a relatively pure water, such as reconstituted water.

During this same stage, the steps leading to the final concentrate should be checked for toxicity recovery. The objective is to place a good estimate on how much of the whole effluent toxicity is contained in the final concentrate. This is best done by testing the toxicity of the concentrate at concentrations near those of whole effluent, correcting for volume losses due to toxicity testing SPE column fractions (which was previously ignored). If the toxicity of the final concentrate is similar to that of whole effluent, allowing for losses, and if the concentration of the suspect(s) is sufficient to account for the concentrate's toxicity, it is time to begin Phase III (EPA, 1993A). If multiple toxicants occur, the toxic units of each are compared to the whole effluent toxic units.

If the concentrations from quantitation and toxicity measurements are close to one another, Phase III procedure should be started, recognizing that other toxicants may yet be identified. If no suspects are found, more concentration, more separation, and possibly different or more sophisticated analytical methods must be used. In some of the effluents we have tested, finding other candidates has taken months and concentration factors of >100,000 have been required. Since few laboratories will have all the needed analytical equipment, instrumentation from other sources should be considered.

Because artifactual toxicity that equals toxicity due to lost or unidentified toxicants can be created, as one progresses to Phase III the suspect toxicant should be identified. One purpose of Phase III is to identify such errors. Should this error occur, one must start again at the beginning of Phase II, or even return to Phase I. If several different effluent samples were evaluated during Phase II, redoing Phase I on additional samples may be time well spent since the effluent may have changed in the interim.

In practice there is no sharp boundary between Phases II and III. In general, as soon as a probable suspect is identified, confirmation procedures of Phase III should begin. If a toxicant has been assumed to have been identified when it has not, the identification of other suspected toxicants can be hampered.

A final suggestion is to investigate the additivity of toxicity for several constituents, if all toxicity is not accounted for. Enhancement of toxicity by methanol should also be checked.

2.5. *I Identifying Organophosphate Pesticides*

Certain compounds must be metabolically activated by the test organism before they become toxic. These activation reactions consist of oxidative metabolism by a family of enzymes collectively known as cytochrome P-450. Compounds such as piperonyl butoxide (PBO) can block the toxicity of metabolically activated toxicants making it a useful tool in the TIE. PBO is a synthetic methylenedioxyphenyl compound that effectively binds to and blocks the catalytic activity of cytochrome P-450. Thus, when a nontoxic amount of PBO is coadministered with the effluent or the effluent fractions that exhibited toxicity, the toxicity of the compound requiring metabolic activation is greatly reduced or completely blocked (Ankley et al., 1991).

Phosphorothioates are organophosphates known to require cytochrome P-450 activation before expressing toxicity and include common insecticides such as diazinon, malathion, parathion, methyl parathion and fenthion. There also are a number of organophosphates that are toxic in the absence of metabolic activation; these include insecticides such as dichlorvos, mevinphos and chlorfenvinphos.

We have found organophosphate insecticides present in effluents and ambient waters at acute and chronic toxicity levels (Amato et al., 1992; Norberg-King et al., 1991). The toxicity of most organophosphates will be removed from the sample by the C, SPE column, and they are typically recovered in the methanol eluates (see EPA, 1991A; EPA, 1992). The addition of PBO to the effluent before addition of the test organisms was used as a subsequent test in Phase I (EPA, 1991A; EPA, 1992). In addition to the C_{18} SPE column removing the toxicity, a reduction in toxicity with the addition of PBO would suggest the presence of metabolically activated compounds such as organophosphates. PBO has similar utility in Phase II of the TIE in that either SPE fractions (Sections 22.8 and 2.3.8) or HPLC fractions (Sections 2.2.11 and 2.3.11) can be tested for toxicity both in the presence and absence of PBO. A reduction in toxicity of the test fraction would suggest the presence of a metabolically activated chemical, and together with chemical analyses, can provide powerful evidence along with GC/MS data, for specific organophosphates as the toxicant(While PBO should be useful for both acute and chronic TIE work, most of our experience has been in the area of acute toxicity. Thus, guidance presented below is based mainly on acute tests.

Toxicity values for PBO are presented in Phase I (EPA, 1991A; EPA, 1992). In acute toxicity tests, concentrations of PBO ranging from 250-500 µg/l have effectively blocked the acute toxicity of relatively large concentrations of metabolically activated organophosphates to cladocerans (Ankley et al., 1991). In chronic toxicity tests with C. *dubia,* PBO concentrations of 50 µg/l have been effective in blocking toxicity in the SPE fractions. Detailed information on stock solution preparation is presented in the Phase I documents and is not repeated here.

When toxicity tests are conducted on SPE fractions or HPLC fractions, aliquots of the PBO solution are added to the test solutions and mixed well before the test organisms are added. As for any TIE manipulation, the successful use of PBO is dependent upon the use of appropriate controls and blanks. Effluent fractions and blank fractions with and without the addition of PBO must be tested simultaneously. A reduction in toxicity of the effluent fraction occurring with the PBO added, and no toxicity exhibited in either of the blanks, indicates that the toxicant requires metabolic activation to exhibit toxicity. If toxicity associated with the PBO addition is observed in the blank fraction, either PBO was present at toxic concentrations or the methanol concentration (from fraction and/or PBO stock addition) in the test was too high. If toxicity is observed in the effluent fraction with PBO added, but not in the effluent fraction without the PBO or in either of the blank fractions, this result is essentially meaningless. In the latter situation it is possible that the PBO has interacted in a synergistic fashion with another compound present in the test effluent that normally would not be toxic.

2.5.2 *Identifying Surfactants*

The goal in this section of Phase II is to identify the toxicants when surfactants are implicated by Phase I and Phase II results. The Phase I procedures of filtration, aeration, and C_{18} SPE all affect surfactant toxicity, and effluent samples that exhibit several or all of these behaviors may contain toxic concentrations of surfactants (EPA, 1991A).

Surfactants are surface active agents that have a molecular structure that includes a polar, hydrophilic segment (either ionic or nonionic) and a relatively large nonpolar, hydrophobic, hydrocarbon segment. Surfactants are used for a variety of household and industrial purposes and therefore are ubiquitous in effluents, particu-

larly in untreated wastewater, and potentially could be present at toxic concentrations in effluents (Ankley and Burkhard, 1992). Some examples of surfactants are soaps, detergents, charged stabilization polymers, and coagulation polymers used in chemical manufacturing processes. The molecular structure of surfactants causes them to congregate at interfaces between water and other phases such as air, oily liquids and particulate matter. This congregative characteristic is responsible for the cleansing and dispersive properties of surfactants.

There are many different kinds of surfactants and they are classified by the nature of their polar segment. When in aqueous solution, the polar segment of a surfactant molecule can be either nonionic (not charged) or ionic (charged). The ionic polar segment can be either negatively charged (anionic), positively charged (cationic), or both negatively and positively charged (amphoteric). Based on this, surfactants are classified into the following major classes: nonionic, anionic, cationic, and amphoteric.

Surfactants physical/chemical properties set them apart from both strictly polar or non-polar organic compounds and these properties uniquely influence the results of Phases I and II procedures for surfactants.

Experiments were conducted with a small sample of surfactants from nonionic, anionic, and cationic categories with the Phase I procedures of filtration, aeration, and C_{18} SPE (Ankley et al., 1990A). In these experiments, filtration removed the toxicity of most of the surfactants tested to some degree, and the degree of removal is most probably dependent on sample matrix, especially solids concentration. Aeration removed the toxicity of all the surfactants tested to some degree while the C_{18} SPE column removed the toxicity completely for all surfactants regardless of class. Surfactants behave unpredictably with regard to eiution from C_{18} SPE columns. For example, toxicity from surfactants of the nonionic and anionic classes, eluted in all fractions 80% to 100% methanol/water (Ankley et al., 1990A). Elution in several fractions rather than eluting in one or two fractions may be caused by the polar/non-polar nature inherent in surfactants. The toxicities from the cationic surfactants were either not recovered in any of the fractions or were recovered to only a small degree in the 100% methanol fraction.

Important indicators of surfactant toxicity are the toxicity test results from aeration experiments. If volatility can be eliminated and toxicity is reduced by aeration, this is strong evidence that a surfactant might be contributing to effluent toxicity (EPA, 1991 A). During aeration, surfactants are most probably removed from solution by the process of sublation. Sublation occurs because surfactant molecules tend to congregate at the interface between the aqueous sample and the aerating nitrogen or air bubbles and are brought to the surface of the liquid sample by the bubbles. At the liquid surface the bubbles break releasing the surfactant, which then adheres to the aeration vessel walls. A compound that can be removed by sublation is by definition a surfactant. It might be possible to recover surfactants from glassware after the sublation process. The glassware can be rinsed with a solvent such as methanol, which can then be toxicity tested and analyzed in the same manner as methanol SPE fractions (Sections 2.2.8 and 2.3.8).

Overall, most surfactants exhibit some of the behavior that is common to non-polar organic compounds such as removal from the effluent by the C_{18} resin and recovery in the methanol/water SPE fractions. While surfactants in general can be considered to be non-polar organics, GC/MS analysis will probably not provide successful sutfactant identification. Most surfactants are not readily chromatographed because of the polar segment of the surfactant molecule. One exception is a class of surfactants in common use that can be analyzed directly by GC/MS, the alkylphenol ethoxyiates. Gieger et al. (1981), provides mass spectral data for the nonylphenol mono-, di- and tri-ethoxylates, which can be used to help identify these compounds. Techniques such as derivatization can make some other specific surfactants compatible with GC and GC/MS, but it is necessary to know the specific identity of the surfactant.

It is difficult to positively identify an unknown surfactant. Although there are many analytical methods available for accurately quantifying specific surfactants, these methods are useful only if the identity of the surfactant is known, or at least suspected. It is not reasonable or practical to analyze a sample using numerous intricate methods, in the hope that one of these methods will detect the surfactant in the sample. Unfortunately, there is no analytical technique available that can readily provide the identity and quantity of an unknown surfactant. Environmental samples (such as municipal and industrial effluents) contain numerous substances that can interfere with available analytical methods. Also, pure surfactants are actually mixtures of homologous and oligomers with varying chain lengths and, in the case of many nonionic surfactants, varying degrees of ethoxylation. The composition and therefore the toxicity of such a mixture might vary. In the course of a TIE, it might become necessary not only to identify the surfactant causing toxicity, but also to learn which particular homologue or oligomer is the most toxic.

One approach to reducing the complexity of identifying an unknown sutfactant is to determine whether the unknown surfactant falls into the anionic or nonionic class. APHA (1989) describes a method for determining anionic surfactants as methylene blue active substances (MBAS). MBAS method can successfully measure the concentration of anionic surfactants of the sulfonate type, the sulfate ester type, and sulfated nonionics type. Unless the identity of the anionic surfactant is known, the analytical measurement is calculated and expressed in terms of the anionic surfactant linear alkylbenzene sulfonate (LAS). APHA (1989) also describes a method for determining nonionic surfactants as cobalt thiocyanate active substances (CTAS). This method is applicable to a wide

range of polyether nonionic surfactants, which includes the widely used alkyl and alkylphenol ethoxylated alcohols.

With these methods the relative amount of anionic or nonionic surfactant can be estimated, but the exact nature or molecular composition of the unknown surfactant will not be determined. These analyses can be conducted on the SPE fractions, HPLC fractions, fraction concentrates, and the whole effluent. Determining the class can be significant progress toward identifying the unknown surfactant. With the class known, specific analyses for the more common surfactants in that class can be performed as a subsequent effort. Unless the identity of the nonionic surfactant is known, the analytical measurement is expressed in terms of an arbitrarily chosen reference nonionic surfactant.

The type of discharge being processed by the wastewater treatment plant might provide information that would enable one to target specific surfactants for analysis. For example, industries feeding into the treatment plant might be discharging certain surfactants or a particular kind of surfactant that is being used in the manufacturing or housekeeping processes. An analytical method suitable for that particular surfactant could then be used to determine whether toxic concentrations can be found in the toxic effluent, fractions, or concentrates.

2.6 Alternate Fractionation Procedures

If toxicity is not recovered in the methanol procedures described above (Sections 2.2 and 2.3), and toxicity is not observed in the post-column effluent, alternative elution procedures can be used. These procedures are not as widely used as the methanol/water elutions discussed above but are effective for highly hydrophobic compounds.

2.6.7 *Modified Elution Method*

The current Phase II method for fractionating non-polar organic toxicants in aqueous samples does not effectively fractionate compounds that are highly hydrophobic. Modifications made to the method have been successful in overcoming this limitation (Schubauer-Berigan and Ankley, 1991; Durhan et al., 1993). Hydrophobic compounds probably are more prevalent in sediment pore waters than in treated effluents. Tracking toxicity caused by these kinds of compounds will be more difficult because of the potential for artifactual toxicity from the solvents required to elute them. An elution scheme incorporating water, methanol, and methylene chloride has been designed that effectively fractionates compounds over a log K_{ow} range from 2.5 to 6.9. The higher log K_{ow} compounds, however, elute in the same set of fractions. Further fractionation by HPLC might be necessary to achieve better resolution of these kinds of compounds. Substituting other sorbents for the currently 'used C SPE resin have also produced encouraging results. 'Both the C_8 SPE and XAD-7 (Rhom and Haas, Philadelphia, PA) sorbents might have utility with particular kinds of toxicants.

The modified elution scheme eliminates the 100% methanol fraction used in the original method, and replaces it with one 50% methylene chloride/methanol, and three 100% methylene chloride fractions (v/v). The composition of the resulting eleven 3 ml (when using a 1,000 mg C_{18} SPE column) fractions is shown in Table 2-8. The methylene chloride containing fractions are combined, then solvent exchange is conducted as described below. The modified elution scheme would be used when the original methanol/water elutions did not effectively elute toxicity in the SPE fractions. In addition, if the suspect toxicants were known to be highly hydrophobic, as in sediment pore water, then the modified elution scheme would be indicated. Blank toxicity should provide insight concerning artifactual methylene chloride toxicity; however, slight reductions in young production might occur in both the blanks and sample fractions. Development of this alternate procedure for chronic toxicity is underway for the C. *dubia* and should be used with caution at this time. If this procedure is used, it is important to accompany the solvent exchanged methanol blank with a methanol only blank.

When toxicity testing SPE fractions, it is always a concern that the matrix of the effluent has been changed and that chemicals might become bioavailable, whereas they were not in the original sample. If this were to happen, the fractions might be more toxic than expected and chemicals might be added to the suspect toxicant list erroneously. This kind of mistake should be caught by obtaining a good toxicity value for the suspect toxicant in an appropriate matrix. For instance, if the suspect toxicant is highly insoluble in water, then when tested in an effluent matrix it should have low toxicity because it is unavailable to the organism. The alternate solvent elution might enhance this problem because the solvent is more likely to solubilize the more hydrophobic compounds than

Table 2-8. Composition of 11 Recommended Fractions in Modified Eiution Scheme

Fraction	Composition of Eluting Solutions (% v/v)		
	Water	Methanol	Methylene Chloride
	75	25	0
2	50	50	0
3	25	75	0
4	20	80	0
5	15	85	0
6	10	90	0
7	5	95	0
8	0	50	50
9	0	0	100
10	0	0	100
11	0	0	100

methanol. Therefore, additional confirmation steps might be needed to eliminate the false suspects.

2.6.2 Solvent Exchange

Since methylene chloride is quite toxic to aquatic organisms, even at very low concentrations (NOEC for *C. dubia* is 0.03%), it must be removed from SPE fractions before the fractions can be tested for toxicity. The exchange of the methylene chloride fraction into methanol is a relatively easy process because of methylene chloride's volatility. The combined fractions to be exchanged (e.g., 15 ml) are placed in a centrifuge tube with a teflon stir bar and an additional 15 ml of methanol. The tube is placed in a 30°C water bath and stirred while a gentle stream of nitrogen is passed over the solution surface. When the volume of the solution reaches 3 ml, the sides of the tube are carefully rinsed with an additional 3 ml of methanol, and the solution is reduced again to a final 3 ml volume. Adjust the volume of methanol used in this procedure to reflect the total volume of combined fractions. The final volume of methanol may then be tested as suggested previously in Sections 2.2.8. and 2.3.8. It is important to obtain and toxicity test a methanol-only blank in addition to the solvent exchanged methanol blank.

2.6.3 Alternative SPE Sorbents and Techniques

In the SPE method described above, C_{18} bonded silica is used as the solid phase for fractionating and isolating non-polar organic toxicants. C_{18} bonded silica was selected because, with proper conditioning, it does not usually contribute artifactual toxicity to sample or sample fractions, it often achieves the required degree of separation and isolation of non-polar organic compounds, and it is commercially available in inexpensive, easy to use, disposable columns. There is, however, no restriction on the solid phase that is used in the TIE procedure, as long as it results in the isolation and separation of non-polar organic toxicants and at the same time does not contribute artifactual toxicity. We have evaluated several sorbents other than C, bonded silica to use for this purpose (Durhan et al., 6993).

We evaluated two prepurified XAD sorbents, XAD-4 and XAD-7 (Rohm and Haas, Philadelphia PA) and a C_8 bonded silica sorbent. Of these sorbents, only XAD-4, a non-polar styrene-divinyl benzene copolymer performed as well as C_{18} bonded silica in the fractionation of non-polar organic compounds. One disadvantage of using an XAD sorbent such as XAD-4 is that it is not commercially available in prepacked disposable columns. In addition, it is important to obtain prepurified XAD-4 sorbent that is free of toxic artifacts, otherwise extensive, time consuming cleanup procedures are required before the sorbent can be used in a toxicity based fractionation. We found that on XAD-7, an acrylic ester copolymer, non-polar organic compounds were inadequately fractionated because of resolution and co-elution problems. The C_8 bonded silica yielded results that were similar but significantly inferior to those obtained with C_{18} bonded silica.

Traditionally, SPE is carried out with the solid phase particles packed in a cylindrical column or cartridge. An alternative form of SPE has been developed, the Empore™ Extraction Disk, in which C_{18} bonded silica particles are enmeshed in an inert PTFE matrix which is then formed into a disk. The manufacturer (3M, St. Paul, MN) claims good recovery of non-polar organics with flow rates as high as 100 ml/min, which would make this an attractive alternative form of SPE. We have evaluated this technique to a limited degree with acutely toxic effluents and sediment pore waters and feel it has great potential in a toxicity based fractionation scheme. Especially attractive is the high flow rate which would allow for large volumes of sample to be processed quickly. However, a procedure for eluting non-polar organics from the disk into several fractions has not yet been developed and could prove to be a challenge.

Section 3
Ammonia

3.1 General Overview

Unlike Phase II procedures for non-polar organic compounds or metals, the toxicant identification methods described in this section are specific for ammonia. The procedures used in this phase of the study assume that Phase I tests and ammonia measurements (see below) have implicated the pH sensitive toxicant, ammonia as causing the acute or chronic toxicity (see Phase I; EPA, 1991A; EPA, 1992). Other compounds with toxicities that increase directly with pH may lead to confounding results or may give results similar to ammonia. For instance, experiments at our laboratory have shown that C. *dubia* are more acutely sensitive to cadmium, nickel, and zinc in acute tests at high pH levels (Section 4). The testing in Phase II should help to discern the toxicity caused by ammonia from that caused by other compounds that might also become more toxic as pH increases. The methods described below can be used to identify ammonia as the toxicant and these data could also be used in Phase III confirmation.

Ammonia is relatively unique in its behavior as pH changes. When ammonia (NH_3) dissolves in water, some of the molecules react to form the ammonium ion NH_4^+, and the equilibrium between these two species is affected by both pH and temperature (EPA, 1985A). The term "total ammonia" refers to the sum of the un-ionized (NH_3) and the ionized (NH_4^+) forms and is referred to as N^+. The toxicity of ammonia to some aquatic species appears to be primarily caused by the un-ionized form. The equilibrium shifts to increase the un-ionized ammonia concentration with increasing pH and increasing temperature. In a constant temperature situation, Table 3-1 shows that as pH increases by one unit, there is nearly a lo-fold increase in the percent of un-ionized ammonia NH_3 present in aqueous solutions at pH 6.0-8.5. The data in Table 3-I are calculated using the dissociation constants for ammonia (EPA, 1979). There are two effects to consider for ammonia as the pH increases; first, the concentration of NH_3 increases (Table 3-1) and second, the toxicity of NH_3 decreases (Tables 3-2 and 3-3). One possible explanation for the second effect is that NH_4^+ is contributing to the toxicity (EPA, 1985A). Measuring and maintaining the pH of the test solution and understanding the effect of pH on the toxicity of ammonia are very important.

As discussed in EPA's ammonia water quality criteria document (EPA, 1985A), the slope of the LC50-pH curve for acute toxicity is similar for different aquatic species (i.e., an average slope can be used for many species). A model was developed to describe the pH dependence of ammonia toxicity, primarily with data for fishes and cladocerans (i.e., daphnids, fathead minnows, rainbow trout, and coho salmon, see EPA, 1985A). This model has been used with acute toxicity data generated at a pH of 8 and a temperature of 25°C for both C. *dubia* and fathead minnows, to predict the LC50 of NH_3 at other pH values (Tables 3-2 and 3-3). It is apparent that the toxicity of NH_3 is about seven times less at pH 7.0 than at pH 6.0, but the amount of NH_3 is ten times greater at pH 7.0 than at pH 6.0. Similarly, at pH 8.0, NH_3 is three times less toxic than at pH 7.0 but ten times more is available at pH 8.0. Ammonia can be implicated as the cause of toxicity if the effluent toxicity and the suspect toxicant exhibit both of these pH effects. Acute toxicity test data generated at ERL-D indicate that this model is not appropriate for all species. For example, the trend of pH-dependence has not been observed in acute tests conducted with the amphipod, *Hyalella azteca,* over a range of pH values in reconstituted waters (EPA, 1991 B) until the hardness is greater than 160 mg/l. In hard or very hard waters, *H. azteca* is more sensitive to NH_3 at higher pHs (P. Monson, personal communication, University of Wisconsin, Superior, WI). We recommend that the effect of pH on the toxicity of ammonia be characterized for the TIE organism, if it has not been done, so that accurate predictions can be made for the organism.

It has not yet been determined whether the pH dependence of ammonia toxicity described for acute toxicity is appropriate for chronic toxicity. The chronic toxicity of ammonia to species typically used in effluent tests, at temperatures similar to those used in TIEs and a variety of pHs is presented in Table 3-4. If chronic ammonia toxicity has not been characterized with respect to pH for the TIE species, it is prudent for the investigator to generate the ammonia toxicity data for at least three distinct pH levels.

Generally, three procedures are used to implicate ammonia in addition to measuring the ammonia in the effluent. These are 1) the graduated pH test (in place of

Table 3-1. Percent Un-Ionized Ammonia in *Aqueous Solutions* for Selected *Temperatures and* pH Values[1]

	Temperature(%)								
pH	*15*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*
6.0	*0.0274*	0.0397	*0.0427*	0.0459	0.0493	*0.0530*	*0.0568*	*0.0610*	*0.0654*
6.1	*0.0345*	*0.0500*	*0.0537*	*0.0578*	*0.0621*	*0.0667*	*0.0716*	*0.0768*	*0.0823*
6.2	*0.0434*	*0.0629*	*0.0676*	*0.0727*	*0.0781*	*0.0839*	*0.0901*	*0.0966*	*0.104*
6.3	*0.0546*	*0.0792*	*0.0851*	*0.0915*	*0.0983*	*0.106*	*0.113*	*0.122*	*0.130*
6.4	*0.0687*	*0.0865*	*0.107*	*0.115*	*0.124*	*0.133*	*0.143*	*0.153*	*0.164*
6.5	*0.0865*	*0.125*	*0.135*	*0.145*	*0.156*	*0.167*	*0.180*	*0.193*	*0.207*
6.6	*0.109*	*0.158*	*0.170*	*0.182*	*0.196*	*0.210*	*0.226*	*0.242*	*0.260*
6.7	*0.137*	*0.199*	*0.214*	*0.230*	*0.247*	*0.265*	*0.284*	*0.305*	*0.327*
6.8	*0.172*	*0.250*	*0.269*	*0.289*	*0.310*	*0.333*	*0.358*	*0.384*	*0.411*
6.9	*0.217*	*0.314*	*0.338*	*0.363*	*0.390*	*0.419*	*0.450*	*0.482*	*0.517*
7.0	*0.273*	*0.396*	*0.425*	*0.457*	*0.491*	*0.527*	*0.566*	*0.607*	*0.650*
7.1	*0.343*	0.497	*0.535*	*0.575*	*0.617*	*0.663*	*0.711*	*0.762*	*0.817*
7.2	*0.432*	*0.625*	*0.672*	*0.733*	*0.776*	*0.833*	*0.893*	*0.958*	*1.027*
7.3	*0.543*	*0.786*	*0.845*	*0.908*	*0.975*	*1.05*	*1.12*	*1.20*	*1.29*
7.4	*0.683*	*0.988*	*1.06*	*1.14*	*1.22*	*1.31*	*1.41*	*1.51*	*1.62*
7.5	*0.858*	*1.24*	*1.33*	*1.43*	*1.54*	*1.65*	*1.77*	*1.89*	*2.03*
7.6	*1.08*	*1.56*	*1.67*	*1.80*	*1.93*	*2.07*	*2.21*	*2.37*	*2.54*
7.7	*1.35*	*1.95*	*2.10*	*2.25*	*2.41*	*2.59*	*2.77*	*2.97*	*3.18*
7.8	*1.70*	*2.44*	*2.62*	*2.82*	*3.02*	*3.24*	*3.46*	*3.71*	*3.97*
7.9	*2.13*	*3.06*	*3.28*	*3.52*	*3.77*	*4.04*	*4.32*	*4.62*	*4.94*
8.0	*2.66*	*3.82*	*4.10*	4.39	*4.70*	*5.03*	*5.38*	*5.75*	*6.14*
8.1	*3.33*	*4.76*	*5.10*	*5.46*	*5.85*	*6.25*	*6.68*	*7.14*	*7.61*
8.2	*4.16*	*5.92*	*6.34*	*6.78*	*7.25*	*7.75*	*8.27*	*8.82*	*9.40*
8.3	*5.18*	*6.43*	*7.85*	*8.39*	*8.96*	*9.56*	*10.2*	*10.9*	*11.6*
8.4	*6.43*	*9.07*	*9.69*	*10.3*	*11.0*	*11.7*	*12.5*	*13.3*	*14.1*
8.5	*7.97*	*11.16*	*11.90*	*12.7*	*13.5*	*14.4*	*15.2*	*16.2*	*17.1*
8.6	*9.83*	*13.6*	*14.5*	*15.5*	*16.4*	*17.4*	*18.5*	*19.5*	*20.7*
8.7	*12.07*	*16.6*	*17.6*	*18.7*	*19.8*	*21.0*	*22.2*	*23.4*	*24.7*
8.8	*14.7*	*20.0*	*21.2*	*22.5*	*23.7*	*25.1*	*26.4*	*27.8*	*29.2*
8.9	*17.9*	*24.0*	*25.3*	*26.7*	*28.2*	*29.6*	*31.1*	*32.6*	*34.2*
9.0	*21.5*	*28.4*	29.9	*31.5*	*33.0*	*34.6*	*36.3*	37.9	39.6

[1]Data from *EPA, 1979.*

the equitoxic solution test as described in the first Phase II document; EPA, 1989A); 2) use of the zeolite resin to remove the ammonia; and 3) air-stripping the ammonia from the sample at a high pH (i.e., pH 11). For both the zeolite resin method and the air-stripping method, subsequent toxicity tests and ammonia measurements are performed on whole effluent and the post-treatment samples.

Depending on the presence of other toxicants in the effluent, additional sample manipulations may be needed before proceeding with the three basic tests. For example, if toxic oxidants such as chlorine are also present in the effluent, sodium thiosulfate must be added to the sample before conducting the Phase II ammonia tests. To date we have not seen ammonia and chlorine as co-occurring toxicants in chronic tests, probably because chlorine degrades rapidly in a test system at 25°C, while ammonia does not. If the additional toxicant(s) can be removed by the C_{18} SPE column, it may be possible to conduct Phase II tests for ammonia on post-C,, SPE column effluent sample. However, the problem of artifactual toxicity associated with the post-C,, SPE column effluent may prevent the use of the graduated pH test (EPA, 1992) and/or the air-stripping test (see Section 8 of EPA, 1991A) on post-column samples.

The results of the graduated pH test, the post-zeolite column test, and the air-stripping test, all will be important in identifying ammonia as a toxicant in acutely or chronically toxic samples. Use of pH changes where graded responses are observed are particularly useful for data evaluation in Phase III correlation steps. Some of the Phase II tests for ammonia are the same steps that are used for Phase III confirmation procedures; therefore, tests such as spiking the effluent with ammonia and then performing the graduated pH test or spiking the post-zeolite effluent samples and then testing the samples simultaneously with the Phase II tests will support the confirmation steps in Phase III.

3.2 Toxicity Testing Concerns

A key issue in interpreting acute or chronic test results for a pH dependent toxicant such as ammonia is monitoring pH changes during the test period. Toxicity differences in Phase I manipulations may be misinterpreted simply because differences in NH, toxicity can occur with only a slight pH change. To illustrate, the change in pH from 8.0 to 7.9 lowers the concentration of NH, 20%, as does a change in pH from 6.1 to 6.0, but a 20% difference is much more important to the toxicity

Table 3-2. Calculated Un-ionized Ammonia LC50s (mg/l) Based on 24-h and 48-h Results of a *Ceriodaphnia dubia* Toxicity Test Conducted at pH 8.0 and 25°C[1]

PH	Percent Dissoc. at 25°C	Unionized Ammonia Expected 24-h LC50	Total Ammonia 24-h LC50	Un-ionized Ammonia Expected 48-h LC50	Total Ammonia 48-h LC50
6.0	0.0568	0.09	158	**0.07**	123
6.1	0.0716	0.12	168	**0.09**	126
6.2	0.0901	0.14	155	0.11	122
6.3	0.1134	0.18	159	0.14	123
6.4	0.143	0.22	154	0.17	119
6.5	0.180	0.27	150	0.21	117
6.6	0.226	0.33	146	0.25	111
6.7	0.284	0.40	141	0.31	109
6.8	0.358	0.48	134	0.38	106
6.9	0.450	0.58	129	0.45	100
7.0	0.566	0.69	122	0.53	94
7.1	0.711	0.81	114	0.62	87
7.2	0.893	0.93	104	0.72	81
7.3	1.12	1.06	95	0.82	73
7.4	1.41	1.21	86	0.93	66
7.5	1.77	1.34	76	1.04	59
7.6	2.21	1.48	67	1.14	52
7.7	2.77	1.61	58	1.24	45
7.8	3.46	1.73	50	1.33	38
7.9	4.32	1.83	42	1.42	33
8.0[2]	5.38	**1.93**	36	1.49	28
8.1	6.68	2.01	30	1.55	23
8.2	8.27	2.08	25	1.61	20
8.3	10.2	2.14	21	1.65	16
8.4	12.5	2.19	18	1.69	14
8.5	15.2	2.23	15	1.73	11
8.6	18.5	2.27	12	1.75	9.5
8.7	22.2	2.30	10	1.77	8.0
8.8	26.4	2.32	8.8	1.79	6.8
8.9	31.1	2.34	7.5	1.81	5.8
9.0	36.3	2.35	6.5	1.82	5.0

[1]LC50s for each pH interval were calculated using EPA's water quality criteria document formula (EPA, 1985A) shown below.

$$\text{Formula } LC50 = \frac{(LC50[pH = 8.0])(1.25)}{1 + 10^{7.4 - pH}}$$

[2]The 24 h and 48 h LC50s to C. *dubia* are 1.93 mg/l and 1.49 mg/l, respectively, at pH 8.0. The formula was used to generated expected LC50s for pH values above 8, though the model is not recommended above pH 8, because generally we have found C. *dubia* data to track with these predictions.

expressed by the ammonia at pH 8.0 than at pH 6.0. For this reason frequent pH monitoring (at least daily) must be performed on tests conducted to determine the trend of ammonia toxicity. Ideally, continuous monitoring of pH is desired. The pH should be measured on each test concentration and each replicate. Experience has shown that the choice of pH meters and probes is critical to produce reliable results. The pH meter used must read accurately to two decimal places and should lock-on the stabilized reading after the rate of change has diminished to a specified rate. Routine cleaning of the probe and a standardized calibration procedure should be established. The pH values can also be recorded after an elapsed time of 60-90 sec. The pH readings should be made using a constant and reproducible stirring rate. The stirring should not result in excessive loss (or gain) of CO_2 which will of course change the pH. The choice of the pH electrode is important. We have found that the glass-bodied combination electrodes provide the most consistent pH readings. However, these should not be left in the test solutions for longer than is needed to obtain constant readings of pH because ions from the electrode reference solution can leak into the test solution, potentially causing artifactual toxicity.

For the Phase II ammonia toxicity tests more replicates (at least double that used in Phase I) must be used and tighter QA/QC procedures must be adhered to than those described in the acute or chronic Phase I manuals (see Section 1.2). For example, a control and at least four effluent dilutions using concentrations that more closely bracket the effect and no effect concentrations (that were determined in Phase I) are used. While parameters such as time to mortality or onset of symptoms in the acute and chronic tests are not an integral part of the tests described below, these observations may be very

Table 3-3. Calculated Un-Ionized Ammonia LC50s (mg/l) Based on 24-h, 48-h, 72-h, and 96-h Results of a Fathead Minnow (*Pimephales promelas*) Toxicity Test Conducted at pH 8.0 and 25°C[1]

pH	Percent Dissoc. at 25°C	Un-ionized Ammonia Expected 24-h LC50	Total Ammonia 24-h LC50	Un-ionized Ammonia Expected 48-h LC50	Total Ammonia 48-h LC50	Un-ionized Ammonia Expected 72-h LC50	Total Ammonia 72-h LC50	Un-ionized Ammonia Expected 96-h LC50	Total Ammonia at 96-h LC50
6.0	0.0568	0.075	131	0.064	113	0.049	86	0.036	63
6.1	0 0716	0.093	130	0.080	112	0.061	85	0.045	63
6.2	0.0901	0.12	128	0.10	111	0.076	84	0.056	62
6.3	0.1134	0.14	127	0.12	109	0.094	83	0.069	61
6.4	0.143	0.18	124	0.15	107	0.12	81	0.086	60
6.5	0.180	0.22	121	0.19	104	0.14	80	0.11	58
6.6	0.226	0.27	118	0.23	102	0.18	77	0.13	57
6.7	0.284	0.32	114	0.28	98	0.21	75	0.16	55
6.8	0 358	0.39	109	0.34	94	0.26	72	0.19	53
6.9	0.450	0.47	104	0.40	90	0.31	68	0.23	50
7.0	0.566	0.56	98	0.48	85	0.36	64	0.27	47
7.1	0.711	0.65	91	0.56	79	0.43	60	0.31	44
7.2	0.893	0.76	85	0.65	73	0.50	56	0.36	41
7.3	1.12	0.87	77	0.75	67	0.57	51	0.42	37
7.4	1.41	0.78	55	0.67	48	0.51	36	0.38	27
7.5	1.77	1.09	62	0.94	53	0.72	40	0.53	30
7.6	2.21	1.20	54	1.03	47	0.79	36	0.58	26
7.7	2.77	1.30	47	1.12	40	0.85	31	0.63	23
7.8	3.46	1.39	40	1.20	35	0.91	26	0.67	19
7.9	4.32	1.48	34	1.27	29	0.97	22	0.71	17
8.0	5.38	1.56	29	1.34	25	1.02	19	0.75	14

[1]LC50s for each pH interval were calculated using EPA's water quality criteria document formula (EPA, 1985A) shown below. The 24-h, 48-h, 72-h, and 96-h LC50s to fathead minnows are 1.56 mg/l, 1.34 mg/l, 1.02 mg/l, and 0.75 mg/l, respectively, at pH 8.0.

$$Formula\ LC50 = \frac{(LC50[pH = 8.0])(1.25)}{1 + 10^{7.4 - pH}}$$

Table 3-4. Un-Ionized Ammonia Toxicity Values for Species Frequently Used in Effluent Testing

Species	Method[1]	pH	Temp (°C)	LC50[2] (mg/l)	ChV[3]
		Acute Data			
C. dubia[4]	S, M	6.2	25	0.12	
C. acanthia[5,6]	FT, M	7.1	24	0.77	
Simocephalus ve tulus[5,6]	FT, M	7.1	24	0.61	
C. dubia[4]	S,M	7.2	25	0.78	
C. dubia[4]	S,M	8.2	25	1.73	--
Daphnia *magna*[6]	S,M	8.2	25	2.08	
P. promelas[6]	FT, M	7.8	25.6	1.87	
P. promelas[6]	FT, M	8.0-8.3	25.2	1.65	
P. promelas[6]	FT, M	8.1	26.1	2.55	
		Chronic Data			
C. dubia[7]	4d-R, M	6.03	*25*	--	0.065
C. dubia[7]	4d-R, M	7.05	*25*	--	0.28
C. acanthia[5,6]	7d-FT, M	7.0-7.5	*24-25*	--	0.34
D. magna[6]	NR	7.6	*20.2*	--	0.63
C. dubia[7]	4d-R, M	8.03	*25*	--	0.62
P. promelas[6]	FT, M	8.0	*24.0*	--	0.13
P. promelas[8]	7d-R, M	7.5-7.6	*25.0*	--	0.48
P. promelas[8]	7d-R, M	7.5-7.7	*25.0*	--	0.45
P. promelas[4]	7d-R, M	8.4	*25*		0.66

[1]FT - flow-through; S = static; R = renewal of solutions at 24 or 48 h; M = measured concentration; NR = not reported.
[2]48-h LC50 for invertebrates and 96-h LC50 for fish.
[3]ChV = chronic value which is the geometric mean of the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) or an IC25.
[4]Data generated at ERL-D.
[5]*C. acanthia* is equivalent to C. dubia.
[6]Data from EPA, 1985A.
[7]Data from 4-d C. *dubia* tests conducted at ERL-D; the effect level is an IC25 (mg/l)
[8]Data from Beigger, 1990.

useful in describing the identification steps used in confirming ammonia as the cause of toxicity.

3.3 Measuring Ammonia Concentration

We have found that the ammonia-selective electrode method has been satisfactory for measuring the ammonia concentrations in most samples, (EPA, 1983; APHA, 1992). Other methods for measuring ammonia are available (such as distillation, nesslerization, and titration) and can be used successfully for determining ammonia concentrations in effluents (EPA, 1983; APHA, 1992). The level of detection for total ammonia generally need not be below 0.5-1.0 mg/l, since concentrations of ≤1.0 mg/l of total ammonia have not been found to be toxic to fathead minnows and C. *dubia.* If ammonia measurements are below 1 mg/l and the sample is toxic, it is likely that the toxicant is not ammonia and other identification procedures should be pursued.

The most reliable ammonia measurements are obtained on fresh samples. However, samples can be preserved by adding concentrated sulfuric acid and storing the samples at 4°C. The pH of the preserved samples should be in the range of 1.5 to 2.0 (EPA, 1983; APHA, 1992). In recent experiments, we have used samples that were stored without acidification at 10°C or refrigerated at 4°C for short periods with good success.

During several effluent tests, the amount of ammonia in the test solutions (see test details below) has decreased over the duration of the test. When levels are in the range of O-30 mg/l, it is prudent to measure the initial concentration of ammonia in the test solution and again after animals were exposed.

3.4 Graduated pH Test

The purpose of the Phase II graduated pH test is to provide more definitive toxicity test data to implicate ammonia as the toxicant in Phase II. In turn, this data may be used in Phase III to confirm the role of ammonia in the toxicity of the effluent. More stringent pH control and pH monitoring will be needed to interpret test results and more precise toxicity estimates (i.e., more replicates, more dilutions, larger number of organisms; see Section 1.2; EPA, 1991A; EPA, 1992) are needed in Phase II than in Phase I. When it is important to predict the impact of the toxicant in the receiving water, the pH of the dilution water should be maintained at receiving water pH. The test procedures discussed below provide good pH control for the graduated pH test. Greater detail is provided for some of the procedures in Phase I (EPA, 1991A; EPA, 1992).

The test chamber size, number of dilutions, species to be tested, type of test (acute or chronic), and the degree of toxicity of the effluent will dictate the volume of effluent needed for the graduated pH test. As a general guide for acute toxicity tests, 300 ml of effluent should suffice for any of three pH adjustment tests described below. The volume for chronic tests will vary based on the type of chronic test performed, the species used, the number of concentrations tested, and number of solution renewals in addition to the items discussed above (Section 1.2 and EPA, 1992).

The procedure for conducting the graduated pH test is to evaluate and determine the toxicity of the effluent at three different pHs (e.g., 6.0, 7.0, and 8.0). The pH should be measured in all of the chambers. If the pH drifts 0.2 pH units or more, the results may not be usable and better pH control must be achieved. However, if pH fluctuates more than 0.2 pH units and toxicity is present only at one pH, the toxicity results may still be useful. The pH levels selected must be within the physiological tolerance of the test species used (which generally is a pH range of 6 to 9). We recommend use of two methods of pH control and comparison of these results to determine that the pH adjustment itself did not introduce an artifact of toxicity. This type of testing may be critical to explaining effects in Phase III (EPA, 1993A).

Regardless of the pH control method chosen, the selection of the appropriate blank is difficult. The change in pH of the dilution water or surface water is not comparable to that of the effluent because the composition of the solutions are different. For some effluents, the addition of either acid or base can be used to adjust and hold the pH within 0.1 pH unit. If this is possible, this technique can be used to compare the results with either the CO_2-pH controlled test or the buffer-pH controlled test. Test results should be similar and these comparisons can be used as a basis for identifying ammonia as a toxicant.

3.4. I *pH Control: Acid/Base Adjustments*

The first method of pH adjustment is the acid/base adjustment described in Phase I (EPA, 1991A; EPA, 1992). For this manipulation, the adjustment of pH is relatively easy and quick, and the loss of volatile compounds is minimized. However, the drawbacks are that: toxicity enhancement from the additives may occur (especially in a chronic TIE), the addition of strong acid or base disrupts the carbonate system equilibrium, the effects of the pH change in the blanks may not serve as a toxicity control for the effluent, the pH stabilization time is lengthy, and pH tends to drift in longer term tests. In the pH range 6 to 9, the amount of high quality acid or base added is usually negligible, and the likelihood of toxicity caused by increased salinity levels is low.

The pH of each concentration and replicate must be frequently monitored because a constant pH during the toxicity test must be maintained. Larger test volumes may be useful to prevent rapid pH fluctuations. The amount of acid and/or base added should be recorded for each pH adjustment to track the additive amount in the effluent samples and the blanks. If toxicity increases dramatically, the concentrations of salts should be calculated to be sure the salinity has not increased above the tolerance level for the TIE species.

3.4.2 *pH Control: CO, Adjustments*

The second method uses CO, to adjust and control test solution pH. The pH is adjusted by varying and controlling the CO, concentration of the gas phase over the water or effluent sample in closed headspace test chambers. It is necessary to maintain a constant pH throughout the test period. The pH of most natural waters and some effluents is controlled by the bicarbonate buffering system and surface waters normally contain <10 mg/l of CO,. Therefore, the amount of CO, to add depends on the desired pH and the chemistry of each test solution. The CO, adjustment has the advantages that the pH is controlled without placing additives directly into the effluent test solutions, the pH change is easy to make, and the pH is generally stable for at least 24 h if the gas-tight container is not opened. Frequent pH measurements are still possible because the headspace can be reflushed with a predetermined concentration of CO_2/air. The disadvantages are that toxicity can occur from the CO,, the concentration of CO_2/air varies for each dilution and effluent (which requires sample specific experimentation) and the manipulations for chronic tests can be time-consuming relative to the acid/base adjustment method. We have not observed any increased toxicity from the addition of CO, unless the concentration in the chamber is over 10%.

Adjustments of the pH to 6.0 or 7.0 can be made by using CO, with or without first adding HCl to the test concentrations. The CO, is purchased in pure form through local commercial gas suppliers, and if particular concentrations of CO_2/air are frequently used, a cylinder of gas of the desired concentration may prove to be resource-efficient. The amount of CO, needed to adjust the pH of the solution is dependent upon sample volume, the test container volume, the desired pH, the temperature, and the effluent constituents (e.g., dissolved solids). Some preliminary work is needed to determine the concentration of CO, to add to achieve the desired pH. When dilutions of an effluent have the same hardness and initial pH as the effluent, about the same amount of CO_2 will usually be needed for each dilution. Sometimes, higher concentrations of CO, are needed for the higher test concentrations. Use of a dilution water of similar hardness as the effluent may make the CO, volume adjustments easier. A different dilution water may only be used in these tests if the toxicity has not been shown to be dependent on water hardness at any pH.

In our laboratory, we have found that glass canning jars with rubber seals and metal balers work well as a gas-tight testing chamber. The testing chamber should be large enough to hold the desired number of test cups, with sufficient headspace to ensure proper DO levels. For example, a 2-quart glass canning jar lying on its side will easily hold 6-l oz cups. We simultaneously test *C. dubia* and fathead minnows in the same chamber using the test solution volumes described in Section 1.2. Since many plastics are permeable to CO,, glass containers are recommended. When CO_2/air is flushed into the headspace of the test chamber, the pH of the test solutions will usually reach equilibrium in about 1 h and a reliable pH can be achieved. Generally, as the alkalinity increases, the concentration of CO, that is needed to maintain the pH also increases. After 1 h, check the pH of the solutions and flush the chambers again. Check the pH again after 2-3 h and from these data determine the concentration of CO, to add for initial pH adjustment for the actual toxicity test and the amount needed for reflushing after the chamber is opened for feeding or pH measurements. In most instances, the amount of CO, produced by the test organisms will not cause further pH shifts. When testing with fish, which usually increase in size during the test, a pH fluctuation may occur that would require flushing with different (e.g., slightly lower) concentrations of CO,.

When the concentration of CO, to inject for the target pH values has been determined, prepare test solutions, add test organisms (and food if necessary) and inject the appropriate concentration of CO, in air using a l-liter gas tight syringe, and quickly close the test chamber. The chambers should be flushed with the CO_2/air mixture several times to ensure the displacement of air currently in the chambers. Place the chamber out of direct laboratory light, as temperatures tend to rise out of the desirable test range in the closed chambers.

For effluents that have initial pH values from 7.8 to 8.5, O-l 0% CO, concentration in the chamber has been used to lower the pH to 6.0. Experiments in hard reconstituted water have shown that up to 8% CO, can be tolerated by C. *dubia* and fathead minnows in acute tests, but 8% has been toxic to C. *dubia* and fathead minnows in the 7-d tests. About 2-3.5% usually will lower the pH of most effluents to 6.5-7.0. If more than 10% CO, for acute tests or 5% CO, for chronic tests is needed to lower the pH of the test solutions, before adding test animals adjust the pH with high quality acid (EPA, 1991 A; EPA, 1992) and then flush the headspace with CO_2/air. The necessary concentration of CO, to use must first be determined experimentally with effluent test solutions adjusted to the appropriate pH with acid solutions. Sometimes >5% CO_2 cannot be used for the dilution water pH adjustment test without the CO, causing toxicity.

The use of a single enclosed test chamber for controlling the pH at all test concentrations may allow the transfer of volatile compounds among treatments. We have experienced volatilization of ammonia in tests and therefore, individual test chambers for each effluent concentration are preferable. Methods that use continuous flow of a CO_2/air mixture, such as tissue cell incubators, may be preferable and give better pH control provided that volatilization or cross contamination is not a problem. At this time we have not attempted to use a continuous flow of CO_2/air mixture and therefore cannot recommend a system to use.

Maintaining pH above the air equilibrium pH (generally above pH 8.3) is difficult without buffers (Section 3.4.3). The pH control in this high range is much more difficult because the concentration of CO, must be very low and microbial respiration can increase the CO, levels in the test chamber. Use of CO,-free air in the headspace may work or flushing a mixture of CO,-free air and normal air through the headspace or test solution may be successful. Because such small CO_2 concentrations are needed and because CO, evolution by microorganisms or test organisms can significantly alter the CO, concentration, frequently flushing (two to four times a day) of the headspace in static tests will probably be required to adequately control pH. For the chronic tests, we have not attempted to use the CO,-free air bubbled through the test solution, because more CO, evolution tends to occur during the chronic tests and the need for reflushing makes the test labor intensive.

For the CO_2-pH controlled tests, the pH should be measured at least every 24 h for both acute and chronic tests and ideally, continuously during pH controlled tests. At each reading, flush the headspace with the CO_2/air mixture. A small amount of experimentation will confirm whether the concentration of CO, previously determined is adequate, or whether the amount required for flushing will be less than that used for the initial pH adjustment.

For chronic tests, daily renewal solutions should be prepared, pH adjusted with HCI if necessary, dispensed into test cups, and placed into a second glass jar chamber and flushed with appropriate concentration of CO,. These should be left to equilibrate at least 1-2 h. Measure the pH quickly and transfer the animals to new test cups and place them into the glass jar. Flush the headspace again with the appropriate CO_2/air mixture.

Table 3-5. Percent Un-Ionized Ammonia in Aqueous Solutions at 25°C and Various TDS Levels'

TSD (mg/l)	pH			
	6.0	7.0	8.0	9.0
0	0.0568	0.566	5.38	36.2
250	0.0521	0.519	4.96	34.3
500	0.0505	0.503	4.81	33.6
750	0.0494	0.492	4.71	33.1
1000	0.0485	0.483	4.63	32.7
1500	0.0471	0.469	4.50	32.0
2000	0.0460	0.458	4.40	31.5
3000	0.0443	0.441	4.24	30.7

[1]Data from Skarheim (1973).

For the 7-d tests with fathead minnows, the chambers must be opened once more each day to accommodate the feeding schedule. The experimenter can take advantage of this by making a pH reading prior to placing food into the test cups. CO_2/air must again be flushed into the chamber. It is important to note that in the fathead minnow test, the pH most likely will be lower after 24 h than in the *C. dubia* test because of the food added and the respiration of the fish which is considerably greater than that of C. *dubia.*

Measurements of pH must be made rapidly to minimize the CO, exchange between the sample and the atmosphere. Avoid vigorously stirring unsealed samples because at lower pH values, the CO, lost during the measurement can cause a substantial pH rise. If possible, measure the DO at the same time because ammonia may have different toxicities when DO is decreased (EPA, 1985A). Keep in mind that if the test animals have been dead for awhile, the pH and/or DO of the test water most likely will have changed.

The controls in the CO, chamber and the baseline test act as checks on the general health of the test organisms, the dilution water and most test conditions. If the effluent pH in the baseline test is close to the pH of the adjusted test solutions (at their respective LC50s, IC25s or IC50s), the toxicity expressed in the two tests should be similar. Significantly greater toxicity in the pH-adjusted test may suggest interference from other factors such as the ionic strength related toxicity if the pH was adjusted with either HCI or NaOH, or possibly CO, toxicity. Dilution water blanks at the various pH levels may or may not be appropriate since the effluent matrix may differ from that of the dilution water. The dilution water blank will be useful in checking the acids and bases that are added for artifactual toxicity. Monitoring the acid and base additions may be useful in determining if artifactual toxicity resulted from the increase in salt content. Monitoring conductivity of the effluent solutions after the addition of the acids and bases may also be helpful in determining artifactual toxicity. The ionic strength of hardwaters or saline waters results in a decreased level of un-ionized ammonia (Table 3-5). For values of TDS from O-500 mg/l, the dissociation constants are expected to be more accurate than values above 500 mg/l that were based on somewhat tenuous assumptions (Skarheim, 1973; see Table 3-5).

3.4.3 *pH Control: Buffer pH Aa'justments*

The third method of pH control uses the addition of standard buffers to the effluent and dilution water to adjust the pH. This method has the advantage in that pH is stable with the buffer addition, the pH change during a test is slow, frequent pH measurements are possible because test vessels are not in air-tight chambers, and the test method set-up is rapid. The disadvantages are that toxicity enhancement or interference from buffers may occur, not all buffers can be used without additional

acid/base adjustments, and the pH stabilization time may be lengthy.

Hydrogen ion buffers are used to maintain the pH level in the graduated pH test (EPA, 1991A; EPA, 1992). Three hydrogen ion buffers were used by Neilson et al. (1990) to control pH in toxicity tests in concentrations ranging from 2.5 to 4.0 mM. These three buffers were chosen based on the work done by Ferguson et al. (1980). These buffers are: 2-(N-morpholino) ethane-sulfonic acid (Mes) (pK_a = 6.15), 3-(N-morpholino) propane-sulfonic acid (Mops) (pK_a = 7.15), and piperazine-N,N'-bis (2-hydroxypropane) sulfonic acid (Popso) (pK_a = 7.8). We have also used two additional buffers: N-(2-hydroxyethyl) piperazine-N'-2-hydroxypropanesulfonic acid (Heppso) (pK_a = 7.8) and N-tris-(hydroxymethyl) methyl-3-amrnopropanesulfonic acid (Taps) (pK_a = 8.4). The Taps buffer is more frequently used than the Heppso buffer. We have experienced problems of having to add an excessive amount of base to obtain the desired pH with the Popso buffer. The Taps buffer effectively maintains the pH above 7.8. Keep in mind that pH is best maintained at or near the pK_a of the buffer.

The acute toxicity of these buffers is low to both C. *dubia* and fathead minnows (EPA, 1991A) and 4 mM concentration or less of all five buffers has not caused chronic toxicity to *C. dubia* or the fathead minnow. The buffers are added at sublethal (e.g., NOEC) levels to maintain the pH of test solutions. While these buffers serve to prevent the pH from drifting during the test, pH adjustment to the desired level is required in the preparation of the solution. A portion of the buffer compound is weighed out and added to the aliquots of whole effluent and dilution water, and both are then pH adjusted with acid or base solutions to the desired pH values. Serial dilutions are made, replicates prepared, and test organisms are added. Care should be taken to ensure equilibrium of buff ered solutions, which may take at least l-2 h. Dilutions should also be left to equilibrate and minor pH adjustments should be made. In certain situations, it may be desirable to prepare the solutions the day before tests begin. At present, we have found we can use batch solutions prepared ahead of time for solution renewals. Our experience also indicates that the amount of any buffer needed to hold any pH is effluent specific. Experimentation with effluents will be required to determine the lowest concentration of buffer needed to maintain the desired pH. The test solutions need not be covered tightly to maintain pH; however, pH should be measured at each test reading at all dilutions.

Use of the buffers is still being developed and the effects caused by interferences from the buffers themselves have not been fully studied. It is possible that the buffers may reduce the toxicity of some toxicants, but this has not generally been seen.

3.5 Zeolite Resin Method

Zeolite is composed of naturally occurring or synthetically created crystalline, hydrated alkali-aluminum silicates. The general formula is $M^{n+}O{\cdot}Al_2O_3{\cdot}ySiO_2{\cdot}zH_2O$; M = group IA or IIA element, n = +2 for group IA, +1 for group IIA, y>2, and z = the number of water molecules contained in the interconnected voids or channels within the zeolite (Windholz, et al., 1983). When zeolite is placed in aqueous solutions, the positively charged group IA or IIA elements (M^{n+}) of the zeolite are mobile and can undergo exchange with other cations in the water. As such, zeolite has frequently been employed as ion exchange resins to remove the ammonium ion (NH,+) from aqueous solutions in TIE work (Ankley et al., 19906; Burkhard and Jensen, 1993). Because of its ability to exchange other cations such as heavy metals, and its use as molecular sieves, filter adsorbents and catalysts, zeolite was not suggested for use in Phase I, except as a subsequent test (EPA, 1991 A). Zeolite can be effective in Phase II, if Phase I results implicate ammonia as the toxicant and establish that other types of toxicants (such as non-polar organics and metals) play no role in the effluent toxicity.

For the acute TIE procedure, zeolite particles should be screened to be in the range of 32 to 95 mm, to ensure efficient ion exchange while preventing channeling or excessive resistance to flow. Extremely large or small particles can be removed by screening the zeolite with sieves or mesh screens. The zeolite column can be prepared by taking 30 g of aquarium zeolite (Argent Chemical Laboratories, Redmond, WA) and adding it to 60 ml of high-purity water. The zeolite slurry is poured into a chromatography column (11 mm i.d. x 15 cm) and three bed volumes of dilution water are passed through the column. The last 10 ml of dilution water is collected for use as a zeolite blank and should not be toxic. Next, 200 ml of 100% effluent is passed through the column at a rate of 2 ml/min. The post-column effluent that is collected will be toxicity tested and its ammonia concentration measured. Temperature and pH should be recorded at test initiation to provide the means to calculate both total and un-ionized ammonia in the sample.

For chronic toxicity tests larger amounts of zeolite should be used. This can be scaled up proportionally from the amounts used in the acute zeolite work. The amounts of solution needed for testing and ammonia measurements will dictate the amount of sample to prepare. Typically a slurry of 60 g of zeolite and 120 ml of high purity water is sufficient for levels of ammonia in the range of 5-50 mg/l and for processing 2,000 ml of effluent. The post-zeolite effluent is collected in aliquots, then each is toxicity tested. In this manner, break-through of ammonia can be measured and toxicity of the various samples with different ammonia levels can be estimated.

Toxicity tests and ammonia measurements are conducted on the effluent and post-zeolite column effluent. Removal of toxicity by the zeolite column and removal of the ammonia concentration will add to the evidence implicating ammonia as the toxicant. An aliquot of the effluent sample (not having passed through the zeolite column) is used for ammonia analysis and the baseline toxicity test. These data will be compared with the same data for the post-zeolite column effluent to determine if the post-column reduction in effluent toxicity is consistent with ammonia removal by the zeolite. The control for test organism survival, dilution water quality and other test conditions will be provided through toxicity tests on dilution water. Dilution water (at the same hardness as the effluent) should be passed through the zeolite column, and will act as a blank for toxic artifacts leached from the zeolite. Increased toxicity in the post-zeolite effluent, relative to the whole effluent, indicates the presence of toxic artifacts. Since many cations will be exchanged, adding solids in the acute tests, such as the YCT food (yeast-Cerophyl®-trout food) fed to C. *dubia*, might improve control survival. Additional clean-up techniques for the zeolite (such as Soxhlet extraction) or alternate uncontaminated sources of zeolite might be needed. Column packing, effluent pH, ammonia levels, and flow rate through the column can all affect the efficiency of the cation exchange process. Lowering effluent pH prior to zeolite treatment and/or lowering flow rate through the column might also result in greater removal of ammonia. Occluded gas between zeolite particles might also impair the column's capacity to remove ammonia. If this appears to be a problem, the zeolite slurry should be degassed by using a vacuum prior to pouring it into the column.

Zeolite columns can be regenerated, but fresh zeolite should be used to pack columns the first time. If the graduated pH test and the zeolite test results are consistent with ammonia toxicity, Phase III confirmation procedures should be started.

Once ammonia is identified and confirmation is initiated, the post-zeolite samples can be spiked with ammonia at the same concentrations as are present in the effluent. These tests are an integral part of the Phase III confirmation process (EPA, 1993A).

3.6 Air-Stripping of Ammonia

This method of ammonia removal takes advantage of the fact that the relatively volatile un-ionized ammonia (NH_3) predominates in a solution with a pH above 9.3. For this reason, one might expect that ammonia would be removed during the Phase I pH 11 adjustment/aeration test (acute testing) or the pH 10 adjustment and aeration test (chronic testing). Based on our experience ammonia is not removed by this method, most likely because the Phase I aeration manipulation is done in a graduated cylinder, which has a low surface-to-volume ratio. By stirring the sample for a longer period of time (>1 h) at a high pH (pH 9.0 or higher) in a container that allows a large surface area to volume ratio, most of the ammonia can be removed from aqueous samples.

A measured amount of effluent for subsequent analysis and testing is pH adjusted to 10 or 11 and placed into a large shallow glass container (e.g., 1000 ml crystallizing dish). The solutions are then agitated (stirred) continuously. The length of time the sample must be stirred is dependent on the concentration of total ammonia in the sample. We have found that for most samples of 10-100 mg/l of total ammonia, 1-6 h is adequate to remove most of the ammonia. After air-stripping is completed, the volume of effluent should be measured and any appreciable loss replaced with high purity water or toxicity might be caused by the concentration of other components in the effluent. The ammonia concentration should be measured immediately after air-stripping and after volume adjustment is complete to ensure ammonia levels are reduced before toxicity tests are initiated. Toxicity tests on the air-stripped solution can then be conducted for both acute and chronic TIE work. Dilution water blanks at the various pHs may or may not be appropriate since the effluent matrix will probably differ from that of the dilution water. Monitoring the acid and base additions may be useful to determine if artifactual toxicity resulted from the increase in salt content and subsequent evaporation that occurred during the air-stripping process. Monitoring conductivity of the effluent solutions after the addition of the acids and bases may also be helpful in determining artifactual toxicity. The dilution water blank should be treated in the same manner as the effluent although it may not serve as a true toxicity control for the effluent.

If the ammonia is decreased and the toxicity is reduced or absent after air-stripping, ammonia is strongly implicated as a contributing factor to the toxicity of the effluent. The results of this test should be compared with the aeration test results of Phase I, the baseline effluent test and the other graduated pH tests. Other compounds could precipitate as a result of the pH adjustment and during the air-stripping procedure. Precipitates may form during the air-stripping process and not dissolve after the volume is readjusted, leaving these compounds unavailable.

Section 4
Metals

4.1 General Overview

This section contains procedures that can be used to identify suspect metal toxicants. The initial evidence used to implicate metallic toxicants is obtained from the Phase I characterization tests, with the results of the EDTA (ethylenediamine tetraacetic acid) addition test providing the best indication of the presence of a metal toxicant. When certain cationic metal toxicants are present, a reduction in sample toxicity with the addition of EDTA should be observed. Other Phase I manipulations that remove or reduce sample toxicity and suggest the presence of a cationic metal include the sodium thiosulfate addition test, the use of a C_{18} SPE column, and filtering the sample when combined with minor pH adjustments. One additional indication of metal toxicity may be when the organisms' response in the toxicity test is atypical of the expected dose response relationship (i.e., partial mortalities in several test concentrations, Schubauer-Berigan et al., 1993B).

Subsequent Phase I tests such as using ion exchange resins might also lead one to the conclusion that a metal is the toxicant. Toxicity removal or reduction after a sample. is treated with an anion exchange resin might implicate toxicants that exist as anionic oxides in water, such as arsenic, chromium, and/or selenium. These anionic oxides will not be specifically removed or rendered biologically unavailable by the routine Phase I tests. Therefore, when the Phase I tests do not seem to show any toxicity reduction, toxicants such as these might be suspected and subsequent tests as discussed above could be useful (see Phase I, EPA, 1991A; EPA, 1992). These situations should be approached on an individual basis since other classes of toxicants might demonstrate the same behavior in Phase I (e.g., total dissolved solids (TDS)).

Further discussion and interpretation of the Phase I results which would lead to the conclusion that a cationic metal toxicant was present in a sample are provided in the Phase ! TIE documents (EPA, 1991A; EPA, 1992).

Other information, such as process details from the discharger and infcrmation from past TREs and/or TIEs, might also help to implicate cationic metals as the toxicants. However, this type of information should be interpreted and used with caution as it might bias the TIE efforts.

If the EDTA addition test in Phase I showed that toxicity was removed or reduced one should proceed to the metal analysis section (Section 4.2). This section provides guidance and recommendations for analyzing samples for metals so that a list of suspect metal toxicants can be obtained. This section also discusses clean metal techniques, detection limits, a prioritization process for analyzing for specific metals, dissolved vs biologically available metals, and provides the rationale for assembling the list of suspect metal toxicants. Prioritizing metals to analyze from Phase I results is strongly recommended in order to save money and time in the TIE process.

If other Phase I tests implicate a metal but EDTA does not, it may be helpful to acquire additional test information through the use of EDTA addition tests, sodium thiosulfate addition tests, graduated pH tests, and ion-exchange resins. This additional toxicity testing (Section 4.3) may be useful in certain situations before analyzing for metals, even when EDTA additions reduced toxicity. These situations include: when the addition tests of EDTA and sodium thiosulfate in Phase I were performed using a single sample concentration (i.e., no dilutions), when the time it takes to obtain results of metal analyses is lengthy, or when Phase I results indicate another type of toxicant (non-metal) is present. The data obtained from the additional testing can then be included in the prioritization process for metals analysis. Professional judgement is required to decide when you have sufficient and appropriate toxicity testing data to proceed to metals analysis.

After processing one sample, a list of suspects may be generated. As future samples are evaluated, the correlation between toxicity of a sample and the concentration(s) of metal(s) over time may also be used to narrow the list of suspect toxicant(In Phase III, the suspect metal toxicant is implicated based upon the correlation of effluent toxicity and metal concentrations, reference suspect metal toxicity data, the use of additives that chelate metal toxicants, and changes in toxicity observed during manipulations of water quality characteristics.

The procedures in this chapter are generally applicable for both acute and chronic toxicity. The main differences between the acute and chronic procedures are the concentrations of additives used in the EDTA and sodium thiosulfate addition tests, lower analytical detection limits, and generating non-toxic blanks for the ion exchange resins for chronic toxicity testing. The use of species other than *C. dubia* or fathead minnows will require consideration of appropriate test volumes and additive concentrations.

4.2 Analysis of Metals

4.2.1 Prioritizing Metals for Analysis

Many cationic metals can be analyzed in a specific sample, but to simplify the amount of analytical effort needed for metals analysis, we suggest a prioritizing process be performed before analyzing any samples. The prioritization process is more valuable when the metal analyses are performed by AA instrumentation since each metal requires an individual analysis. Conversely, with ICP (inductively coupled plasma) instrumentation, numerous metals can be analyzed at once, and the prioritization process is less valuable in this instance. With both ICP and AA methods, a list of metals and required levels of detection will be needed before the samples are analyzed.

This prioritization is based primarily upon acute toxicity data with C. *dubia.* Its applicability to chronic toxicity and other species is expected to be similar but has not yet been determined. The toxicity test results from the EDTA additions, sodium thiosulfate additions, and graduated pH tests performed in Phase I form the basis for prioritization. When available, Phase II results from using the procedures in Section 4.3, should be included in this evaluation. Because we do not have a complete understanding of the effects of these procedures for each metal, the following should be taken as a starting point for metals analysis.

Information regarding historical discharge monitoring data, past or current TRE and/or TIE information, or process information may be useful in prioritizing metals for analysis. For example, if a discharger uses zinc in their manufacturing process and EDTA removed the toxicity, it would be logical to analyze for zinc first. If zinc was present at nontoxic concentrations or at concentrations too low to cause the observed toxicity, analysis for additional metals would be performed. If zinc was present at concentrations high enough to cause the observed toxicity, Phase III procedures (EPA, 1993A) should then be started to confirm zinc as the identified suspect toxicant.

When EDTA additions reduce or remove the toxicity of the sample, initially copper, lead, cadmium, nickel, and zinc should be measured. When sodium thiosulfate additions reduce or remove the toxicity of the sample, copper, cadmium, and silver should be measured.

Phase I results would not normally lead to the conclusion that an anionic toxicant was present (i.e., cationic metals that exist in aqueous samples as anionic oxides). If additional Phase I tests had been performed which characterized anionic toxicants or other specific discharger information was available, measurements of arsenic, chromium, and selenium should be made.

As stated above, these metals should be a starting point for metals analysis. Further interpretation of the Phase I results could be done by including the results of the graduated pH test and by jointly examining the results of the EDTA addition, thiosulfate addition, and graduated pH tests.

When multiple toxicants of different classes are present, Phase I data are often difficult to interpret. One should try to identify and confirm as soon as possible the role of one toxicant when multiple toxicants are present. By defining the role of one toxicant, efforts can be better focused on the remaining unidentified toxicants.

4.2.2 Metal Analysis Methods

There are three types of chemical instrumentation available for the analysis of cationic elements; these are AA, inductively-coupled plasma-atomic emission spectroscopy (ICP-AES), and inductively-coupled plasma-mass spectrometry (ICP-MS).

EPA methods using ICP-AES, ICP-MS, and AA (EPA, 1983; EPA, 19910) are available for quantifying cationic metals in aqueous samples. Tables 4-l and 4-2 summarize method detection limits for the analysis of cationic metals in aqueous samples using AA with direct aspiration, AA with the furnace procedure, ICP-AES, and ICP-MS.

The detection limits required in Phase II for the identification of suspect cationic metal toxicants will be determined by the toxicity of metals for the TIE species. In some cases, especially for chronic toxicity, the effect level might be lower than the detection limits listed in Tables 4-1 and 4-2. Detection limits should be improved to obtain optimal levels of detection (i.e., at least two times lower than the effect level).

Toxicity data for some species and test types for many metals have not been determined, especially for 7-d chronic toxicity tests. Therefore, to determine the needed levels of detection, effect levels for specific metals may have to be determined.

The required level of detection will often dictate the method needed for performing the metal measurement. It will be beneficial for laboratories to compile a database containing method detection limits and toxic effect levels for cationic metals using data from their organisms, analytical methods, and toxicity testing conditions. These data are not necessary in advance but this

Table 4-1. Atomic Absorption Detection Limits and Concentration Ranges'

Metal	Direct Aspiration		Furnace Method[2]	
	Detection Limit (mg/l)	Optimum Concentration Range(mg/l)	Detection Limit (µg/l)	Optimum Concentration Range (µg/l)
Aluminum	0.1	5 - 50	3	20 - 200
Antimony	0.2	1 - 40	3	20 - 300
Arsenic3	0.002	0.002 - 0.02		5 - 100
Beryllium	0.005	0.05 -2	0.2	I - 3 0
Cadimum	0.005	0.05 -2	0.1	0.5 - 10
Calcium	0.01	0.2 -7		
Chromium	0.05	0.5 - 10	1	5 - 100
Cobalt	0.05	0.5 -5		5-100
Copper	0.02	0.2 -5		5 - 100
Lead	0.1	1 -20		5 - 100
Magnesium	0.001	0.002 - 0.5		
Manganese	0.01	0.1 -3	0.2	I - 3 0
Mercury4	0.0002	0.0002 - 0.01		
Molybdenum (p)	0.1	1 - 40		3 - 60
Nickel(p)	0.04	0.3 - 5		5 - 50
Potassium	0.01	0.1 -2		
Selenium[2]	0.002	0.002 - 0.02	2	5 - 100
Silver	0.01	0.1 - 4	0.2	1 - 25
Sodium	0.002	0.03 - 1		
Tin	0.8	10 -300	5	20 -300
Vanadium (p)	0.2	2 - 100	4	10 - 200
Zinc	0.005	0.85 - 1	0.05	0.2 - 4

[1]The estimated detection limits and concentration ranges were taken from EPA, 1983.
[2]The listed furnace values are those expected when using a 20 µl injection and normal gas flow except in the case of arsenic and selenium where gas interrupt is used. The symbol (p) indicates the use of pyrolytic graphite with the furnace procedure.
[3]Gaseous hydride method.
'Cold vapor technique.

Table 4-2. Estimated Instrumental Detection Limits for ICP-MS and ICP-AES

Element	Estimated Detection Limit, ICP-MS (µg/l)	Estimated Detection limit, ICP-AES[2] (µg/l)
Aluminum	0.05	45
Arsenic	0.9	53
Antimony	0.08	32
Beryllium	0.1	0.3
Cadmium	0.1	4
Calcium		10
Chromium	0.07	7
Cobalt	0.03	7
Copper	0.03	6
Lead	0.08	42
Magnesium		30
Manganese	0.1	2
Molybdenum	0.1	8
Nickel	0.2	15
Potassium		[3]
Selenium	5	75
Silver	0.05	7
Sodium		29
Vanadium	0.02	8
Zinc	0.2	2

[1] The estimated instrumental detection limits are taken from EPA, 1991 D. They are given as a guide for instrumental limits, the actual detection limits are sample dependent and may vary as the sample matrix vanes.
[2]The estimated instrumental detection limits as shown are taken from EPA, 1983. They are given as a guide for an instrumental limit. The actual method detection limits are sample dependent and may vary as the sample matrix varies.
[3]Highly dependent on operating conditions and plasma.

type of information will be very useful for future TIE efforts.

When toxicity effect levels appear to be below the detection limits of current analytical methods, the use of "clean" analytical techniques may be required through all steps in the analysis of the sample because background contamination is the major cause of inadequate levels of detection. Some general principles of clean metal techniques include the use of contamination free reagents, acid cleaned plastic labware, acid cleaned membrane filters (not glass fiber), class 100 benches for sample preparation, proper sample collection, preservation, and storage procedures; and proper QA/QC procedures using blanks, spiked matrixes, and replicate analyses. A summary of clean metal techniques and procedures for lowering the levels of detection can be found in Nriagu et al., 1993; Patterson and Settle, 1976; and Zief and Mitchell, 1976.

Some cationic metals, such as arsenic, selenium, and chromium, have different stable oxidation states in aqueous samples and more importantly the different oxidation states may have different toxicities. In Section 4.23, procedures to determine the concentration of the different oxidation states are provided.

In some TIEs, a measurement of the metals associated with the suspended solids may be needed

(Section 4.2.4). Procedures for preparing suspended solids removed by filtration for metals analysis are available; see EPA method 200 (EPA, 1983) and EPA method 200.2 (EPA, 19910).

4.2.3 *Metal Speciation*

The procedures suggested above (Section 4.2.2) are used to determine the total concentration of a metal in an effluent. Many metals exist in water in different forms due to the various stable oxidation states of the metal. Arsenic (As^{3+}, As^{5+}), chromium (Cr^{3+}, Cr^{6+}), and selenium (Se^{4+}, Se^{6+}) are important metals that exist in different forms in water. Determining the speciation for these metals may be important in the TIE since the toxicities are different for the various forms of each metal. For example, Cr^{6+} is the form that is of toxicological concern while Cr^{3+} is generally not toxic (EPA, 1985D).

For chromium, methods for measuring the hexavalent form (Cr^{6+}) such as method 218.5 (EPA, 1983) are available. The amount of the trivalent form of chromium (less toxic form) is determined by taking the difference between the concentrations for total and hexavalent chromium.

For arsenic, the method of Ficklin (1983) is suggested for speciation measurements. This method uses an anion-exchange resin to separate the arsenite (As^{3+}) and arsenate (As^{5+}, more toxic form) species. Graphite furnace atomic-absorption spectroscopy is then used to measure the concentrations of each form.

For selenium, the method of Oyamada and lshizaki (1986) is recommended for speciation. This method (like that for arsenic) uses column chromatography with an anion-exchange resin to separate the selenite (Se^{4+}) and selenate Se^{6+} (more toxic form) and graphite furnace atomic-absorption spectroscopy to measure each form.

Ion chromatography can also be used to determine the different forms of the above metals (EPA, 1991 D), but we have not used this technique to date.

4.2.4 *Identification of Suspect Metal Toxicants*

Initial implication of suspect metals based on a comparison of total metal analyses data and effluent toxicity test results should be made. Then analysis for dissolved and suspended metals can be made if necessary. These metal values should be compared to available toxicity values, but tests on reference metals might have to be conducted with matching effluent conditions, such as pH and hardness to obtain comparable toxicity values. Side-by-side tests with individual reference metal standards and effluent samples might prevent being mislead by different test designs and are worth the effort. Literature summaries of metal toxicity data are also available (EPA, 1980; EPA, 19858; EPA, 1985D; EPA, 1985E; EPA, 1985F; EPA, EPA, 1986; EPA, 1987; EPA, 19888; and AQUIRE, 1992). In addition to matching the hardness and pH of the dilution water to the effluent sample by the addition of the appropriate ratios of magnesium carbonate and calcium carbonate, it might be possible in some cases to mimic the wastewater total suspended solids (SS) and total organic carbon (TOC) in the water used to test the metal. For example, TOC and SS from the addition of the YCT food can be at levels such that the total SS level in the dilution water might be similar to that found in the effluent. TOC may also be modified by the addition of humic acid. If the dilution water does not closely match the effluent, nonstandard dose-response relationships are observed in the toxicity test, i.e., several test concentrations exhibit partial mortality. In addition, a trend is noticed that as metal concentrations decrease at the effluent LC50s or IC25s, toxicity of the effluent increases.

If a sample is to be filtered, a membrane filter(s), such as a 0.45 µm polycarbonate filter should be prepared by rinsing with high purity water, followed by an appropriate volume of dilution water needed for blank toxicity tests and analysis. The toxicity test guidance is described in Section 1.2 and in the Phase I documents (EPA, 1991 A; EPA, 1992). An appropriate quantity (<50 ml) of the last portion of the high purity water passing through the filter should be collected as an analytical blank to check for metals contamination from the filter and the filtration apparatus. An aliquot of the effluent is then filtered through the 0.45 µm membrane filter(s). If more than one filter is required for the effluent, a portion of each can be combined for testing.

The filtered and unfiltered effluent samples and the filtration blank should be tested for toxicity to measure the effect of filtration on sample toxicity. The toxicity test techniques are described in the non-polar organic section (Sections 2.2.3 and 2.3.3) unless data are needed for Phase III confirmation and then, greater replication and randomization will be needed (see Section 1.2). The toxicity tests should be performed for the test species using a dilution water (e.g., reconstituted water) of a similar hardness and pH to that of the effluent. If toxicity is reduced or removed upon filtration (and effluent toxicity has not previously been affected by C_{18} SPE or filtration through a glass fiber filter), it is possible that metals were retained by the 0.45 µm filter. Analysis for metals retained by the filter may help in interpreting sample data.

Metals analyses should be performed on the analytical blank collected from the filter and on the filtered and unfiltered effluent samples. The choice of metals to measure will be determined by the prioritization process described above. As stated previously, the level of detection for the metal of interest should be lower than the effect concentration for the metal.

Biologically Available Metals: Traditionally, dissolved metals for aqueous samples have been defined as those that pass through a 0.45 µm membrane filter, i.e.,

polycarbonate filter. The dissolved metals are in no way synonymous with the *biologically available metals.* Other than the use of an aquatic organism there is no technique to determine the biologically available fraction of the total metal. Furthermore, only rudimentary techniques are available to specifically identify the individual species of a metal (e.g., free charged metal ions [M^{n+}], inorganic ion pairs or complexes such as aquoions, [$M(H_2O)^{n+m}$], hydroxoions [$M(OH)_p^{n-p+}$], oxoions [MO_q^{n-2q+}], organic complexes and chelates [M x EDTA], metal species bound to high molecular weight organic material [M x lipid] or metal species in the form of highly dispersed colloids or sorbed on colloids [M x clay]). Stumm and Morgan (1981) have listed some general methods for assisting in identification of individual species. In some cases, binding of metals to inorganic and organic ligands in effluents will reduce the bioavailability of the metals and cause the metal concentration at the effluent LC50, IC50, or IC25 to be larger than the metal concentration determined in the metal dilution water toxicity test. For a set of effluent samples with a wide range of toxicities, better agreement should occur between the effect concentration of the metal in a dilution water toxicity test and the more toxic effluent samples (where the toxicity testing matrix of the effluent more closely matches that of the dilution water). Methods for determining the bioavailable fraction of the total metal are limited.

Some indication of the binding of metals to organics in the effluent may be arrived at through hexane extraction of an aliquot of the sample (Stary, 1964). Theoretically, metals bound to organic materials that are soluble in hexane should be extracted from the effluent. The hexane can then be evaporated and the residue reconstituted and analyzed for metals. Additionally, the loss of metals can be estimated by repeating the metal analysis on the extracted effluent and comparing this result to the hexane extract results. The toxicity attributed to metals associated with organics might be estimated by performing a toxicity test on the solvent extracted effluent. Traces of hexane must be removed from the extracted effluent by aeration prior to toxicity testing. The effects of aeration on sample toxicity must also be considered in this analysis. In any case, metals strongly suspected of causing or contributing to sample toxicity should be tested in dilution water as described above with the TIE test species.

The effects of variable water quality characteristics on metal toxicity must be evaluated over the effluent sampling period. One way to assess this is to collect several samples over a short time span. As an example, for an acutely toxic effluent, collect six grab samples in 24 h, and calculate the correlation coefficient for sample metal concentration (or summed toxic units of metals) versus sample toxicity for each sample. The set of correlation coefficients for multiple sampling events might give results less affected by hardness, SS, and TOC, assuming that water quality characteristics affecting metal toxicity will vary less during short time periods. For chronic toxicity, it might be useful to measure concentrations of metals in several daily samples and conduct separate chronic tests on each sample. Obviously, metal concentrations must vary enough to provide a sufficient range for correlation. When one reaches this stage, Phase III work should start using Phase III methods. Symptoms, species sensitivity, spiking, water quality adjustments and correlation are all applicable Phase III approaches to confirm the cause of toxicity.

4.3 Additional Toxicity Testing Methods

Guidance on EDTA addition tests, sodium thiosulfate addition tests, graduated pH tests, and the use of ion-exchange resins for use in Phase II are presented in this section. These procedures might be used before performing analyses for cationic metals, but most often they will be used to refine a list of suspect metal toxicants and to provide data to support the identified suspect in Phase III.

In the acute Phase I, EDTA and sodium thiosulfate addition tests can be conducted by adding incremental amounts of EDTA or sodium thiosulfate to a, single effluent concentration. To provide further evidence in Phase II, these two tests should be conducted with effluent dilutions to assess the toxicity reduction (see EPA, 1992). The data generated from these procedures provide a powerful tool for identifying the cause of toxicity in samples containing mixtures of cationic metals. For example, toxicity caused by either copper or zinc could be determined by using the following test information: toxicity of both metals would be removed by EDTA addition (Section 4.3.1), sodium thiosulfate can remove toxicity caused by copper but not zinc (Section 4.3.2), and copper is more toxic at higher pH levels while zinc is not (Section 4.3.3). Depending on how the toxicity of the sample changes with these tests, one could eliminate one of these metals from the list of suspect metal toxicants.

Results of this type of testing will be used to develop evidence implicating the identified suspect metal. These tests would be performed on a number of samples over time to demonstrate the consistency of the cause of toxicity. In addition, when a mixture of toxicants is present, additions of EDTA or thiosulfate could be used to remove the cationic metal toxicity after performing other Phase II manipulations, e.g., C_{18} SPE.

4.3.1 *EDTA Addition Test*

Any reduction in effluent toxicity effected by the addition of EDTA suggests that certain cationic metals might be present in the effluent at toxic levels. Background information and discussion of the behavior of EDTA and cationic metals can be found in Phase I (EPA, 1991A; EPA, 1992).

Ideally, the amount of EDTA added would be just enough to chelate the toxicant(s) without causing EDTA

toxicity or substantially changing the water quality. For either C. *dubia* or fathead minnows, we have found it useful to add two different EDTA concentrations to two separate effluent tests (with dilutions). Controls without EDTA must be included. The EDTA stock solution is added after the effluent dilutions are prepared so that the EDTA concentration is the same at each effluent dilution (see Phase I, EPA, 1991A; EPA, 1992).

In Phase II, conducting simultaneous EDTA addition tests on effluent and the suspect metal in matching test water can provide evidence supporting the suspect metal as the toxicant if the results of these two tests are similar. If the metal is chelated by EDTA in the dilution water test but not in the effluent test then either there is a strong matrix effect from the effluent or it is the incorrect suspect metal. It is important to use the same pH in both tests in case there is any pH effect on the metal's toxicity.

In addition to removing toxicity caused by metals, EDTA reduces the acute toxicity of some cationic surfactants. This reduction of toxicity might also occur in chronically toxic effluents, and the toxicity reduced by EDTA should not be assumed to be due only to cationic metals.

4.3.2 *Sodium **Thiosulfate** Addition Test*

The acute Phase I oxidant reduction test (EPA, 1991A) or the chronic sodium thiosulfate addition test (EPA, 1992) is used to determine to what extent constituents reduced by the addition of sodium thiosulfate ($Na_2S_2O_3$) are responsible for the effluent toxicity. Although the use of the sodium thiosulfate test was designed to determine if oxidative compounds (such as chlorine) were responsible for effluent toxicity, experience has also shown that thiosulfate can also form a stable non-toxic complex with some metals. Since the complexing ability of thiosulfate is more metal specific than EDTA, this reagent can be used to determine if a specific metal is responsible for the effluent toxicity. Recent work by Mount (1991) has shown that in acute toxicity tests with C. *dubia* in moderately hard water that Cu^{2+}, Cd^{2+}, Hg^{2+}, Ag+, and Se^{6+} can be complexed using sodium thiosulfate (see EPA, 1991 A for more details). This complexing ability might not be applicable to chronic toxicity. For example, in a C. *dubia* 7-d test with copper, the toxicity was not reduced with sodium thiosulfate addition but was reduced with EDTA addition.

Table 4-3. Metal LC50s with Respect to Test pH[1]

Metal	Species	LC50 (µg/l) pH 6.2	pH 7.2	pH 8.2
Zn	C. dubia	>530	360	95
	P. promelas	830	333	502
Ni	C. dubia	>200	137	13
	P. promelas	>4000	3360	3080
Pb	C. *dubia*	*280*	>2700	>2700
	P. promelas	*810*	>5400	>540
cu	*C. dubia*	10	*28*	*201*
	P. promelas	*15*	*44*	>200
Cd	C. *dubia*	*563*	*350*	*121*
	P. promelas	*54*	*74*	<5

[1]LC50 values were determined at 48-h for *C. dubia* and 96-h for *P. promelas.* Data taken from Schubauer-Berigan et al., 1993A.

If the addition of sodium thiosulfate does not reduce the effluent toxicity thought to be related to metals, the use of SO, (EPA, 1991A) additions followed by the addition of sodium thiosulfate is recommended. In some situations, the thiosulfate concentration may be reduced by non-toxic oxidants and thus, not be available for complexing the toxic metal. The addition of SO should preferentially reduce these non-toxic oxidants which will allow the now available thiosulfate to complex the toxic metals. Depending upon the complexation ability of sodium thiosulfate for a specific metal, it might or might not complex the toxic metal. If the suspected metal toxicant can be complexed (e.g., cadmium, copper, selenium (as selenate), mercury; see EPA, 1991A; EPA, 1992), then a reduction in sample toxicity should occur with the addition of sodium thiosulfate. If the suspected metal cannot be complexed (e.g., zinc, lead, manganese, and nickel), then no reduction in sample toxicity should occur with the addition of the sodium thiosulfate.

As with the EDTA addition test, sodium thiosulfate additions should be conducted concurrently on the effluent and on dilution water spiked with the suspect metal toxicant. Care must be used to conduct these tests at similar pH levels. When toxicity test results are consistent with the expected behavior, strong evidence relating the suspected metal toxicant to the cause of the effluent toxicity has been obtained. These results in conjunction with the ion exchange test, analytical measurements for toxic metals, and the EDTA addition test provide evidence sufficient for one to proceed to toxicant confirmation, Phase III, of the TIE.

Both sodium thiosulfate and EDTA can reduce the toxicity of some metals and this information can be helpful in identifying the toxicant. However to date, this effect of thiosulfate/metal complexation has not been demonstrated for chronic toxicity. Knowing which metals are bound by both sodium thiosulfate and EDTA and which metals are complexed with only one or the other additive can be very helpful in narrowing down the possible toxicant.

*4.3.3 Metal Toxicity Changes with **pH***

In Phase I, the graduated pH test is performed to evaluate the presence of compounds whose toxicity varies with pH. For ammonia, toxicity is greatest at pH 8.5 and least at pH 6.5 for some species. Therefore, as suggested in the first Phase II document, that for samples in which toxicity is enhanced at elevated pH, the identification effort should focus on ammonia. However, some

effluent and sediment pore water TIEs have indicated that some toxicity caused by metals can be affected by pH within the range of pH 6 to 9 (Schubauer-Berigan et al., 1993A). Some metals, notably zinc, nickel and cadmium, exhibit greater toxicity at elevated pH, which could confuse their characterization with that of ammonia (Table 4-3), while copper and lead show elevated toxicity at pH 6.2. These pH-dependent toxicities can be used as a tool for the identification (and confirmation) of toxicity caused by these metals. For example, toxicity to *C. dubia* in a sediment pore water sample was completely removed by additions of EDTA. The sample also exhibited greater toxicity at pH 6.5 than at 8.5, and metal concentrations indicated that only copper was present at toxicologically significant concentrations. The pH dependent toxicity of the sample along with the EDTA addition results and metal analysis supported the identification of copper as the toxicant.

4.3.4 *Ion-Exchange Test*

Ion-exchange resins have been used in TIEs for generating supporting evidence for identifying the cause of toxicity in effluents (Doi and Grothe, 1989; Phase II zeolite test). For cation exchange resins, removal of toxic cations such as NH,+, Cd*+, and Pb^{2+} from the effluent occurs with the corresponding release of cations (i.e., counter ions) such as H^+ and Na^+ into solution. Similarly, for anion exchange resins, removal of toxic anions such as $Cr_2O_7^{2-}$ and AsO_4^{2-} from the effluent occurs with the corresponding release of anions such as OH- and Cl- into solution. For both cation and anion exchange resins, charge neutrality exists between the resin and aqueous phase and therefore, if the resins remove 5 µmoles of Cd^{2+} from solution, 10 µmoles of H^+ would be released into solution. The exchange process is concentration dependent and is reversible. Cations removed from the solution may then be recovered from the exchange resin by passing an acidic solution over the resins (e.g., 1 N HCl for analysis of metals).

We have had limited experience with ion exchange resins but the following general guidance can be provided. First, ion exchange resins are not chemical specific but rather remove a wide range of cations or anions, metallic and non-metallic. We have observed that anion exchange resins can remove cations (e.g., Zn^{2+}) from solution quite efficiently. The reasoning that only cationic materials are removed by cation ion exchange resins is not always reliable. Experimental verification of which materials were removed by the resin will be necessary on a case-by-case basis. Second, wide changes in the pH of the post-column effluent can occur depending upon the type of cation or anion released by the resin. These changes in pH will cause problems in interpreting toxicity tests if the pH is not adjusted prior to the toxicity test. Third, many of the ion exchange resins are based upon a styrene or acrylic divinylbenzene backbone and this material can remove other types of toxicants such as non-polar organics. Consequently, because of its non-specificity the removal of toxicity by an ion exchange column should not be used as the only piece of evidence to implicate a metal as the toxicant.

Resins under evaluation and/or those which have been used include IRA-35, IRA-68, IRA-94, IRA-900, IRC-718, and GT-73 (Rohm and Haas, Philadelphia PA) and aquarium zeolite (Argent Chemical Laboratories, Redmond WA). The key to obtaining useable data from an ion exchange test is to obtain non-toxic blanks. Since numerous ion exchange resins exist, guidance for preparing all resins for TIE work cannot be provided. A variety of procedures have been used in our laboratory to condition the columns and to obtain non-toxic blanks.

Effluent volumes ranging from 1,000 to 10,000 ml have been used, and the volume is dependent on the hardness of the dilution water, bed volume of the column, strength and type of the ion change resin, which ions were being exchanged, the toxicity of the effluent, and the species being tested. For example, for acutely toxic effluents, glass chromatography columns (11 mm i.d.) are packed with about 10 cm of resin and the solutions are pumped up through the column at a flow rate of 4 to 5 ml/min. First, a small volume of high purity water (e.g., 200 ml) is passed through the column, and discarded. Next, the dilution water (volumes are variable, i.e., 1 ,000-5,000 ml) is passed through the column until the pH of the post-column dilution water is above 7.0.

Following this procedure, the necessary volume of dilution water to use for toxicity testing is passed through the column and collected. The type of dilution water to use is effluent specific and in general, should be the same as the dilution water used in the toxicity test for the effluent. The pH of the post-column dilution water should be monitored and the pH adjusted to return the water solution to its original pH. Toxicity tests are then performed on the post-column dilution water sample (column blank). After obtaining non-toxic blanks for a particular batch of resin, the conditioning process can be used on other aliquots of the resin with a similar procedure; however, column toxicity blanks must always be tested.

To identify acute toxicity, we generally begin by using 200 ml of effluent (filtered or unfiltered) and collect the post-column effluent. The pH of the post-column effluent is checked and if necessary the pH is adjusted to that of the baseline test, and tested for toxicity. For chronic toxicity, the volume of effluent needed for the toxicity test will dictate the amount of resin and the size of the column. When evaluating a new resin, use proportions of water, effluent, and resin, similar to those described above for acutely toxic effluents. New aliquots of resins should be prepared and used for each ion exchange test. By doing so, artifactual toxicity problems

from other effluents and sample manipulations can be avoided.

We have had limited success in the elution of the ion exchange resin to recover the exchanged toxicant(therefore, we cannot provide specific guidance. in theory, cations and anions can be eluted **from ion exchange** resins using a strong acidic (HCI) or basic (NaOH) solution. Performing successful toxicity testing on these solutions is extremely difficult because of artifactual toxicity problems.

When toxicity is removed by the ion exchange test, useful information about the toxicant(s) may be obtained. However, as discussed above, the removal of a toxicant by the column may not be as straightforward as first perceived. The use of other manipulations and analytical measurements on the pre- and post-column effluents will be required to establish the significance of the results of the manipulation.

When toxicity is not removed by the ion exchange test and non-toxic blanks are obtained, the conclusion that the toxicant is not a cation or anion can be made. However, the slight possibility exists that the resin may not be able to exchange the toxicant because of steric and size considerations.

Section 5
Chlorine

5.1 General Overview

One of the first analytical measurements recommended in the Phase I documents (EPA, 1991A; EPA, 1992) upon arrival at the laboratory is for total residual chlorine (TRC) in each effluent sample. Chlorine is a commonly used biocide and oxidant and is frequently found at acutely toxic concentrations in municipal effluents (EPA, 19856). Sublethal chronic toxicity from chlorine in effluent samples is not as likely to occur due to the degradation of chlorine (see below) with holding of the sample. Chlorine is unstable in aqueous solutions and decomposition is more rapid in solutions when chlorine is present at low concentrations. From the TRC measurement and the Phase I tests (sodium thiosulfate addition and aeration tests), further steps to identify the effects that might be due to chlorine can be taken. Oxidants other than chlorine occur in effluents and the removal of toxicity by the addition of sodium thiosulfate does not prove that chlorine was the cause of effluent sample toxicity.

Molecular chlorine or hypochlorite dissociates into free aqueous chlorine, hypochlorous acid, and hypochlorite ion when added to effluents. Chlorine can also combine with ammonia to form chloramines, i.e., mono- , di-, and tri-chloramines and with organic compounds, especially organic nitrogen (APHA, 1992). The measured total residual chlorine (TRC) of an effluent is the concentration of free and combined forms (mentioned above) added together. The portion of the TRC associated with an individual form is matrix dependent. Chlorinated industrial and wastewater effluents normally contain only the combined form of chlorine (APHA, 1992).

These various forms of combined chlorine may have different effect concentrations for toxicity, and the toxicities of these individual forms are not all known for acute or chronic toxicity to *C. dubia* or fathead minnows. However, while the TRC level in the effluent samples may be the same, the concentration of the various forms may be different because of the matrix inherent to the effluent. This matrix of TRC may also be variable from sample to sample for the same discharger.

Another complication is that current analytical methods for measuring TRC are not chlorine specific. Other oxidizing compounds, e.g., bromine, iodine, hydrogen peroxide, ozone, and manganese, will be quantified as chlorine by the analytical methods for measuring TRC and may provide the analyst with a false positive for chlorine.

5.2 Tracking Toxicity and TRC Levels

Several methods are available for measuring total TRC (EPA methods 330.1, 330.2, 330.3, 330.4, and 330.5 (EPA, 1983)). Measurements of TRC in the effluent upon arrival of the sample at the laboratory should always be made. If TRC is not detected, chlorine should not be considered a suspect toxicant since the analytical methods do not yield false negatives.

For acutely toxic effluents, grab samples both before and after the chlorination process should be collected simultaneously (i.e., within minutes of each other). Upon arrival of these samples at the laboratory, a baseline toxicity test should be initiated and at pre-determined intervals after day 1 (e.g., day 2, day 3, day 5, day 8) to evaluate whether the toxicity is degrading. TRC determinations should be performed in conjunction with each toxicity test.

Generally the TRC in most effluent samples stored at 4°C degrades in 2 to 5 d after collection. Therefore, if residual chlorine is a toxicant the toxicity of the post-chlorination sample should decrease as TRC levels decrease, and pre- and post-chlorination samples should have the same toxicity after the decay of TRC.

The toxicity of chlorine in an effluent sample will be dependent on the matrix of the effluent and the species tested. If chlorine toxicity data does not exist for the species being used, it will be necessary to measure the LC50 or IC25 of chlorine using the TIE organisms and dilution water. Using those LC50 and/or IC25 values, the comparison of TUs of the effluent to the TUs of residual chlorine is useful to evaluate the effects of the TRC. When the TU comparison data and pre- and post-chlorination toxicity data indicate TRC as a suspect toxicant Phase III procedures should be initiated.

With the measurable levels of TRC at sample collection, the loss of toxicity with the corresponding decreasing levels of TRC, and the pre- and post-chlorina-

tion samples exhibiting similar toxicity with the decrease in TRC, Phase III confirmation should begin (EPA, 1989.6; EPA, 1993A). However, these steps will not insure that the toxicant is chlorine since other oxidants will be detected by the TRC measurement techniques.

Section 6
Identifying Toxicants Removed by Filtration

6.1 General Overview

If the results of Phase I tests indicate that the filtration manipulation removed or reduced toxicity, the investigator should carefully compare these results to those of the other manipulations before trying to identify the toxicants that might be on the filter. We have observed that metals, non-polar organic compounds and volatile compounds can all be removed under certain filtering conditions, but these observations have been dependent on the individual effluent or the sediment pore water samples. Other Phase I manipulations (e.g., EDTA, C_{18} SPE extraction) can lead to subsequent Phase II identification steps. However, for toxicity reductions effected by filtration, more intermediate steps of Phase I type manipulations must be done before analytical procedures are used to identify the toxicant(In addition, some other manipulations may provide specific information regarding the identity of toxicants that may have been removed by filtration; these include additions of PBO (Section 2.5.1), the graduated pH test (Phase I tests for determining toxicity caused by ammonia, metals and ionizable organic compounds), and the sodium thiosulfate test (Phase I test for detecting toxicity caused by volatile oxidants such as chlorine or metals). If one or more of these manipulations removes toxicity, then identification work should proceed as described in the previous sections to identify the cause of toxicity.

It is important to consider that all toxicity removed by filtration may not be actually removed by the process of filtering. For example, when the pH of the sample is altered, the mechanism(s) for removal by filtration can change. While ammonia is predominantly ionized at a sample pH of 8.3, the ammonia would not tend to be removed through volatilization if a vacuum was applied for filtration purposes. Yet by adjusting the sample pH to 11, the ionized ammonia concentration decreases to 1.7% at 25°C. When a sample is adjusted to a pH of 11, volatilization of a toxicologically significant amount of the un-ionized ammonia could occur and the toxicity results would indicate that filtration removed toxicity. Also, changes in speciation at elevated pH render many metals insoluble, which could result in their removal by the filter at pH 11 (Schubauer-Berigan et al., 1993B).

If the toxicant is thought to be a non-polar organic toxicant, and filtration partially removes toxicity, it may be useful and save toxicity testing time to eliminate the filtration step altogether before applying the sample to the C_{18} SPE column (discussion in Section 2.2.2 and Section 2.3.2).

6.2 Filter Extraction

When filtration has been the only manipulation to affect the toxicity, then extraction of the filters and tracking the toxicity of the extracts should be attempted. In addition, the use of other types of filters should be evaluated (i.e., nylon, teflon, and polycarbonate) to see if toxicity removal is a function of the filter type. In using the extraction procedures, the idea is to separate the toxic compounds associated with the filter by extracting them into a solvent. Next, efforts are made to concentrate the toxic compounds in the filter extract and test them at a concentration that can be related to the original sample and evaluate the efficiency of the extraction. Identifying the filter-removable contaminants without additional information can be difficult because of the lack of specificity of the filtration process. But once a suspect candidate has been discovered, then measurements can be made to determine whether a toxicologically significant concentration of the suspect toxicant(s) had indeed been removed by filtration. If this is the case, then it may not be necessary to consider further extractions of the filters. If, however, the concentrations of the suspect toxicant(s) are not decreased after filtration then it may be useful to attempt additional identifications by solvent extraction of the filters as described below.

One technique we have used with filterable toxicity is to extract the filters with either polar or non-polar solvents. To remove toxicants from the filter we have used either organic solvents (methanol, methylene chloride) or pH 3 high purity water as the extraction solvent. The solvent is then toxicity tested to track toxicity (methylene chloride must first be exchanged into methanol), additional Phase I tests are performed to characterize the filter extract, and then chemically analyzed using Phase II procedures. It is important to remove all of the methylene chloride before toxicity testing a filter extract and these procedures are described in detail in Section 2.6.2. To date, methanol has been used to extract toxicity from filters used with effluent samples and methylene chloride/ methanol solutions have been used to extract filters from

sediment pore water. The experiences described below are based on acute toxicity experiments, and efforts to recover filterable toxicity for chronically toxic effluent have not yet been needed.

To isolate a toxicant removed through filtration, several filters can be combined and extracted simultaneously if necessary. **The volume of sample passed** through the filters is important for calculating concentration factors, and should be recorded. The filtrate should also be reserved **for toxicity comparisons and analytical** testing. Sufficient sample should be passed through the filter to allow for both toxicity testing and chemical analysis on both the filtered sample and the filter-extract solution (generally >200 ml). Carefully move the filters to a glass (acid leached) or plastic beaker, then soak the filters (l-5) in 20 ml of solvent for 1 h. Cool water sonication is optional to attempt to recover particle-associated compounds. Carefully remove the filters and save (store at 4°C) in case additional extractions are necessary. If pH 3 high purity water is used as the extraction solvent, the extract should be readjusted to the initial pH of the sample, then toxicity tested. If methanol is used, it is evaporated to -2 ml under a stream of nitrogen. Be careful to rinse the sides of the containers with methanol to ensure complete solubilization of organic compounds. This methanol solution can then be toxicity tested using SPE fraction testing procedures (Section 2.2.8). Alternatively, if a methylene chloride/methanol solvent is used, the solvent should first be exchanged into pure methanol (Section 2.6.2), then treated as the methanol extract described above. The concentration of the solvent extract will depend on the volume originally passed through the filters, which depends on the desired high test concentration, and the volume of extract and filtered sample required for analytical purposes. Blank filters (through which has been passed a volume of dilution water) should be extracted and tested identically to the sample filters to ensure that the solvents do not introduce artifactual toxicity.

For any of the extraction techniques, the solutions should be tested at the same time as the baseline test (unfiltered) and filtered sample test to compare the toxicity recovered by the filter extraction with that removed from the sample.

Another option for toxicants removed by filtration is to try other techniques to remove the toxicants which avoid filtration. For example, sediment **pore water samples have been centrifuged at relatively high speeds** (10,000-20,000 g) for 30 min prior to passing the sample over the C_{18} SPE column and filtration could thus be eliminated.

Filter extractions (EPA, 1991 A) have been **used** in several sediment TIE studies, with procedures suggested for both non-polar organics and metals (Schubauer-Berigan et al., 1990; Schubauer-Berigan and Ankley, 1991). In some effluent and pore water samples, toxicity thought to be caused by non-polar organic compounds (e.g., PAHs and polymers) has also been removed by filtration. These compounds may be associated with particulate material, and be physically filtered from the sample, or removed by association with oil and grease that sorbs to the filter.

In some cases, binding of metals or organic compounds to inorganic and organic ligands in effluents or sediment pore waters will reduce their bioavailability and when toxicity testing filter extracts, it is always a concern that the matrix of the sample has been removed and that chemicals might become available when they were not in the original sample. If this were to happen, the extracts might be more toxic than expected and chemicals might be added to the suspect toxicant list erroneously. This kind of mistake should be caught by obtaining a good toxicity value for the suspect in an appropriate matrix (more detailed discussion in Section 26.1). Therefore, additional confirmation steps might be needed to eliminate the false suspects.

Section 7
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Appendix A
Effluent Volume Calculation Worksheets

Table A-l. Effluent Volume Calculation Worksheets

SPE Fractionation of the Effluent

1) Volume of effluent: See Table 2-6 for initial suggestions for the volume of effluent.	ve	_____	ml
2) SPE fractionation:			
Eluate volume from the SPE column: See Table 2-l for approximate eluate volume or measure volume.	**a**	_____	ml
Concentration factor for eluate: **b, = ve ÷ a**	b_1	_____	x
3) Testing organism and conditions:			
Toxicity test volume/replicate:	**c**	_____	ml
C. *dubia* 1 O-l 5 ml/replicate (acute/chronic)			
D. magna 1 O-l 5 ml/replicate (acute)			
D. pulex 1 O-l 5 ml/replicate (acute)			
P. piomelas 1 **O-200** ml/replicate (acute)			
P. promelas 50-250 ml/replicate (chronic)			
Number of replicates:	**d**	_____	
Initial sample + number of renewals	**e**	_____	
Highest test concentration:	**f**	_____	x
Is the methanol concentration okay?' ml eluate = **f x c + b,** %methanol = ml eluate x 100 ÷ c			
4) Volume of eluate needed for toxicity testing:			
If no dilutions: $g = c \times d \times e \times f \div b_1$	**g**	_____	ml
If using 0.5 dilution factor: $h = 2 \times c \times d \times e \times f \div b_1$	h	_____	ml
If using dilutions by spiking each concentration[2] directly: $i = f + f \div 2 + f \div 4 + f \div 8$ $j = i \times c \times d \times e \div b_1$	**j**	_____	**ml**
Total volume of eluate used: **k = g, h,** or **j**	**k**	_____	ml
5) Volume of eluate remaining after toxicity testing: $m_1 = a - k$	m_1	_____	ml

'Acceptable levels of methanol for C. *dubia* and fathead minnows are ≤0.6% and 1%, respectively.
[2]An example of using four test concentrations, the number of dilutions may vary.

Table A-I. Continued

Toxicity Testing and GC/MS Analysis of the SPE Concentrate

1) SPE Concentration of the SPE fraction:
Eluate volume from the SPE column: **a** _____ **ml**
See Table 2-3 for approximate **eluate** volume or measure **volume.**
Concentration factor for the eluate: $b_2 = b, x\ m, + a$ b_2 _____ x

2) Testing organism and conditions:
Toxicity test volume/replicate: **c** _____ ml
C. dubia **1** O-l 5 ml/replicate (acute/chronic)
D. magna **1 O-25** ml/replicate (acute)
D. pulex 1 **O-25** ml/replicate (acute)
P. promelas **1 O-200** ml/replicate (acute)
P. promelas **50-250** ml/replicate (chronic)
Number of replicates: **d** _____
initial sample + number of renewals: **e** _____
Highest test concentration: **f** _____ x

3) Volume of eluate needed for toxicity testing:
If no dilutions: $g = c \times d \times e \times f + b_2$ **g** _____ ml

If using 0.5 dilution factor:
$h = 2 \times c \times d \times e \times f + b_2$ **h** _____ ml

If using dilutions by spiking each concentration directly:
$i = f + f{+}2 + f{+}4 + f{+}8$
$j = i \times c \times d \times e + b_2$ **j** _____ ml

Total volume of eluate **used: k = g, h,** or **j** **k** _____ ml

4) Amount of eluate used for GC/MS analysis: **l** _____ ml

5) Volume of eluate remaining after toxicity testing:
$m_2 = a - k - l$ m_2 _____ ml

Table A-l. Continued

HPLC Fractionation of the SPE Concentrate

1) HPLC Fractionation of the SPE concentrate:			
HPLC Fraction volume:		**a**	_____ ml
See Sections 2.2.10 and 2.3.10			
Concentration factor for the eluate: $b_3 = b_2 \times m_2 + a$		b_3	_____ x
2) Testing organism and conditions:			
Toxicity test volume/replicate:		**c**	_____ ml
C. *dubia*	1 O-l 5 ml/replicate (acute/chronic)		
D. magna	1 O-25 ml/replicate (acute)		
D. pulex	1 O-25 ml/replicate (acute)		
P. promelas	1 O-200 ml/replicate (acute)		
P. promelas	50-250 ml/replicate (chronic)		
Number of replicates:		**d**	_____
Initial sample + number of renewals:		**e**	_____
Highest test concentration:		**f**	_____ x
3) Volume of eluate needed for toxicity testing:			
If no dilutions: $g = c \times d \times e \times f \div b_3$		**g**	_____ ml
If using 0.5 dilution factor:			
$h = 2 \times c \times d \times e \times f \div b_3$		h	_____ ml
If using dilutions by spiking each concentration directly:			
$i = f + f \div 2 + f \div 4 + f \div 8$			
$j = i \times c \times d \times e \div b_3$		**j**	_____ ml
Total volume of eluate used: **k = g, h,** or **j**		**k**	_____ ml
5) Volume of eluate remaining after toxicity testing:			
$m_3 = a - k - l$		m_3	_____ ml

Table A-1. Continued

Concentration of the HPLC Fraction for Toxicity Testing and GC/MS Analysis

1) SPE Concentration of the SPE fraction:			
Eluate volume from the SPE column:		**a**	_____ ml
See Table 2-3 for approximate eluate volume or measure volume.			
Concentration factor for the eluate: $b_4 = b_3 \times m_3 + a$		b_4	_____ x
2) Testing organism and conditions:			
Toxicity test volume/replicate:		**c**	_____ ml
C. *dubia*	*1* O-l 5 ml/replicate (acute/chronic)		
D. magna	1 O-25 ml/replicate (acute)		
D. pulex	1 O-25 ml/replicate (acute)		
P. promelas	1 O-200 ml/replicate (acute)		
P. promelas	50-250 ml/replicate (chronic)		
Number of replicates:		**d**	_____
Initial sample + number of renewals:		**e**	_____
Highest test concentration:		**f**	_____ x
3) Volume of eiuate needed for toxicity testing:			
If no dilutions: $g = c \times d \times e \times f \div b_4$		**g**	_____ ml
If using **0.5** dilution factor: $h = 2 \times c \times d \times e \times f \div b_4$		**h**	_____ ml
If using dilutions by spiking each concentration directly: $i = f + f \div 2 + f \div 4 + f \div 8$ $j = i \times c \times d \times e \div b_4$		**j**	_____ ml
Total volume of eluate used: **k = g, h,** or **j**		**k**	_____ ml
4) Amount of eluate used for GC/MS analysis:		**l**	_____ ml
5) Volume of eluate remaining after toxicity testing: $m_4 = a - k - l$		m_4	_____ ml

Table A-2. Effluent Volume Calculation Worksheets (Example)

SPE Fractionation of the Effluent

1) Volume of effluent: See Table 2-6 for initial suggestions for the volume of effluent.	ve	20,000 ml
2) SPE fractionation:		
Eluate volume from the SPE column: See Table 2-1 for approximate eluate volume or measure volume.	a	40 ml
Concentration factor for eluate: **b, ▫ ve ÷ a**	b_1	500 x
3) Testing organism and conditions:		
Toxicity test volume/replicate:	c	10 ml
C. *dubia* 1 O-l 5 ml/replicate (acute/chronic) *D. magna* 1 O-l 5 ml/replicate (acute) *D. pulex* 1 O-1 **5** ml/replicate (acute) *P. promelas* **1 O-200** ml/replicate (acute) *P. promelas* 50-250 ml/replicate (chronic)		
Number of replicates:	d	5
Initial sample + number of renewals	e	7
Highest test concentration:	f	2 x
Is the methanol concentration okay?[1] ml eluate = **f x c ÷ b,** %methanol = ml eluate x 100 ÷ c		
4) Volume of eluate needed for toxicity testing:		
If no dilutions: **g = c x d x e x f ÷ b_1**	g	ml
If using 0.5 dilution factor: **h = 2 x c x d x e x f ÷ b_1**	h	ml
If using dilutions by spiking each concentration* directly: **i = f + f÷2 + f÷4** **j = i x c x d x e ÷ b_1**	j	2.45 ml
Total volume of eluate used: **k = g, h,** or **j**	k	2.45 ml
5) Volume of eluate remaining after toxicity testing: **m_1 = a - k**	m_1	m37.55 l

'Acceptable levels of methanol for *C. dubia* and fathead minnows are 10.6% and 1%, respectively.
*Example uses three test concentrations.

Table A-2. Continued

Toxicity Testing and **GC/MS** Analysis of the SPE Concentrate

1) SPE Concentration of the SPE fraction:		
Eluate volume from the SPE column: See Table 2-3 for approximate eluate volume or measure volume.	**a**	0.44 ml
Concentration factor for the eluate: $b_2 = b, \times m_1 \div a$	b_2	42,670 x
2) Testing organism and conditions:		
Toxicity test volume/replicate:	**c**	10 ml
C. dubia 10-15 ml/replicate (acute/chronic)		
D. magna 10-25 ml/replicate (acute)		
D. pulex 10-25 ml/replicate (acute)		
P. piomelas 10-200 ml/replicate (acute)		
P. promelas 50-250 ml/replicate (chronic)		
Number of replicates:	d	5
Initial sample + number of renewals:	e	7
Highest test concentration:	f	4 x
3) Volume of eluate needed for toxicity testing:		
If no dilutions: $g = c \times d \times e \times f \div b_2$	**g**	ml
If using 0.5 dilution factor: $h = 2 \times c \times d \times e \times f \div b_2$	h	ml
If using dilutions by spiking each concentration directly: $i = f + f \div 2 + f \div 4$ $j = i \times c \times d \times e \div b_2$	**j**	0.057 ml
Total volume of eluate used: k = g, h, or **j**	**k**	0.057 ml
4) Amount of eluate used for GC/MS analysis:	**l**	0.018 ml
5) Volume of eluate remaining after toxicity testing: $m_2 = a - k - l$	m_2	0.365 ml

Table A-2. Continued

HPLC Fractionation of the SPE Concentrate

1) HPLC Fractionation of the SPE concentrate:
HPLC Fraction volume:
See Sections 2.2.10 and 2.3.10
Concentration factor for the eluate: $b_3 = b_2 \times m_2 \div a$

2) Testing organism and conditions:
Toxicity test volume/replicate:

C. *dubia*	1 O-l 5 ml/replicate (acute/chronic)
D. magna	1 O-25 ml/replicate (acute)
D. pulex	1 O-25 ml/replicate (acute)
P. promelas	1 O-200 ml/replicate (acute)
P. *promelas*	50-250 ml/replicate (chronic)

Number of replicates:	d	5	
Initial sample + number of renewals:	e	7	
Highest test concentration:	f	8	x

3) Volume of eluate needed for toxicity testing:
If no dilutions: $g = c \times d \times e \times f \div b_3$ — g _____ ml

If using 0.5 dilution factor:
$h = 2 \times c \times d \times e \times f \div b_3$ — h _____ ml

If using dilutions by spiking each concentration directly:
$I = f + f \div 2 + f \div 4$
$j = I \times c \times d \times e \div b_3$ — j 0.315 ml

Total volume of eluate used: $k = g$, h, or j — k 0.315 ml

5) Volume of eluate remaining after toxicity testing:
$m_3 = a - k$ — m_3 0.685 ml

Table A-2. Continued

Concentration of the HPLC Fraction for Toxicity Testing and GC/MS Analysis

1) SPE Concentration of the SPE fraction:		
Eluate volume from the SPE column: See Table 2-3 for approximate eluate volume or measure volume.	**a**	0.22 ml
Concentration factor for the eluate: $b_4 = b_3 \times m_3 \div a$	b_4	48.495 x
2) Testing organism and conditions:		
Toxicity test volume/replicate:	**c**	10 ml
C. ***dubia*** ***1*** O-l 5 ml/replicate (acute/chronic)		
D. magna 1 O-25 ml/replicate (acute)		
D. pulex 1 O-25 ml/replicate (acute)		
P. promelas 1 O-200 ml/replicate (acute)		
P. promelas 50-250 ml/replicate (chronic)		
Number of replicates:	**d**	5
Initial sample + number of renewals:	**e**	7
Highest test concentration:	**f**	16 x
3) Volume of eluate needed for toxicity testing:		
If no dilutions: $g = c \times d \times e \times f \div b_4$	**g**	ml
If using 0.5 dilution factor: $h = 2 \times c \times d \times e \times f \div b_4$	h	ml
If using dilutions by spiking each concentration directly: $i = f + f \div 2 + f \div 4$ $j = i \times c \times d \times e \div b_4$	j	0.202 ml
Total volume of eluate used: **k = g, h,** or **j**	k	0.202 ml
4) Amount of eluate used for GC/MS analysis:	l	0.018 ml
5) Volume of eluate remaining after toxicity testing: $m_4 = a - k - i$	m_4	0 ml

*U.S. GOVERNMENT PRINTING OFFICE:1993-750-002/80303

United States
Environmental Protection
Agency

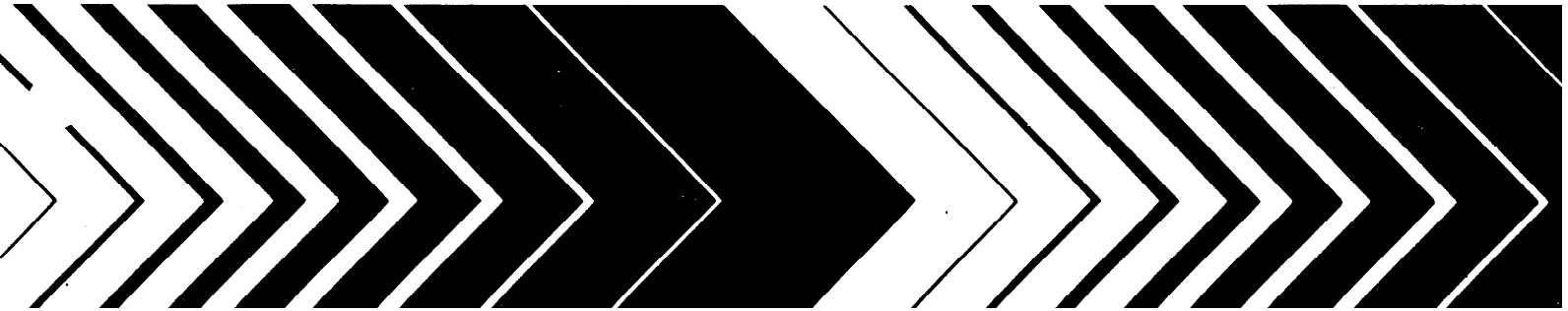
Office of Research and
Development
Washington DC 20460

EPA/600/R-92/081
September 1993

EPA

Methods for Aquatic Toxicity Identification Evaluations

Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity



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Methods for Aquatic Toxicity Identification Evaluations

Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity

by

D. I. Mount[1]
T. J. Norberg-King[2]

With Contributions from:

G. T. Ankley[2]
L. P. Burkhard[2]
E. J. Durhan[2]
M. K. Schubauer-Berigan[1]
M. T. Lukasewycz[1]

[1]ASci Corporation - Contract No. 68-C0-0058
*U.S. Environmental Protection Agency

Previous Phase III Methods
by D. I. Mount
EPA-600/3-88/036

National Effluent Toxicii Assessment Center
Technical Report 02-93

Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Duluth, MN 55804

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Foreword

This Phase III document is the last in a series of guidance documents intended to aid dischargers and their consultants in conducting aquatic organism toxicity identification evaluations (TIEs). TIEs might be required by state or federal agencies as the result of an enforcement action or as a condition of a National Pollutant Discharge Elimination System (NPDES) permit. These documents should aid individuals in overseeing and determining the adequacy of effluent TIEs as a part of toxicity reduction evaluations (TREs).

There are two major reasons to require the confirmation procedures. First the effluent manipulations used in Phase I characterizations (EPA, 1988; EPA, 1991 A; EPA, 1992) and Phase II identifications (EPA, 1989A; EPA, 1993A) might (with some effluents) create artifacts that might lead to erroneous conclusions about the cause of toxicity. Therefore in Phase III confirmation steps, manipulations of the effluent are avoided and/or are minimized, therefore artifacts are far less likely to occur. Sometimes, toxicants will be suspected through other approaches (such as the treatability route) which on their own are not definitive and in these instances, confirmation is necessary. Secondly, there is the probability that the substances causing toxicity might change from sample to sample, from season to season or some other periodicity. As toxicity is a generic measurement, measuring toxicity cannot reveal variability of the suspect toxicant whereas the Phase III confirmation procedures are designed to indicate the presence of variable toxicants. Obviously, this crucial information is essential so that remedial action may be taken to remove toxicity.

Confirmation, whether using the procedures described in this document or others, should always be completed because the risk is too great to avoid or eliminate this step. Especially for discharges where there is little control over the influent or for discharge operations that are very large or complex, the probability that different constituents will cause toxicity over time is great. Most of the approaches in Phase III are applicable to chronically toxic effluents and acutely toxic effluents.

In this confirmation document, guidance is included when the treatability approach (EPA, 19898; EPA, 1989C) is taken. Use of the treatability approach requires confirmation as much as or more than the toxicant identification approach (Phase II). The reader is encouraged to use both the acute Phase I characterization (EPA, 1991 A) and the chronic Phase I characterization (EPA, 1992) documents for details of quality assurance/quality control (QA/QC), health and safety, facilities and equipment, dilution water, sampling and testing. The TIE methods are written as general guidance rather than rigid protocols for conducting TIEs and these methods should be applicable to other aqueous samples, such as ambient waters, sediment elutriate or pore waters, and leachates

Abstract

In 1989, the guidance document for acutely toxic effluents entitled *Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures* was published (EPA, 19890). This new Phase Ill manual and its companion documents (EPA, 1991 A; EPA, 1992; EPA, 1993A) are intended to provide guidance to aid dischargers in confirming the cause of toxicity in industrial and municipal effluents. The toxicity identification evaluation (TIE) starts with a characterization of the effluent toxicity using aquatic organisms to track toxicity; this step is followed by identifying a suspect toxicant(s) and then confirming the suspect toxicant as the cause of toxicity.

This Phase III confirmation document provides greater detail and more insight into the procedures described in the acute Phase III confirmation document (EPA, 1989D). Procedures to confirm that all toxicants have been correctly identified are given and specific changes for methods applicable to chronic toxicity are included. Adifficult aspect of confirmation occurs when toxicants are not additive, and therefore the effects of effluent matrix affecting the toxicants are discussed. The same basic techniques (correlation, symptoms, relative species sensitivity, spiking, and mass balance) are still used to confirm toxicants and case examples are provided to illustrate some of the Phase III procedures. Procedures that describe the techniques to characterize the acute or chronic toxicity (EPA, 1988) and to identify (EPA, 1989A) toxicants have also been rewritten (EPA, 1991 A; EPA, 1992; EPA, 1993A).

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Acknowledgments

This document presents additional information acquired since the document entitled *Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures* (EPA-60013-88-036; EPA, 1989D) was prepared by Donald Mount and published in 1989. This manual reflects new information, techniques, and suggestions made since the Phase III confirmation methods for acute toxicity were developed. The suggestions, techniques and cautions contained in this document are based on a large database generated by the staff of the National Effluent Toxicity Assessment Center (NETAC) at the U.S. Environmental Protection Agency (EPA), Environmental Research Laboratory, Duluth (ERL-D), MN. NETAC staff that provided technical support consisted of Penny Juenemann and Shaneen Schmidt (ERL-D staff), Joe Amato, Lara Anderson, Steve Baker, Tim Dawson, Nola Englehorn, Doug Jensen, Correne Jenson, Jim Jenson, Elizabeth Makynen, Phil Monson, Greg Peterson, and Jo Thompson (contract staff). Their collective experience has made this document possible and the contributions are gratefully acknowledged. The support through EPA's Office of Research and Development (ORD) and Office of Water made this research possible at ERL-D.

Section 1
Introduction

The final confirmation phase of a toxicity identification evaluation (TIE) consists of a group of steps intended to confirm that the suspect cause(s) of toxicity is correctly identified and that all the toxicity is accounted for. Typically this confirmation step follows experiments from the toxicity characterization step (Phase I) and analysis and additional experiments conducted in toxicity identification (Phase II) (EPA, 1991A; EPA, 1992; EPA, 1993A). However, there often may be no identifiable boundary between phases. In fact, all three phases might be underway concurrently with each effluent sample and depending on the results of Phase I characterization, the Phase II identification, and Phase III confirmation activities might begin with the first sample evaluated. Phase III confirmation procedures should also follow after toxicants have been identified by other means or when treatability approaches are used. Rarely does one step or one test conclusively prove the cause of toxicity in Phase III. Rather, all practical approaches are used to provide the weight of evidence that the cause of toxicity has been identified. The various approaches that are often useful in providing that weight of evidence consist of correlation, observation of symptoms, relative species sensitivity, spiking, mass balance estimates and various adjustments of water quality.

The approaches described in this document have been useful in TIEs at ERL-D. While the guidance provided in this manual is based largely on experience with wastewater effluents, in general the methods discussed are applicable to ambient waters (Norberg-King et al., 1991) and sediment pore or elutriate water samples as well (EPA, 1991B). However, specific modifications of the TIE techniques might be needed (e.g., sample volume) when evaluating these other types of samples.

Confirmation is important to provide data to prove that the suspect toxicant(s) is the cause of toxicity in a series of samples and to assure that all other toxicants are identified that might occur in any sample over time. There may be a tendency to assume that toxicity is always caused by the same constituents, and if this assumption carries over into the data interpretation but the assumption is false, erroneous conclusions might be reached. That is why the correlation step (Section 2) is accompanied by other approaches (i.e., Sections 3-9) because each approach aids in revealing any changes in the toxicant(s) in the confirmation phase of the TIE.

Seasonal trends in toxicants have been observed in publicly owned treatment works (POTW) effluents and some sediment samples. For example, organophosphate pesticides have been observed to increase in concentrations in wastewaters during the late winter and spring months (Norberg-King et al., 1989). Therefore, the confirmation steps of Phase III might need to include seasonal samples. This effort cannot always be pre-determined. The presence of a different toxicant(s) must be considered throughout the TIE, and when samples are collected over several months the seasonality of a suspect toxicant should be carefully considered and studied. When remedial action requires treatment changes, one must be certain that toxicity from specific toxicant(s) is consistently present and that the suspect toxicant(s) accounts for all the toxicity. Treatment modifications will not necessarily result in removal of all toxicants to acceptable concentrations. If toxicity is caused by a variety of toxicants present at varying intervals, the remedial actions that are practical might differ from the remedial action required when toxicity is caused by the same constituents consistently.

TIEs conducted at ERL-D have shown that toxicants often are not additive or toxicants are present in ratios such that the toxicity contribution by one might be diluted out in the range of the effluent effect concentration (e.g., LC50 or ICp value). Thus, the toxicant present at lower yet toxic concentrations may not be readily discerned. The frequency of occurrence and impact on data interpretation of either of the above cases was not addressed previously (EPA, 1989D) but are now discussed in Section 2. Toxicants that do not express their toxicity because of the presence of other toxicants (either the toxicants are non-additive or the toxicants occur in disparate ratios) are referred to as hidden toxicants (Section 9). Detection of hidden toxicants is one of the most difficult aspects of confirmation. It is a mistake to search for a concentration of any chemical present in the effluent at a toxic concentration and to declare any found as the cause

of toxicity. Matrix effects of the effluent samples make conclusions such as these subject to error without further work as either the hidden toxicant(s) or the principal toxicant(s) are likely to be missed using such an approach.

There is a strong tendency to shorten or eliminate the confirmation steps because by the time Phase III confirmation has been reached, the investigators might be convinced of the cause of toxicity and the confirmation steps seem redundant. However, one cannot expect to concentrate the effluent on a C_{18} solid phase extraction (SPE) column and not change a complex mixture such as effluents, and arrive at some false conclusions about the toxicants in the earlier phases.

Not all approaches discussed in the following sections will be applicable to every effluent, and additional approaches might need to be developed during the TIE. The various approaches need not be performed in any particular sequence, and the list of possible approaches will get larger as experience is gained. To effectively evaluate effluent samples from one particular discharger to obtain a correlation, substantial calendar time could be required and any steps for correlation should be initiated at the beginning stages of Phase III. Judgement must be made as to how many of the approaches described in Phase III confirmation should be used and how many samples for each should he completed. How completely Phase III confirmation is done will determine the authenticity of the outcome. The amount of confidence in the results of the TIE that is required is dependent at least in part on the significance of the decision that will be based on the results. For example, if a suspect toxicant can be removed by pretreatment or by a process substitution, a higher degree of uncertainty may be acceptable than if an expensive treatment plant is to be built. Such considerations are subjective and cannot be reduced to a single recommended decision making process with a specified number of samples.

Time and resources might be conserved if identification (Phase II) and confirmation (Phase III) can be started on the very first effluent sample used in the Phase I characterization. However, this is only possible when the results from the Phase I characterization are definitive enough to allow the investigators to proceed to identification and confirmation. In the acute Phase III confirmation document (EPA, 1989D), although perhaps not explicitly stated, performing Phase I characterizations on several samples before attempting Phases II and III was implied. Initiating the Phase III confirmation steps earlier in the TIE is often particularly useful. In addition, many regulatory agencies have adopted a policy that requires that the previous TIE approach be modified. For some dischargers, action might be required after the first exceedence in toxicity, which means that each effluent sample collected for toxicity testing is of equal regulatory concern when the toxicity is greater than the permit allows. This regulatory practice was not in place in 1989 when the earlier TIE guidance was available (EPA, 1989D) and at that time we did not expect that the cause of toxicity in one sample could be sufficiently deduced as we have been able to do. The importance of confirmation on several samples is not reduced by the importance of conducting confirmation steps on single samples; rather, the cause of toxicity for each sample must be confirmed.

In addition to the importance of each sample with toxicity greater than the allowable amount specified in a permit, a sample that is quite different from the previous samples must be evaluated to determine if the data point must be included in the Phase III correlation final data analyses. For each effluent sample, the data points must be explainable. If one sample is quite different than other samples it can cause the correlation to be less useful; however, if it can be shown to have a different toxicant the data point for that sample can be eliminated from the correlation. For example, suppose five consecutive samples during a Phase III evaluation exhibited toxicity that correlated well with a suspect toxicant. Then a sixth sample exhibits greater toxicity than previous samples while the measured concentration of the suspect toxicant is much lower than measurements on previous samples. In this sixth sample, the greater toxicity is thought to be caused by a different toxicant. Now in plotting the data for the correlation (Section 2), the datum point for the sixth sample will not be similar to the points for the existing regression and could render the correlation non-significant. If however, when the sixth sample is then subjected to intensive study using Phase I characterization and Phase II identification techniques, and if another toxicant is identified (or even if Phase I only shows that the toxicity has very different characteristics), datum for the sixth sample can legitimately be excluded from the correlation. This preserves the worth of the data for the previous five samples. In confirmation, every effort should be made to determine why a particular sample shows different responses in the various TIE steps from other samples.

This is not to imply that multiple effluent samples need not be subjected to Phase I manipulations, even if Phase II and/or Phase III are initiated on the first sample. Most effluent samples tend to be representative of the routine effluent discharge. However, determining what is the characteristic discharge for each effluent is important to the final success and completeness of the TIE.

When Phase III is completed, all results that were obtained during the TIE should be explainable. Unless the results make sense for all samples (aside from an occasional aberrant data point) something has been missed or is wrong. If so, the confirmation is not complete. Many techniques used in Phase III require keen observations and extensive or broad knowledge of both chemistry and toxicology but above all the ability to synthesize small bits of evidence in a logical sequence is essential. This TIE work is most effective when scientists interact daily.

A note of caution. If data obtained on early samples during Phase I are to be used for Phase III purposes, quality control will have to be suitable to provide defensible data (cf., EPA, 1991A; EPA, 1992; EPA, 1993A). In Phases I and II, the permissibility of using small numbers of animals and replicates, and omitting measurements such as pH, DO, and temperature that are required for routine monitoring tests or single chemical tests was discussed (EPA, 1989E; EPA, 1991 A; EPA, 1992; EPA, 1993A). These modifications were made to reduce cost and allow more testing, but at this point shortcuts must be avoided because definitive data that constitute the basis for important decisions are generated in Phase III. For Phase III testing, the effluent test protocols that triggered the TIE (EPA, 1991C; EPA, 1993B) should be followed, paying careful attention to test conditions, replicates, quality of test animals, representativeness of the effluent samples tested, and strict QA/QC analytical procedures including blanks and recovery measurements. Analytical work must be selective for the identity of the toxicant and its concentration measurement. When small differences in toxicity must be detected, concentration intervals should be smaller to obtain partial effects (e.g., use dilution factors of 0.60 or 0.65 versus 0.5). Remember, all of the data from Phases I and II (for either acute or chronic toxicity) are considered preliminary relative to Phase III data. However, if a suspect toxicant is identified and Phases I and II data may be necessary for confirmation, stricter QA/QC can be applied for each of the subsequent Phases I and II techniques so that the data can be used in Phase III.

For samples exhibiting chronic toxicity, modifications or 'changes to some of the TIE procedures are required for confirming the cause of chronic toxicity. Remember that for confirmation (as well as for Phases I and II), only a single sample of effluent should be used for each renewal in any chronic test (cf., EPA, 1992; EPA, 1993A). This is important because one cannot correlate a measured concentration of a toxicant with the toxicity measured in a test if multiple samples are used for each renewal and the toxicant is not present in some samples but other toxicants appear. Even more likely, the ratios of the toxicants, when more than one is present, might change from sample to sample. In these instances, there is no valid way to calculate the toxicity of a given toxicant. Overall, considerations for chronic toxicity tests in Phase III are not much different than acute toxicity tests in Phase III. At present, permit requirements specify the 7-d test and unless data are gathered to show that the 4-d and 7-d tests yield the same results and that the same toxicants are involved, the 7-d test should be used for confirmation (cf., EPA, 1993A). If the 4-d *Ceriodaphnia dubia* test has been used instead of the 7-d *C. dubia* test (see EPA, 1992) during Phases I and II, serious consideration should be given to returning to the 7-d test for Phase III.

When identification of the toxicant(s) causing chronic toxicity is desired, and the effluent also exhibits acute toxicity, it might be possible to use acute toxicity as a surrogate measure to characterize the toxicity in Phase I and assist in an identification in Phase II. It must be demonstrated that the cause of the acute toxicity is the same toxicant(s) as the toxicant(s) causing the chronic toxicity. Yet for confirmation, use of chronic toxicity endpoints to confirm the cause of the chronic toxicity is strongly recommended to avoid misleading the TIE results when using acute toxicity as a surrogate for chronic toxicity. As discussed in the chronic Phase I manual (Section 5.8; EPA, 1992), effect levels for chronic tests should be calculated using the linear interpolation method rather than the hypothesis test (EPA, 1992). In order to get more precise estimates of endpoints, test concentration intervals might have to be narrowed (see above). However, when point estimation techniques for other than survival endpoints (such as the inhibition concentration (ICp); EPA, 1993B) are used, a point estimate effect concentration can be estimated. The effect concentration estimates will also be more accurate when intermediate concentrations are used (i.e., use dilution factors of 0.6 or 0.65).

Section 2
Correlation Approach

2.1 Correlation

The purpose of the correlation approach is to show whether or not there is a consistent relationship between the concentration of suspect toxicant(s) and effluent toxicity. For the correlation approach to be useful, the toxicity test results with the effluent must demonstrate a wide range of toxicity with several effluent samples to provide an adequate range of effect concentrations for the regression analysis. For sediment samples, spatial variability might be used to perform correlation analyses (EPA, 19916).

The effluent effect concentration (i.e., LC50 or ICp) data and the measured toxicant concentration data must be transformed to toxic units (TUs) for the regression analysis to evaluate whether or not a linear relationship exists. Effluent TUs are obtained by dividing 100% by the effect concentration expressed in percent of the effluent (cf., EPA, 1991A; EPA, 1992). The suspect toxicant concentration is converted to TUs by dividing the measured toxicant concentration by the LC50 or ICp for that toxicant (data to make this comparison might have to be generated; EPA, 1993A). If more than one toxicant is present, the concentration of each one is divided by the respective LC50 or ICp value and the TUs can then be summed (cf., discussion below for non-additive toxicants).

Most of the effluents we have tested have exhibited a wide range of toxicity with several different samples and therefore the data can be used in the correlation approach. Typically for the correlations that we have conducted, the data used are from toxicity tests without any manipulations and from chemical measurements on the effluent samples for the concentrations of the suspect toxicant. However for effluents where ammonia was the cause of the toxicity, the effluent toxicity. results have not varied in toxicity enough, nor have the ammonia concentrations fluctuated enough to use the data in a correlation. Also, when the effect concentration is greater than 1 00%, this information is not useful since the data point cannot be included in the regression analysis. However, when samples are marginally toxic or when the suspect toxicant concentrations do not vary enough from sample to sample (i.e., ammonia is cause of toxicity), changes in toxicity can be induced by sample manipulation (cf., EPA, 1993A) and this toxicity data can be used to develop a different type of correlation. For example, the toxicity of a given amount of total ammonia can be changed by over an order of magnitude by altering the pH of aliquots of the effluent within an acceptable physiological range (e.g, pH 6 to 9). For some metals and some species, the toxicity can also be changed by adjusting the pH and using dilution waters of varying hardness. This type of data is useful in the correlation step as providing additional weight of evidence. Therefore, the idea of minimal manipulation(s) and any risk of creating artifactual toxicity are off set by the utility of the data.

An example of the regression from an effluent from a POTW in which the suspect toxicant was diazinon is given in Figure 2-l. The independent variable (x-axis) is the TUs of diazinon and the dependent variable (y-axis) is the effluent TUs. The solid line is the observed regression line obtained from the data points, and the dashed line is the expected or theoretical regression line. If there is 1 .O TU of the toxicant in 100% effluent, then the effluent should have 1 .0 TU (i.e., the LC50 =100%). Likewise for 2.0 TUs of suspect toxicant, the effluent TUs should be 2.0, et cetera. Thus, the expected line has a slope of one and an intercept of zero. In Figure 2-1, the intercept (0.19) is not significantly different from zero and the slope is very close to 1 (1.05). The r^2 value is 0.63 which, while not high, indicates that the majority of the effluent toxicity is explained by the concentration of the toxicant. As the r^2 becomes lower, less confidence can be placed on slope and intercept. In a small data set such as this, one datum point that had 5.0 TUs for the effluent toxicity lowered the r^2 value substantially. As discussed in Section 1, if an intensive effort had been expended on that sixth sample and another toxicant(s) had been found, this particular datum point could have been excluded and the r^2 value would have been higher.

In another POTW effluent, diazinon was also the suspect toxicant. For these data (Figure 2-2), the slope is 1.38, the intercept is 1.24 and the r^2 value is only 0.15, which all indicate poor fit for diazinon as the only toxicant. The low r^2 value indicates a large amount of scatter, therefore little can be inferred from the slope and the intercept. Based on this correlation, we returned to Phase II analytical procedures and identified two other organophosphates (chlorfenvinphos (CVP) and malathion). Toxicity data indicated that CVP was present at toxic

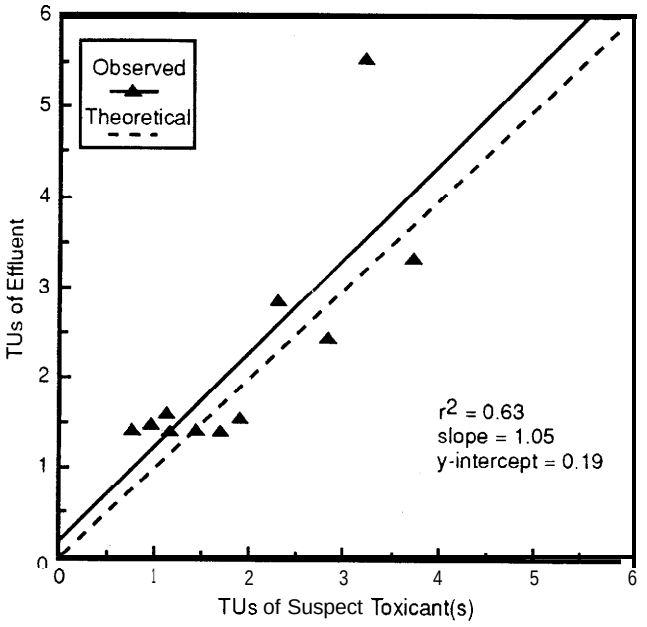

Figure 2-1. Correlation of toxic units (TUs) for an effluent and one suspect toxicant in POTW effluent.

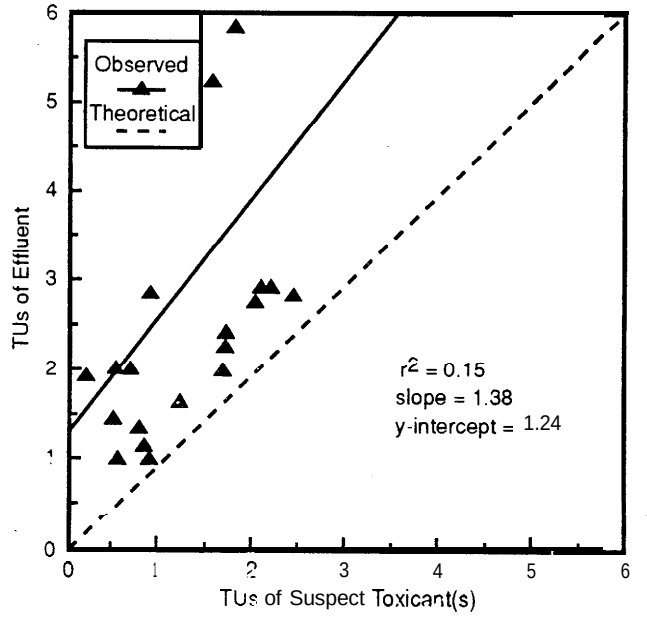

Figure 2-2. Correlation of toxic units (TUs) for an effluent and cne suspect toxicanr in a POTW effluent when two toxicants are the cause of toxicity.

concentrations while malathion was no?. After testing each compound both separately and as a mixture, the toxicity from all three chemicals was determined to be additive, so a new correlation was begun with analytical measurements made for all three chemicals. CVP and diazinon have nearly identical LC50 values for the species (C. *dubia*) used in this TIE. Malathion is about one-fourth as toxic as CVP or diazinon. Since the measured concentrations of malathion were lower than its toxicity, it was not included in the regression analysis. In a new correlation with data for the TUs summed for CVP and diazinon versus the effluent TUs, the data show a much better fit to the expected slope and intercept and a high r^2 value (Figure Z-3). Malathion TUs could also have been included in the regression (although its contribution to toxicity was minimal) because it was additive with other toxicants. This type of situation is discussed below.

In addition to slope and intercept, some judgement of the scatter about the regression line must be made. This can be done statistically, but when the sample size is large, the scatter can be very large and yet not negate the relationship. A-suggested approach to avoid the effect of sample size on the significance of scatter is to set a lower limit on r^2. This value (often expressed as percent) provides the measure of how much of the observed effluent toxicity is correlated to the measured toxicant. It is not dependent on choosing the correct effect concentration of the toxicant. The specific choice of the minimum value of r^2 should be made based upon the consequences of the decision. It is important to recognize that experimental error makes an r^2 value greater than 0.80 or 0.85 difficult to obtain. Therefore, where minimal chance of an incorrect decision is required, an r^2 value of nearly 0.80 may be used. Where an increased risk of an incorrect decision (i.e., a lesser amount of the toxicity accounted for) is acceptable, a lower value such as 0.60 may be used.

Since <1.O TU cannot be directly measured in the effluent, such values are, of necessity, excluded from the regression. (This comment is exclusive of the use of concentrates such as the C_{18} SPE fractions' where TUs of <1.O are possible.} However in some instances, when the TUs based on chemical analyses are <1.O TU and effluent effect values are <1.0 TU, the data support the validity of the regression provided a suspect toxicant has been found in several previous samples. In the correlation for the effluent toxicity depicted in Figure 2-2, toxicity was present in a different fraction (Phase II non-polar organic identification) than where the pesticides were identified. A specific toxicant was not identified in that fraction and toxicity was not always measurable in that fraction. However, this additional toxicity may have decreased the r^2 value.

Correlation might be more definitive when two or more toxicants are present. For example, suppose three toxicants are involved. If each toxicant has the same LC50 and each is strictly additive with the ratio of their concentrations remaining the same, the slope will be the expected but the intercept will be positive if all toxicants

[1]TUs can be calculated from toxicity tests with the fractions, the concentrate or the HPLC fractions as described in Phase II (EPA,' 1993A).

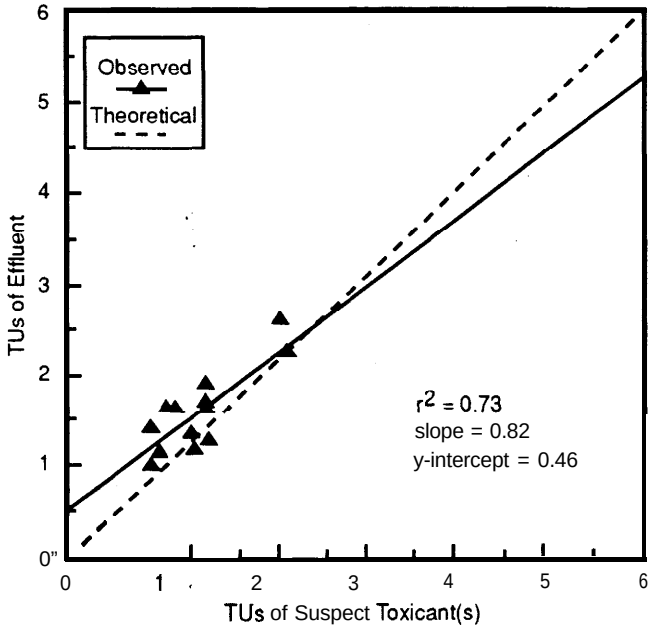

Figure 2-3. Correlation of toxic units (TUs) for an effluent and two toxicants in a POTW effluent.

are not identified. If the relative amounts (ratios) of each toxicant vary from sample to sample, the slope, intercept and r^2 will be different from the expected if only one toxicant is identified. If the toxicity of one of the toxicants is substantially different, and if the ratios of the three toxicants vary from sample to sample, then the slope, intercept, and r^2 value will all be different from expected if all are not identified. Much can be learned from studying the interrelationship of slope, intercept and the r^2 value. For example, a high r^2 value and an intercept near zero with a slope larger than 1 can be caused by using an effect concentration for the toxicant that is not appropriate for the toxicant in the effluent matrix (e.g., suspect toxicant is more toxic in effluent matrix than in single chemical test). This error causes the toxicant TUs to be too few relative to the effluent TUs (Figure 2-4) (cf., discussion below on non-additive toxicants). If toxicant concentrations and effluent toxicity show a wide distribution, a significant correlation will be easier to demonstrate than for a narrow range.

Great care must be taken to understand whether or not toxicants are additive or if the TUs for each toxicant are so different that only one toxicant determines the effect level. For either situation, the resulting data will have to be interpreted as though the toxicants are non-additive. For example, suppose the ratio of TUs is so disparate that at the effluent effect concentration, the toxicant with fewer TUs is always present at a fraction of a TU (e.g., 0.25 of a TU). Whether the two toxicants are additive or not is irrelevant because the major toxicant will set the effluent effect concentration. While 0.25 TUs of the minor toxicant appear to be relatively unimportant in view of experimental variability, this affects the regression. If in one sample the effect concentration is 25% and the 4 to 1 ratio of toxicants occurs, there are 4 TUs of the major toxicant and 1 TU of the minor toxicant. If the toxicant concentrations are summed, 5 TUs will be plotted against 4 effluent TUs, and this results in a 25% error. When secondary toxicants are present in concentrations that will not contribute to the effect concentration of the effluent, they should not be included in the correlation data set. Obviously if an effluent had several toxicants in dissimilar ratios, the error of including the minor TUs in a correlation plot could be large and may negate the correlation significance. The investigator should evaluate the data in regression plots to consider the significance of the contribution of the secondary toxicant especially if the toxicants appear to be additive.

Unfortunately the minimum fraction of a TU that is detectable will depend on the precision of the laboratory performing the testing. And of course the precision of the testing is not only dependent on the quality of the work, but the inherent precision of measuring specific toxicant TUs. That is, the toxicity measurement for some chemicals is more precise than for some other chemicals. In general, a chemical such as NaCl whose toxicity is generally not affected by pH, alkalinity, hardness, total organic carbon (TOC), suspended solids or solubility, can be measured more precisely than a chemical whose toxicity is affected by these factors, such as lead or copper. Therefore, each laboratory must determine which fractional value of a TU at the effect concentration is unmeasurable, thus indicating which TUs contributed by the minor toxicant should be deleted from the correlation data set.

Clearly, if two or more toxicants are strictly non-additive, then only the major one (the one present in the most TUs) should be included in the correlation data set. Since additivity might be easier to measure than the minimum measurable contribution of a fraction of a TU, it may be preferable to first determine if additivity occurs. If substances appear to be partially additive, then very careful work is required to properly add TUs.

Some very unusual decisions are required in accepting data into the correlation database when toxicants are strictly non-additive. For example, consider zinc and ammonia in the same effluent sample; we have found them to be strictly non-additive. Also consider that in some samples zinc and ammonia occur in TU ratios of 3 to 1 and in other samples the ratio is 1 to 2. In the regression for the 3 to 1 ratio samples, only zinc TUs should be plotted. In the regression for the 1 to 2 ratio samples, only ammonia TUs should be plotted. For this particular example, 3 TUs for the first sample and 2 TUs for the second sample would be used if the data is interpreted correctly (i.e., plotting total TUs) or 4 and 3 TUs would be used respectively, if the data is interpreted

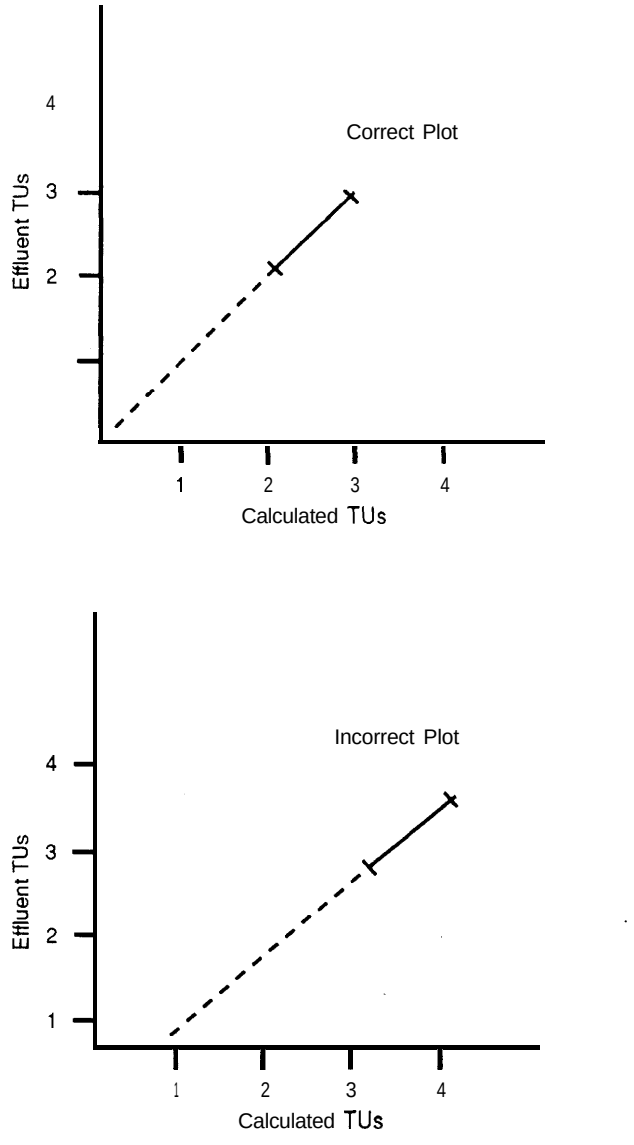

Figure 2-4. Correct (top) and incorrect (bottom) plots of toxic units (TUs) for non-additive toxicants.

incorrectly. The slopes for both plots would be 1 but a negative intercept instead of an intercept of 0 would be obtained for the incorrect plot. The more similar the TUs of each toxicant are to each other, the greater the error in the correlation will be.

2.2 Correlation Problems Caused by Matrix Effects

Correlation becomes much more difficult when the toxicants interact with the other effluent constituents in ways that change their toxicity and we refer to these changes as matrix *effects*. There are numerous matrix effects and all of them will not be discussed here; instead a framework is provided to aid in designing tests or test conditions to validly incorporate matrix effects in such a manner that useable correlation data can be obtained.

Matrix effects generally fit into one of two categories. One category is when the toxicants change form in some manner which exhibit a different toxicity. A very common example is ammonia which changes from NH_3 to NH_4^+ as pH decreases. NH_4^+ is so much less toxic than NH_3 that it is often considered nontoxic? Another example is HCN whose most toxic form is as un-dissociated HCN, a form predominating at low pH values. As pH increases the equilibrium shifts to more H^+ and CN^-. If metals are present, metal-cyanide complexes form which are often less toxic than HCN but metal-cyanide complexes might vary in toxicity depending on the metal. For example, iron-cyanide complexes are much less toxic than some of the other metal complexes. Metal-cyanide complexes might also photodecompose in sunlight releasing HCN or H^+ and CN^-, depending on pH.

A second category of matrix effects involves such physical changes as sorption or binding in some manner so as to make the toxicant unavailable to the organism. For example, non-polar organics sorb onto suspended solids, and some metals, such as copper, also sorb onto suspended solids. The presence of organic matter on suspended solids might increase the sorbtive capacity. Predictably, changes in water chemistry often change the sorption/solution equilibrium and thereby, change the portion of total toxicant that is available to the organism.

To further complicate matters, biological characteristics of the test organisms might change the availability of the same toxicant form. For example a non-polar organic sorbed on suspended solids such as bacterial cells, might be unavailable to a fish but readily available to daphnids because cells might be ingested and digested by daphnids. The uptake route then is through the digestive tract but the toxicant has entered the body none-the-less.

From the above discussion, it is obvious that one method of correlation will not be applicable for all toxicants. A temptation may be to remove the toxicant from the effluent and then use the effluent as a diluent to measure toxicity. However, because effluents are so complex and undefined, there is virtually no way to remove one or a few constituents and still be certain other characteristics have not been changed. For example, zeolite removes ammonia but it also removes some metals and non-polar organics; the C_{18} resin removes metals as well as non-polar organics; ion exchange columns remove ionized constituents, but non-polar organics also are retained by the columns. Toxicant removal procedures have utility but require very complicated simultaneous testing of the effluent and proper blanks (cf., EPA, 1992; EPA,

[2]See specific discussion in Section 3, Phase II (EPA, 1993A).

1993A) is necessary to properly interpret results (cf., Section 9 on hidden toxicants).

In Phase III, quantitative comparisons are being made between toxicity and concentrations of toxicants rather than qualitative comparisons as in Phases I and II (EPA, 1991A; EPA, 1992; EPA, 1993A). In the correlation approach, such comparisons are the essence of the technique. Therefore even small changes in form or availability might be unacceptable. This means that manipulations and changes must be minimized when effluent toxicity and toxicant concentrations are to be compared.

Solvent extraction, so commonly used for organic analyses, is likely to extract biologically unavailable organics as well as soluble forms. The total measured concentration may be larger than the true exposure concentration. Use of the C_8 SPE column also is not free from problems as the C_{18} SPE column is a finer filter than the glass fiber filters commonly used for pre-column filtration. Therefore solids are likely to be physically retained on the upper part of the column. When the column is eluted with methanol, the methanol extracts toxicant(s) from the solids (which might not be biologically available) as well as elutes the C_{18} sorbent itself. For Phases I and II, this might be unimportant, but for the Phase III correlation step where careful quantitative comparison is necessary, the effect might be unacceptable. Such problems probably reach a maximum when working with samples such as highly organic sediment pore water (with high organic characteristics) where much of the chemical might be biologically unavailable.

The central problem for either type of matrix effect is the difficulty of analytically measuring the biologically available portion of the specific toxic form. A correlation for a POTW effluent where for nickel was suspected of causing the toxicity is shown in Figure 2-5. During Phase I, the acute toxicity was removed with EDTA additions, and in Phase II the nickel was measured at toxic concentrations to C. *dubia.* The toxicity correlated very well with total nickel concentration (r^2 = 0.89 and a slope of 1 .17) and it appeared that only nickel seems to be involved. But the intercept of -12.34 is quite different from the expected zero. Such an intercept would be expected if there were a relatively fixed amount of nickel which was not biologically available in all samples. In this example, because all other confirmation data corroborated nickel as the toxicant, a constant concentration of nontoxic nickel was thought to provide the explanation for the unexpected intercept value. However, there is no obvious reason to think that the quantity, or even the percentage of total toxicant, is the same across samples for other toxicants, or for nickel in other matrices.

For the effluent samples that lose their toxicity in a short time, the nontoxic effluent can be used for the suspect toxicant(s) tests as a diluent in parallel tests using a standard dilution water to elucidate matrix effects on toxicity. Toxicity test results with quite different toxicity would reflect matrix effects. If toxicity is persistent, developing two separate correlations using pure chemical additions on two different effluent samples, each with substantially different toxicant concentrations, might be useful. If the toxicity test results indicate that the biologically unavailable portion changes with measured concentrations, the slope should be different than one. This approach requires careful work and the investigator must consider incorporating equilibrium time experiments (cf., EPA, 1993A).

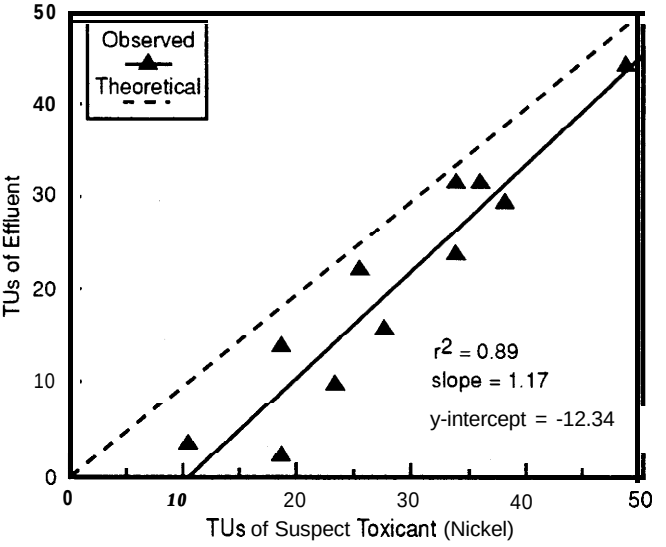

Figure 2-5. Correlation of toxic units (TUs) for a POTW effluent and the suspect toxicant, nickel.

Metals can be especially difficult toxicants to implicate using correlation because the toxicity of metals is typically very matrix dependent. When the knowledge of these characteristics is extensive for a chemical, as it is with ammonia (see Phase II), testing can be tailored to the chemical and a very powerful correlation obtained. The large amount of available information on ammonia does not exist for most metals. In these instances, the logic pattern should to be reversed where the approach has to become: if x *is the toxicant, what are the matrix effects?.* These can be found by pure chemical testing combined with Phases I or II manipulations. Once an adequate understanding of matrix effects is obtained, the information can be used to answer the question: Is the *effluent toxicant behavior consistent with the matrix effects for the suspect toxicant?*

Matrix effects will have varying impacts on toxicant behavior that also depends on the effluent effect concentration. For effluents which have effect concentrations in the <10% range, the test solutions will more closely resemble the diluent water matrix than the effluent. If the effluent has effect concentrations in the 50% to 100% range, the matrix effects of the test solution will most likely resemble those of the effluent, not of the dilution water. Since effluent TUs are calculated from

responses occurring in the dilution near the effect concentration, the matrix characteristics of that concentration are of the most concern for correlation. Thus the importance of the effluent matrix effects diminishes as the toxicity of the effluent is greater (i.e., matrix at effect level is more like dilution water).

One can safely say that the difficulty of simulating the matrix effects with a simulated effluent is quite large so that the choice is clearly to use the actual effluent when possible. An important reason for this choice is that so few matrix effects have been studied extensively, and beyond pH and hardness little data exists. Even then the interrelationship between pH, alkalinity and hardness were often ignored.

The above discussion does not provide all of the options on how to handle matrix effects. However, it should provide convincing evidence that more than the correlation step alone is necessary to provide adequate confirmation!

In summary, the TIE research experience has revealed two major areas of potential problems in using the correlation approach. The lack of additivity for toxicants found in effluents requires careful analysis when calculating TUs for regression purposes. Secondly, when there are matrix effects, correlation becomes difficult because the effluent matrix might change from sample to sample and because there are no analyses specific for the toxic forms. For such effluents, other confirmation techniques should be used more extensively to better support the overall confirmatory efforts.

Section 3
Symptom Approach

Different chemicals may produce similar or very different symptoms in a test species. Probably no symptom of intoxication is unique to only one chemical. Therefore, while similar symptoms observed between two samples means the toxicant(s) could be the same or different, different symptoms means the toxicant(s) is definitely different, or there are multiple toxicants in the two samples. By observing the symptoms displayed by the test organisms in the effluent and comparing them to the symptoms displayed by test organisms exposed to the suspect toxicants, failure to display the same symptoms means the suspect toxicant(s) is probably not the true one or the only one.

Behavior of most test species is difficult to put into words so that a clear image of behavior is obtained. Behavioral and morphological changes of 30-d old fathead minnows (*Pimephales promelas*) were used as diagnostic endpoints in 96 h flow-through single chemical tests. Organic chemicals of various modes of action were tested and video recordings were used to monitor the behavioral response (Drummond et al., 1986; Drummond and Russom, 1990). Substances within a single chemical classification did not necessarily cause the same type of response (Drummond and Russom, 1990). Therefore, it is difficult to predict chemical classification using behavioral monitoring alone.

This type of behavioral monitoring data does not exist for the cladocerans or the newly hatched fathead minnows or other species that are most frequently used in the TIE process. However, noting various symptoms is useful in the TIE. This is done by simply exposing the test species to the suspect toxicant(s) and observing how they react. By the time confirmation is initiated, toxicity tests with the suspect toxicants will have been conducted using pure compounds and symptoms may have been observed. It is important to note the symptoms observed during all testing because such characteristics can be very helpful in confirmatory work.

The intensity of exposure concentrations might change the symptoms observed with the suspect toxicant in the effluent. Therefore, it is important to compare symptoms at concentrations that require about the same period of onset. This can be done by comparing symptoms at exposure concentrations that have similar TUs. In this way both the unknown (sample) and the known toxicants (pure compound) can be set at the same toxicity level.

Observations of the organisms should not be delayed until the normal length of the test has elapsed. With some toxicants, the test organisms will show distinctive symptoms soon after the exposure begins, whereas later, symptoms are often more generalized and less helpful. For some other toxicants, a sequence of different symptom types are displayed by the test organism over the exposure period and the sequence may be more definitive for a given chemical than the individual symptoms. In few cases will the symptoms be unique enough to specifically identify the toxicant, but symptoms different from those caused by the pure suspect toxicant are convincing evidence that the suspect toxicant is not the true or only one.

A second caution is needed regarding mixtures of toxicants. Mixtures of toxicants can produce symptoms in test animals different from the symptoms of the individual toxicants comprising the mixture. When more than one toxicant is involved, the investigator must not only include all the toxicants, but include them in the same ratio as measured in the effluent. Often the toxicant of the mixture at the highest concentration relative to its effect concentration will cause most of the symptoms. As for single toxicants, the mixture concentration causing the same endpoint in a similar exposure period should be compared. Spiking effluent with the suspect toxicants and comparing the results of the spiked effluent sample and the unspiked effluent sample toxicity tests, both near their effect concentrations, is a good approach to take (Section 5).

Symptoms caused by the toxicant(s) might be quite different among different species of organisms; therefore the use of two or more species provides increased definitiveness of the observations. For both species, the researcher must compare symptoms at concentrations that are equitoxic. The greater the difference in sensitivity, the more important this becomes, The chemical concentration is unimportant; the important consideration is that equitoxic concentrations are compared.

Suppose, for example, species A and B have LC50 values for a suspect toxicant of 1 and 80 mg/l. Then concentrations of 2 and 160 mg/l may be used to compare symptoms of species A and B, respectively. If the onset of symptoms is rapid, then perhaps 1.25 and 100 mg/l (1.25xLC50) should be tried. Since symptoms vary with the exposure intensity, using various multiples of the LC50 (i.e., 0.5, 1, 2x) can add additional confirmation data, if the same set of symptoms are seen in both series. If more than one toxicant is involved, and the ratio of the two species' LC50 values for toxicant A is markedly different than for toxicant B, C, D, ... then the definitiveness of using symptoms is even greater.

For acute toxicity, time-to-mortality at equitoxic concentrations can be used as a symptom type of test. Some chemicals cause mortality quickly and some cause mortality slowly. If for two effluent samples, toxicity is expressed quickly for one and for the other very slowly, the toxicants are probably not the same.

In chronic testing, use of symptoms is also applicable. For example, adult mortality, number of young/female, death of young at birth, growth retardation, abortion, or time to onset of symptoms, all can also be monitored and such observations may be useful. The shape of the dose response curve may also be a determinant in assisting in confirmation. Some chemicals show an all or none type of response (diazinon) while others (i.e.. NaCl) display a relatively flat concentration-response slope for chronic toxicity.

Section 4
Species Sensitivity Approach

The effect concentrations can be compared for the effluent of concern and the suspect toxicants, using species of different sensitivities. If the suspect toxicant(s) is the true one(s), the effect levels of effluent samples with different toxicity to one species will have the same ratio as for a second species of different sensitivity. Also the ratio for each species should be the same as for known concentrations of the pure toxicant. The same ratio of effect values for two species implies the same toxicant in both samples of effluent. Obtaining the same effluent toxicity ratio among various effluent samples for each species as is obtained by exposure to comparable concentrations of known toxicants, implies that the suspect toxicants are the actual ones present. However, if other effluent characteristics affect toxicity and if they vary, the ratios could also be affected.

The common notion that goldfish are resistant to most toxicants and trout are sensitive to most toxicants is not readily substantiated (AQUIRE, 1992). Many species are more sensitive to certain groups of toxicants than trout. Of course, there are generalizations that can be made. For example, sunfish (Centrarchids), frequently are much more resistant to metals than goldfish, minnows, and daphnids (AQUIRE, 1992). Daphnids tend to be more resistant to chlorinated hydrocarbon insecticides than many fish species and more sensitive to organophosphate insecticides (AQUIRE, 1992). These differences must always be verified for the suspect toxicants; generalities can only be used as an initial guide to species selection. Sensitivity differences of 1 O-i 00x may occur in some chemical groups and not in others. If several toxicants are involved, interpreting the results and designing the ancillary experiments is more difficult. If successful, the power of the result for multiple toxicants is much greater than for a single toxicant. The difference in sensitivity between *Ceriodaphnia* and fathead minnows has, on several occasions, revealed either a change in the suspect toxicants present in a series of effluent samples, or the presence of other toxicants in addition to those suspected.

Comparison of sensitivity among species has another very important use. Some species may evidence toxicity from an effluent constituent that the TIE test species did not. If this happens, then the above comparison will be confused, but at least there will be a warning that the suspect toxicant may not be the cause of toxicity. In order to determine what is happening, the investigator should step back to Phase II, and possibly step back to Phase I to characterize the additional toxicant and then identify the toxicant using the new species. A second Phase III effort might be necessary for this toxicant and species. It is important not to assume that the resident species have the same sensitivity as the TIE test species. Especially for freshwater discharges into saltwater this concern is critical when a saltwater organism triggered the TIE, because at present the techniques and procedures described in Phases I and II are most likely to be done using freshwater organisms especially since the effluent is freshwater. If the concern is for marine organisms and their protection cannot be assumed (cf., Section 8, Phase I; EPA, 1991A), confirmation must be conducted with marine organisms.

In chronic testing, chemical and physical conditions might differ more among tests on different species because food must be provided during the test period and different foods are used for each species. For example, the final pH of fathead minnow 7-d tests might be lower than in acute fathead minnow tests and both are likely to be lower than in *Ceriodaphnia* chronic tests due to greater respiration rates for fish than cladocerans and food in fish tests. If the investigation was to confirm ammonia toxicity, this pH difference could result in confusing results by showing the *Ceriodaphnia* to be more sensitive than the fathead minnows when the reverse should be true (cf., EPA, 1993A; Phase II). The above example illustrates reasons to maintain careful quality control in Phase III work.

Section 5
Spiking Approach

In spiking experiments, the concentration of the suspect toxicant(s) is increased in the effluent sample and then toxicity is measured to see whether toxicity is increased in proportion to the increase in concentration. While not conclusive, if toxicity increases proportionally to an increase in concentration, considerable confidence is gained about the true toxicant(Two principles form the basis for this added confidence. To get a proportional increase in toxicity from the addition of the suspect toxicant when it is in fact not the true toxicant, both the true and suspect toxicants would to have 1) very similar toxicity and 2) to be strictly additive. The probability of both of these coinciding by chance is small.

Removing the suspect toxicants from the effluent without removing other constituents or in some way altering the effluent is usually not possible. The inability to do this makes the task of establishing the true toxicity of the suspect toxicants in the effluent difficult. For many toxicants, effluent characteristics, such as TOC, suspended solids, or hardness, affect the toxicity of a given concentration, Some characteristics, such as hardness, can be duplicated in a dilution water, but certainly not TOC or suspended solids because there are many types of TOC and suspended solids, and generic measurements do not distinguish among the different types. For example, effluent TOC occurs as both dissolved and suspended solids. In POTW effluents, the source of the TOC is likely to be largely from biological sources, both plant and animal (e.g., bacteria) and bacteria are likely to make up a large component of suspended solids. If there have been recent storms, oily materials from stormwater runoff might be high. Simulating TOCs from such variable sources is next to impossible because TOC is not solely the result of man-made organic chemicals. For suspended solids, shape, porosity, surface-to-volume ratio, charge and organic content (all or any), will impact sorption characteristics. None of these qualities are measured by the standard methods for measuring suspended solids nor can they be reproduced in a simulated effluent.

In a simple system, such as reconstituted soft water, it is reasonable to expect that for most chemicals a doubling of the chemical concentration will double the toxicity, at least in the effect concentration range. If the solubility of the toxicant is being approached or there are effects from water characteristics such as suspended solids, then the toxicity might not double or conceivably could more than double. For example, if a chemical with a large n-octanol/water partition coefficient (log P) is largely sorbed on solids, doubling the total concentration might more than double the toxicity because the added chemical might remain in solution. Another important issue is that equilibrium might not be established during the entire test period and is probably unlikely to occur before the test organisms are added. For example, in our TIE research, we found various surfactants sorb to solids and can be removed by filtration (Ankley et al., 1990). In these experiments, however, filtration failed to remove surfactants immediately after they were spiked in an effluent but surfactants were removed after a few days equilibrium time. Other chemicals are likely to show similar behavior in regard to equilibrium time.

If several toxicants are involved, then their interaction (additivity, independent action, synergism) must be measured or otherwise included in the confirmation process (cf., Section 2). Since ratios might be as important as concentration, the best way to spike when multiple toxicants are involved is to increase each toxicant by the same number of TUs (e.g., by doubling each). In this way the ratios of the toxicities remain constant.

The fact that two or more toxicants fail to show additivity is useful evidence in confirmation. Interpreting spiking data might require a very high level of competence in both toxicology and chemistry; otherwise the data could be very misleading. Using more than one species of differing sensitivity is effective in adding confidence to the results. When matrix effects are complicated, other types of spiking can be done to reduce the effects of the effluent matrix characteristics. If a method exists for removing the toxicants from the effluent, such as the C_{18} SPE procedures (EPA, 1993A), the extracts or methanol fractions can be spiked with pure chemicals in addition to spiking effluent, using the same principles as described for effluents. The advantage in this approach is that matrix characteristics such as suspended solids and TOC will be absent or much reduced and will not affect spiking experiments as much. The disadvantage is that proof that the extracts or fractions contain the true toxicants must be generated. Some approaches for doing

this are given in Section 6. The use of the spiking approach is especially applicable to fractions from the C_{18} SPE column or the high performance liquid chromatography (HPLC) column used for the isolation of non-polar organics. In these procedures, the constituents are separated from much of the TOC, suspended solids and hardness, so that spiked additions might be strictly additive where they might not be in the effluent. Suggestions and precautions about ratios and all other previously discussed concerns apply here too. In addition, concerns about the methanol percentages in the toxicity tests, the amount of SPE or HPLC eluate required for the toxicity tests and the issue of toxicity enhancement by methanol must be considered in order to generate the appropriate toxicity data. Spiking the methanol fractions with suspect toxicants, however, does not provide the same confidence about the cause of toxicity in the effluent as spiking the effluent directly. The mass balance approach described in Section 6 could be coupled with spiking the effluent with a portion of the fractions to make the data more relevant to whole effluent toxicity.

For chronic testing spiking a portion of the methanol fractions, such as C_{18} SPE methanol fractions into dilution water to mimic the effluent, requires some special considerations as discussed in the chronic Phase I (EPA, 1992) and the new Phase II (EPA, 1993A). For any test species, the effects of the methanol at the effluent spiking concentration for the test species must either be essentially non-existent or clearly established so that proper interpretation is applied. The use of spiking for chronic toxicants of the methanol fractions is not as easy as the spiking for acute toxicants due to the limitations in the quantity of methanol that would be added with each fraction for the toxicity test. If the chronic toxicity effect level is around or <25% effluent and the highest fraction tested is 4x higher than the chronic effect level, add-back tests can be conducted similar to the acute add-backs but the quantity of methanol required for the testing and analysis must be considered (cf., Section 2; EPA, 1993A). As discussed in Phase II, once a suspect toxicant has been tentatively identified, the steps of confirmation should be started although sample volumes of methanol eluates might limit the amount of testing (see Phase II, Section 2; EPA, 1993A) with chronically toxic samples. Spiking of appropriate levels for chronic toxicity for single chemicals (or mixtures) is limited as sublethal data are not as plentiful as acute data. The acute toxicity of some chemicals might be altered by methanol (i.e., surfactants). The possibility that this is occurring must be checked and a correction applied if warranted. Spiking fractions also has applicability for hidden toxicants; refer to Section 9 for further details.

Spiking can also be done effectively when the suspect toxicant(s) of concern can be removed. However, since other toxicants might also be removed, the data must be carefully interpreted. Ammonia is a good example (cf., Phase II; EPA, 1993A) to use with this technique where one toxicant can be removed. Ammonia can be removed from the effluent by passing samples over the zeolite resin, after which the concentration can be restored in the post-zeolite effluent by the addition of ammonia. If toxicity is also restored, then it is likely that there is sufficient ammonia to cause the toxicity observed. However, it cannot be concluded from these data alone, that ammonia is the cause of toxicity because the zeolite can also remove substances other than ammonia. Another substance which is non-additive with ammonia yet present at a lesser or the same number of TUs could cause the initial effluent toxicity but not be discernable by this removal technique. This is an example of a hidden toxicant (see Section 9). For acute toxicity, zinc could behave exactly this way because it is non-additive with ammonia yet zinc is also removed by zeolite. Using other ammonia removal methods, such as high pH stripping, followed by spiking to the initial ammonia concentration will enhance confidence that a hidden toxicant is not present. Other examples involving the C_{18} SPE column and various ion exchange resins would be approached and interpreted similarly.

Section 6
Mass Balance Approach

This approach is applicable only to those situations in which the toxicant(s) can be removed from the effluent and recovered in subsequent manipulation steps. The objective is to account for all toxicity to assure that small amounts of toxicity are not being lost. This concern is partly covered by the correlation approach (Section 2); however, a totally different toxicant present at a small concentration could appear as experimental variability in the correlation and go unnoticed.

The mass balance concept is best described by illustration for acutely toxic effluents and the C_{18} SPE fractions. As described in Phase II (Section 2.2.7; EPA, 1993A) for acutely toxic effluents, the effluent has been passed over a C_{18} SPE column which is then eluted with the methanol/water fractions. After the toxicity tests on the individual fractions are completed, add-back tests can be initiated to determine whether all of the toxicity in the original sample was accounted for in the SPE fractions. For this step, there are three separate tests (with dilutions and replicates to calculate effect endpoints) that must be conducted which consist of the all-fraction test, the toxic-fraction test, and the nontoxic-fraction test. Assuming a complete recovery of all non-polar organics from the SPE column, this should yield a solution of non-polar organic compounds equal to the original sample concentrations, In the mass balance approach, these add-back tests are conducted using an aliquot of the effluent that has passed through the C_{18} SPE column (post-SPE column nontoxic effluent) or an aliquot of dilution water. Each toxic fraction is added back to the post-SPE column effluent, so that each is present at original effluent concentrations (i.e., lx effluent concentration). For example for acutely toxic effluents, the toxic-fraction test solution is prepared using methanol concentrations as described in Phase II (i.e., Section 2.2.7; EPA, 1993A) and for each fraction where toxicity was observed in the fraction toxicity test, 30 µl of each is added to the same 10 ml of nontoxic post-C_{18} SPE column effluent (or dilution water). A portion of each of the remaining fractions where toxicity was not demonstrated are now added to a second post-SPE column aliquot at effluent concentrations for the nontoxic-fraction test. Finally portions of all the fractions (e.g., n= 8 for acutely toxic effluents) are added to a third post-SPE column aliquot at effluent concentrations for the all-fraction test. If all the toxicity is exhibited in the toxic-fraction test, then the all-fraction test results and the toxic-fraction test results should be the same as in the unaltered effluent. Results from the nontoxic-fraction test should indicate that no toxicity is present. This mass balance (or add-back) approach allows the researcher to ascertain whether or not the toxicity in the toxic-fraction test equals the effluent toxicity. Small amounts of toxicity can be undetectable in the toxic-fractions when tested separately or the toxicant(s) might not have been eluted from the C_{18} SPE columns. Unless mass balance experiments are conducted, such loss of toxicity might not be detected. In the effluent example discussed in Section 2, the toxicity was contained usually in the 75%, 80%, and 85% fractions and occasionally in the 70% fraction! The r^2-value, slope, and intercept were all close to the expected values if two toxicants (diazinon and CVP) were causing the effluent toxicity (Figure 2-3). However, in Table 6-l the results of mass balance tests indicate that toxicity from the all-fraction test was greater than the toxicity of the toxic-fraction test. While this difference is small, it did seem to be real and was attributed to a small amount of another toxicant in the 70% fraction. In 11 of 12 samples, the results from the all-fraction tests indicate there was greater toxicity than was found in the toxic-fraction tests. On the few occasions when the 70% fraction was toxic, it did not contain any of the three suspect toxicants. Without the mass balance data, consistent presence of the additional toxicant would not have been discovered.

At the stage where the toxic-fractions have been identified, the test of the fractions in a mass-balance test is highly desirable. For chronic toxicity testing, the amount of eluate available might be limited following the fraction toxicity tests. Using eluate for the add-back tests might be a trade-off between tracking toxicity and having sufficient eluate to concentrate for further analysis. This limits the add-back tests broad applicability for chronic toxicity TI Es unless the effluent is toxic enough that at 4x the chronic effect level, the methanol concentrations do not exceed

[3]During development of the non-polar organic procedures, various elution profiles were used that included the 70% methanol/water fraction.

Table 6-1. Comparison of Effluent Toxicity and Toxicity Measured in Effluent Fraction Add-back Tests

Sample	Toxic Units (TUs)		
	Effluent	All-fractions	Toxic-fractions
12/03/87	1.18	1.64	1.43
01/12/88	2.00	2.94	3.13
01/13/88	1.93	2.86	2.53
02/03/88-I[a]	cl .oo	1.15	<1.oo
02/03/88-II	2.00	1.75	1.64
03/03/88-I[a]	1.15	1.06	<1.00
03/03/88-II	1.33	1.52	1.13
03/23/88-I	3.70	3.03	2.86
03/23/88-II	2.86	2.86	2.44
04/28/88	2.27	1.72	1.64
05/17/88	2.27	2.04	2.00
05/17/88	2.27	1.67	1.59
Mean	2.13	2.18	2.00

[a]Values excluded from mean calculations due to less than values.

the organisms tolerance. For chronically toxic samples, the all-fraction add-back test with C. *dubia* is not possible due to high methanol concentrations in test cups unless chronic toxicity is below 25% and add-backs are done using 25% effluent as the high test concentration (cf., Phase II; EPA, 1993A). The data from the individual methanol/water tests may be summed; however this approach must be considered more tentative than add-back tests (see below).

A deficiency in the above approach to mass balance is that there can be some toxicity in the post-SPE column effluent which has not been removed by the C_{18} SPE but which is not present in concentrations high enough to detect. The above mass balance approach alone will not identify this. However, if the add-back tests described above are repeated using a standard dilution water, residual toxicity in the post-SPE column effluent should cause the toxic-fraction test and all-fraction test to show more toxicity when added to the post-SPE column effluent than when added to dilution water. A confounding effect of this approach is that if the toxicity is changed by matrix effects (suspended solids or TOC), then the toxicity will be different in the clean water test. Matrix effects can be discerned, in part, by a third spiking experiment where a portion of all of the fractions and a portion of each toxic-fraction test are spiked into whole filtered effluent (which has not passed through the C_{18} SPE column). If the addback tests in dilution water indicates greater toxicity than the addback tests with the post-SPE column effluent, and the same type of addback test experiment with filtered effluent (i.e., 1 µm filter) indicate that the fractions are exactly additive, then matrix effects are indicated.

Some post-SPE column effluent samples develop fungal or bacterial growth or perhaps a precipitate forms after the effluent passes through the column. For the fungal type of growth, this is thought to occur when some methanol bleeds into the effluent as it passes through the column and more rinsing will not eliminate this problem. Some effluents consistently develop this type of growth in the post-column effluent while others exhibit this pattern in only an occasional sample. To alleviate this problem, conditioning the column with acetonitrile has helped (cf., the acute Phase I (EPA, 1991A) and chronic Phase I (EPA, 1992) for details). When methanol fractions are spiked into the effluent this problem might or might not be enhanced; we have found this to be an effluent-specific occurrence.

Caution is warranted in situations where toxicity is contained in more than one SPE fraction. The researcher should not necessarily expect the toxicity expressed by each individual fraction that is tested separately to add up to the total effluent toxicity. First, toxicants may not be additive and second, some toxicity which cannot be detected in individual fractions may add to the whole toxicity. For example, any one C_{18} SPE fraction may not show toxicity but may contain some of the toxicant that is in the adjacent toxic-fraction. In this case, the toxicity of the toxic-fraction test would be less than expected. If this happens in more than one pair of fractions, the sum of the toxicity from the toxic-fraction test will be less than the effluent toxicity or all-fraction test. These concerns are especially important when several toxicants are involved and one or more occur in more than one fraction.

For effluents where the C_{18} SPE column is not used, but where the toxicants can be removed from the sample, the same objectives should be achievable, but the methods will be different. For example, if an effluent appears to contain a volatile toxicant, the mass balance could be done on the trap and on the purged sample. Since we have not yet done mass balance on samples such as these we have no experience from which to offer additional guidance or advice.

Some of the mass balance process begins in Phase II, and there is a subtle difference in the purpose of mass balances in Phases II and III. In Phase II, usually only a few samples are used and mass balances are necessary to determine the need for more identification in those few samples. The mass balance is useful in early stages of Phase II as well before toxicants are identified at all, because it allows the investigator to decide if the toxicants present at 2x or 4x whole effluent concentrations are also expressing toxicity at lower concentrations.

In Phase III as many samples are tested, the mass balance approach can provide information over time with many samples whether or not the suspect toxicants consistently account for all or the majority of the toxicity. As illustrated above, the power of the mass

balance approach to detect small degrees of toxicity is better than for the correlation approach.

When a portion of the toxicant is not biologically available and therefore does not contribute to toxicity, care must be taken to assure that removal of the toxicant from the sample does not remove biologically non-available portions. An example of this situation may be the alternative solvent extraction procedures which may remove a bound toxicant(s) sorbed on suspended solids with the solvent and is now toxic, yet it was not toxic in the unaltered sample.

Section 7
Deletion Approach

In some situations, particularly for industrial discharges, keeping the suspect toxicants out of the waste stream influent or effluent for short periods of time and also conducting toxicity tests on the wastewater simultaneously may be practical. When this approach can be used, it offers the most convincing evidence obtainable that the suspect toxicants are the true ones. Care must be taken however, that other substances are not deleted or that some characteristic such as pH does not change also. If a researcher can be certain that all changes are known, then this approach is definitive. Changes in the toxicants with time are as much of a concern here as in any other approach. These can be handled by the approaches outlined in earlier sections and the deletion approach need not be done repeatedly; however, if it were practical to do so, it would certainly be effective. If some samples do not contain one or more suspect toxicants, these effluent samples can be used to the advantage in confirmation in much the same way as intentional deletions described in this section can be used to confirm toxicity.

Section 8
Additional Approaches

This section mentions only a few of many steps that can be used to further confirm the cause of toxicity. The steps mentioned are mostly those that we have used and found helpful and practical.

The pH is one of the most important effluent characteristics that changes toxicity. The pH of POTW effluents, sediment pore or elutriate waters, and ambient waters will almost always rise when they are exposed to air, especially in the small test volumes used in TIE work. Commonly, pH in an effluent sample at 25°C will rise from 7.1-7.3 to 8.3-8.5 during a 24 h period. That pH change is enough to increase ammonia toxicity (based on total ammonia) about three fold. Such pH changes can destroy work for some purposes, but by regulating these pH changes, the pH fluctuations can be used to great advantage for other purposes.

Phase II (EPA, 1993A) describes the use of pH change to identify ammonia toxicity. The toxicity of some metals, hydrogen cyanide and hydrogen sulfide among others, is altered by pH change. Other characteristics, such as hardness, can also be varied to see if the changes in toxicity follow a predictable pattern. The toxicity of some metals could be approached in this way. Not all equilibria are as rapid as the ammonia equilibrium, so the amount of time for equilibria to occur should be controlled and standardized (cf., Phase II; EPA, 1993A). Various time . periods may have to elapse before the expected changes occur and this may differ with each effluent. With the improved methods of pH control described in the Phase I documents (EPA, 1991; EPA, 1992), much more use can be made of pH manipulation.

Often chemicals in effluent samples may not be biologically available, and if they are not, then they are not likely to cause toxicity. They may be made biologically available through some manipulation in Phase I and subsequently identified in Phase II. Through confirmation, the toxicity due to such a toxicant will become apparent when the correlation indicates a poor fit (cf., Section 2). For many toxicants, biological availability can be demonstrated by measuring body uptake. If the constituent of concern enters the body from the effluent, it is certainly biologically available. Exposure to pure compounds may be necessary to establish which particular organ should be evaluated for the toxicant. In acute metal exposures using fish, most metals concentrate first in the gills while non-polar organics concentrate in fatty tissues such as the liver. When a chemical is metabolized by the organism, a residue measurement for that compound is not a valid measure of the lethal body burden because it is unknown whether the metabolite is more of less toxic than the parent compound. If the suspect toxicant has a known mode of action, such as the acetylcholinesterase inhibition produced by organophosphate pesticides, this exposure effect can be measured to assess if toxic effects conform with the predicted effect. The use of enzyme blockers such as piperonyl butoxide (PBO) is also an aid in confirming toxicity caused by specific classes of toxicants (cf., Phase II; EPA, 1993A).

As additional steps are needed for confirming the cause of toxicity, combinations of various Phase I and Phase II procedures should always be used whenever practical. When several results are combined and all results are indicating the same type of toxicant, the data are more conclusive than when only one procedure yields predicted results.

Total dissolved solids (TDS) are a common problem in certain areas of the country and for certain industries. TDS will not cause toxicity from osmotic stress (this can easily be shown because their toxicity is not related to osmotic pressure) but rather TDS acts as a set of specific toxicants. For toxicity caused by TDS, the ratios and concentrations of the major cations and anions can be measured analytically. A similar mix of these major ions can be added to a dilution water to see if the expected toxicity is present. By testing various mixtures, the researcher can ascertain which of the TDS components contribute most to the toxicity.

Section 9
Hidden Toxicants

In the previous section, references were made to the problem of hidden toxicants. Essentially there are two situations which may produce the problem of hidden toxicants. The first situation occurs when disparate ratios of TUs of two toxicants are present in the effluent sample. Since the effect concentration is measured by diluting the effluent, when disparate ratios occur, the TUs of the toxicant present in fewer TUs in 100% effluent are so low at the effect diluent, that its contribution if any, is not measurable. This problem exists whether the toxicants are additive or non-additive. This situation generally will not be encountered in effluents that have very slight toxicity (i.e., effect concentration 75% to 100%) because little or no dilution is required to achieve the effect concentration. For those toxicants present in disparate ratios in effluents with marginal toxicity, the chemical present at the low levels may be nontoxic even in 100% effluent.

The second situation where hidden toxicant(s) occurs is when the toxicants are non-additive or partially additive in the effluent sample. These toxicants may occur at approximately equal TUs or at disparate ratios of TUs, as long as those present at lesser TUs are present at 1 TU in the 100% effluent (cf., discussion of performing correlation on these types of toxicants, contained in Section 2).

If confirmation is being conducted for both acute and chronic toxicity or if acute toxicity is being used as a surrogate for chronic toxicity, the acute to chronic ratio must also be considered. For example, consider an effluent with toxicants A and B for which the acute-to-chronic ratios are 3 and 12, respectively and the TUs for acute toxicity are 2 and 1 in an effluent sample for A and B, respectively. By definition, 1 acute TU (TU,) for toxicant A equals 3 chronic TUs (TU_c) and for B, 1 TU, = 12 TU,. In this example, the acute toxicity of the effluent will be determined by A and the chronic toxicity will be determined by B. If in another situation, the acute-to-chronic ratios for two compounds were similar, then one of the toxicants would determine the effect concentration for both acute and chronic toxicity. These examples illustrate the importance of acute-to-chronic ratios for non-additive toxicants. Acute-to-chronic ratios have special importance for additive toxicants when acute toxicity is being used as a surrogate measure for chronic toxicity. If acute toxicity is being used as a surrogate it must be demonstrated that the cause of the acute toxicity is the same as the chronic toxicity. When acute toxicity is used as a surrogate for chronic toxicity in Phases I and II, interpretation of the results can easily be biased and these considerations are important.

When a toxicant can be removed from the effluent and recovered, the identification of the presence of a hidden toxicant is more readily known. For example, the use of the C_{18} SPE column may remove hidden toxicants. The toxicant(s) is recovered in the eluate a&measured both analytically and toxicologically. This type of hidden toxicant may be observed if ammonia is present at concentrations that could cause toxicity. For example, in an effluent sample ammonia is present at 3 TUs. Ammonia will not be removed by the C_{18} SPE column and yet an additional 1.5 TU of a non-polar organic toxicant is evident when the C_{18} SPE eluate test is conducted. If the discharger applied remedial treatment they would be able to remove the ammonia toxicity yet the effluent would still be toxic. The same concept of hidden toxicants can be found when toxicants are removed by sublation which is followed by recovery and concentration of toxicity (cf., Phase I; EPA, 1991 A; EPA, 1992). For example, sublation can separate some surfactants, resin or fatty acids, and polymers from such constituents as metals and ammonia. Hydrogen sulfide can be removed by a purge and trap method, thereby separating it from other effluent constituents.

Specific blockers of toxicity such as EDTA for metals and PBO for organophosphates are also useful in establishing the cause of toxicity. The more specific the blocker, the more definitive are the results, However, present knowledge does not allow us to be certain that compounds such as EDTA do not also affect the toxicity of other chemicals. Use of two specific blockers such as EDTA and sodium thiosulfate for copper, allows more definitive conclusions (cf., Phase I; EPA, 1992).

Manipulating characteristics such as pH is useful but can easily mislead thinking. For example, if the effluent has ammonia toxicity, the toxicity due to ammonia should disappear if the pH is lowered appropriately. These results do not allow a conclusion that there are no hidden

toxicants. If, however, the pH is lowered so as to eliminate ammonia toxicity but the effluent toxicity exists or even increases, then the likelihood of a hidden toxicant is high. Unfortunately a complication to this rationale is that the toxicity expressed at the lower pH may be totally artifactual due to mechanisms of pH adjustments.

The best approach to find hidden toxicants is to first use, those methods that alter the effluent the least, can remove and recover removed hidden toxicants, and are most specific for a few toxicants. This advice is most applicable where the effort is to try to find out if some specified type of toxicant is a hidden one, e.g., is there a non-polar organic as a hidden toxicant.

If, however, the search is for any type of hidden toxicant then every conceivable technique should be used that would help to distinguish a hidden toxicant from the suspect toxicant(Hidden toxicants are very hard to find when ammonia is the primary toxicant. Various tests used to identify ammonia as the toxicant, i.e., use of the zeolite resin, graduated pH tests and air-stripping (EPA, 1993A), all have a reasonable probability of changing the toxicity of many other potential toxicants. For instance, it is known that zeolite removes some non-polar organics and metals. Air-stripping (at pH 11) could also remove or destroy many other chemicals as it often must be done for a extended period of time to achieve good ammonia removal. The graduated pH test results might also implicate a metal as a toxicant (EPA, 1993A). If these tests were conducted in Phase II (EPA, 1993A) and the results consistently indicated ammonia toxicity, these data indicate that there are no hidden toxicants. The required characteristics for a hidden toxicant to behave exactly as ammonia are very specific and obtaining results like those described above for a toxicant other than ammonia is unlikely.

If the hidden toxicant is additive with the suspect toxicant but occurs in a disparate ratio, the confirmation effort must first emphasize confirming the cause of toxicity (or remove the toxicity) of the primary toxicant. Then toxicity from the hidden toxicant should be measurable. The probability a hidden toxicant that has additive toxicity will not express its toxicity using several Phase I or Phase II techniques is less than the probability that a non-additive toxicant will express its toxicity using several of the same techniques.

If the remedial action for a primary toxicant is specific and easy, such as a product substitution, the search for hidden toxicants perhaps should be done after the remedial action has reduced or eliminated the primary toxicant from the effluent. The remedial action (especially if it is treatment) may also eliminate the hidden toxicant(What must be avoided if at all possible, is to carry out expensive remedial action only to find that the effluent is still toxic.

The problem of hidden toxicants is a major reason a researcher should not accept the presence of toxic concentrations of suspect toxicant as sufficient confirmation (cf., Section 1). The presence of biologically unavailable forms (cf., Section 8) is a compelling reason not to do so.

A thorough confirmation is resources well spent in most instances. Non-additivity and disparate ratios complicated by non-availability occur too frequently to bypass confirmation. Seasonal changes or changes without a pattern, in effluent toxicants are further reasons to perform the confirmation over a period of time to assure that the entire suite of toxicants has been found.

Section 10
Conclusions

Often the most laborious and difficult part of the TIE is developing data to adequately establish the cause of toxicity. In our experience, frequently the suspect cause of toxicity is found without difficulty but developing a convincing case to prove that the suspect cause is the true toxicant is the challenge.

Especially for POTW plants, this confirmation phase must be performed over a considerable period of time to be certain that the cause of toxicity is not changing. TIEs on POTWs and some industrial categories are not likely to be a one time event but will have to be repeated as long as the inputs to the plant change. Our current wastewater treatment plants were not designed to remove specific chemicals, so there is no reason to expect that they will remove everything which they receive. Especially where the control over the influent is not complete, as is the case with POTW plants, a solid case must be developed to assure that the cause of toxicity is not changing.

Section 11
When the Treatability Approach Has Been Used

As discussed in Phase I, two main approaches may be used to remove a toxicity problem--toxicant identification and source control or treatability. Phases I and II involve the first approach while treatability procedures accompanied by toxicity testing are used in the second (EPA, 19898; EPA 1989C).

In the second approach, treatment methods are varied to determine which will remove toxicity without identifying the specific toxicants. The treatability approach requires as much confirmation as the toxicant identification approach. Since the treatability approach should remove toxicity, the confirmation procedures are somewhat different.

Repeat samples should be tested to ensure that toxicity has been successfully removed. This should be done over a sufficient length of time to assure that the range of conditions are included during the confirmation phase. Such events as seasonal changes, production changes, storms, and intermittent operations all should be included during the confirmation phase. Toxicity should be consistently removed or appropriately reduced, as required. Either acute or chronic toxicity removal can be confirmed this way.

One must be absolutely sure that the toxicity to resident species has been successfully removed. As has been pointed out in Phases I and II, the effluent constituents producing toxicity to one species may not be the same for other species. Toxicity by a given treatment method may remove all toxicity for one species but not for another. The species of concern must be tested in the effluent from the treatment method selected. If chronic toxicity is the concern, this testing may be more difficult because chronic testing methods may not be available for resident species. In selected cases, symptoms may be substituted for the usual endpoints of chronic tests but their use would be case specific.

Section *12*
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☆U.S. GOVERNMENT PRINTING OFFICE:1993 - 750-002/80298

EPA/600/R-96/054
September 1996

Marine Toxicity Identification Evaluation (TIE)

Phase I Guidance Document

Edited by

Robert M. Burgess
Kay T. Ho
George E. Morrison
National Health and Environmental Effects Research Laboratory
Narragansett, Rhode Island 02882

Gary Chapman
National Health and Environmental Effects Research Laboratory
Newport, Oregon 97365

Debra L. Denton
Region IX: U.S. Environmental Protection Agency
San Francisco, Calfornia 94105

National Health and Environmental Effects Research Laboratory
Atlantic Ecology Division
U.S. Environmental Protection Agency
Narragansett, Rhode Island 02828

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Abstract

During the last ten years Toxicity Identification Evaluation (TIE) methods have been used extensively with freshwater effluents, receiving waters, and sediments. TIEs may be required by state or federal agencies as a result of enforcement actions, as a condition of the discharger's National Pollutant Discharge Elimination System (NPDES) permit, or may be conducted voluntarily by permittees. This guidance document, using the freshwater TIE approach as a model, has been developed to aid in conducting acute and chronic marine TIEs. It focuses on Phase I of the TIE: Toxicity Characterization. Phase I of a TIE characterizes the classes of toxicants causing adverse biological effects. These classes may include metals, organics, pH dependent toxicants, volatile toxicants, filterable toxicants, and oxidants. In this document, information is provided for: (1) salinity adjustment of freshwater effluents with brine, (2) general guidance for the performance of small volume marine toxicity tests with Atlantic, Gulf, and Pacific Coast species used in NPDES permit or as a NPDES permit testing requirement, (3) tolerances to the chemicals added during a TIE, and (4) the conduct of TIE manipulations. These acute/chronic TIE procedures have been developed for a number of specific macroalgas, echinoids, mysids, bivalves, an amphipod, gastropods, and fishes. Recommended manipulations described in this document include filtration, aeration, EDTA chelation, oxidant reduction, graduated pH, C_{18} solid phase extraction (SPE), cation exchange SPE, and sea lettuce *Ulva lactuca* addition.

Foreword

The Marine Toxicity Identification Evaluation (TIE): Phase I Guidance Document focuses on methods for characterizing toxicity associated with discharges to marine waters including effluents and receiving waters. Its purpose is to provide guidance to dischargers, testing laboratory staff, and local, state, and regional personnel in conducting Phase I of a marine TIE. Methods for conducting freshwater toxicity tests and TIEs have been produced (EPA 1991a, 1991b, 1993a, 1993b, 1993c); however, these methods were not directly applicable to marine samples. As stated in EPA 1993c:

> These methods are not mandatory but are intended to aid those who need to characterize, identify or confirm the cause of toxicity in effluents or other aqueous samples such as ambient waters, sediments, and leachates. Where we lack experience, we have indicated this and have suggested avenues to follow. All tests need not be done on every sample; the tests are, in general, independent. However, experience has taught us that skipping tests may result in wasted time, especially in the early stages of Phase I. An exception to this is when one wants to know only if a specific substance, for example ammonia, is causing the toxicity or if toxicants other than ammonia are involved. Otherwise, we urge the whole battery of tests.

We assume the reader is familiar with the following documents describing (1) TIE methods: *Toxicity Identification Evaluation: Characterization of Chronically Toxic Effluents, Phase I* (EPA 1991a), *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures*, Second Edition (EPA 1991b), *Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA 1993b), *Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA 1993c); 2) toxicity testing methods: *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms* (EPA 1994), *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA 1993a), *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA 1995); and 3) Toxicity Reduction Evaluations (TREs): *Toxicity Reduction Evaluation Protocol for Municipal Wastewater Treatment Plants* (EPA 1989a), and *Generalized Methodology for Conducting Industrial Toxicity Reduction Evaluations (TREs)* (EPA 1989b). Methodologies for both acute and sublethal (chronic) toxicity testing have been included in this manual. We invite comments on this document in order to improve future editions.

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Acknowledgments

This document is the result of the hard work and dedication of several individuals. In the area of adapting existing toxicity test methods for TIE application the following persons are acknowledged: Pamela Comeleo (formerly of Science Application International Corporation (SAIC), Narragansett, RI), Randy Comeleo (Signal Corporation), Anne Kuhn, (EPA-AED), Glen Modica (formerly SAIC, Narragansett, RI), Mark Tagliabue (EPA-AED), Diane Griffin and Paul Krause (MEC Analytical Systems, Inc., Tiburon, CA), Bryn Phillips, John Hunt, and Brian Anderson (University of California-Santa Cruz). The insightful internal technical reviews of Anne Kuhn (EPA-AED), Rick McKinney (EPA-AED), Peg Pelletier (EPA-AED), Sherry Poucher (SAIC, Narragansett, RI), Richard Steele (EPA-AED), and Richard Voyer (EPA-AED) are appreciated. The data furnished by Bob Berger of the East Bay Municipal Utility District (Oakland, CA) on Pacific coast species is much appreciated. Further, the financial support of EPA Regions IX and X for developing other Pacific coast species tolerance data is noted. The input of Anne Dailey (EPA, Region X, Seattle, WA) is also acknowledged. We also thank the personnel at the EPA Mid-Continent Ecology Division (MED) in Duluth, MN for their hospitality during our visits, willingness to answer our various questions, and sections of their TIE documents; specifically we thank: Teresa Norberg-King, Gary Ankley, Marta Lukasewycz, and Elizabeth Durhan of the EPA-MED, Mary Schubauer-Berigan (University of South Carolina, Georgetown, SC), Tim Dawson (Integrated Laboratory Systems, Duluth, MN), Don Mount and Joe Amato (Asci, Duluth, MN), and Doug Jensen (Sea Grant, Duluth, MN). We also wish to thank the reviewers of the draft and current editions of this document for their comments: Teresa Norberg-King (EPA-MED) , David Hutton (D.G. Hutton, Inc. Newark, DE), Stephen Bainter, Allen Chang, Phillip Jennings, Michael Morton, and Wren Stenger (EPA, Region VI, Dallas, TX), and Patti Tenbrook (East Bay Municipal Utility District, Oakland, CA). Further, we thank the multitude of individuals we have discussed TIE related issues with at scientific meetings and during phone conversations. Finally, the excellent services of Edward Shear (Department of English, University of Rhode Island), our technical writer, are also acknowledged.

NHEERL, AED, Narragansett Contribution No. 1788

Abbreviations

AED	Atlantic Ecology Division, EPA, Narragansett, Rhode Island
C_{18}	Octadecyl
CWA	Clean Water Act
DI	Deionized Water
DO	Dissolved Oxygen
EC_{50}	Median Effect Concentration
EDTA	Ethylenediaminetetraacetic Acid
EPA	U.S. Environmental Protection Agency
GP2	General Purpose Medium Number 2
LC_{50}	Median Lethal Concentration
MEOH	HPLC Grade Methanol
MED	Mid-Continent Ecology Division, EPA, Duluth, Minnesota
MSDS	Materials Safety Data Sheets
$Na_2S_2O_3$	Sodium Thiosulfate
NPDES	National Pollutant Discharge Elimination System
QAP	Quality Assurance Plan
SDS	Sodium Dodecyl Sulfate
SLP	Standard Laboratory Procedure
SOP	Standard Operating Procedure
SPE	Solid Phase Extraction
TIE	Toxicity Identification Evaluation
TRC	Total Residual Chlorine
TRE	Toxicity Reduction Evaluation
WQC	Water Quality Criteria

Section 1
Introduction

1.1 Background

The Clean Water Act (CWA 1972), in its original and all subsequent versions, established a "national policy that the discharge of toxic pollutants in toxic amounts be prohibited." The goal of the CWA is to eliminate the discharge of pollutants into waters in the U.S.; however, this goal is not immediately attainable. Consequently, the CWA allows for National Pollutant Discharge Elimination System (NPDES) permits for wastewater discharges. In order to insure that the CWA's prohibition on toxic discharges are met, an integrated system of testing procedures has been developed. This document presents additional methods for the conduct of Toxicity Identification Evaluation (TIE) which are part of this testing system.

During the last several years, TIE methods were developed and applied to freshwater effluents and receiving waters (Parkhurst et al. 1979; Walsh and Garnas 1983; Gasith et al. 1988; EPA 1991a, 1991b, 1993b, 1993c; Burkhard and Ankley 1989; Norberg-King et al. 1991). Methods for freshwater sediment TIEs have also been drafted (Ankley et al. 1992a). Implementation of these methods has demonstrated the regulatory and scientific utility of the TIE approach. For example, TIEs have identified specific 'problem toxicants' in effluents (Schimmel et al. 1988; Goodfellow et al. 1989; Ankley et al. 1990a; Jop et al. 1991a; Norberg-King et al. 1991; Amato et al. 1992; McCulloch et al. 1993; Ankley and Burkhard 1992; Burkhard and Jenson 1993; Schubauer-Berigan et al. 1993) receiving waters (Galassi et al. 1988; Schimmel et al. 1988; Norberg-King et al. 1991; Kszos et al. 1992), and freshwater sediments (Ankley et al. 1990b; Schubauer-Berigan and Ankley 1991; Ankley et al. 1992b; Hoke et al. 1992; Krantzberg and Boyd 1992; Schubauer-Berigan et al. 1993; Wenholz and Crunkilton 1995; Gupta and Karuppiah 1996). Furthermore, improvements have been incorporated as methods were applied (Doi and Grothe 1989; Ankley et al. 1990b; Durhan et al. 1990; Burkhard et al. 1991; Jop et al. 1991b; Mount and Mount 1992; Wong et al. 1996; Bailey et al. 1996; Hewitt et al. 1996).

1.2 Related Documents

As stated in the forward, this report assumes that the reader is familiar with several related documents. The report, *Methods for Aquatic TIEs: Phase I Toxicity Characterization Procedures, Second Edition* (EPA 1991b), contains essential background information on Phase I TIE procedures that is not duplicated in this report; and in addition, that report describes the related freshwater TIE procedures. Also, this report assumes that the reader is familiar with the following related documents: *Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA 1993b), *Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity* (EPA 1993c), *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms* (EPA 1994), *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA 1993a), *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA 1995), *Toxicity Reduction Evaluation Protocol for Municipal Wastewater Treatment Plants* (EPA 1989a), and *Generalized Methodology for Conducting Industrial Toxicity Reduction Evaluations (TREs)* (EPA 1989b), and that this report will be used in conjunction with these related documents. Methodologies for both acute and sublethal toxicity testing have been included in this manual.

1.3 Development of Marine TIE Methods

Research conducted at the U.S. Environmental Protection Agency's (EPA) Atlantic Ecology Division (AED) in Narragansett, RI has focused on the development of marine TIEs for saline samples using freshwater TIE methods as models. In addition, two new TIE manipulations are described: a cation exchange manipulation and macroalga *Ulva lactuca* addition (Burgess et al. submitted; Ho et al. in prep.). Marine TIEs are performed using marine species on waters discharging into or from marine environments. The marine TIE methods described in this document are designed specifically for use with the marine species listed in Table 1-1. Other TIE or toxicity testing directed fractionation studies performed in marine waters and sediments used mutagenic (Grifoll et al.1988; Grifoll et al.1990; Grifoll et al. 1992; Samiloff et al. 1983; Ho and Quinn 1993a; Ho and Quinn 1993b) and whole organism assays (Walsh and Garnas 1983; Quilliam and Wright 1989; Higashi et al. 1992; Svenson et

al. 1992; Weis et al. 1992; Burgess et al. 1993; Bailey et al., 1995; Burgess et al., 1995; Ho et al., 1995).

Table 1-1. Marine Species Discussed in This Document.

Region	Organism Type	Species
Atlantic and Gulf Coast	Macroalga	*Champia parvula*
	Echinoid	*Arbacia punctulata*
	Bivalve	*Mulinia lateralis*
	Mysid	*Mysidopsis bahia*
	Amphipod	*Ampelisca abdita*
	Fishes	*Menidia beryllina*
		Cyprinodon variegatus
Pacific Coast	Macroalga	*Macrocystis pyrifera*
	Echinoids	*Strongylocentrotus purpuratus*
		Dendraster excentricus
	Bivalves	*Crassostrea gigas*
		Mytilus californianus
		Mytilus galloprovincialis
	Gastropod	*Haliotis rufescens*
	Fish	*Atherinops affinis*

Two fundamental questions addressed during the development of this manual were: (1) can marine species tolerate the chemicals used in TIE manipulations and (2) are freshwater TIE chemical manipulations directly applicable to saline effluent samples? The tolerance of marine species was addressed with most of the species in Table 1-1 using TIE additives (e.g., ethylenediaminetetraacetic acid (EDTA), sodium thiosulfate ($Na_2S_2O_3$), and methanol). A series of Phase I TIEs, conducted with several marine species on four industrial (electrical equipment) and municipal effluents and several mock effluents and single chemicals, were used to address whether the freshwater manipulations were compatible with saline samples (Burgess et al. 1995; Ho et al. 1995; Ho et al. in prep.). It should be noted that the Atlantic and Gulf coast species in Table 1-1 have undergone fairly extensive TIE research with "real" effluents for the preparation of this document. The Pacific coast species have not undergone similar research; however, they have been used in the private sector for the past few years.

Results of tolerance tests for EDTA and $Na_2S_2O_3$ readily demonstrated that these marine species can tolerate TIE manipulations at concentrations sufficient to alter toxicant effects. Generally, the effect concentrations for various additives by these marine species were similar to those for freshwater species (EPA 1991b).

The feasibility of using TIE chemicals and manipulations, such as EDTA, cation exchange solid phase extraction (SPE), and C_{18}, to characterize toxicity in a seawater matrix has been illustrated through several studies. For example, experiments with the chelator EDTA investigated the toxicity of metals in seawater (Sunda and Guillard 1976; Anderson and Morel 1978). Cation exchange has been used extensively for isolating divalent metals from seawater (e.g., McLaren et al 1985; Pai and Fang 1990). Similarly, C_{18} reverse-phase chromatography has been applied to measure the marine partitioning behavior of chemicals between dissolved organic carbon and aqueous phases (Mills et al. 1982; Hanson et al. 1988).

As the procedures in this manual illustrate, the majority of the freshwater methods (EPA 1991a, 1991b) functioned acceptably when used with marine samples. Two primary exceptions were the graduated pH procedures designed to characterize pH dependent toxicants and the conduct of each manipulation at pHs 9 and 11 (EPA 1991b). Seawater has a strong carbonate buffering system that makes any long-term pH adjustments difficult to maintain. Alteration of seawater pH with acids, bases, or organic buffers, while often initially successful, does not permanently repress the natural carbonate buffering and prevent the return to initial seawater pH. We found the most effective way to successfully adjust and maintain the pH of seawater samples (for the durations required for toxicity testing) was to conduct exposures in controlled atmospheric chambers. Unlike the variety of procedures used in the chronic and acute freshwater TIE methods(EPA 1991a, 1991b, 1993b, 1993c), we found that controlling pH in atmospheric chambers was the least intrusive, and only efficient, method of those we tested.

The use of 'closed chambers' was also investigated. In this approach, exposure chambers were completely filled with the sample, adjusted to the desired pH with acid or base, and the test organisms added. Tight-fitting lids sealed the chambers from the atmosphere. Closed chambers, while useful in some applications (i.e., where dissolved oxygen was not low) were not as universally applicable as the controlled atmospheric chambers.

Unlike the freshwater graduated pH procedure which is conducted at three distinctly different pHs (e.g., 6.0, 7.0 and 8.0 (EPA 1991b)), exposures on saline waters are performed at pHs 7, ambient seawater (8.2-8.4), and 9. These pH values were adopted because: (1) some marine test species demonstrated unacceptable control survival at pHs less than 7 and (2) maintaining sample pHs at levels two pH units above or below ambient pH levels was difficult and often ineffective. Additionally, shifting sample pHs to 11 resulted in the precipitation of some seawater hydroxides (Stumm and Morgan 1981) and severely altered seawater composition.

Section 2
Health and Safety

The following section has been reprinted, with minor modifications from *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures,* Second Edition (EPA 1991b).

Since TIEs involve, by definition, working with effluents of unknown composition, the accompanying safety measures must be adequate for a wide spectrum of chemical and biological agents. Often, one may be able to judge probable concerns from the type of treatment used. For example, extended aeration is likely to minimize the presence of volatile chemicals and chlorinated effluents are less likely to contain viable pathogens.

Exposure to water samples during collection and its use in the laboratory should be kept at a minimum. Inhalation and dermal absorption can be reduced by using laboratory hoods and wearing rubber gloves, laboratory aprons or coats, safety glasses, and respirators. Further guidance on health and safety for toxicity testing is described in Walters and Jameson (1984).

In addition to taking precautions with effluent samples, a number of the reagents that might be used during the tests described in this manual are known or suspected to be toxic to humans. Analysts should familiarize themselves with safe handling procedures for these chemicals (DHEW, 1977; OSHA 1976), as well as the manufacturer's Materials Safety Data Sheets (MSDS). Use of the compounds may also necessitate specific waste disposal practices.

Section 3
Quality Assurance

The following section has been reprinted, with minor modifications from *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures,* Second Edition (EPA 1991b).

Quality assurance is composed of two aspects, quality verification and quality control. Quality verification entails a demonstration that the proposed study plan was followed as detailed and that work carried out was properly documented. Some of the aspects of quality verification include chain of custody procedures, statements on the objective of the study and what is known about the problem at its outset, instrumental log books, and work assignments. This aspect of quality assurance ensures that a "paper trail" is created to prove that the work plan has been covered completely. The quality control aspect of quality assurance involves the procedures which take place such as the number of samples to be taken and the mode of collection, standard operating procedures for analyses, and spiking protocols.

No set quality assurance program can be dictated for a TIE; the formula to a successful study will be unique to each situation. However, adherence to some general guidelines in formulating a Quality Assurance Plan (QAP) may increase the probability of success.

In preparing a QAP, enough detail should be included so that any investigator with an appropriate background could take over the study at any time. Cross checking of results and procedures should be built into the program to the extent possible. Records should be of a quality that can be offered as evidence in court. Generally, the QAP should be provided in a narrative form that encourages the user to think about quality assurance. To be effective, the QAP must be more than a paper exercise simply restating standard operating procedures (SOPs). It must increase communication between clients, program planners, field and laboratory personnel and data analysts. The QAP must make clear the specific responsibilities of each individual. The larger the staff, the more important this becomes. While QAPs may seem to be an inconvenience, the amount of effort they require is commensurate with the benefits derived.

3.1 TIE Quality Control Plans

A successful TIE is dependent upon a strong quality control program. Obtaining quality TIE data is difficult because the constituents are unknown in contrast to quality control procedures for a standard analytical method for a specific chemical. In such an analysis, one knows the characteristics of the analyte and the implications of the analytical procedure being used. Without knowledge of the physical/chemical characteristics of the analyte, however, the impact of various analytical procedures on the compound in question is not known. Further, quality control procedures are specific to each compound; quality control procedures appropriate to one analyte may be completely inappropriate to another.

The problem of quality control is further aggravated because quality control procedures for aquatic toxicity test may be radically different from those required for individual chemical analyses. This additional dimension to quality control requires a unique framework of checks and controls to be successful. The impacts of chemical analytical procedures on sample toxicity must be included. Likewise, procedures used to insure quality toxicity test results should not impact chemical analyses. For example, in performing a standard aquatic toxicity test, samples with low dissolved oxygen (DO) are usually aerated. This practice may, however, result in a loss of toxicity if the toxicant is volatile or subject to oxidation.

3.2 Cost Considerations/Concessions

The quality control practices required in any given experiment must be weighed against the importance of the data and decisions to be based upon that data. The crucial nature of certain data will demand stringent controls, while quality control can be lessened in other experiments having less impact on the overall outcome.

Effluent toxicant identification evaluations require a large number of aquatic toxicity tests. The decision to use the standard toxicity test methods described in EPA 1993a, 1994, 1995 (involving a relatively high degree of quality control), must be weighed against the degree of complexity involved, the time required and number of tests performed; all of these affect the cost of testing. For this reason, toxicity tests used in the early phases of the evaluation generally do not follow these protocols, nor do they require exacting quality controls because the data are only

preliminary. Phase I, and to a lesser extent, Phase II results are more tentative in nature as compared to tests performed for confirmation of effluent toxicant(s) in Phase III.

The progressions towards increasing definitive results is also reflected in the use of only a few species in the initial evaluation studies and multiple species in the later stages. The use of several species of aquatic organisms to assure that the effluent toxicity has been reduced to acceptable levels is necessary because species may have different sensitivities to the same pollutant. Quality control must relate to the ultimate goal of attaining and maintaining the designated uses of the receiving water. For this reason, final effluent test results must be of sufficient quality to ensure ecosystem protection. The use of dilution water for the toxicity tests that mimics receiving water characteristics (i.e., salinity) will help to ensure that the effluent will remain non-toxic after being discharged into the environment. In the instances where the effluent dominates the receiving water, the dilution water should mimic the characteristics of the effluent. In addition, it is essential that variability in the cause of effluent toxicity be defined during the course of the TIE so that appropriate control actions provide a final effluent safe for discharge.

3.3 Variability

The opportunities to retest any effluent to confirm the quality of initial TIE results will be limited at best. In addition to the shifting chemical and toxicological nature of the discharge over time, individual effluent samples stored in the laboratory change. Effluent constituents degrade at unknown rates, as each toxicant has its own rate of change. The change in a sample's toxicity over time represents the cumulative change in all of the constituents, plus that variation resulting from experimental error. Some guidelines for assessing and minimizing changes in sample chemistry and toxicity are discussed in later sections. Regardless of the precautions taken to minimize sample changes, a sample cannot be retested with certainty that it has not changed.

3.4 Intra-Laboratory Communication

Quality control procedures in chemistry and biology can be quite different. For example, phthalates are a frequent analytical contaminant requiring special precautions that are not of toxicological concern. The toxicological problem presented by zinc levels typically associated with new glassware are of no concern to those performing organic analyses. The difference in glassware cleanup procedures is an example of one of many differences that must be resolved. Cleaning procedures must be established to cover the requirements of both. Time schedules for analyses must be detailed in advance. One cannot assume toxicant stability; therefore, time delays between the biological and chemical analysis of a sample cannot be tolerated.

3.5 Record Keeping

Throughout the TIE, record keeping is an important aspect of quality verification. All observations, including organism symptoms, should be documented. Details that may seem unimportant during testing may be crucial in later stages of the evaluation. Investigators must record test results in a manner such that preconceived notions about the effluent toxicants are not unintentionally reflected in the data. TIEs required by state or federal pollution control agencies may require that some or all records be reviewed.

3.6 Phase I Considerations

Effluent toxicity is "tracked" through Phases I, II, and III using aquatic organisms. Such tracking is the only way to detect where the toxicants are until their identity in known. The organism's response must be considered as the foundation and therefore, the toxicity test results must be dependable. System blanks (blank sampled carried through procedures and analyses identical to those performed on effluent sample) are used extensively throughout the TIE to detect toxic artifacts added during the effluent characterization manipulations. With the exception of tests intended to make the effluent more toxic, or situations in which a known amount of toxicity has been intentionally added, sample manipulation should not cause the effluent toxicity to change.

There are many sources of toxicity artifacts in Phase I. These include: excessive ionic strength resulting from the addition of acid and base during pH adjustment, formation of toxic products by acids and bases, contaminated air or carbon dioxide sources, inadequate mixing of test solutions, contaminants leached from filters, pH probes, solid phase extraction (SPE) columns, and the reagents added and their contaminants. The appropriate toxicity data for the reagent chemicals used in Phase I and common aquatic test organisms are provided as needed in subsequent sections of this document.

Frequently, toxic artifacts are unknowingly introduced. For example, some pH meters with refillable electrodes can act as a source of silver which can reach toxic levels in the solutions being measured for pH. This is especially a problem where there is a need to carefully maintain or track solution pH. Using pH electrodes without membranes avoids the silver problem (which can only be detected by the profuse use of blanks).

Oil in air lines or from compressors is a source of contamination. Simple aeration devices, such as those sold for use with aquaria are better as long as caution is taken to prevent contamination of the laboratory air which is taken in by the pump.

Worst case blanks should be used to better ensure that toxicity artifacts will be recognized. Test chambers should be covered to prevent contamination by dust and to minimize evaporation. Since small volumes are often used, evaporation must be controlled. For some manipulations, plastic disposable test chambers are recommended to avoid problems related to the reuse of test chambers. Cups from the same lot should be spot-checked for toxicity.

Glassware used in various tests and analyses must be cleaned not only for the chemical analyses but so that toxicity is not introduced either by other contaminants or by residues of cleaning agents. Since the organisms are sensitive to all chemicals at some concentrations, all toxic concentrations must be removed and not just those for which analyses are being made.

Randomization techniques, careful observance of organism exposure times and the use of organisms of approximately the same age ensure quality data. Standard reference toxicant tests should be performed with the aquatic test species on a regular basis and control charts should be developed (EPA 1993a, 1994, 1995). During Phase I it will not be known how much the toxicity of the reference toxicants varies over time compared to the toxicant(s). When the toxicants are known, they should be used as the reference toxicant. Reference toxicant tests should be performed to coincide with the TIE testing schedule.

3.7 Phase II Considerations

In Phase II, a more detailed quality control program is required. Interferences in toxicant analysis are for the most part unknown initially but as toxicant identifications are made, interferences can be determined. Likewise instrumental response, degree of toxicant separation, and detector sensitivity can be determined as identifications proceed.

3.8 Phase III Considerations

In Phase III of a TIE, the detail paid to quality control and verification is at the maximum. This phase of the study responds to the compromises made to data quality in Phases I and II. For this reason, confidence intervals for toxicity and chemical measurements must be calculated. These measurements allow the correlation between the concentration of the toxicants and effluent toxicity to be checked for significance based on test variability. Effluent manipulations prior to chemical analyses and toxicity testing are minimized in this phase in an effort to decrease the chance for production of artifacts. Field replicates to validate the precision of the sampling techniques and laboratory replicates to validate the precision of analyses must be included in the Phase III quality control program. System blanks must be provided. Calibration standards and spiked samples must also be included in the laboratory quality control program. Because an attempt will be made to correlate effluent toxicity to toxicant concentration, spiking experiments are important in determining recovery for the toxicant(s). These procedures are feasible because the identities of the substances being measured are known.

The toxicants being analyzed can be tested for using pure compounds, thereby alleviating the need for a general reference toxicant. Because the test organism also acts as an analytical detector in the correlation of effluent toxicity with toxicant(s) concentration, changes in the sensitivity of the test organism must be known. This is best achieved by using the same chemicals identified for the reference toxicants.

Section 4
Equipment, Supplies, and Facilities

Equipment necessary to perform each of the Phase I procedures is listed in Section 9 under each manipulation. In addition, basic analytical laboratory equipment such as pH meters, pumps (vacuum and fluid), pipettors, and the capacity for maintaining compressed gas cylinders and regulators are required.

A reliable source for large numbers (hundreds) of test organisms is essential for TIE work. It is recommended that on-site culturing facilities be used to prevent TIE activity from being subject to seasonal availability of field collected organisms or delays in shipping from suppliers.

A supply of "clean" saline water is necessary as a diluent, a natural seawater control, a performance control for reference toxicant testing (EPA 1994), and as a source of hypersaline brine. Large supplies of brine solutions (100‰) can be prepared, stored, diluted with deionized water (DI) to desired salinities, and used in batches to insure seawater consistency and to avoid seasonal fluctuations in water quality. At AED, saline water has been prepared from both natural seawater and GP2 synthetic seawater (e.g., EPA 1994). In addition, water used for test organism culturing should come from the same source (EPA 1994). For a discussion of acceptable source waters and their quality control, one should consult the reports: *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Marine and Estuarine Organisms*, Second Edition (EPA 1994) and *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to West Coast Marine and Estuarine Organisms* (EPA 1995). Further discussion will be found in Section 5.4: Salinity Adjustments and Dilution Water.

Section 5
Sample Collection, Handling, Salinity Adjustment, and Dilution

5.1 General Collection

Effluents should be collected in clean plastic or glass containers. Generally, the collection site should be the same as the monitoring site specified in the NPDES permit unless a specific concern suggests otherwise (cf. EPA 1994). Examples of when it would be appropriate to use alternate or additional collection sites include: (1) better access to a sampling point between the final discharge and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge and it is desired to obtain a sample prior to chlorination; or (3) there is a desire to evaluate the toxicity of the influent to municipal waste treatment plants prior to their being combined with other wastewater streams or non-contact cooling water. It may be possible to collect enough additional sample at the time of compliance sampling if a TIE is to be done. EPA (1991b) provides further guidance on sample handling and includes a discussion of the choice between plastic and glass containers that is useful, since certain types of toxicants may absorb to certain surfaces. Additionally, the documents (EPA 1994, 1995) should be consulted for collection requirements.

The time, date, location, duration and procedures used should be recorded for effluent sample collection. During collection, aeration and transfer of effluents should be minimized to reduce the loss of volatile chemicals. Any additional observations such as color, turbidity, chlorine odor, or unusual sampling conditions (i.e., heavy rain) should be noted. If an industrial effluent is to be tested, it may be useful to record any available information on the current production levels and types of operating processes. The condition of the facilities treatment system should also be determined by the individual collecting the sample. In addition, it is recommended that total ammonia, total residual chlorine (TRC), pH, dissolved oxygen (DO), salinity/conductivity, and temperature be recorded upon arrival of the sample. At AED, salinity is usually measured using a refractometer for marine samples. Figure 5-1 provides a sample log book page for recording of sampling data.

Stored or shipped samples should be kept at 4°C and tested for toxicity within 36 hours. Limited observations on a single industrial effluent suggest that the timing of salinity adjustment (i.e., at time of collection or immediately before testing) was not critical. Parallel tests showed no toxicity differences over a 16 day period (Ho et al. 1995). However, this observation is not universal and it is suggested that an initial toxicity test be conducted on the day that the sample arrives.

The volume requirements for performing Phase I of a TIE will vary according to the toxicity of the sample. The more toxic the sample, the less effluent sample will be needed. To a certain extent, the choice of tests to be performed may also affect the desired sample volume. Table 5-1 provides estimates of the volumes of sample needed for the Phase I marine TIE tests.

5.2 Composite versus Grab Samples

There are several factors to consider when designing a sample collection scheme (EPA 1994). A 24-hour composite sample is more representative of total effluent toxicity and is more likely to collect the toxic fraction if it is intermittent (i.e., timed with an industrial process). However, a composite sample may make the toxic fraction more difficult to detect because of dilution. In addition, compositing is expensive and time consuming. The simpler and less expensive grab sample is a "snap shot" of effluent toxicity at the time of collection. A grab sample, however, has the disadvantage that it may miss intermittent toxicity altogether, or conversely, collections synchronized to a suspected manufacturing process or seasonal discharge can result in a very toxic sample. The choice of sampling method consequently will depend on the goals of a given TIE and the nature of the plant from which it is being collected. For example, if the sample is being taken from a wastewater treatment plant with a two-day detention time, there is little need for the use of composite samples. Please consult EPA 1991b,1993a for further discussion of this issue.

5.3 Pre- or Post- Chlorinated Samples

The decision to sample a municipal effluent before or after the addition of chlorine will depend on the objectives of the study. While addition of sodium thiosulfate helps determine how much of the toxicity is due to chlorine, it may also remove other oxidants and some metals, thus complicating the interpretation of results. Further, the presence of chlorine will often mask the effects of other less abundant toxicants. It is recommended to test both pre- and post- chlorinated samples to determine what portion of toxicity is attributable to chlorine.

Sample Log No.:_____

Date of Arrival:_____

Date and Time
of Sample Collection:_____

Facility:_____

Location:_____

NPDES No.:_____

Contact:_____

Phone Number:_____

Sampler:_____

Sample Type: ☐ Grab ☐ Composite

☐ Glass ☐ Plastic

☐ Prechlorinated

☐ Chlorinated

☐ Dechlorinated

Specific Sampling Information:

Sample Conditions Upon Arrival:

Temperature:_____
pH:_____
Total Alkalinity:_____
Total Hardness:_____
☐ Conductivity:_____
or
☐ Salinity:_____
Total Residual Chlorine:_____
Total Ammonia:_____
Dissolved Oxygen:_____

Conditions of treatment system at time of sampling:

Status of process operations/production (if applicable):

Comments:

Figure 5-1. Example Data Sheet for Logging in Samples.

Table 5-1. Estimated Volumes for Phase I Marine TIE Tests.*

Characterization Step	Volume Needed (ml)†	Total (ml)
Chemistry	~ 500 ‡	
Initial	~ 100	
Baseline	~ 120	
Filtration	~ 100	
Aeration	~ 100	
EDTA Addition	~ 100	
$Na_2S_2O_3$ Addition	~ 100	
Ulva lactuca Addition	~ 200	
C_{18} Solid Phase Extraction	~ 100	
Cation Solid Phase Extraction	~ 100	
Graduated pH	~ 100	
pH 7	~ 100	
Ambient pH	~ 100	
pH 9	~ 100	
		~2000

* Values are for three replicates for initial and baseline tests and two replicates in the manipulations. Test volumes are assumed to be 20 ml/replicate. Values are directly applicable to Atlantic and Gulf Coast species, Pacific Coast species may require greater volumes.

† Assumed sample tested at 100% and diluted by 50% splits. Initial and baseline include five treatments, and manipulations include three treatments.

‡ Includes physical measures (e.g., temperature, salinity), pH, ammonia, chlorine, and dissolved oxygen.

5.4 Salinity Adjustments and Dilution Water

Dilution water for marine TIEs is hypersaline brine (100‰) adjusted to the desired salinity with DI water. Brine is made by slowly evaporating filtered natural seawater until the salinity reaches 100‰ (do not exceed this level), filtering it through a one micron filter, and storing it in 20 liter cubitainers® or polycarbonate water cooler jugs (EPA 1994). The seawater should be of high quality and collected on an incoming tide to minimize the possibility of contamination. The brine and DI mixture is a very consistent dilution water as any given "batch" of brine can be used for a year or more.

Directions for the use of hypersaline brine for salinity adjustment is also described in EPA 1993a. Basically, for freshwater salinity adjustment (0‰), the volume of brine (V_{brine}) added is described by the relationship: $V_{brine}=(S_{test} \times V_{test})/S_{brine}$, where S_{test} is the desired test salinity, V_{test} is the test sample volume, and S_{brine} is the brine salinity.

Using hypersaline brine for effluent salinity adjustment causes a degree of sample dilution that is dependent upon the initial sample salinity and the desired test salinity. For example, the greatest concentration of a freshwater effluent (i.e., 0‰) adjusted to 30‰ with 100‰ hypersaline brine is 70%. For purposes of continuity and simplicity, all further discussion of effluent concentration in this document refers to salinity adjusted samples. Therefore, 100% salinity adjusted sample means the effluent concentration is between 70% and 100%.

An alternative approach to adjust effluent salinity is the addition of artificial seawater salts like GP2. Although this method has not been tested at AED with Phase I Marine TIEs, this method has the advantage that it does not dilute the effluent sample, and consequently may be useful in certain circumstances. It is not recommended that the artificial seawater be substituted for brine as dilution water, as brine contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for the adequate growth, survival, and/or reproduction of marine and estuarine organisms (EPA 1994). Consequently, the use of artificial seawater salts may be problematic in some cases. Conversely, for a very weakly toxic samples, where brine dilution would be problematic, the addition of sea salts may be required. Finally, if a sample is hypersaline (i.e., >34‰), dilution with DI water may be needed. In general, a TIE should be performed using dilution waters similar to that used in the toxicity test(s) which triggered the TIE.

Concentrations selected for testing should be bracketed around known or estimated LC_{50} and EC_{50} values. Determining test concentrations for initial testing requires some estimations, unless the effluent has been previously tested. Starting at the highest possible concentration and using logarithmic splits results in a wide distribution of concentrations. Concentrations for the baseline and the manipulations testing should be established by bracketing the LC_{50} or EC_{50} values generated in the initial test.

Section 6
Toxicity Testing

6.1 Test Species

The toxicity testing species described in this document are listed in Table 6-1. The table indicates species recommended for use in Pacific, Atlantic, and Gulf Coast testing. The reader may note small changes to these methods compared to methods reported elsewhere (EPA 1993a, 1994, 1995). Changes were made to adapt methods for TIE use.

Both acute and chronic (i.e., sublethal) endpoints are presented. In the table, endpoints are labeled as "mortality" for acute toxicity tests while short-term chronic tests specify an endpoint other than mortality. The chronic tests include the macroalga sexual reproduction and germination and growth test using *Champia parvula* and *Macrocystis pyrifera*, and the echinoid sperm cell test using sea urchins *Strongylocentrotus purpuratus* and *Arbacia punctulata*, and the echinoid fertilization test with the sand dollar *Dendraster excentricus*. Bivalve and gastropod development tests with *Mulinia lateralis*, *Crassostrea gigas*, *Mytilus californianus*, *Mytilus galloprovincialis*, and *Haliotis rufescens* are used. The acute tests include those for fishes: *Menidia beryllina*, *Cyprinodon variegatus*, and *Atherinops affinis*, the mysid *Mysidopsis bahia*, and the amphipod *Ampelisca abdita*.

Table 6-1. Marine Species Recommended for Use in Marine TIEs

Organism	Species	Region	Endpoint*	Exposure (hr.)
Macroalga	*Champia parvula*	Atlantic and Gulf Coasts	sexual reproduction	48
	Macrocystis pyrifera	Pacific Coast	germination/growth	48
Echinoid	*Arbacia punctulata*	Atlantic and Gulf Coasts	fertilization	1
	Strongylocentrotus purpuratus	Pacific Coast	fertilization	~1
			or development	72
	Dendraster excentricus	Pacific Coast	fertilization	~1
			or development	72
Bivalve	*Mulinia lateralis*	Atlantic and Gulf Coasts	mortality/development	24
	Crassostrea gigas	Pacific Coast	development	48
	Mytilus californianus	Pacific Coast	development	48
	Mytilus galloprovincialis	Pacific Coast	development	48
Gastropod	*Haliotis rufescens*	Pacific Coast	development	48
Mysid	*Mysidopsis bahia*	Atlantic and Gulf Coasts	mortality	48
Amphipod	*Ampelisca abdita*	Atlantic and Gulf Coasts	mortality	48
Fish	*Menidia beryllina*	Atlantic and Gulf Coasts	mortality	48
	Cyprinodon variegatus	Atlantic and Gulf Coasts	mortality	48
	Atherinops affinis	Pacific Coast	mortality/growth	48-168

* Acute tests are indicated by an endpoint of mortality, chronic tests by an endpoint other than mortality.

6.2 Test Methods

This section provides brief descriptions of the marine Phase I TIE toxicity tests. The TIE toxicity testing methods are very similar to conventional methods described in EPA 1993a, 1994, and 1995 except for minor changes to account for exposure volume reductions and feeding protocols. The Appendix provides test parameters of the methods.

In addition to the noted tests, we have conducted sediment interstitial water TIEs with the marine amphipod *Ampelisca abdita* and bivalve *Mulinia lateralis*. Further, we have used conventional NPDES toxicity tests, using the mysid *Mysidopsis bahia* and sea urchin *Arbacia punctulata*, in sediment interstitial water TIEs.

6.2.1 Macroalga Sexual Reproduction or Germination/Growth Tests

These methods use sexual reproduction of the macroalga *Champia parvula* and the germination and growth of the kelp *Macrocystis pyrifera* to measure toxicity. The *Champia parvula* procedure involves measuring the development of cystocarps on female plants. The *Macrocystis pyrifera* procedure quantifies the germination of settled zoospores and length of the germination tube.

Changes to the *Champia parvula* method (EPA 1994) for TIE purposes include a reduction in test solution volume from 100 mL to 20 mL and use of 50 mL petri dishes as the exposure chambers. Further, when conducting the Graduated pH Procedure, photosynthesis will increase pH by approximately 0.1 - 0.4 units. This is to be expected but should not exceed 0.5 pH units. Test parameters of these methods are presented in the Appendix.

6.2.2 Echinoid Sperm Cell Tests

The echinoid sperm cell tests have reduced fertilization of exposed gametes as an indication of toxicity. Dilute sperm solutions are exposed to test samples for 20 to 60 minutes. Following this exposure eggs are added to the samples and fertilization is allowed to occur. Twenty minutes after egg addition the test is terminated by the addition of a fixative. Fertilization is determined by microscopic examination of an aliquot from each treatment, and is shown by the presence of a membrane surrounding the egg.

Little has been changed in the sperm cell test methods to accommodate TIE applications. The existing method (EPA 1994, 1995) is extremely useful for TIE applications due to its use of very small exposure volumes (i.e., 5 mL), demonstrated sensitivity, and relatively rapid exposure. For conducting the Graduated pH Procedure, we have found it useful to keep the test scintillation vials in the atmosphere controlled chambers during the 20-60 minute sperm exposure to maintain desired pH values (cf. Section 9.9). Test parameters are presented in the Appendix.

6.2.3 Echinoid, Bivalve, and Gastropod Development Tests

The development tests involve several marine species and developmental endpoints (EPA 1994, 1995). Echinoid procedures assess the formation of the larval test. Bivalve and gastropod tests evaluate the growth of the larval shell. Microscopic analysis is used to determine test and shell condition. All tests are performed in small volumes (5-10 mL) and are amenable for TIEs. Test parameters of the methods are found in the Appendix.

6.2.4 Acute Mysid and Fish Tests

For TIEs, three Atlantic and Gulf Coast test methods are conducted similarly and use a mortality endpoint. Experimental designs consist of static 48-hour exposures with five organisms in 10 to 20 mL of test solution (i.e., 30 mL exposure cups). Mysid (*Mysidopsis bahia*) toxicity tests use 1-5 day animals. For fish testing, 9 to 14 day old *Menidia beryllina*, and 1-14 day old *Cyprinodon variegatus* are used. A TIE method for using 9-15 day old fish *Atherinops affinis* with small test volumes has not been fully developed. Test parameters are given in the Appendix.

Noteworthy changes to the standard marine acute methods (EPA 1993a) are the reduction in sample volume from approximately 100 mL to 10 or 20 mL and reduction in exposure period from 96 hours to 48 hours. When conducting the Graduated pH Procedure the organisms will add CO_2 to the exposure chambers resulting in decreases in sample pHs. Also, feeding test organisms *Artemia* will further reduce chamber pHs. To avoid drastic reductions in sample pH, especially in the pH 9.0 treatment, feed test organisms small rations. The Appendix details these and other changes to the standard methods.

6.2.5 Other Marine Species

Included in various sections of this document are references to other marine species, besides some of the common marine NPDES toxicity testing species, which can be incorporated into the marine TIE. Currently, these species are the amphipod *Ampelisca abdita* and the bivalve *Mulinia lateralis*. At AED, they have proven valuable in developing marine sediment TIE methods, but they can also be used to assess effluent toxicity. At the time this document was prepared, insufficient information was available to include the West Coast survival and growth method using the mysid *Holmesimysis costata.*

As with the other marine toxicity tests that use "whole organisms," major changes to the current methods with *Ampelisca abdita* (Scott and Redmond 1989) include reducing exposure volumes to approximately 10 mL and exposure duration to 48 hours. An evaluation of a 24-hour embryo-larval development test using the bivalve *Mulinia lateralis* is continuing.

Section 7
Statistical Methods

Test results are used to calculate point estimates (e.g., LC_{50}s and EC_{50}s). EPA recommends probit, Spearman-Karber, trimmed Spearman-Karber, and Inhibition Concentration (IC_p; p is the percent effect, e.g., mortality, reduced growth, etc.) as means to calculate point estimates (EPA 1993a, 1994, 1995).

Conversion of point estimates to toxic units (e.g., Toxic Units = $100/LC_{50}$ or $100/IC_p$) eliminates the inverse relationship between toxicity and LC_{50} or EC_{50} values making TIE interpretation easier. Furthermore, if the concentration of toxicants are known for a given sample, the toxic units for the individual toxicants can be compared to the total sample toxic units. The sum of the toxic units of the individual toxicants should be similar to the total toxic units of the sample, assuming they are all measured, bioavailable, and that their toxicities are additive.

Section 8
Ion Imbalance

The methods in this document do not directly address toxicity caused by ion imbalance as recorded in some types of effluents (e.g., McCulloch et al. 1993). If an ion imbalance is suspected in a sample, several studies are available that discuss how to characterize and identify such toxicity (McCulloch et al. 1993; Mount et al. in press; Douglas and Horne in press; Douglas et al. in press; Tietge et al. in press). It should be noted that although an ion imbalance may impart an apparent 'salinity' to a sample, in most cases the sample is not truly marine. Marine salinity has a specific composition of ions at relatively consistent proportions to one another. Effluents with ion imbalances seldom will have truly marine composition.

An approach for determining if an ion imbalance may be present in a given sample is to perform an anion and cation analysis for major elements (e.g., sodium, calcium, potassium, magnesium, chloride, sulfate, and bromide). Measured values can be compared to toxicity information (Douglas et al. in press), marine Water Quality Criteria (WQC), and known marine background levels (Millero and Sohn 1992) to assess if an imbalance may occur.

Section 9
Toxicity Identification Evaluation Procedures

A Phase I marine TIE characterization consists of the following recommended components (see also Figure 9-1):

- Initial Toxicity Test (§9.1, §6, Appendix)
- Baseline Toxicity Test (§9.2, §6, Appendix)
- Filtration Procedure (§9.3)
- Aeration Procedure (§9.4)
- EDTA Procedure (§9.5)
- $Na_2S_2O_3$ Procedure (§9.6)
- C_{18} Solid Phase Extraction (SPE) Procedure (§9.7)
- C_{18} SPE Methanol Elution Test (§9.8)
- Graduated pH Procedure (§9.9)
- Cation Exchange SPE Procedure (§9.10)
- Cation Exchange SPE Acid Elution Test (§9.11)
- *Ulva lactuca* Procedure (§9.12)

Figures 9-1 and 9-2 give an overview of the design of a typical marine Phase I TIE. One should note, however, that because of the varying durations of the toxicity tests used in a marine Phase I TIE that the indications of 'DAY 1' and 'DAY 2' may not always be appropriate.

While the Initial and Baseline Toxicity Tests are based on routine toxicity testing exposures, the other procedures (e.g., EDTA and $Na_2S_2O_3$) are specialized and require some knowledge of the sensitivity of the testing organisms to specific chemicals. The following sections describe the objectives and general procedures for conducting the TIE manipulations. Familiarity with the freshwater TIE procedures (EPA 1991a, 1991b) is recommended.

Specific information concerning numbers of treatments, types of species to test, volumes of effluent to prepare, and duration of exposures are only recommendations and may require modification depending upon each application. Blanks are described for each procedure and involve using the control seawater (often brine and DI) in the manipulations before the sample.

9.1 Initial Toxicity Test

9.1.1 General Approach

The objective of an Initial Toxicity Test for a TIE is to determine the toxicity of a given sample. The Initial Toxicity Test is performed on DAY 1 of the marine TIE process, while the Baseline Toxicity Test and procedures are generally conducted on DAY 2 (Figure 9-1 and Figure 9-2).

9.1.2 Materials

- Materials, organisms and apparatus necessary to conduct toxicity test (See Section 6 and Appendix).

9.1.3 Procedural Overview

Design of Initial Toxicity Test

Initial Toxicity Tests have a serial dilution design. We recommend five concentrations (post-salinity adjusted): 100%, 50%, 25%, 12.5%, 6.25% and a control (i.e., 0%) with one to three replicates (three preferred) per concentration (Figure 9-2). However, if a sample is very toxic, this range of concentrations will be too high and a set of lower concentrations will be needed. Therefore, if data from compliance testing suggests high toxicity, one should adopt a different set of concentration ranges including the necessary lower non-toxic concentrations.

Results of Initial Toxicity Test

Initial Toxicity Test results are used to judge how toxic the sample is toxic and if a TIE on the given sample is warranted. If so, Initial Toxicity Test results will be used to establish effluent test concentrations for subsequent TIE manipulations.

From our experience, it may be difficult, but not impossible, to conduct a TIE when the toxic units of a sample from the Initial Toxicity Test using the most sensitive species are <2 (i.e., LC_{50} > 50%). It is critical, however, to insure that the toxic units are <2 by repeating toxicity tests and using smaller concentration intervals (i.e., bracketing the effect concentrations more closely). Table 9-1 provides some other criteria as to when decisions can be made about proceeding with the Baseline Toxicity Test and TIE procedures.

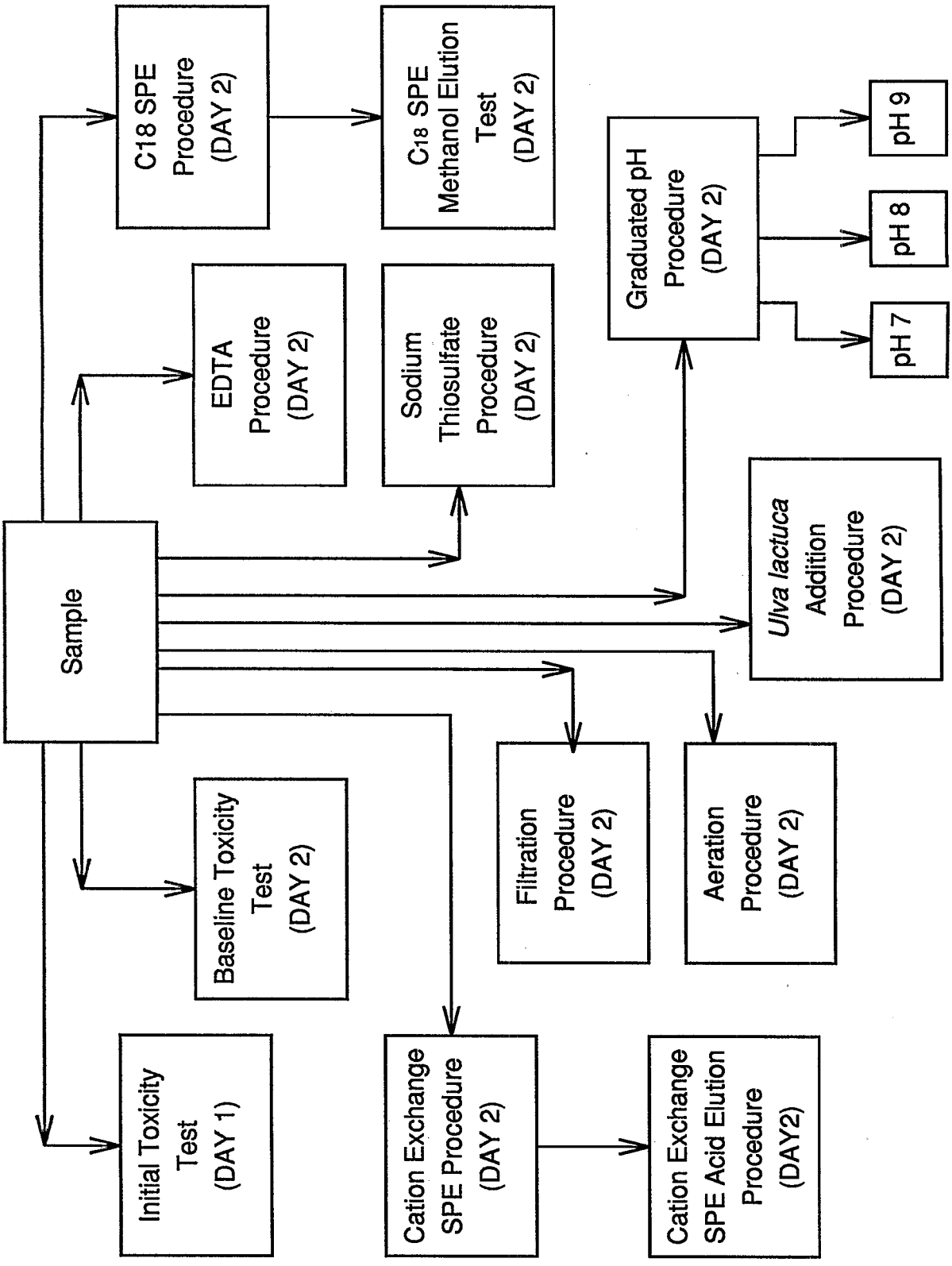

Figure 9-1. Overview Flowchart of a Typical Complete Phase I Marine TIE Characterization.
(NOTE: As a result of toxicity test durations, DAY 2 manipulations may occur later than the true DAY 2)

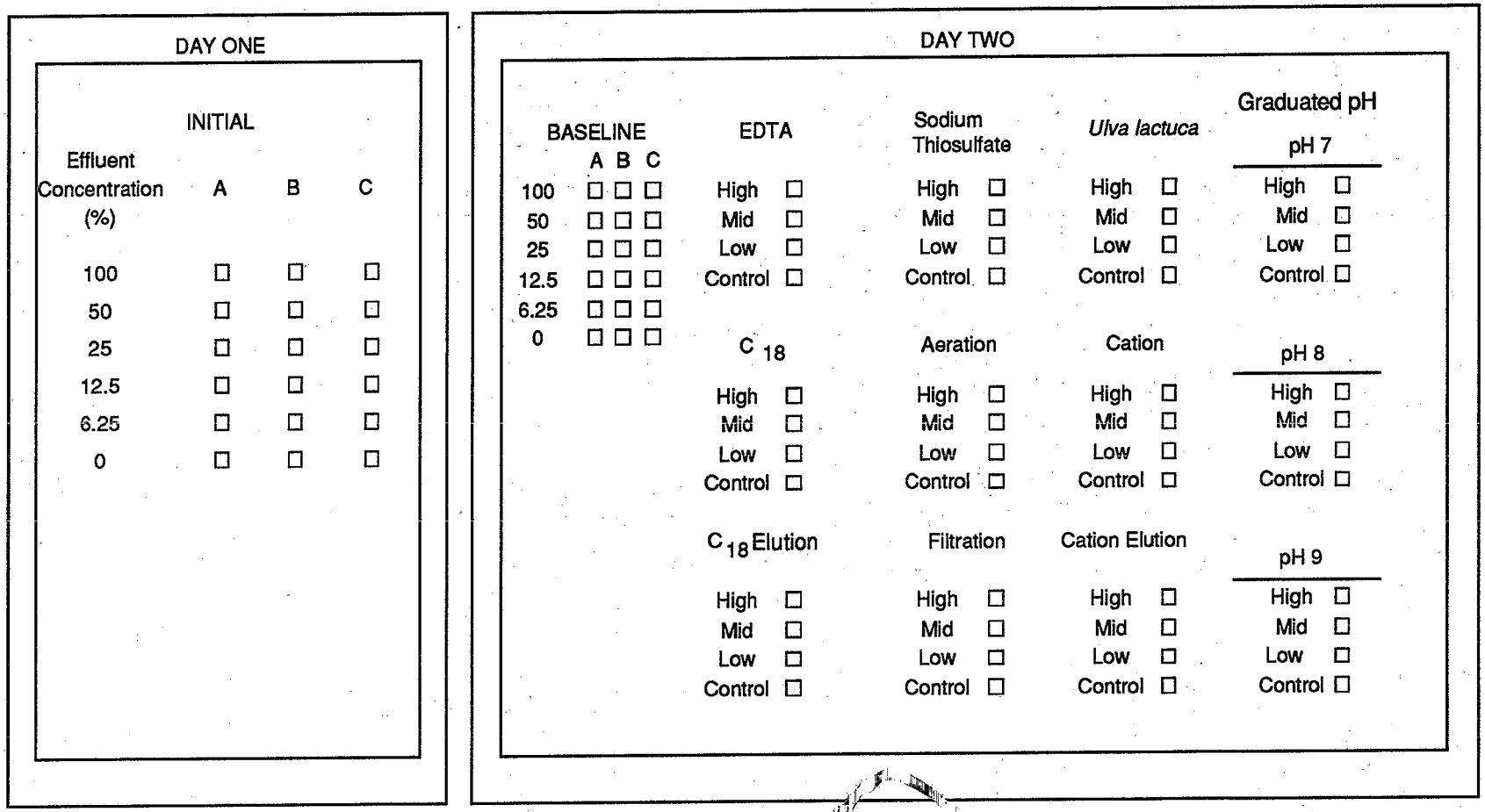

Figure 9-2. Schematic of Marine TIE Experimental Design. (NOTE: Squares represent replicates; Effluent concentrations a[illegible]ample

Table 9-1. Guidance on Conduct of Baseline Toxicity Test and TIE Procedures

Toxicity Test Species	Guidelines to Make Decision to Proceed
Champia parvula	Due to duration of exposure, one may have to use results of other tests or delay initiation of TIE
Arbacia punctulata	Results of initial toxicity test (Day 1)
Mulinia lateralis	48 hr. results
Mysidopsis bahia	24 hr. results; if no toxicity, use 48 hr. results
Ampelisca abdita	24 hr. results; if no toxicity, use 48 hr. results
Menidia beryllina	24 hr. results; if no toxicity, use 48 hr. results
Cyprinodon variegatus	24 hr. results; if no toxicity, use 48 hr. results
Macrocystis pyrifera	48 hr. results
Strongylocentrotus purpuratus	**fertilization:** Day 1 results; **development:** 72 hr. results
Dendraster excentricus	**fertilization:** Day 1 results; **development:** 72 hr. results
Crassostrea gigas	48 hr. results
Mytilus californianus	48 hr. results
Mytilus galloprovincialis	48 hr. results
Haliotis rufenscens	48 hr. results
Atherinops affinis	24 hr. results; if no mortality, use 48 hr. results, up to 168 hr.

Because of the long duration of the algal *Champia parvula* reproduction test, it is difficult to follow the standard TIE format. Therefore, it is necessary to use test results from other species to predict *Champia parvula*'s response or perform the initial test five to seven days earlier than the other species (assuming no alterations in toxicity due to storage). *Champia parvula* is often the most sensitive NPDES toxicity testing species when tested with municipal and industrial effluents (Schimmel et al. 1989) and therefore, a prediction of high toxicity is warranted. Conversely, because of the short duration of the sea urchin *Arbacia punctulata* sperm cell test, an entire TIE can often be conducted in two days, or even one day, if prior information about the toxicity of the sample is available and appropriate dilutions can be prepared. The fertilization endpoints of toxicity test using *Strongylocentrotus purpuratus* and *Dendraster excentricus* can be used similarly.

9.2 Baseline Toxicity Test

9.2.1 General Approach

Results of the Baseline Toxicity Test are used for comparison with the Initial Toxicity Test and TIE manipulations. Objectives are to: (1) determine if sample toxicity has changed relative to Initial Toxicity Test and (2) provide a baseline for comparison with results of TIE procedures. A Baseline Toxicity Test is performed following the Initial Toxicity Test, in conjunction with the TIE Manipulations (Figures 9-1 and 9-2). In Figure 9-2, we indictate the use of one replicate per test concentration and three concentrations per procedure. These values for the study design are not recommendations but must be determined according to study objectives, logistics and economic constraints.

9.2.2 Materials

- Materials, Organisms and Apparatus necessary to conduct toxicity test (See Section 6 and Appendix).

9.2.3 Procedural Overview

Design of Baseline Test

Baseline Toxicity Tests have a serial dilution design. Usually five concentrations: 100%, 50%, 25%, 12.5%, 6.25% and a control (0%) with three replicates/concentration are used. However, if the Initial Toxicity Test demonstrates greater toxicity, lower dilutions may be justified.

Results of Baseline Toxicity Test

Because of the variety of species potentially being tested, Baseline Toxicity Test results will be dependent on the toxicity test being used. However, regardless of species, the questions being answered are the same for each toxicity test, "Did sample toxicity change relative to the Initial Toxicity Test and did the TIE procedures decrease or increase toxicity compared to the Baseline Toxicity Test?" Quantitatively, these questions are answered by comparing toxic units between the various procedures. Sources of toxicity are implied from the magnitude of difference between the baseline and TIE procedures results. However, statistical evaluations of significance may be precluded, for most TIE tests, because of insufficient replication within TIE experimental designs. See Section 10 for further discussion of the interpretation of TIE results.

9.3 Filtration Procedure

9.3.1 General Approach

Filtration is used to determine whether toxicants pass through a filter or are associated with particles. Note for effluents, samples can be filtered before being passed through the C_{18} column (See Section 9.7). However, filtration may create artifacts (e.g., toxicant sorption to filter) that may need to be addressed in evaluating results. Filtrates are the substances that pass through the filter.

9.3.2 Materials

- Oil-free air pump and tubing—to force sample through filtration apparatus.
- 0.45 µm (or similar size) glass fiber filters and filtration apparatus—to separate particles from sample. For samples that are suspected to contain toxic metals, organic membrane filters may be used instead of glass filters. However, a comparison of filter types may be necessary.

9.3.3 Procedural Overview

(1) Filter brine and DI blank; remove brine and DI blank filtrate for testing (Figure 9-3).
(2) Without changing filters, filter the effluent. Change filters as often as necessary to prevent clogging, repeating step 1 as needed. Save all filters for possible later analysis (i.e., wrap in aluminum foil or Parafilm® and store at 4°C). Remove filtrate for testing.
(3) Use filtered brine and DI blank as diluent.

9.4 Aeration Procedure

9.4.1 General Approach

Samples are aerated to determine if toxicity is due to volatile toxicants (e.g., H_2S or volatile hydrocarbons).

9.4.2 Materials

- Oil-free air pump and tubing—to aerate sample.
- Graduated cylinders—to hold sample while aerating.
- 1-10 mL pipettes—attached to tubing and placed in sample during aeration. Fritted end on pipettor tubing will improve aeration.

9.4.3 Procedural Overview

(1) Samples should be aerated in a hood.
(2) Separately pour sample, and brine and DI blank into graduated cylinders (Figure 9-4).
(3) Connect 1-10 mL pipettes to air pump tubing and place pipettes into graduated cylinders.
(4) Turn pump on, adjust air flow to establish many small bubbles, and let sample aerate for 1 hour.
(5) Test aerated sample using aerated brine and DI as diluent.

9.5 EDTA Procedure

9.5.1 General Approach

EDTA (ethylenediaminetetraacetic acid) is an organic chelating agent that preferentially binds with divalent cationic metals, such as copper, nickel, lead, zinc, cadmium, mercury, and other transition metals (Garvan 1964). Studies have demonstrated that when a metal is bound to the EDTA molecule, the toxicity of the metal is greatly reduced (e.g., Sunda and Guilliard 1976). In this procedure, EDTA is added to samples to evaluate metal toxicity. Table 9-2 provides recommended exposure concentrations and Tables 9-3 and 9-4 report results of tolerance testing with Atlantic, Gulf, and Pacific Coast species.

9.5.2 Materials

- EDTA stock solution (25 g EDTA/L DI (74.4 mmols EDTA/L) refrigerated)
- Glass Erlenmeyer flask (100-250 mL), microbalance, weighing pan, and Teflon®-coated stirbar—for preparing EDTA stock solution.
- Adjustable microvolume pipetter (10-1000 µL range) and tips—for dispensing EDTA stock solution to exposure chambers.

Table 9-2. Volumes of EDTA Stock Solution for Additions (25g EDTA/L stock solution)

Replicate Volume (mL)	Volume (µL) EDTA Solution/Replicate	Volume (µL) EDTA Solution/Replicate for *M. pyrifera*
5	12	10
10	24	20
20	48	40
40	96	80
100	240	200
200	480	400

9.5.3 Procedural Overview

(1) Prepare EDTA stock solution: weigh-out 2.78 g of $EDTA \circ 2H_2O$ reagent (sodium salt) and add to 100 mL of DI. Mix with a Teflon®-coated stirbar until EDTA is completely in solution. This stock solution is stable and can be stored refrigerated (Figure 9-5).
(2) Set-up dilution series with sample. Generally, a TIE dilution series consists of three effluent concentrations and a blank (brine and DI), however, the statistical design of the TIE should be based on the objectives of the study, logistics, and economic constraints. The concentrations tested should bracket observed toxicity, based on the Initial Toxicity Test. **Do not add the organisms yet!**
(3) Tolerance testing of several Atlantic, Gulf, and Pacific Coast species indicates that most organisms can tolerate 60 mg EDTA/L (0.22 mmols EDTA/L) (Table 9-3, 9-4). Given the EC_{50} of 100 mg/L for *M. pyrifera*, it is advisable to use 50 mg/L (0.14 mmol/L) for the EDTA Procedure with that species. This concentration of EDTA is sufficient to chelate about 22 mg Total M^{2+}/L (equal molarity of metals). Use Table 9-2 to determine the volume of EDTA stock (25 g EDTA/L) to add to test containers:
(4) Add specified volume, mix thoroughly and allow EDTA and sample to interact for about 3 hours. **Do not add the organisms yet!**
(5) After 3 hours, add test organisms to dilution series.

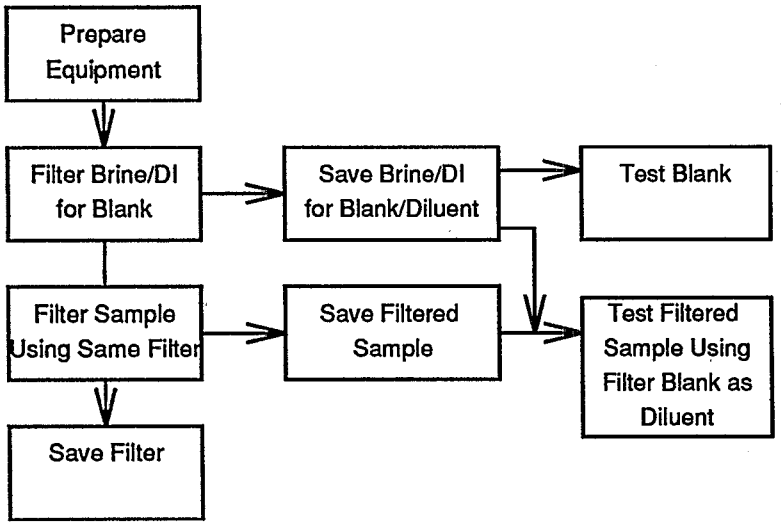

Figure 9-3. Overview Flowchart of Filtration Procedure.

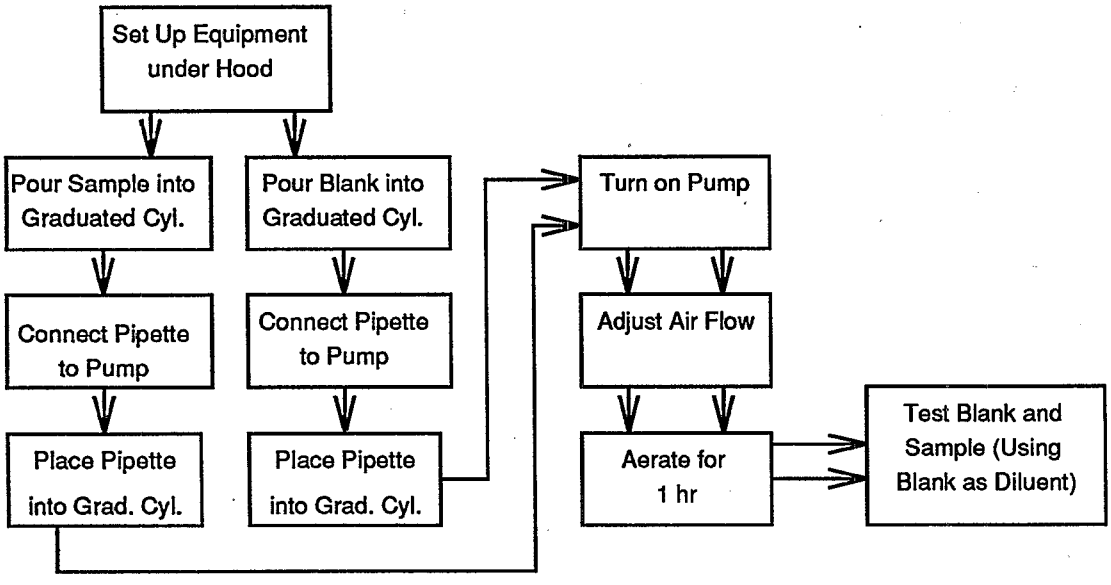

Figure 9-4. Overview Flowchart of Aeration Procedure.

Table 9-3. Atlantic and Gulf Coast Species Tolerance to EDTA (mg/L) (see Appendix for specific salinity and temperature).

Duration (hr)	Species LC_{50} or EC_{50} (± 95% Confidence Intervals)						
	Champia parvula	*Arbacia punctulata*	*Mulinia lateralis*	*Mysidopsis bahia*	*Ampelisca abdita*	*Menidia beryllina*	*Cyprinodon variegatus*
	(EC_{50})	(EC_{50})	(EC_{50})	(LC_{50})	(LC_{50})	(LC_{50})	(LC_{50})
1.2	-	300 (300-300)	-	-	-	-	-
24	-	-	-	318 (309-323)	240 (150-350)	362 (354-369)	> 600 (-)
48	165 (94.2-240)	-	288 (283-295)	313 (300-326)	175 (65.6-205)	353 (347-359)	542 (534-547)
72	-	-	-	318 (303-327)	164 (50-200)	353 (344-359)	348 (345-349)
96	-	-	-	315 (298-325)	150 (28.2-188)	350 (344-359)	346 (344-349)

- Not Available

Table 9-4. Pacific Coast Species Tolerance to EDTA (mg/L) (see Appendix for specific salinity and temperature).

Duration (hr)	Species LC_{50} or EC_{50} (± 95% Confidence Intervals)							
	*Macrocystis pyrifera**	*Strongylocentrotus purpuratus*†	*Dendraster excentricus*†	*Crassostrea gigas*	*Mytilus californianus*	*Mytilus galloprovincialis*	*Haliotis rufescens*	*Atherinops affinis*‡
	(EC_{50})	(EC_{50})	(EC_{50})	(EC_{50})	(EC_{50})	(EC_{50})	(EC_{50})	(EC_{50})
<1.0	-	>750	>750	-	-	-	-	-
24	-	-	-	-	-	-	-	-
48	100 (-)	-	-	>750	>750	>750	300 (-)	-
72	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	300 (-)

- Not Available
* Germination Endpoint
† Fertilization Endpoint
‡ 7 Day Growth Endpoint

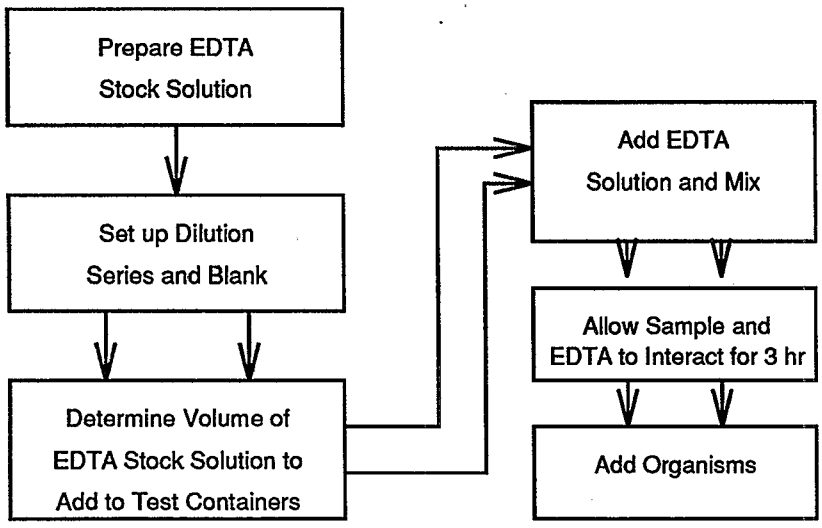

Figure 9-5. Overview Flowchart of EDTA Procedure.

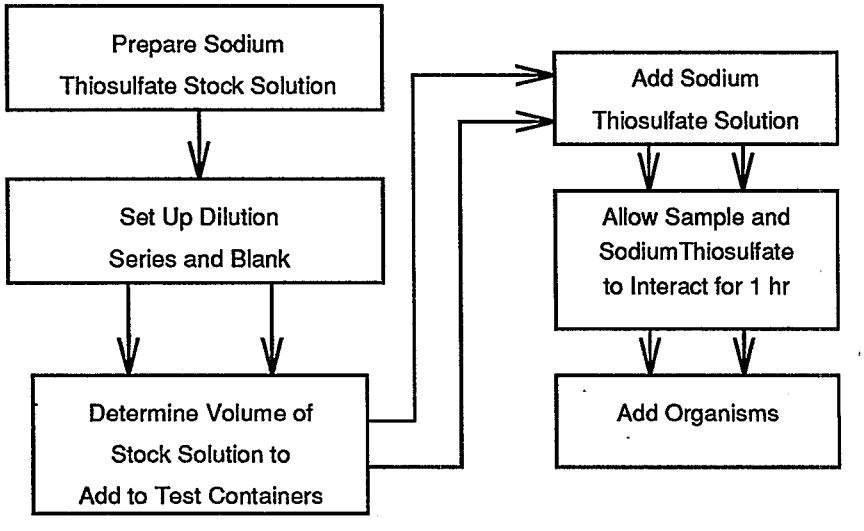

Figure 9-6. Overview Flowchart of Sodium Thiosulfate Procedure.

9.6 Sodium Thiosulfate Procedure

9.6.1 General Approach

Addition of sodium thiosulfate ($Na_2S_2O_3$), a reducing agent, to a sample containing oxidants (e.g., chlorine or bromine), results in a reduction reaction (White 1972) that may decrease sample toxicity. For example, chlorine (Cl_2) added to sewage effluent prior to release would undergo the following reaction:

$$Cl_2 + 2e^- \xrightarrow{2S_2O_3^{2-}} 2Cl^-$$

where the 2 electrons (e^-) provided by the thiosulfate (S_2O_3) reduce the toxic diatomic chlorine (Cl_2) to nontoxic chlorine ions (Cl^-). In this test, $Na_2S_2O_3$ is added to effluent samples to evaluate whether toxic oxidants are present. Table 9-5 provides recommended exposure concentrations and Tables 9-6 and 9-7 report the results of tolerance testing with Atlantic, Gulf, and Pacific coast species.

9.6.2 Materials

- $Na_2S_2O_3$ Stock Solution (15 g $Na_2S_2O_3$/L DI (94.9 mmols $Na_2S_2O_3$/L)). **This solution cannot be stored. Make up prior to use.**
- Glass Erlenmeyer flask (100-250 mL), microbalance, weighing pan, spatula and Teflon®-coated stirbar—for preparing $Na_2S_2O_3$ stock solution.
- Adjustable microvolume pipetter (10-1000 µL range) and tips—for dispensing $Na_2S_2O_3$ stock solution to exposure chambers.

9.6.3 Procedural Overview

(1) Make-up $Na_2S_2O_3$ Stock Solution

- Weigh-out 2.35 g of $Na_2S_2O_3 \circ 5H_2O$ reagent, add to 100 mL of DI in a flask with a Teflon®-coated stirbar, and allow to mix until all the $Na_2S_2O_3$ is completely in solution (Figure 9-6).

(2) Use of $Na_2S_2O_3$ in TIE

- (a) Set up dilution series with sample. Generally, a TIE dilution series will consist of three effluent concentrations and blank (brine and DI). Concentrations should bracket observed toxicity, based on the Initial Toxicity Test. **Do not add organisms yet!**
- (b) Use Table 9-5 to determine the volume of $Na_2S_2O_3$ stock to add to test chambers. Tolerance testing of several Atlantic, Gulf, and Pacific coast toxicity testing species indicates that all organisms can tolerate 50 mg $Na_2S_2O_3$/L (0.32 mmol $Na_2S_2O_3$/L) (Table 9-5).

(3) Add $Na_2S_2O_3$ and allow to interact for about one hour. **Do not add organisms yet!**

(4) After one hour, add test organisms to exposure chambers.

Table 9-5. Volumes of $Na_2S_2O_3$ Stock Solution for Additions (15g $Na_2S_2O_3$/L stock solution)

Replicate Volume (mL)	Volume (µL) $Na_2S_2O_3$ Solution/Replicate
5	17
10	34
20	68
40	136
100	340
200	680

9.7 C_{18} SPE Procedure

9.7.1 General Approach

The C_{18} solid phase extraction (SPE) column manipulation is used to determine if toxic components are nonionic organic compounds. In the manipulation, reverse phase liquid chromatography is applied to extract nonionic organic toxicants from the aqueous sample. Operationally, filtered test solutions (i.e., samples and controls) are passed through a disposable C_{18} column and the post-column effluent tested for toxicity (Figure 9-5). Absence of toxicity in the post-column effluent suggests that organic toxicants were active in the original sample. Elution of the column with methanol can return toxicants to aqueous solution to confirm toxicity (see Section 9.8).

Tables 9-8 and 9-9 provide information on the tolerance of Atlantic, Gulf, and Pacific coast species to methanol.

9.7.2 Materials

- Disposable C_{18} column(s)—for performing C_{18} manipulation (e.g., Waters (Sep-Pak Environmental Plus 1000 mg / 2.0 mL column))
- HPLC Grade Methanol (MEOH)—for activating C_{18} column(s).
- Low flow metering pump (~10 mL/min) and tubing—for forcing sample through C_{18} column.
- Separatory funnel—to serve as a sample reservoir.
- Erlenmeyer flasks—for collecting post-C_{18} effluent.

Table 9-6. Atlantic and Gulf Coast Species Tolerance to $Na_2S_2O_3$ (mg/L) (see Appendix for specific temperature and salinity)

Duration (hr)	Species LC_{50} or EC_{50} (± 95% Confidence Intervals)						
	Champia parvula (EC_{50})	*Arbacia punctulata* (EC_{50})	*Mulinia lateralis* (EC_{50})	*Mysidopsis bahia* (LC_{50})	*Ampelisca abdita* (LC_{50})	*Menidia beryllina* (LC_{50})	*Cyprinodon variegatus* (LC_{50})
1.2	-	>15000 (-)	-	-	-	-	-
24	-	-	-	>200	>300 (-)	12200 (11300-13000)	>15000 (-)
48	181 (141-773)	-	9400 (8990-9760)	164 (155-169)	>300 (-)	12000 (11500-12600)	>15000 (-)
72	-	-	-	121 (116-126)	223 (122-283)	11500 (10700-12400)	>15000 (-)
96	-	-	-	119 (113-125)	150 (87.5-214)	9550 (8330-10600)	>15000 (-)

- Not Available

Table 9-7. Pacific Coast Species Tolerance to $Na_2S_2O_3$ (mg/L) (see Appendix for specific temperature and salinity).

Duration (hr)	Species LC_{50} or EC_{50} (± 95% Confidence Intervals)							
	*Macrocystis pyrifera** (EC_{50})	*Strongylocentrotus purpuratus*† (EC_{50})	*Dendraster excentricus*† (EC_{50})	*Crassostrea gigas* (EC_{50})	*Mytilus californianus* (EC_{50})	*Mytilus galloprovincialis* (EC_{50})	*Haliotis rufescens* (EC_{50})	*Atherinops affinis*‡ (EC_{50})
<1.0	-	>1000	>1000	-	-	-	-	-
24	-	-	-	-	-	-	-	-
48	200 (-)	-	-	>500	>500	>500	10000 (-)	-
72	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	10000 (-)

- Not Available
* Germination Endpoint
† Fertilization Endpoint
‡ 7 Day Growth Endpoint

9.7.3 Procedural Overview

(1) Preparation of Tubing
- (a) Connect pump, sample reservoir and column with tubing. Do not attach column. Pump 25 ml of DI water followed by 25 ml of MEOH through the entire system to remove any contamination. Throughout this procedure a flowrate of 10 mL/min is used (Figure 9-7).

(2) Preparation of C_{18} Column
- (a) Attach C_{18} column to tubing (check manufacturer's recommendations for wetting volumes and total capacity of the column). Pass recommended volume of MEOH through the column. **Do not let the column dry out.**
- (b) Pass recommended volume of DI through the column. **Do not let the column dry out;** to avoid drying the column, leave a small volume of DI in the tubing.

(3) Blank Sample
- (a) Pass the brine and DI filtered blank through the wet prepped column.
- (b) Allow first 10-20 ml of brine and DI to pass into waste container before collecting sample. Collect enough post-column brine and DI to conduct toxicity tests (the column can now go dry).

(4) Re-prepare Column
- From Step 2, the same column may be used. **Do not let the column dry out** in between the preparatory steps or before adding the filtered sample.

(5) Sample
- (a) Pass the filtered sample through the wet prepped column.
- (b) Collect enough post-column sample to perform toxicity tests. Column can now go dry.

(6) Toxicity Testing
- (a) Prepare test dilutions using post-column sample and post-column brine and DI.
- (b) Add organisms.

9.8 Methanol Elution Test

9.8.1 General Approach and Materials

If following the C_{18} Column SPE Procedure (Section 9.7), and the post-column effluent shows reduced toxicity, it is recommended that the column be eluted with methanol to attempt to verify sample toxicity is due to an organic toxicant. Tables 9-8 and 9-9 provide information on the tolerance of several marine species to methanol.

Materials are the same as in the C_{18} Column SPE Procedure (Section 9.7.2) except the column is now "loaded."

9.8.2 Procedural Overview

(1) Preparation of Tubing
- Same as C_{18} Column SPE Procedure, Section 9.7.3.(1).(a) (Figure 9-7).

(2) Elution of Column
- (a) The reader is advised to consult EPA 1993b for specific details of column elution. The information here is only cursory.
- (b) Attach loaded column to tubing. Pass at least one column bed volume of methanol through column twice using a flowrate of 10 mL/min. Volume reduce eluate if necessary.
- (c) Collect methanol in container and return to initial sample volume with clean brine and DI. Use only enough methanol to be well below toxicity values in Table 9-8 and 9-9.

(3) Toxicity Testing
- (a) Prepare test dilutions using reconstituted sample and brine and DI.
- (b) Add organisms.

9.9 Graduated pH Procedure

9.9.1 General Approach

The pH of marine waters is largely controlled by the concentration of dissolved CO_2 present:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons H^+ + CO_3^=$$

As the concentration of CO_2 increases, the carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-) dissociate and the reaction goes to the right, generating an excess of hydrogen ions (H^+) which decreases sample pH. Conversely, if CO_2 is absent the hydrogen ions are found in an associated form and sample pH increases. In this procedure, sample pH is manipulated to determine if pH dependent toxicants are responsible for observed toxicity. For example, if sample toxicity increases with increasing sample pH, toxicants such as ammonia (NH_3) are suspected (Miller et al. 1990). Conversely, if sample toxicity increases with decreasing sample pH, toxicants such as hydrogen sulfide (H_2S) are suspected. Also, in freshwater, the toxicity of some metals is known to change as a function of pH (Schubauer-Berigan et al. 1993). For marine samples, exposures are conducted at three pHs: 7, ambient (7.9-8.4), and 9 using atmosphere-controlled chambers (Figure 9-8).

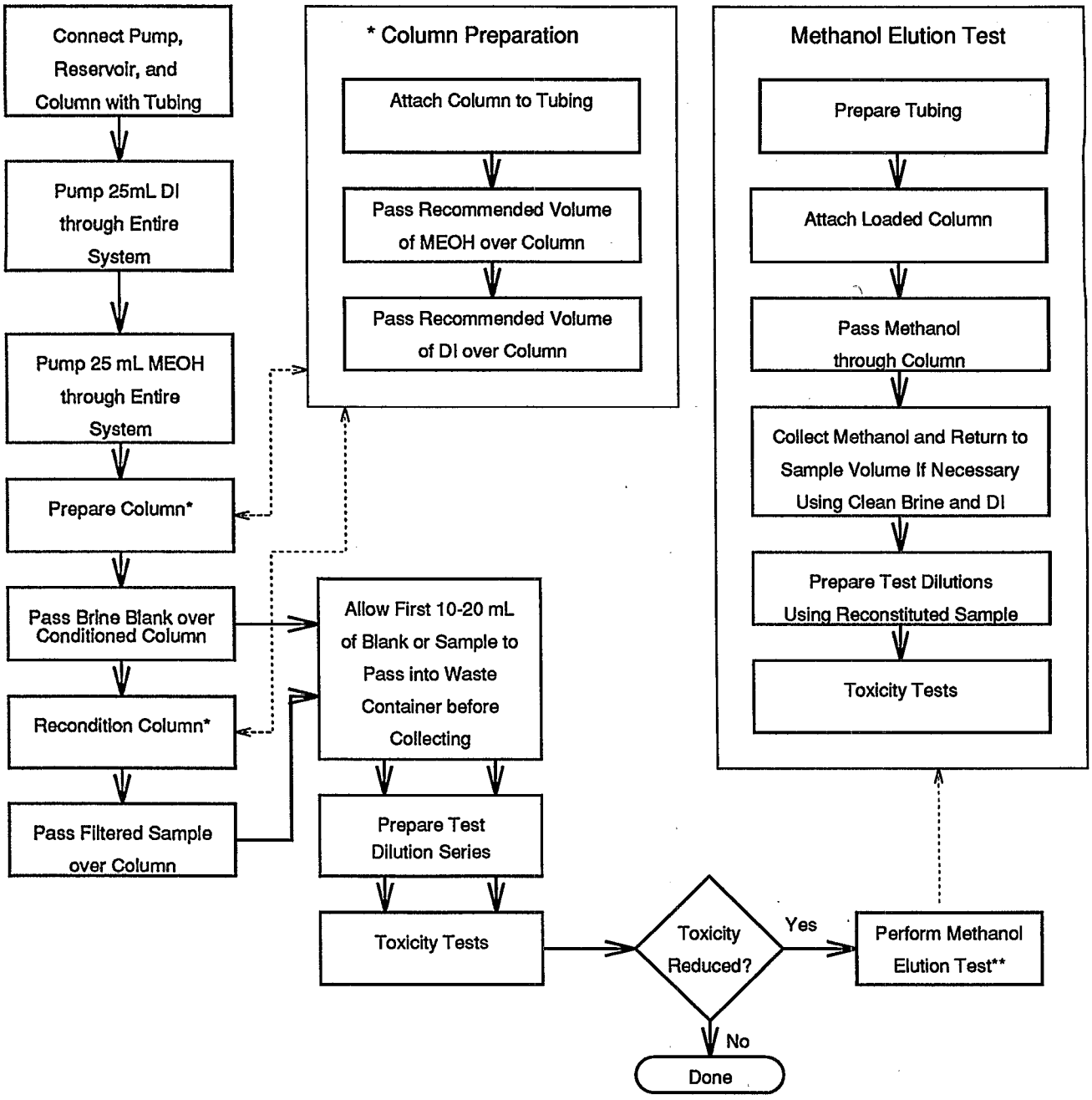

Figure 9-7. Overview Flowchart for C_{18} SPE Procedure and Methanol Elution Test (** Consult EPA 1993b).

Table 9-8. Atlantic and Gulf Coast Species Tolerance to Methanol (%v/v) (see Appendix for specific temperature and salinity)

Duration (hr)	Species LC_{50} or EC_{50} (± 95% Confidence Intervals)						
	Champia parvula (EC_{50})	*Arbacia punctulata* (EC_{50})	*Mulinia lateralis* (EC_{50})	*Mysidopsis bahia* (LC_{50})	*Ampelisca abdita* (LC_{50})	*Menidia beryllina* (LC_{50})	*Cyprinodon variegatus* (LC_{50})
1.2	-	9.31 (9.30-9.33)	-	-	-	-	-
24	-	-	-	2.43 (2.37-2.46)	3.75 (3.75-3.75)	2.56 (2.44-2.63)	3.89 (3.81-3.95)
48	0.13 (0.10-0.26)	-	2.18 (2.14-2.25)	2.35 (-)	3.21 (3.01-3.33)	2.33 (2.14-2.50)	3.67 (3.38-3.94)
72	-	-	-	2.35 (-)	1.25 (0.98-1.91)	1.77 (1.50-2.17)	3.39 (2.90-3.93)
96	-	-	-	2.30 (2.26-2.33)	0.75 (0.59-0.86)	1.55 (1.32-181)	3.33 (2.85-3.75)

- Not Available

Table 9-9. Pacific Coast Species Tolerance to Methanol (%v/v) (see Appendix for specific temperature and salinity).

Duration (hr)	Species LC_{50} or EC_{50} (± 95% Confidence Intervals)							
	*Macrocystis pyrifera** (EC_{50})	*Strongylocentrotus purpuratus*† (EC_{50})	*Dendraster excentricus*† (EC_{50})	*Crassostrea gigas* (EC_{50})	*Mytilus californianus* (EC_{50})	*Mytilus galloprovincialis* (EC_{50})	*Haliotis rufescens* (EC_{50})	*Atherinops affinis*‡ (EC_{50})
<1.0	-	3.78 (3.47-4.11)	3.50 (3.32-3.69)	-	-	-	-	-
24	-	-	-	-	-	-	-	-
48	-	-	-	3.14 (2.69-3.59)	2.26 (2.02-2.57)	3.55 (3.34-3.74)	-	-
72	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	-

- Not Available
* Germination Endpoint
† Fertilization Endpoint
‡ 7 Day Growth Endpoint

9.9.2 Materials

- pH 7.0 and pH 9.0 atmospheric chambers—for maintaining sample pHs at desired levels. Our atmospheric chambers were constructed from plexiglass in two sizes: 30 cm wide x 25 cm deep x 16 cm high and 80 cm x 40 cm x 30 cm. These chambers are not completely sealed from the ambient atmosphere but do maintain a positive pressure ensuring atmospheric gases do not enter. Locating the gas ports in the center of the chambers is advised to improve gas mixing.
- pH meter, stir plate, Teflon®-coated stirbars and calibration buffers—for monitoring sample pHs.
- Cylinders of CO_2, air, low CO_2 or low hydrocarbon air (e.g., Zero-Grade® or CO_2-Free®, (M.G. Industries, Valley Forge, PA)), and regulators for above cylinders (CGA 320 (CO_2), CGA 346 (Air) & CGA 590 (low CO_2))—to flow into pH chambers.
- CO_2 Scrubber—to remove CO_2 contamination from low CO_2 air (e.g., Merck, Damstadt, Germany).
- Precision flow meters (CO_2 meter should be capable of 2 mL/min)—for metering gas flow to chambers.

9.9.3 Sample Preparation

Samples are prepared for testing as described in the other TIE procedures, but with the following special preparations (Figure 9-9).

pH 7

(1) Approximately 24 hours before the manipulations are to be conducted, initiate CO_2 and air flow into the pH 7.0 chamber. Adjust the CO_2 flow to approximately 2% of the air flow (e.g., ~2 mL/min CO_2 to 98 mL/min of air).
(2) Approximately 18 hours before toxicity testing is to begin, check gas flow and place separate containers of the sample and blank (brine and DI) into the chamber. Let equilibrate overnight.

pH 8 (Initial)

Generally, pH 8 is the blank (brine and DI) and sample under initial atmospheric conditions. Because of the strong carbonate buffering capacity of seawater, the pH of these samples will usually range from 7.90 to 8.40. Set up this series at the same time as the pH 7 and 9.

pH 9

(1) Approximately 24 hours before manipulations are to begin, adjust the low CO_2 air flow to the pH 9.0 chamber to 150 - 300 mL/min.
(2) Adjust needed volumes of blank (brine and DI) and sample with 1 M sodium hydroxide (NaOH) to pH 9.0±0.3. CAUTION! The amount of NaOH needed varies based on the sample; overshooting pH 9.0 can result in excessive toxicity due to high salinity from excess sodium addition. After adjusting the pH, place the blank and sample volumes into the pH 9 chamber and close tightly.
(3) Approximately 18 hours before toxicity testing is to begin, check the pHs of the blank and sample to ensure that pH 9 is being maintained.

9.9.4 Procedural Overview

(1) Before conducting the toxicity test, check pHs of test solutions. For tests with marine animals (except for bivalves), pHs should be 7.0±0.3 for pH 7, ambient pH for pH 8, and 9.0±0.3 for pH 9 (Table 9-10). When testing marine plants, pHs should be 7.5±0.2 for pH 7, ambient pH for pH 8 and 9.0±0.3 for pH 9 (Table 9-10). Adjusted pH samples can be maintained outside of the chambers for short time periods (e.g., 5 - 10 minutes) to allow for preparing and monitoring the test.
(2) Set up toxicity test with test solutions and place dilution series in the appropriate chambers for the duration of test. Table 9-10 provides acceptable pH ranges for exposing Atlantic, Gulf, and Pacific coast marine organisms. Note that bivalve species are particularly sensitive to low pHs.
(3) Check gas flow and pH at least every 24 hrs. NOTE: Because of organism respiration or photosynthesis, pHs in the respective chambers will decrease or increase from nominal values, but changes should not exceed ± 0.3 pH units. If necessary, adjust gas flow to maintain desired pHs.

Table 9-10. Operational Species Tolerance Ranges to pH*

Species	pH Range
Atlantic and Gulf Coasts	
Champia parvula	7.4-9.2
Arbacia punctulata	7.2-9.1
Mulinia lateralis	8.0-8.8
Mysidopsis bahia	6.8-8.8
Ampelisca abdita	7.1-9.0
Menidia beryllina	Insufficient Data
Cyprinodon variegatus	6.6-8.8
Pacific Coast	
Macrocystis pyrifera	7-9
Strongylocentrotus purpurtatus	~7.8-8.5
Dendraster excentricus	Insufficent Data
Crassostrea gigas	7.5-8.5
Mytilus californianus	8.0-8.5
Mytilus galloprovincialis	7.5-8.5
Haliotis rufescens	7-9
Atherinops affinis	7-9

* See Appendix for specific salinity and temperature.

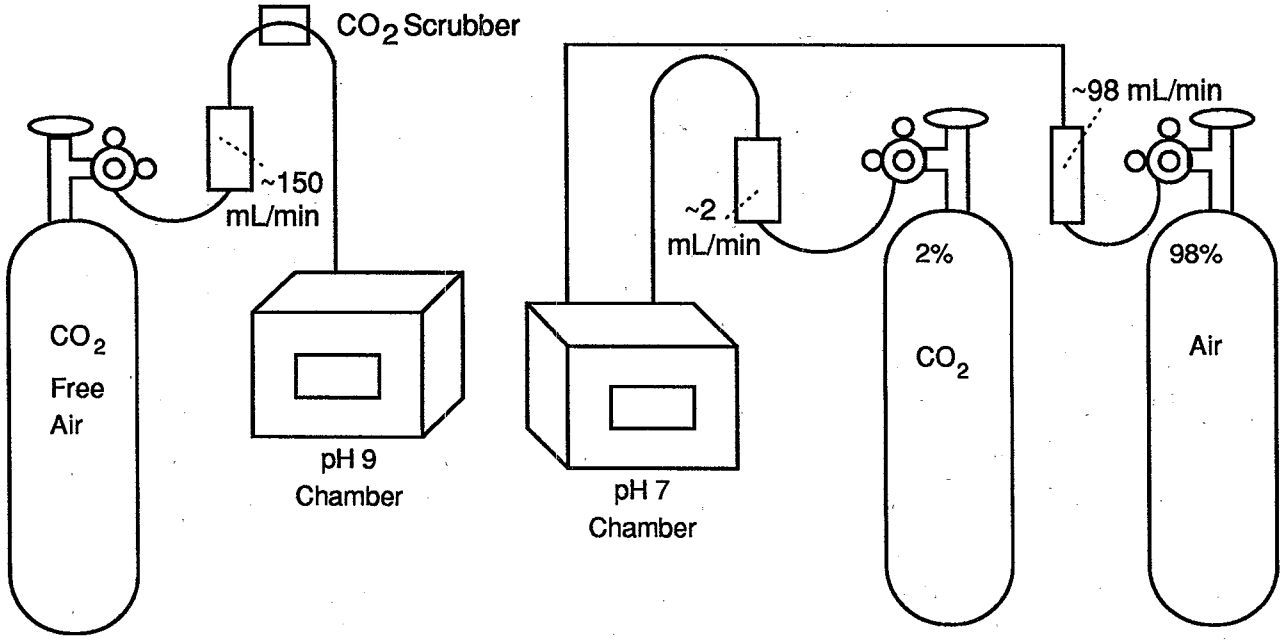

Figure 9-8. Apparatus Schematic for Graduated pH Procedure.

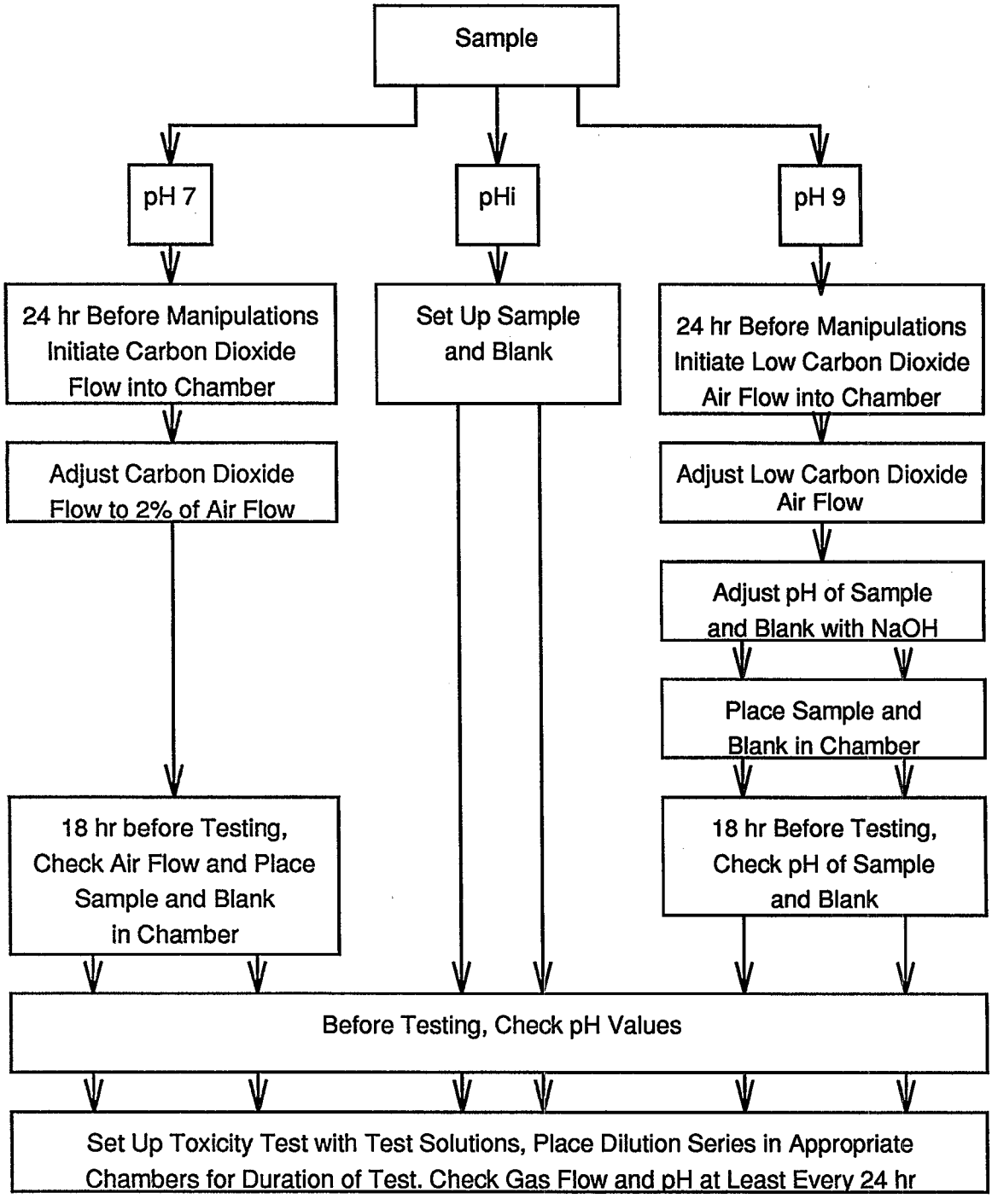

Figure 9-9. Overview Flowchart for Graduated pH Procedure.

9.10 Cation Exchange SPE Procedure

9.10.1 General Approach

The cation exchange manipulation is used to determine if toxic components are cationic in nature (e.g., metals). Cation exchange chromatography is applied to remove cationic toxicants from the aqueous sample. This manipulation can be used to support the EDTA manipulation (cf. Section 9.5) and with elution verify potential metal toxicity. Operationally, filtered test solutions (i.e., samples and controls) are passed through a disposable cation exhange column and the post-column sample tested for toxicity (Figure 9-10). Reduced toxicity in the post-column sample suggests that cationic toxicants are active (Burgess et al. submitted). Not all interferences with the cation exchange SPE procedure have been identified; therefore, it is important to perform the acid elution to verify metal toxicity.

Resulting post-cationic exchange column effluent is then tested to determine if the toxicity has been removed. The cation exchange column is activated with a combination of methanol and DI.

9.10.2 Materials

- Disposable cation exchange column(s)—for performing cation exchange manipulation (e.g., Supelco LC-WCX (500 mg/3 mL tube))
- 1M HCl Acid
- 1M NaOH
- Low flow metering pump (~0.5-10 mL/min) and tubing—for forcing sample through cation exchange column.
- Separatory funnel—to serve as effluent sample reservoir.
- Erlenmeyer flasks—for collecting post-column effluent.

9.10.3 Procedural Overview

(1) Preparation of Tubing
- (a) Connect pump, sample reservoir and column to tubing. Do not attach column. Pump 10 mL of 1 M HCl followed by 25 mL of DI through the entire system to remove any contamination. Throughout column preparation a flow of 7-10 mL/min is used.

(2) Preparation of Cation Exchange Column
- (a) Attach cation exchange column to tubing. For Supelco LC-WCX (3 mL/500 mg) column, the following procedure is recommended; for other types, check manufacturer recommendations. Using a flow rate of 2.5 mL/min, pass 2 mL of methanol through column. **Do not let the column dry out.**
- (b) Pass 6 mL of DI through the column. **Do not let the column dry out.** To avoid drying the column, leave a small volume of DI in the tubing.

(3) Blanks
- (a) Pass the brine and DI filtered blank through the wet prepared column.
- (b) Allow first 5 mL of brine and DI to pass into a waste container before collecting blank. Collect enough post-column brine and DI to conduct toxicity tests. Check pH to insure residual acid is not contaminating the sample. **Do not let the column dry out.**

(4) Effluent Sample
- (a) Pass the filtered sample through the wet prepared column.
- (b) Collect enough post-column sample to perform toxicity test. Column can now go dry. Check pH to insure residual acid is not contaminating sample.

(5) Toxicity Testing
- (a) Prepare test dilutions using post-column sample and post-column brine and DI.
- (b) Add organisms.

9.11 Cation Exchange SPE Acid Elution Test

9.11.1 General Approach and Materials

If following the Cation Exchange SPE procedure (Section 9.10), the post-column sample is non-toxic, it is recommended that the column be eluted with 1 M HCl to verify sample toxicity due to metal toxicants.

Materials for this test are the same as the Cation Exchange SPE Procedure (Section 9.10.2).

9.11.2 Procedural Overview

(1) Preparation of Tubing
- Same as Cation Exchange SPE Procedure, Section 9.10.3.(1).(a).

(2) Elution of Column
- (a) Attach loaded column to tubing. Pass 6 mL 1 M HCl through column using a flowrate of 0.5 mL/min.
- (b) Collect HCl in container and return sample to original volume with clean brine and DI and adjust pH with sodium hydroxide (Figure 9-10).

(3) Toxicity Testing
- (a) Prepare test dilutions using reconsituted sample and DI.
- (b) Add organisms.

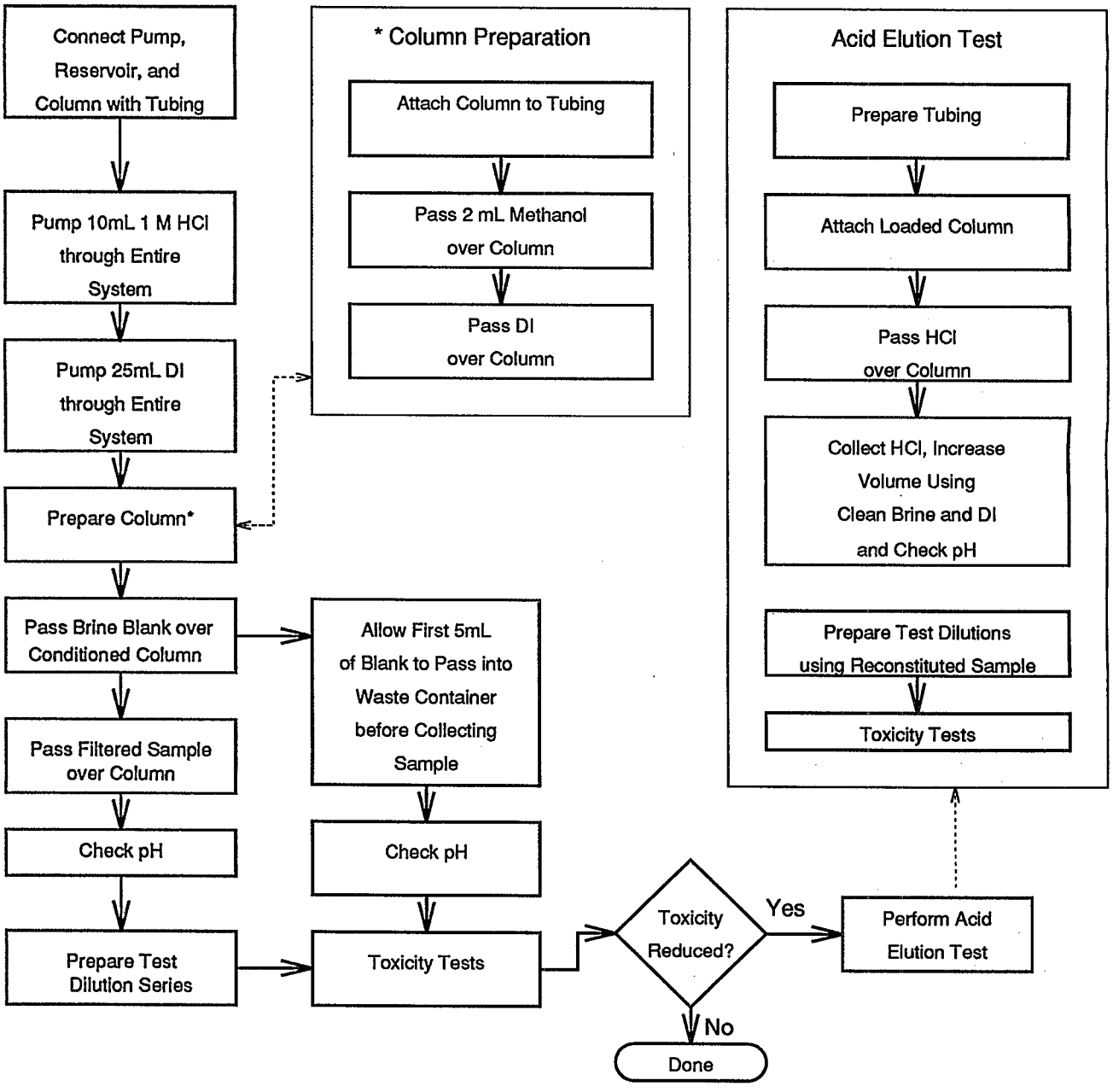

Figure 9-10. Overview Flowchart for Cation Exchange SPE Procedure and Acid Elution Test.

9.12 *Ulva lactuca* Procedure

9.12.1 General Approach

The objective of this manipulation is to remove ammonia from seawater samples by addition of a marine macrophyte *Ulva lactuca*, commonly known as sea lettuce. *Ulva lactuca* is a macrophyte that has the ability to uptake, store, and utilize large amounts of ammonia. *Ulva lactuca* has historically been used to clean-up effluents in aquaculture (Cohen and Neori 1991; Neori et al. 1991) and has proven effective in removing environmental concentration of ammonia from seawater (Ho et al. in prep.).

9.12.2 Materials

- *Ulva lactuca* 5g/60mL of sample
- Oil-free air pump, tubing, and pipettes
- Containers—to hold 60 mL sample, *Ulva lactuca*, and allow for aeration
- Light source (~75 µE/m²/s)
- Temperature 15-20°C. Temperatures over 20°C hasten the degradation of *Ulva lactuca* during storage.

9.12.3 Procedural Overview

(1) *Ulva lactuca* Collection and Storage

- Collect *Ulva lactuca* from a clean site. Sort through plants and discard any with white or yellowing tips. Remove any surficial organisms and hold static in 30‰ clean seawater in aerated jars under 16:8 light:dark condition until use. Sea lettuce is held in static systems, not flow-through conditions to minimize the exposure of the plant to nutrient concentration. Presumably, if the plant is "starved", it will uptake ammonia more quickly when placed in the sample. Maximum holding time for *Ulva lactuca* is four days but should be used within 24 hr for optimal results (Figure 9-11).

(2) *Ulva lactuca* Addition

- Remove *Ulva lactuca* from holding jars using forceps, gently pat dry and place in salinity adjusted sample under lights with gentle aeration for five hours.
- (b) Remove *Ulva lactuca* from sample.

(3) *Ulva lactuca* Removal

- (a) Remove *Ulva lactuca* from sample.
- (b) Prepare toxicity dilutions with *Ulva lactuca* treated brine and DI and sample.

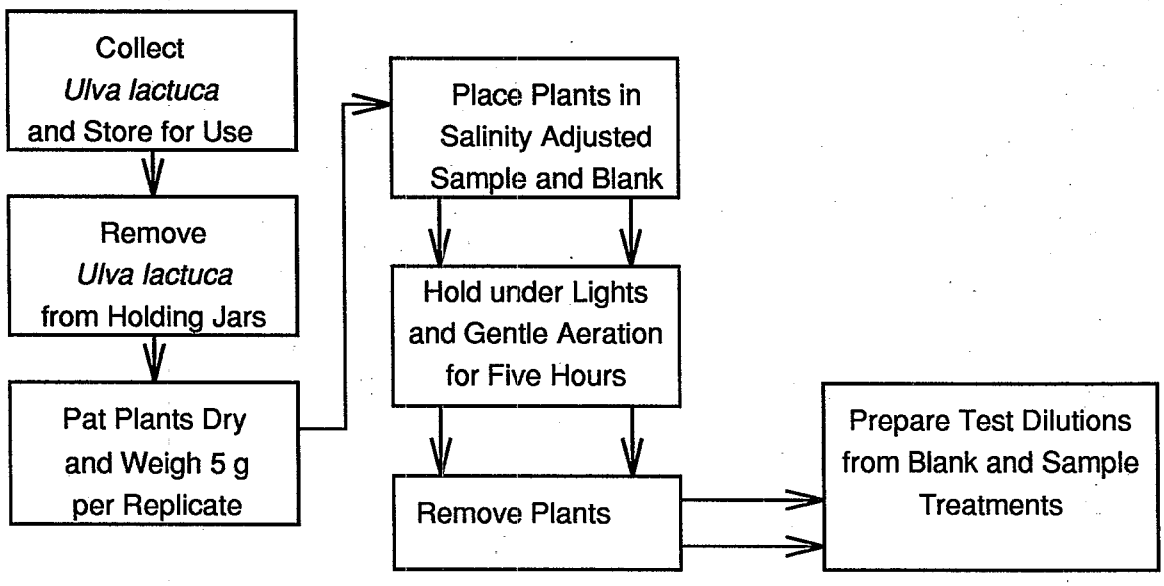

Figure 9-11. Overview Flowchart for *Ulva lactuca* Procedure.

Section 10
TIE Interpretation

To determine the efficacy of these methods in characterizing unknown toxicants, we performed some marine TIE manipulations on two spiked brine and DI samples (i.e., mock effluent). One sample contained 40 mg/L of the reference toxicant sodium dodecyl sulfate (SDS) and the other copper sulfate (1.0 mg copper/L). Results from these TIEs conducted on very simple samples provide insight into the complexity of interpreting marine TIE data.

10.1 Sodium Dodecyl Sulfate (SDS)

In this TIE, tests were conducted with the mysid *Mysidopsis bahia*. Results are presented in Table 10-1.

Table 10-1. Results of Toxicity Test with Sodium Dodecyl Sulfate-Spiked Brine and DI Using Mysid, *Mysidopsis bahia*. Conditions: 30‰, 21°C.

Manipulation	Toxic Units
Initial	Not Performed *
Baseline	6.8
EDTA Addition	6.7
$Na_2S_2O_3$ Addition	7.5
Filtration	6.7
Post C_{18}	No Toxicity †

* Historic data used to determine baseline exposure concentration.
† 0% Mortality in highest concentration (40 mg SDS/L)

As these data demonstrate, the C_{18} column removed all toxicity, and there was no significant change in toxicity in the other manipulations except for the possible increase in toxicity caused by sodium thiosulfate. These results should be interpreted that organic compounds are responsible for all or most of the toxicity. Although C_{18} column elution data for this example analysis is not available, the reader is reminded that that procedure is highly recommended (cf. Section 9.8).

10.2 Copper

Copper toxicity tests were conducted with the sea urchin *Arbacia punctulata*, mysid *Mysidopsis bahia*, and fish *Menidia beryllina*. Results are presented in Table 10-2

Table 10-2. Results of Toxicity Test with Copper-Spiked Brine and DI Using Sea Urchin, *Arbacia punctulata*, Mysid, *Mysidopsis bahia*, and Fish, *Menidia beryllina*. Conditions: 30‰, 21°C.

Manipulation	Toxic Units		
	Arbacia punctulata	*Mysidopsis bahia*	*Menidia beryllina*
Initial	5.0	2.4	8.6
Baseline	11.9	1.7	5.3
EDTA Addition	<2.0 *	<2.0 †	<4.0 §
$Na_2S_2O_3$ Addition	2.2	5.3	<4.0 #
Filtration	5.0	2.1	<4.0 **
Aeration	14.5	5.8	6.4
Post C_{18}	3.1	<2.0 ‡	<4.0 §

* 100% Fertilization at 50% effluent.
† 100% Survival at 50% effluent.
‡ 60% Survival at 50% effluent.
§ 100% Survival in 25% effluent.
\# 90% Survival in 25% effluent.
** 60% Survival in 25% effluent.

Results of this TIE are not as easily evaluated as was SDS; clearly, EDTA removed the most toxicity in all cases with all three species, but other manipulations removed toxicity as well. Toxicity to *Arbacia punctulata* increased between the Initial Toxicity Test and the Baseline Toxicity Test by 6.9 toxic units. This significant variablility in the response of the sea urchin sperm cell test is not uncommon when measuring copper toxicity. Morrison et al. (1989) reports a coefficient of variation of 46% for *Arbacia punctulata* in reference toxicant tests with copper.

All manipulations removed some amount of toxicity to *A. punctulata* except aeration, which increased toxicity about 2.5 toxic units. Toxicity to the mysid was fairly low but both the sodium thiosulfate and aeration manipulations increased toxicity. Exposures to the fish demonstrated a small reduction in toxicity between the Initial and Baseline Toxicity Tests and all manipulations reduced toxicity except for aeration.

Possible reasons for these results are: 1) sodium thiosulfate reduces the toxicity of some metals (EPA 1991b; MED, Duluth, personal communication), 2) filtration of metals through a glass fiber filter may result in adsorption of copper to the filter surface, and 3) C_{18} chelates some metals like copper. Aeration results that were consistent for all species suggest that the sample volume was reduced, and consequently, metal concentrations increased. However, it has been observed that EDTA seldom reduces the toxicity of any other toxicants except metals (MED, Duluth, personal communication); therefore, Table 10-2 results strongly support the presence of metals toxicity. If this sample had been a complex mixture of toxicants from an industrial or municipal plant, evaluation of these initial results would have suggested a combination of metals and organics as being the sources of toxicity.

10.3 Summary of Results

Phase I as described in this guidance document is dedicated to toxicity characterization. In Phases II and III, the TIE includes more advanced approaches: for example, the use of analytical chemistry (EPA 1993b, 1993c). For the exercise with copper above, analytical chemistry would progress the characterization from types of toxicants to specific toxicants by demonstrating the presence of elevated levels of copper. In general, comparison of these concentration data for various contaminants to the sensitivities of the test species in the scientific literature, including EPA WQC, may help to elucidate which types of toxicants to include or exclude from consideration. Specifically, toxicity information on toxic metals, organics and ammonia are readily available from these sources. Use of this information will help individuals conducting marine TIEs to establish sensitivity patterns for the various marine species (e.g., *Arbacia punctulata* is very sensitive to most divalent transition metals and insensitive to most organics and ammonia). These sensitivity patterns in turn become diagnostic TIE tools contributing to the determination of what toxicants are active. Any complementary data (e.g., historical, collection, site) will assist in the characterization.

The investigator needs to keep in mind potential interferences to the TIE manipulations; although the methods are designed to be specific to single classes of toxicants, they may not be so in practice. Documented interferences or 'side effects' include: the pH manipulations changing the toxicity of both metals and ionic organic toxicants (Schubauer-Berigan et al. 1993; Spehar et al. 1984); and the C_{18} SPE can sorb certain metals from seawater; filtration may remove metals and nonionic organic toxicants from solution while *Ulva lactuca* removes nonionic toxicants (Ho et al. in prep.). Also, not all possible interferences associated with the cation exchange SPE have been determined. Despite the problems interferences can create when interpreting a TIE, advantage may be taken of interferences to aid in the characterization of toxicants.

Following the Phase I of a marine TIE are Phases II (Identification) and III (Confirmation). The reader is advised to refer to EPA 1991b, 1993b, and 1993c for guidance in performing these phases.

Section 11
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Appendix
Summary of Test Conditions and Acceptability

The tables in this appendix summarize test conditions and acceptability for the Phase I Marine TIE characterization tests. Because routine TIE toxicity testing methods are not currently available for all Pacific Coast species, the standard test conditions are provided. Tables correspond to those in EPA 1993a, 1994, 1995. Readers should refer to these references for detailed procedural outlines of the toxicity tests, and use the tables in this appendix for Marine Phase I TIE-specific variations.

Table A.1. Summary of TIE Test Conditions and Test Acceptability Criteria for Amphipod, *Ampelisca abdita*, Acute Toxicity Tests.

1. Test Type	Static non-renewal
2. Salinity	30±2‰
3. Temperature	20±2°C
4. Light quality	Ambient laboratory light
5. Light intensity	10-20 µE/m²/s (50-100-ft-c) (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	25 mL chambers
8. Test solution volume	10-20 mL
9. Size of test organisms	0.5-0.7 mm
10. No. of organisms per chamber	5
11. No. replicate chambers per concentration	1-3 (TIE manipulations) 3 (Initial and Baseline)
12. Feeding regime	none
13. Dilution water	Natural seawater or hypersaline brine
14. Test concentrations	6 (Initial and Baseline toxicity tests) 4 (TIE procedures)
15. Dilution series	0.5
16. Test duration	24, 48, or 96 h
17. Endpoints	Mortality (LC_{50})
18. Test acceptability criteria	≥90% survival in controls

Table A.2. Summary of TIE Test Conditions and Test Acceptability Criteria for Sea Urchin, *Arbacia punctulata*, Fertilization Test.

1. Test Type	Static
2. Salinity	30±2‰
3. Temperature	20±1°C
4. Light quality	Ambient laboratory light during test preparation
5. Light intensity	10-20 µE/m²/s, or 50-100 ft-c (ambient laboratory levels)
6. Test chamber size	Disposable (glass) liquid scintillation vials (20 mL capacity), presoaked in control water
7. Test solution volume	5 mL
8. No. of sea urchins	Pooled sperm from four males and pooled eggs from four females are used per test
9. No. egg and sperm cells per chamber	About 2000 eggs and 5,000,000 sperm cells per vial
10. No. replicate chambers per concentration	4 (minimum of 3)
11. Dilution water	Uncontaminated source of natural seawater; deionized water mixed with hypersaline brine or artificial sea salts (HW Marinemix®, FORTY FATHOMS®, GP2, or equivalent)
12. Effluent concentrations	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Test dilution factor	Effluents: ≥0.5 Receiving waters: None, or ≥0.5
14. Test duration	1 hour and 20 min
15. Endpoints	Fertilization of sea urchin eggs
16. Test acceptability criteria	70%-90% egg fertilization in controls

Table A.3. Summary of Standard Test Conditions and Test Acceptability Criteria for the Topsmelt, *Atherinops affinis*, Larval Survival and Growth Test. (NOTE: for Phase I TIE, conditions may need to be altered (e.g., test volume)).

1. Test Type	Static-renewal
2. Salinity	5 to 34‰ (± 2‰ of the selected test salinity)
3. Temperature	20±1 °C
4. Light quality	Ambient laboratory illumination
5. Light intensity	10-20 µE/m²/s (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	600 mL
8. Test solution volume	200 mL/replicate
9. Renewal of test solutions	Daily
10. Age of test organism	9-15 days post hatch
11. No. of larvae per test chamber	5
12. No. replicate chambers per concentration	5
13. Source of food	Newly hatched *Artemia* nauplii
14. Feeding regime	Feed 40 nauplii per larvae twice daily (morning and night)
15. Cleaning	Siphon daily, immediately before test solution renewal and feeding
16. Aeration	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/min.
17. Dilution water	Uncontaminated 1 µm-filtered natural seawater or hypersaline brine prepared from natural seawater
18. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
19. Dilution factor	effluents: ≥0.5 Receiving waters: None, or ≥0.5
20. Test duration	7 days
21. Endpoints	Survival and growth (weight)
22. Test acceptability criteria	≥80% survival in controls, 0.85 mg average weight of control larvae (9 day old), LC_{50} with copper must be ≤205 µg/L, <25% MSD* for survival and 50% MSD for growth†

* MSD Mean Standard Deviation
† Provisional, check with appropriate Region or State for latest guidance.

Table A.4. Summary of TIE Test Conditions and Test Acceptability Criteria for the Red Macroalga, *Champia parvula*, Sexual Reproduction Test.

1. Test type	Static, Static non-renewal
2. Salinity	30±2‰
3. Temperature	23±1 °C
4. Light source	Cool-white flourescent lights
5. Light intensity	100 µE/m²/s (500 ft-c)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	50 mL polystyrene or borosilicate petri dishes or 125 mL Erlenmeyer flasks
8. Test solution volume	20 mL (minimum)
9. No. of organisms per test chamber	5 female branch tips and 1 male plant
10. No. replicate chambers per concentration	4 (minimum of 3)
11. No. of organisms per concentrations	24 (minimum of 18)
12. Dilution water	Uncontaminated source of natural seawater; deionized water mixed with hypersaline brine or artificial sea salts (HW Marinemix®, FORTY FATHOMS®, GP2, or equivalent)
13. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
14. Dilution factor	Effluents: ≥0.5 Receiving waters: None, or ≥0.5
15. Test duration	Two day exposure to effluent, followed by 5 to 7 day recovery period in control medium for cystocarp development
16. Endpoints	Reduction in cystocarp production compared to controls
17. Test acceptability criteria	80% or greater survival, and an average of 10 cystocarps per plant in controls

Table A.5. Summary of Standard Test Conditions and Test Acceptability Criteria for Oyster, *Crassostrea gigas* and Mussels, *Mytilus californianus* and *Mytilus galloprovincialis*, Embryo-Larval Development Test.

1. Test type	Static non-renewal
2. Salinity	30±2‰
3. Temperature	20±1°C (oysters) 15 or 18 ±1°C (mussels)
4. Light quality	Ambient laboratory illumination
5. Light intensity	10-20 µE/m²/s (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	30 mL
8. Test solution volume	10 mL
9. No. of larvae per chamber	150-300
10. No. replicate chambers per concentration	4
11. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
12. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor	effluents: ≥0.5 Receiving waters: None, or ≥0.5
14. Test duration	48 hours (or until complete development up to 54 hours)
15. Endpoints	Survival and normal shell development
16. Test acceptability criteraia	Control survival must be ≥70% for oyster embryos or ≥50% for mussel embryos in control vials; ≥90% normal shell development in surviving controls; and must achieve %MSD* of <25%†

* MSD Mean Standard Deviation
† Provisonal, check with appropriate Region or State for latest guidance.

Table A.6. Summary of TIE Test Conditions and Test Acceptability Criteria for Fish, *Cyprinodon variegatus*, Acute Toxicity Tests.

1. Test type	Static non-renewal
2. Salinity	25±10
3. Temperature	20±2°C
4. Light quality	Ambient laboratory light
5. Light intensity	10-20 µE/m²/s (50-100-ft-c) (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	25 mL chambers
8. Test solution volume	10-20 mL
9. Age of test organisms	1-14 days old at start
10. No. replicate chambers per concentration	1 (TIE manipulations) 3 (Initial and Baseline)
11. No. organisms per chamber	5
12. Feeding regime	Feed one drop of concentrated *Artemia* nauplii suspension daily (approximately 100 nauplii per mysid)
13. Dilution water	Natural seawater or hypersaline brine
14. Test concentrations	6 (Initial and Baseline toxicity tests) 4 (TIE procedures)
15. Dilution series	0.5
16. Test duration	24, 48, or 96 h
17. Endpoints	Mortality (LC50)
18. Test acceptability criteria	≥80% survival in controls

Table A.7. Summary of Standard Test Conditions and Test Acceptability Criteria for Albalone, *Haliotis rufescens*, Larval Development Test. (NOTE: for Phase I TIE, conditions may need to be altered (e.g., sample volume)).

1. Test Type	Static non-renewal
2. Salinity	34±2‰
3. Temperature	15±1 °C
4. Light quality	Ambient laboratory illumination
5. Light intensity	10 µE/m²/s (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	600 mL*
8. Test solution volume	200 mL/replicate*
9. Larvae density per chamber	5-10 per mL
10. No. Replicate chambers per concentration	5
11. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine plus reagent water
12. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor	Effluents: ≥0.5 Receiving waters: None, or ≥0.5
14. Test duration	48 h
15. Endpoint	Normal shell development
16. Test acceptability criteria	≥80% normal shell development in the controls; must have statistical significant effect at 56 µg/L zinc; must acheive a %MSD† of <20%‡

* Successful tests performed at 10 mL volume in 20 mL scintillation vials (Hunt et al. In press).
† MSD Mean Standard Deviation
‡ Provisional, check with appropriate Region or State for latest guidance

Table A.8. Summary of Standard Test Conditions and Test Acceptability Criteria for Giant Kelp, *Macrocystis pyrifera*, Germination and Germ-tube Length Test. (NOTE: for Phase I TIE, conditions may need to be altered (e.g., sample volume)).

1. Test Type	Static non-renewal
2. Salinity	34±2‰
3. Temperature	15±1 °C
4. Light quality	Ambient laboratory light during test preparation
5. Light intensity	50±10 µE/m²/s
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	600 mL
8. Test solution volume	200 mL/replicate
9. Spore density per test chamber	7500 /mL of test solution
10. No. Replicate chambers per concentration	5
11. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
12. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor	Effluents: ≥0.5 Receiving waters: None or ≥0.5
14. Test duration	48 h
15. Endpoints	Germination and germ-tube length
15. Test acceptability criteria	≥70% germination in the controls; ≥ 10µm germ-tube length in the controls and the NOEC must be below 35 µg/L in the reference toxicant test; must achieve a %MSD* of <20 for both germiniation and germ-tube length in the reference toxicant.†

* MSD Mean Standard Deviation
† Provisional, check with appropriate Region or State for latest guidance.

Table A.9. Summary of TIE Test Conditions and Test Acceptability Criteria for Fish, *Menidia beryllina*, Acute Toxicity Test.

1. Test Type	Static non-renewal
2. Salinity	25±10‰
3. Temperature	20±2°C
4. Light quality	Ambient laboratory light
5. Light intensity	10-20 µE/m²/s (50-100-ft-c) (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	25 mL chambers
8. Test solution volume	10-20 mL
9. Age of test organisms	9-14 days old at start
10. No. replicate chambers per concentration	1 (TIE manipulations) 3 (Initial and Baseline)
11. Organisms per chamber	5
12. Feeding regime	Feed one drop of concentrated *Artemia nauplii* suspension daily (approximately 100 nauplii per mysid)
13. Dilution water	Natural seawater or hypersaline brine
14. Test concentrations	6 (Initial and Baseline toxicity tests) 4 (TIE procedures)
15. Dilution series	0.5
16. Test duration	24, 48, or 96 h
17. Endpoints	Mortality (LC_{50})
18. Test acceptability criteria	≥80% survival in controls

Table A.10. Summary of TIE Test Conditions and Test Acceptability Criteria for Bivalve, *Mulinia lateralis*, Embryo-Larval Development Test.

1. Test type	Static non-renewal
2. Salinity	30±2‰
3. Temperature	20±2°C
4. Light quality	Ambient laboratory illumination
5. Light intensity	10-20 µE/m²/s (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	30 mL
8. Test solution volume	10 mL
9. No. of larvae per chamber	~300
10. No. Replicate chambers per concentration	3-4
11. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
12. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor	Effluents: ≥0.5 Receiving waters: None, or ≥0.5
14. Test duration	48 hours
15. Endpoints	Survival and normal shell development
16. Test acceptability criteria	> 70% Survival; >90% Development

Table A.11. Summary of TIE Test Conditions and Test Acceptability Criteria for Mysid, *Mysidopsis bahia*, Acute Toxicity Tests.

1. Test type	Static non-renewal
2. Salinity	25±10‰
3. Temperature	20±2°C
4. Light quality	Ambient laboratory light
5. Light intensity	10-20 µE/m²/s (50-100-ft-c) (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	30 mL chambers
8. Test solution volume	10-20 mL
9. Age of test organisms	48 h old at start
10. Number of organisms per chamber	5
11. No. Replicate chambers per concentration	1 (TIE manipulations) 3 (Initial and Baseline)
12. Feeding regime	Feed one drop of concentrated *Artemia nauplii* suspension daily (approximately 100 nauplii per mysid)
13. Dilution water	Natural seawater or hypersaline brine
14. Test concentrations	6 (Initial and Baseline toxicity tests) 4 (TIE procedures)
15. Dilution series	0.5
16. Test duration	24, 48, or 96 h
17. Endpoints	Mortality (LC_{50})
17. Test acceptability criteria	≥80% survival in controls

Table A.12. Summary of Standard Test Conditions and Test Acceptability Criteria for the Purple Urchin, *Strongylocentrotus purpuratus*, and Sand Dollar, *Dendraster excentricus*, Fertilization Tests.

1. Test Type	Static non-renewal
2. Salinity	34±2‰
3. Temperature	12±1°C
4. Light quality	Ambient laboratory light during test preparation
5. Light intensity	10-20 µE/m²/s (ambient laboratory levels)
6. Test chamber size	16 x 100 or 16 x 125 mm
7. Test solution volume	5 mL
8. Number of spawners	Pooled sperm from up to four males and pooled eggs from up to four females are used per test.
9. No. Egg and sperm cells per chamber	About 1,120 eggs and not more than 3,360,000 sperm per test tube
10. No. Replicate chambers per concentration	4
11. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater or artificial sea salts
12. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
12. Dilution factor	Effluents: ≥0.5 Receiving waters: None or ≥0.5
13. Test duration	40 min (20 min plus 20 min)
14. Endpoint	Fertilization of eggs
15. Test acceptability criteria	≥70% egg fertilization in controls; %MSD* of <25%; and appropriate sperm counts†

* MSD Mean Standard Deviation
† Provisional, check with appropriate Region or State for latest guidance.

Table A.13. Summary of Standard Test Conditions and Test Acceptability Criteria for the Purple Urchin, *Strongylocentrotus purpuratus*, and Sand Dollar, *Dendraster excentricus*, Embryo-Larval Development Test.

1. Test Type	Static non-renewal
2. Salinity	34±2‰
3. Temperature	15±1°C
4. Light quality	Ambient laboratory illumination
5. Light intensity	10-20 µE/m²/s (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h darkness
7. Test chamber size	30 mL
8. Test solution volume	10 mL
9. No. Replicate chambers per concentration	4
10. Dilution water	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
11. Test concentrations	Effluent: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
12. Dilution factor	Effluents: ≥0.5 Receiving waters: 100% receiving water and a control
13. Test duration	72±2 h
14. Endpoint	Normal development; mortality can be included
15. Test acceptability criteria	≥80% normal shell development in the controls; must acheive a %MSD* of <25%†

* MSD Mean Standard Deviation
† Provisional, check with appropriate Region or State for latest guidance.

☆U.S. GOVERNMENT PRINTING OFFICE: 1997 - 549-001/60110

United States Environmental Protection Agency
Office of Wastewater Management (4203)
EPA 833-R-00-003
June 2000
http:\\www.epa.gov\owm\npdes.htm

Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System

Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System Program

June 30, 2000

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NOTICE AND DISCLAIMER

This document provides guidance to NPDES regulatory authorities and persons interested in whole effluent toxicity testing. This document describes what EPA believes to be sources of variability in the conduct of whole effluent toxicity testing under the Clean Water Act. The document is designed to reflect national policy on these issues. The document does not, however, substitute for the Clean Water Act, an NPDES permit, or EPA or State regulations applicable to permits or whole effluent toxicity testing; nor is this document a permit or a regulation itself. The document does not and cannot impose any legally binding requirements on EPA, States, NPDES permittees, and/or laboratories conducting whole effluent toxicity testing for permittees (or for States in the evaluation of ambient water quality). EPA and State officials retain discretion to adopt approaches on a case-by-case basis that differ from this guidance based on an analysis of site-specific circumstances. This guidance may be revised without public notice to reflect changes in EPA policy.

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ACKNOWLEDGEMENTS

This guidance was prepared through the cooperative efforts of the U.S. Environmental Protection Agency's (EPA) Office of Wastewater Management and Office of Science and Technology in the Office of Water, EPA's Office of Research and Development, and EPA's Office of Enforcement and Compliance Assurance. The Cadmus Group, Inc. provided support for the final document production.

EPA Variability Workgroup

Debra Denton, EPA Region 9, San Francisco, CA
John Fox, EPA Office of Science and Technology, Washington, DC
Florence Fulk, EPA Office of Research and Development, Cincinnati, OH
Kathryn Greenwald, EPA Office of Enforcement and Compliance Assurance, Washington, DC
Madonna Narvaez, EPA Region 10, Seattle, WA
Teresa Norberg-King, EPA Office of Research and Development, Duluth, MN
Laura Phillips, EPA Office of Wastewater Management, Washington, DC

EPA Support Outside of the Variability Workgroup

Gregory Currey, EPA Office of Wastewater Management, Washington, DC
Margarete Heber, EPA Office of Wetlands, Oceans, and Watersheds, Washington, DC
Phillip Jennings, EPA Region 6, Dallas, TX
Henry Kahn, EPA Office of Science and Technology, Washington, DC
Marion Kelly, EPA Office of Science and Technology, Washington, DC
James Pendergast, EPA Office of Wetlands, Oceans, and Watersheds, Washington, DC
Stephen Sweeney, EPA Office of General Counsel, Washington, DC
William Telliard, EPA Office of Science and Technology, Washington, DC
Robert Wood, EPA Office of Wastewater Management, Washington, DC
Marcus Zobrist, EPA Region 2, New York, NY

Contractor Support

The Cadmus Group, Inc., Durham, NC

Karalyn Colopy Susan Conbere
Blanche Dean Penelope Kellar

DynCorp Information and Enterprise Technology, Inc., Alexandria, VA

Robert Brent

Science Applications International Corporation, Columbia, MD

Sidina Dedah Ruth Much
Kathleen Stralka

Assistance from States

State Case Example and Chapter 4

EPA especially thanks Larry Ausley and Matt Matthews of the North Carolina Department of Environment and Natural Resources, Division of Water Quality for preparing Chapter 4 (Variability in Context) and Appendix F (Improvements in Minimizing WET Test Variability by the State of North Carolina).

State-Specific WET Program Implementation

EPA appreciates the assistance and support from the following States in providing their State-specific approaches to the WET program implementation used for Appendix E (Examples of Selected State WET Implementation Programs) of this document.

Betty Jane Boros-Russo, State of New Jersey
Kari Fleming, State of Wisconsin
Randall Marshall, State of Washington
Matt Matthews, State of North Carolina
Charlie Roth, State of Kentucky

Additional Assistance

We also thank the EPA Regions, States, and laboratories that provided the WET toxicity data used to develop this guidance document.

EPA Peer Review

Peer review was conducted following the EPA's Science Policy Council Handbook for Peer Review (January 1998). The anonymous review comments are gratefully acknowledged, and the changes were incorporated, as appropriate.

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EXECUTIVE SUMMARY

Background

The Federal Water Pollution Control Act, commonly known as the Clean Water Act, was enacted in 1972 with the objective of "*restoring the chemical, physical, and biological integrity of the Nation's waters*." Among the U.S. Environmental Protection Agency's (EPA's) efforts toward this objective is the National Pollutant Discharge Elimination System (NPDES) program. This program is designed to control toxic discharges, implement water quality standards, and restore waters to "fishable and swimmable" conditions. Point sources that discharge pollutants must do so under the terms and conditions of an NPDES permit. One approach EPA employs to control toxic pollutants under the NPDES permits program is using whole effluent toxicity (WET) controls.

EPA is issuing this document to both address questions raised on WET test method variability and to satisfy a requirement of a July 1998 settlement agreement with litigants for the Western Coalition of Arid States (WestCAS) and Edison Electric Institute et al. This document was developed by an EPA workgroup consisting of EPA's Office of Water's (OW) Headquarters, Office of Enforcement and Compliance Assurance, Office of Research and Development, and Regional staff. The document was externally peer reviewed in accordance with EPA's peer review guidelines. The document addresses WET test method variability by identifying the potential sources of variance associated with WET testing, discusses how to minimize it and, finally, describes how to address it within the NPDES permitting program. The document cites both Agency and external ongoing research on this topic and scientific findings, particularly technical information that support efforts to minimize WET test result variability.

While the document provides recommendations on how to reduce or minimize WET test variability, the document does not supersede current Agency guidance, policy, or regulation, including EPA's promulgated test methods (40 CFR Part 136), which remain in effect. EPA expects that implementation of the NPDES program and NPDES permits will continue to comply with regulatory requirements and follow applicable EPA guidance and policy.

Why WET Testing?

Whole effluent toxicity is the aggregate toxic effect of an aqueous sample (e.g., effluent, receiving water) measured directly by an aquatic toxicity test. Aquatic toxicity tests are laboratory experiments that measure the biological effect (e.g., growth, survival, and reproduction) of effluents or receiving waters on aquatic organisms. In aquatic toxicity tests, organisms of a particular species are held in test chambers and exposed to different concentrations of an aqueous sample, for example, a reference toxicant, an effluent, or a receiving water, and observations are made at predetermined exposure periods. At the end of the test, the responses of test organisms are used to estimate the effects of the toxicant or effluent.

Whole effluent toxicity test results are an integral tool in the assessment of water quality. For the protection of aquatic life, the integrated strategy includes the use of three control approaches: the chemical-specific control approach, the WET control approach, and the biological criteria/bioassessment/bioassay approach. The primary advantage of using WET control over individual, chemical-specific controls is that WET integrates the effects of all chemical(s) in the aqueous sample. Reliance solely on chemical-specific numeric criteria or the narrative criterion of bioassessment controls would result in only a partially effective State toxics control program. These toxicity tests therefore must be performed using best laboratory practices, and every effort must be made to enhance repeatability of the test method. This document presents EPA's approaches to achieve the goals listed below.

Effect of This Guidance

This document clarifies several issues regarding WET variability and reaffirms EPA's guidance in the *Technical Support Document for Water Quality-Based Toxics Control* (TSD, USEPA 1991a). This document provides NPDES regulatory authorities and all stakeholders, including permittees, with guidance and recommendations on how to address WET variability. EPA's recommendations and conclusions are detailed in Chapter 7, and Appendix C provides sample NPDES permit language reflecting these recommendations.

The most significant recommendation is to use and report the values for the percent minimum significant difference (PMSD) with all WET data results. The minimum significant difference (MSD), which is also referred to as error mean square (EMS), represents the smallest difference between the control mean and a treatment mean that leads to the statistical rejection of the null hypothesis (i.e., no toxicity) at each concentration of the WET test dilution series. The MSD provides an indication of within-test variability and test method sensitivity. Using this information, the regulatory authority and permittees can better evaluate WET test results.

This document makes several other recommendations, such as continue to use the TSD statistical approach without adjusting for test method variability, obtain sufficient representative effluent samples, verify effluent toxicity data against reference toxicant data, maintain clear communication between the regulatory authority and permittee, and maintain good laboratory checks and certification programs.

Three Goals of This Document

This document describes three goals EPA has defined to address issues surrounding WET variability. In addition, the document is intended to satisfy the requirements of a settlement agreement to resolve litigation over rulemaking to standardize WET testing procedures.

1. Quantify the variability of promulgated test methods and report a coefficient of variation (CV) as a measure of test method variability (see Chapter 3 and Appendix A).

2. Evaluate the statistical methods described in the *Technical Support Document for Water Quality-Based Toxics Control* (TSD) for determining the need for and deriving WET permit conditions (see Chapter 6 and Appendix G).

3. Suggest guidance for regulatory authorities on approaches to address and minimize test method variability (Chapter 6). In addition, the document is intended to provide guidance to regulatory authorities, permittees, and testing laboratories on conducting the biological and statistical methods and evaluating test effect concentrations (Chapter 5).

Data Evaluated

EPA assembled a comprehensive data base to examine variability in the WET test methods from the EPA Regions, several States, and private laboratories, which represent a widespread sampling of typical laboratories and laboratory practices. EPA applied several criteria to the data before they were accepted, including detailed sample information, strict adherence to published EPA WET test methods, and test acceptability criteria (TAC). The resulting data base contains data from 75 laboratories for 23 methods for tests concluded between 1988 and 1999.

Approach Taken To Evaluate Test Method Variability

The variability that EPA is assessing is associated with replicate tests using reference toxicants and WET testing methods within analytical laboratories. The focus of this guidance is *not* to quantify test variability between laboratories or to quantify the total variability of WET tests conducted on effluents.

Rather, the purpose is to quantify method variability within laboratories (repeatability) to enable NPDES programs to distinguish between variability caused by the testing method and variability associated with toxicity of multiple effluent samples taken from the same facility.

To quantify test method variability within and between laboratories using this data base, EPA examined two key parameters: (1) the effect concentrations [effect concentration (EC25), lethal concentration (LC50), no observed effect concentration (NOEC)] estimated by the test, which are used to derive WET permit limits and evaluate self-monitoring data with those limits; and (2) the minimum significant difference (MSD), which summarizes the variability of organism responses at each test concentration within an individual test. The MSD represents the smallest difference that can be distinguished between the response of the control organisms and the response of the organisms exposed to the aqueous sample. The MSD provides an indication of within-test variability and test method sensitivity.

Principal Conclusions

The principal conclusions of this document follow.

Evaluation of Test Method Variability

- Comparisons of WET method precision with analytes commonly limited in NPDES permits clearly demonstrate that the variability of the promulgated WET methods is within the range of variability experienced in other types of analyses. Several independent researchers and studies also have concluded that method performance improves when prescribed methods are followed closely by experienced analysts (Section 4.1.2).

- This document provides interim CVs for promulgated WET methods in Appendix A, Tables A-1 (acute methods) and A-2 (chronic methods), pending completion of between-laboratory studies, which may affect these interim CV estimates.

Evaluation of Approach To Incorporate Test Method Variability

- EPA's TSD presents guidance for developing effluent limits that appropriately protect water quality, regarding both effluent variability and analytical variability, provided that the WET criteria and waste load allocation (WLA) are derived correctly (Section 6 and Appendix G).

- EPA's analysis of data gathered in the development of this document indicates that the TSD approach appropriately accounts for both effluent variability and method variability. EPA does not believe that current proposals for alternative approaches are available that would discount the effects of method variability using the TSD procedures, because the current proposals would not ensure adequate protection of water quality (Section 6.1.1 and Appendix G).

Development of Guidance to Regulatory Authorities

- EPA recommends that regulatory authorities implement the statistical approach as described in the TSD to evaluate effluent for reasonable potential and to derive WET limits or monitoring triggers (Section 6.1 and Appendix G).

- EPA recommends that regulatory authorities calculate the facility-specific CVs using point estimate techniques to determine the need for and derive a permit limit for WET, even if self-monitoring data are to be determined using hypothesis testing techniques, for example, to determine a "no effect' concentration ("NOEC"). This document describes such facility-specific calculations (Section 3.4.1 and 6.2).

Additional Recommendations and Guidance

This document also provides recommendations and guidance on minimizing variability in three specific areas in order to generate sound WET test results: (1) obtaining a representative effluent sample; (2) conducting the toxicity tests properly to generate the biological endpoints; and (3) conducting the appropriate statistical analysis to obtain defensible effect concentrations (EC25, LC50, NOEC). If these recommendations are addressed, the reliability of the test endpoint values should improve.

- **Regulatory Authorities:** Design a sampling program that collects representative effluent samples to fully characterize effluent variability for a specific facility over time (Sections 6.1.3 and 6.2).

- **Regulatory Authorities:** Ensure proper application of WET statistical procedures and test methods (Sections 5.2 through 5.5).

- **Regulatory Authorities:** Incorporate both the upper and lower bounds using the percent minimum significant difference (PMSD) to control and to minimize within-test method variability and increase test sensitivity. To achieve the PMSD upper bound, either the replication should increase or within-test method variability should decrease, or both (Section 6.4 and Table 3-6).

- **Testing Laboratories:** Encourage WET testing laboratories to maintain control charts for PMSD and the control mean and report the PMSD with all WET test results (Section 5.3.1.1).

- **Regulatory Authorities:** Participate in the National Environment Laboratory Accreditation Program and routine performance audit inspections to evaluate laboratory performance (Section 5.3.1.1).

- **Regulatory Authorities:** Incorporate EPA's guidance on error rate assumption adjustments, concentration-response relationships, confidence intervals, acceptable dilution waters, how to block by parentage for the chronic *Ceriodaphnia dubia* test, and control of pH drift (USEPA 2000a).

LIST OF ACRONYMS AND ABBREVIATIONS[1]

ACR	acute-to-chronic ratio
AML	average monthly limit
ANOVA	analysis of variance
APHA-AWWA-WEF	American Public Health Association-American Water Works Association-Water Environment Federation
ASTM	American Society for Testing and Materials
BSAB	Biomonitoring Science Advisory Board
CCC	criteria continuous concentration
CFR	Code of Federal Regulations
CMC	criteria maximum concentration
CV	coefficient of variation
CWA	Clean Water Act
DMR	discharge monitoring report
EMS	error mean square [also referred to as mean square error (MSE)]
EPA	U.S. Environmental Protection Agency (also, the Agency)
FR	*Federal Register*
IC	inhibition concentration
IWC	instream waste concentration (sometimes referred to as receiving water concentration)
LC50	lethal concentration, 50 percent
LOEC	lowest observed effect concentration
LTA	long-term average (LTAa = acute LTA; LTAc = chronic LTA; LTAa,c = acute-to-chronic LTA)
MDL	maximum daily limit
MSD	minimum significant difference
MSE	mean square error [also referred to as error mean square (EMS)]
MZ	mixing zone
NELAP	National Environment Laboratory Accreditation Program
NOEC	no observed effect concentration
NPDES	National Pollutant Discharge Elimination System
NTRD	National Toxicant Reference Database
PAI	Performance Audit Inspections
PMSD	percent minimum significant difference

[1] Note: These acronyms and abbreviations may have other meanings in other EPA programs or documents.

QA	quality assurance
QC	quality control
rMSE	square root of the mean square error
RP	reasonable potential
RWC	receiving water concentration (sometimes referred to as instream waste concentration)
SCTAG	Southern California Toxicity Assessment Group
SETAC	Society of Environmental Toxicology and Chemistry
TAC	test acceptability criteria
TIE	toxicity identification evaluation
TMDL	total maximum daily load
TRE	toxicity reduction evaluation
TSD	EPA's *Technical Support Document for Water Quality-based Toxics Control* (March 1991, EPA505/2-90-001)
TU	toxic unit (TUa = acute toxicity; TUc = chronic toxicity)
VF	variability factor
WET	whole effluent toxicity
WLA	waste load allocation
WQBEL	water quality based effluent limit

GLOSSARY

Acute Toxicity Test is a test to determine the concentration of effluent or ambient waters that causes an adverse effect (usually death) on a group of test organisms during a short-term exposure (e.g., 24, 48, or 96 hours). Acute toxicity is measured using statistical procedures (e.g., point estimate techniques or a *t*-test).

Acute-to-Chronic Ratio (ACR) is the ratio of the acute toxicity of an effluent or a toxicant to its chronic toxicity. It is used as a factor for estimating chronic toxicity on the basis of acute toxicity data, or for estimating acute toxicity on the basis of chronic toxicity data.

Ambient Toxicity is measured by a toxicity test on a sample collected from a receiving waterbody.

ANOVA is analysis of variance.

Average Monthly Limit (AML) is the calculated average monthly limit of waste load allocation assigned by a State or EPA for a particular facility.

CCC are water quality criteria for chronic exposure (criteria continuous concentrations).

Chronic Toxicity Test is a short-term test in which sublethal effects (e.g., reduced growth or reproduction) are usually measured in addition to lethality. Chronic toxicity is defined as TUc = 100/NOEC or TUc = 100/ECp or ICp.

CMC are water quality criteria for acute exposures (criteria maximum concentration).

Coefficient of Variation (CV) is a standard statistical measure of the relative variation of a distribution or set of data, defined as the standard deviation divided by the mean. It is also called the relative standard deviation (RSD). The CV can be used as a measure of precision within (within-laboratory) and between (between-laboratory) laboratories, or among replicates for each treatment concentration.

Confidence Interval is the numerical interval constructed around a point estimate of a population parameter.

Effect Concentration (EC) is a point estimate of the toxicant concentration that would cause an observable adverse effect (e.g., death, immobilization, or serious incapacitation) in a given percent of the test organisms, calculated from a continuous model (e.g., Probit Model). EC25 is a point estimate of the toxicant concentration that would cause an observable adverse effect in 25 percent of the test organisms.

Hypothesis Testing is a statistical technique (e.g., Dunnett's test) for determining whether a tested concentration is statistically different from the control. Endpoints determined from hypothesis testing are NOEC and LOEC. The two hypotheses commonly tested in WET are:

Null hypothesis (H_0): The effluent is not toxic.
Alternative hypothesis (H_a): The effluent is toxic.

Inhibition Concentration (IC) is a point estimate of the toxicant concentration that would cause a given percent reduction in a non-lethal biological measurement (e.g., reproduction or growth), calculated from a continuous model (i.e., Interpolation Method). IC25 is a point estimate of the toxicant concentration that would cause a 25-percent reduction in a non-lethal biological measurement.

Instream Waste Concentration (IWC) is the concentration of a toxicant in the receiving water after mixing. The IWC is the inverse of the dilution factor. It is sometimes referred to as the receiving water concentration (RWC).

LC50 (lethal concentration, 50 percent) is the toxicant or effluent concentration that would cause death in 50 percent of the test organisms.

Lowest Observed Effect Concentration (LOEC) is the lowest concentration of an effluent or toxicant that results in adverse effects on the test organisms (i.e., where the values for the observed endpoints are statistically different from the control).

Long-term Averages (LTAs) of pollutant concentration or effluent toxicity are calculated from waste load allocations (WLAs), typically assuming that the WLA is a 99^{th} percentile value (or another upper bound value) based on the lognormal distribution. One LTA is calculated for each WLA (typically an acute LTA and a chronic LTA for aquatic life protection). The LTA represents expected long-term average performance from the permitted facility required to achieve the associated WLA.

Maximum Daily Limit (MDL) is the calculated maximum WLA assigned by a State or EPA for a particular facility.

Minimum Significant Difference (MSD) is the magnitude of difference from control where the null hypothesis is rejected in a statistical test comparing a treatment with a control. MSD is based on the number of replicates, control performance, and power of the test.

Mean Square Error (MSE) is the average dispersion of the items around the treatment means. It is an estimate of a common variance, the within variation, or variation among observations treated alike. [Also referred to as error mean square (EMS).]

Mixing Zone is an area where an effluent discharge undergoes initial dilution and is extended to cover the secondary mixing in the ambient waterbody. A mixing zone is an allocated impact zone where water quality criteria can be exceeded as long as acutely toxic conditions are prevented.

No Observed Effect Concentration (NOEC) is the highest tested concentration of an effluent or toxicant that causes no observable adverse effect on the test organisms (i.e., the highest concentration of toxicant at which the values for the observed responses are not statistically different from the controls).

National Pollutant Discharge Elimination System (NPDES) program regulates discharges to the nation's waters. Discharge permits issued under the NPDES program are required by EPA regulation to contain, where necessary, effluent limits based on water quality criteria for the protection of aquatic life and human health.

Power is the probability of correctly detecting an actual toxic effect (i.e., declaring an effluent toxic when, in fact, it is toxic).

Precision is a measure of reproducibility within a data set. Precision can be measured both within a laboratory (within-laboratory) and between laboratories (between-laboratory) using the same test method and toxicant.

Quality Assurance (QA) is a practice in toxicity testing that addresses all activities affecting the quality of the final effluent toxicity data. QA includes practices such as effluent sampling and handling, source and condition of test organisms, equipment condition, test conditions, instrument calibration, replication, use of reference toxicants, recordkeeping, and data evaluation.

Quality Control (QC) is the set of more focused, routine, day-to-day activities carried out as part of the overall QA program.

Reasonable Potential (RP) is where an effluent is projected or calculated to cause an excursion above a water quality standard based on a number of factors.

Reference Toxicant Test is a check of the sensitivity of the test organisms and the suitability of the test methodology. Reference toxicant data are part of a routine QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

Significant Difference is defined as a statistically significant difference (e.g., 95 percent confidence level) in the means of two distributions of sampling results.

Statistic is a computed or estimated quantity such as the mean, standard deviation, or coefficient of variation.

Test Acceptability Criteria (TAC) are specific criteria for determining whether toxicity test results are acceptable. The effluent and reference toxicant must meet specific criteria as defined in the test method (e.g., for the *Ceriodaphnia dubia* survival and reproduction test, the criteria are as follows: the test must achieve at least 80 percent survival and an average of 15 young per surviving female in the controls).

Total Maximum Daily Load (TMDL) is a determination of the amount of a pollutant, or property of a pollutant, from point, nonpoint, and natural background sources, including a margin of safety, that may be discharged to a water quality-limited waterbody.

***t*-Test** (formally Student's *t*-Test) is a statistical analysis comparing two sets of replicate observations, in the case of WET, only two test concentrations (e.g., a control and 100 percent effluent). The purpose of this test is to determine if the means of the two sets of observations are different [e.g., if the 100-percent effluent concentration differs from the control (i.e., the test passes or fails)].

Type I Error (alpha) is the rejection of the null hypothesis (H_o) when it is, in fact, true (i.e., determining that the effluent is toxic when the effluent is not toxic).

Type II Error (beta) is the acceptance of the null hypothesis (H_o) when it is not true (i.e., determining that the effluent is not toxic when the effluent is toxic).

Toxicity Test is a procedure to determine the toxicity of a chemical or an effluent using living organisms. A toxicity test measures the degree of effect of a specific chemical or effluent on exposed test organisms.

Toxic Unit-Acute (TUa) is the reciprocal of the effluent concentration (i.e., TUa = 100/LC50) that causes 50 percent of the organisms to die by the end of an acute toxicity test.

Toxic Unit-Chronic (TUc) is the reciprocal of the effluent concentration (e.g., TUc = 100/NOEC) that causes no observable effect (NOEC) on the test organisms by the end of a chronic toxicity test.

Toxic Unit (TU) is a measure of toxicity in an effluent as determined by the acute toxicity units (TUa) or chronic toxicity units (TUc) measured. Higher TUs indicate greater toxicity.

Toxicity Identification Evaluation (TIE) is a set of procedures used to identify the specific chemicals causing effluent toxicity.

Toxicity Reduction Evaluation (TRE) is a site-specific study conducted in a step-wise process designed to identify the causative agents of effluent toxicity, isolate the source of toxicity, evaluate the effectiveness of toxicity control options, and then confirm the reduction in effluent toxicity.

Variance is a measure of the dispersion in a set of values, defined as the sum of the squared deviations divided by their total number.

Whole Effluent Toxicity (WET) is the total toxic effect of an effluent measured directly with a toxicity test.

Waste Load Allocation (WLA) is the portion of a receiving water's total maximum daily load that is allocated to one of its existing or future point sources of pollution.

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1.0 INTRODUCTION

1.1 Background

The Federal Water Pollution Control Act, commonly known as the Clean Water Act (CWA), was enacted in 1972 with the objective of "*restoring the chemical, physical, and biological integrity of the Nation's waters*." Several goals and policies were established in the Act, including the following:

- Eliminating the discharge of pollutants into navigable waters by 1985;
- Wherever attainable, achieving an interim goal of water quality that provides for the protection and propagation of fish, shellfish, and wildlife, and provides for recreation in and on the water by November 1, 1983; and
- Prohibiting the discharge of toxic pollutants in toxic amounts.

In the 28 years since the CWA was enacted, the U.S. Environmental Protection Agency (EPA) and States authorized to administer EPA's National Pollutant Discharge Elimination System (NPDES) permitting program have made significant progress toward achieving these goals. NPDES is designed to control toxic discharges, implement a water quality standards program, and restore waters to "fishable and swimmable" conditions. A point source that discharges pollutants to waters of the United States must do so under the terms and conditions of an NPDES permit. In setting these terms and conditions, EPA and the States have integrated their control of toxic pollutants through combined use of three approaches [*Technical Support Document for Water Quality-based Toxics Control* (USEPA 1991a, referred to as the TSD)]:

- Chemical-specific controls,
- Whole effluent toxicity (WET) controls, and
- Biological criteria/bioassessments and biosurveys.

The WET approach to protection of water quality is the primary subject of this document.

In 1989, EPA defined whole effluent toxicity as "*the aggregate toxic effect of an effluent measured directly by an aquatic toxicity test*" [54 Federal Register (FR) 23868 at 23895, June 2, 1989]. Aquatic toxicity tests are laboratory experiments that measure the biological effect (e.g., growth, survival, and reproduction) of effluents or receiving waters on aquatic organisms. In aquatic toxicity tests, groups of organisms of a particular species are held in test chambers and exposed to different concentrations of an aqueous test sample, for example, a reference toxicant, an effluent, or a receiving water. Observations are made at predetermined exposure periods. At the end of the test, the responses of test organisms are used to estimate the effects of the toxicant or effluent.

In the early 1980s, EPA published methods (USEPA 1985, 1988, 1989) for estimating the short-term acute and chronic toxicity of effluents and receiving waters to freshwater and marine organisms. WET data gathered in the 1980s indicated that approximately 40 percent of NPDES facilities nationwide discharged an effluent with sufficient toxicity to cause water quality problems. Further reductions in the toxicity of wastewater discharges were necessary to achieve compliance with narrative water quality standards expressed as "no toxics in toxic amounts." In response to these findings, EPA implemented a policy to reduce or eliminate toxic discharges. The *Policy for the Development of Water Quality-based Permit Limitations for Toxic Pollutants* (49 FR 9016, March 9, 1984) introduced EPA's integrated toxics control program. To support this policy, EPA developed the TSD (USEPA 1991a). The TSD provides guidance to

regulators in implementing WET testing requirements in NPDES permits. In 1989, EPA promulgated regulations specifying procedures for determining when water quality-based effluent limitations are required in NPDES permits [40 CFR, 122.44(d)]. On October 26, 1995, EPA promulgated WET test methods (USEPA 1993, 1994a, and 1994b) and added them to the list of EPA methods approved under Section 304(h) of the CWA (40 CFR, 136) for use in the NPDES program. Although the rulemaking was challenged in court, that challenge has been stayed pending completion of a settlement agreement. The rulemaking remains in force and effect unless and until EPA takes further action.

1.2 Effect of This Guidance

This document attempts to clarify several issues regarding WET variability and reaffirms EPA's earlier guidance and recommendations published in the TSD (USEPA 1991a). This document is intended to provide NPDES regulatory authorities and all stakeholders, including permittees, with guidance and recommendations on how to understand and account for measurement variability in WET testing. The document's recommendations and conclusions are detailed in Section 7. Appendix C provides sample NPDES permit language reflecting these recommendations.

The most significant recommendation is to use and report the values for the percent minimum significant difference (PMSD) with all WET data results. The minimum significant difference (MSD) is the smallest difference that can be distinguished between the response of control organisms and the response of test organisms at each concentration of the WET test dilution series. The MSD provides an indication of the within-test variability and test method sensitivity. Using this information, the regulatory authority and permittees can better evaluate WET test results.

This document also recommends the following:

- Continue to use the EPA TSD statistical approach for NPDES permit limit development (no test method variability adjustments are needed);
- Collect and evaluate a sufficient number of representative effluent samples;
- Verify effluent toxicity data carefully along with reference toxicant data;
- Maintain good communication between the regulatory authority and permittee throughout all phases of the permitting process;
- Implement the PMSD to evaluate both WET and reference toxicant data to minimize within-test method variability and increase test sensitivity;
- Maintain laboratory checks with good laboratory certification programs to encourage experienced laboratories and skilled analysts for the toxicity testing program for individual WET laboratory performance.

1.3 Three Goals of This Document

EPA prepared this document to achieve the following three goals:

1. Quantify the variability of promulgated test methods and report a coefficient of variation (CV) as a measure of test method variability (see Chapter 3 and Appendix A).

2. Evaluate the statistical methods described in the *Technical Support Document for Water Quality-Based Toxics Control* (TSD) for determining the need for and deriving WET permit conditions (see Chapter 6 and Appendix G).

3. Suggest guidance for regulatory authorities on approaches to address and minimize test method variability (Chapter 6). In addition, the document is intended to provide guidance to regulatory authorities, permittees, and testing laboratories on conducting the biological and statistical methods and evaluating test effect concentrations (Chapter 5).

This document does not address effluent variability. It does, however, discuss how handling effluent samples can affect tests. Chapter 2 provides definitions of terms used and discusses the ways in which variability can be quantified. Chapter 3 describes the variability of the effect concentration estimates (EC25, LC50, and NOEC) and the variability of endpoint measurements (survival, growth, and reproduction). Chapter 4 discusses WET variability in the context of chemical-specific method variability. Chapter 5 provides guidance to permittees, testing laboratories, and regulatory authorities to minimize test method variability. Chapter 6 provides guidance to regulatory authorities on how to determine reasonable potential (RP) and derive permit limits or monitoring triggers and evaluate self-monitoring data. Chapter 7 presents EPA's principal conclusions. Chapter 8 is a bibliography containing a list of documents cited herein and additional reading material.

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2.0 DEFINITION AND MEASUREMENT OF METHOD VARIABILITY IN WET TESTING

The terms used to express toxicity test results are defined in this chapter, and methods for quantifying WET test method variability are discussed. Additional terms used throughout this document, along with their definitions, are provided in the Glossary as part of the front matter of this document.

2.1 Terms and Definitions

Biological endpoints are the biological observations recorded when conducting toxicity tests. These observations may include the number of surviving organisms or the number of young produced. There are two basic types of biological endpoints: responses recorded as response/no response (e.g., dead or alive) are quantal data; responses recorded as a measured response (e.g., weight) or as a count (e.g., number of young produced) are considered continuous data. For most WET tests, the observations for each tested concentration are combined and then reported as an average or percentage to represent the biological endpoint. For example, the fathead minnow larval survival and growth chronic test method has two biological endpoints (i.e., percent survival and average dry weight for each test concentration).

Effect concentrations are concentrations of a test material (i.e., effluent, referent toxicant, receiving water) derived from the observed biological endpoints followed by data analysis using either hypothesis testing procedures or point estimate techniques. Effect concentrations derived using point estimation techniques represent the concentration of a test material at which a predetermined level of effect occurs. For example, LC50 is the lethal concentration at which 50 percent of the organisms respond. Effect concentrations commonly estimated for WET methods are LC50, EC50 (effect concentration at which a 50-percent effect occurs), and IC25 (inhibition concentration at which a 25-percent effect occurs). Hypothesis test methods are used to determine the no observed effect concentration (NOEC). The NOEC represents the highest effect concentration in the test concentration response that is not significantly different from the control response. Multiple statistical endpoints can be derived for each WET method. For example, the endpoints for the fathead minnow larval survival and growth chronic test can be reported as an EC25 for growth, an NOEC for growth, an LC50 (or EC50) for survival, and an NOEC for survival.

2.2 Defining WET Test Variability

As with any measurement process, WET tests have a degree of variability associated with the test method performance. Three measures of variability related to WET tests are within-test variability, within-laboratory variability, and between-laboratory variability.

- **Within-test (intra-test) variability** is the variability in test organism response within a concentration averaged across all concentrations of the test material in a single test.

- **Within-laboratory (intra-laboratory) variability** is the variability that is measured when tests are conducted using specific methods under reasonably constant conditions in the same laboratory. Within-laboratory variability, as used in this document, includes within-test variability. The American Society for Testing and Materials (ASTM) uses the term “repeatability” to describe within-laboratory variability. Repeatability is estimated (as a sample variance or standard deviation) by repeating a test method under realistically constant conditions within a single laboratory.

- **Between-laboratory (inter-laboratory) variability** is the variability between laboratories. It is measured by obtaining results from different laboratories using the same test method and the same

test material (e.g., reference toxicant). Between-laboratory variability, as used in this document, does *not* include the within-laboratory component of variance. ASTM uses the term "reproducibility" to describe between-laboratory variability. Reproducibility is estimated by having nearly identical test samples (duplicates or splits) analyzed by multiple laboratories using similar standard methods. Although reproducibility is generally synonymous with between-laboratory variability, estimates of reproducibility may combine within-laboratory and between-laboratory components of variance, making between-laboratory variability numerically larger than within-laboratory variability as defined above.

For purposes of consistency, EPA uses the terms within-laboratory and between-laboratory variability throughout this document.

Numerous factors can affect the variability of any toxicity test method. These factors include the number of test organisms, the number of treatment replicates, randomization techniques, the source and health of the test organisms, the type of food used, laboratory environmental conditions, and dilution water quality. The experience of the analyst performing the test, analyzing the data, and interpreting the results may also affect variability (Grothe et al. 1996, Fulk 1996).

2.3 Quantifying WET Test Variability

Historically, information on the variability of toxicity tests has been developed using effect concentrations, such as the NOEC, EC25, EC50, and LC50 for survival, fecundity, and growth. Variability measures should be quantified based on the end use of the data (i.e., effect concentrations) and be directly related to the WET permit requirement. Typically, the effect concentrations are the endpoints used for evaluating self-monitoring results. The variability of the effect concentrations is quantified by obtaining multiple test results under similar test conditions using the same test material. For example, the sample standard deviation and mean for EC25 obtained from multiple monthly reference toxicant tests for the fathead minnow survival and growth chronic test conducted at one laboratory would quantify "within-laboratory" variability for that laboratory. EPA used this approach to evaluate data for the development of this document (see Chapter 3).

Examining variability for each effect concentration of each biological endpoint for each test method is essential. The biological endpoints may be different for various toxicants and effluents. One biological endpoint, such as reproduction, may be more sensitive to a certain toxicant than another endpoint, such as survival. That sensitivity may be reversed for a different toxicant. Alternatively, an endpoint may be more sensitive to one toxicant than another toxicant.

Three other measures of variability (which are not addressed in this document) that have been applied to WET tests are:

1. Determine the variability of the biological endpoint response. For example, the variance of the biological response (e.g., growth and survival) can be calculated. This approach is useful, but does not quantify variability of the WET test effect concentration, which is important in the context of this document.

2. Quantify the uncertainty of each test point estimate (e.g., the EC50, EC25, or LC50) using confidence intervals, which reflect within-test variability.

3. Use the standard deviation to quantify the uncertainty in the mean of the replicate response at each concentration within a particular test. For example, laboratories can compare the standard deviations of the average weight of fathead minnow larvae in four chronic tests at one test concentration, such as 1 mg/L sodium chloride. These standard deviations may be pooled across

all the concentrations when data have been transformed (if necessary) to give similar variances at each concentration. From the pooled variance, one may calculate a minimum significant difference (MSD) value, which is a useful indication of test sensitivity (see Chapters 3 and 5). In this document, the standard deviation at each concentration was not evaluated as a measure of variability. However, the MSD was considered as a measure of WET test variability.

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3.0 VARIABILITY OF WET TEST METHODS

Chapter 3 describes the variability of effect concentration estimates (EC25, LC50, and NOEC) and endpoint measurements (survival, growth, and reproduction). For definitive studies of the variability of WET methods, readers should also refer to the TSD (USEPA 1991a, Part 1.3.3) and to WET methods manuals (USEPA 1993, 1994a, 1994b). EPA will complete and report on a new between-laboratory study of promulgated methods in 2000 or 2001.

3.1 Acquisition, Selection, and Quality Assurance of Data Presented in This Document

EPA solicited data for reference toxicant tests from laboratories that conduct WET tests and use reference toxicant testing as part of their quality control (QC) program. Reference toxicant testing is required, as specified in EPA toxicity test methods, to document laboratory performance over time for laboratories conducting self-monitoring tests. When laboratories are conducting effluent tests, at least one reference toxicant test must be conducted each month using the same toxicant, test concentrations, dilution water, and data analysis methods. These reference toxicant tests must be conducted using the same test conditions (type of dilution water, temperature, test protocol, and species) that are used for WET tests conducted by the laboratory.

Reference toxicant tests were used to characterize method variability because, in contrast to effluent samples, fixed concentrations of known toxicants are used. Only with this standardization is it possible to conclude that variability of the effect concentration estimates is derived from the sources discussed above, rather than from changes in the toxicant.

EPA received reference toxicant test data from several States, private laboratory sources, and the EPA Regions. Data sources used for these analyses include the EPA National Toxicant Reference Database (NTRD), the EPA Region 9 Toxicity Data Base, and laboratory bench sheets voluntarily submitted by independent sources. Although the data do not represent a random sample of laboratories or tests, they do represent a widespread sampling of typical laboratories and practices.

EPA required that reference toxicant tests included in its data base meet the following four criteria:

1. Test records documented the test method, organism, test date, laboratory, reference toxicant, and individual biological responses in the concentration series.

2. Data for each replicate were provided as required in the published method using the current test method.

3. The test used at least five toxicant concentrations and a control for the most commonly reported chronic toxicity test methods—(1) 1000.0, fathead minnow larval survival and growth; (2) 1002.0, *Ceriodaphnia* survival and reproduction; and (3) 1006.0, inland silverside survival and growth. For other chronic toxicity test methods, the test used at least four toxicant concentrations and a control because the methods permitted, in the recent past, the use of only four concentrations.

4. EPA personnel or an EPA contractor calculated the effect concentration, verified that all test acceptability criteria (TAC) had been met, and verified that the statistical flowchart had been followed correctly. Thus, all summary statistics and estimates were calculated from the replicate data and strictly followed the most current EPA test methods.

Details of data quality assurance and test acceptance are provided in a separate document, available at EPA's Office of Water docket, located in the Office of Science and Technology ["Whole Effluent Toxicity (WET) Data Test Acceptance and Quality Assurance Protocol"]. An attachment to that document provides a laboratory-by-laboratory listing of quality assurance flags, test dates, and toxicant concentrations, as well as summary statistics by laboratory for the NOEC, EC25, and LC50 estimates and test endpoints (survival, growth, reproduction, etc.). Laboratories are not identified by name.

The data set of reference toxicant tests includes information from 75 laboratories for 23 methods for tests conducted between 1988 and 1999. This document addresses, and provides specific guidance on, the variability of methods promulgated by EPA in 40 CFR Part 136 (Table 3-1). The data are also used to develop between-laboratory interim estimates of method variability for the promulgated methods (Appendix A). The Agency identifies these CVs as "interim;" EPA may revise some or all of these estimates based on between-laboratory studies to evaluate some of the promulgated test methods.

The next section presents summary statistics for the promulgated methods. Summary statistics for all methods in the data set appear in Appendix B. For methods represented by a few laboratories, summary statistics should not be considered representative of method performance. For example, EPA's Office of Water usually relies on acceptable data from at least six laboratories (USEPA 1996b) when it conducts a multi-laboratory study to quantify method performance. The data used here have not been obtained under conditions as rigorous as those applied to a between-laboratory study and for that reason, may overestimate variability, particularly for the extremes.

Coefficients of variation are used as descriptive statistics for NOECs in this document. Because NOECs can take on only values that correspond to concentrations tested, the distribution (and CV) of NOECs can be influenced by the selection of experimental concentrations, as well as additional factors (e.g., within-test variability) that affect both NOECs and point estimates. This makes CVs for NOECs more uncertain than the CVs for point estimates, and the direction of this uncertainty is not uniformly toward larger or smaller CVs. Despite these confounding issues, CVs are used herein as the best available means of expressing the variability of interest in this document and for general comparisons among methods. Readers should be cautioned, however, that small differences in CVs between NOECs and point estimates may be artifactual; large differences are more likely to reflect real differences in variability (a definition of what is "small" or "large" would require a detailed statistical analysis and would depend upon the experimental and statistical details surrounding each comparison). NOECs can only be a fixed number of discrete values; the mean, standard deviation, and CV cannot be interpreted and applied as they are for a continuous variable such as the EC25 or EC50. For instance, the typical reference toxicant test might result in only three observed NOEC values, most of them at one or two concentrations. The mean will fall between tested concentrations, as will the stated confidence intervals; thus, these do not actually represent expected outcomes, only approximations of the expected outcome.

As an alternative to CVs, ratios are used to quantify variability of EC25, EC50, and NOEC measurements in Appendix B. Ratios of measurements have been used previously to quantify and compare variability of NOEC and EC50 (Chapman et al. 1996b, Dhaliwal et al. 1997).

3.2 Variability of EC25, LC50, and NOEC

3.2.1 Within-Laboratory Variability of EC25, LC50, and NOEC

This section characterizes the within-test and within-laboratory variability of effect concentration estimates. Tables 3-2 through 3-4 summarize variation across laboratories of the within-laboratory coefficients of variation (CVs), without respect to reference toxicant tested. Tables showing more extensive summaries appear in Appendix B (Tables B-1 through B-3).

Table 3-1. Promulgated WET Methods Included in This Report

Test Method No.	Test Method	EPA Data Base		
		Toxicants	Tests	Labs
Freshwater Methods for Chronic Toxicity[a]				
1000.0	*Pimephales promelas*, Fathead Minnow Larval Survival and Growth Test	Cd, Cr, Cu, KCl, NaCl, NaPCP, SDS	205	19
1000.0	*Pimephales promelas*, Fathead Minnow Embryo-Larval Survival and Teratogenicity Test		0	0
1002.0	*Ceriodaphnia dubia*, Water Flea Survival and Reproduction Test	Cd, Cu, KCl, NaCl, NaPCP	393	33
1003.0	*Selenastrum capricornutum*,[b] Green Alga Growth Test	Cu, NaCl, Zn	85	9
Marine & Estuarine Methods for Chronic Toxicity[c]				
1004.0	*Cyprinodon variegatus*, Sheepshead Minnow Larval Survival and Growth Test	Cd, KCl	57	5
1005.0	*Cyprinodon variegatus*, Sheepshead Minnow Embryo-larval Survival and Teratogenicity Test		0	0
1006.0	*Menidia beryllina*, Inland Silverside Larval Survival and Growth Test	Cr, Cu, KCl, SDS	193	16
1007.0	*Americamysis (Mysidopsis) bahia*, Mysid Survival, Growth, and Fecundity Test	Cr, Cu, KCl	130	10
1008.0	*Arbacia punctulata*, Sea Urchin Fertilization Test		0	0
1009.0	*Champia parvula*, Red Macroalga Reproduction Test	Cu, SDS	23	2
Methods for Acute Toxicity [d,e]				
2000.0	Fathead Minnow Survival Test	Cd, Cu, KCl, NaCl, NaPCP	217	21
2002.0	*Ceriodaphnia dubia* Survival Test	Cd, Cu, KCl, NaCl, NaPCP	241	23
2004.0	Sheepshead Minnow Survival Test	SDS	65	3
2006.0	Inland Silverside Survival Test	Cd, KCl, SDS	48	5
2007.0	Mysid (*A. bahia*) Survival Test	Cd, Cu, SDS	32	3
2011.0	Mysid (*H. costata*) Survival Test	Cd, SDS	14	2
2019.0	Rainbow Trout Survival Test	Cu, Zn	10	1
2021.0	*Daphnia magna* Survival Test	Cd	48	5
2022.0	*Daphnia pulex* Survival Test	Cu, NaCl, SDS Cd, Cu, NaCl, NaPCP	57	6

[a] See publications EPA/600/4-89-001 (USEPA 1989) and EPA/600/4-91-002 (USEPA 1994b).

[b] The genus and species names for *Selenastrum capricornutum* have been changed to *Raphidocelis subcapitata*. In this document, however, *Selenastrum capricornutum* is used to avoid confusion.

[c] See publication EPA/600/4-91-003 (USEPA 1994a) and EPA/600/4-87/028 (USEPA 1988).

[d] See publications EPA/600/4-85/013 (USEPA 1985) and EPA/600/4-90/027F (USEPA 1993).

[e] EPA did not assign method numbers for acute methods in EPA/600/4-90/027F. The numbers assigned here were created for use in this document and in related materials and data bases.

Reference toxicant codes:

Cd	cadmium	NaCl	sodium chloride
Cr	chromium	NaPCP	sodium pentachlorophenate
Cu	copper	SDS	sodium dodecyl sulfate
KCl	potassium chloride	Zn	zinc

Table 3-2. Quartiles (25th and 75th) and Median (50th) of the Within-Laboratory Values of CV for EC25 (Chronic Tests)

Test Method[a]	Test Method No.	Endpoint[b]	No. of Labs	Percentiles of CV		
				25th	50th	75th
Fathead Minnow Larval Survival & Growth	1000.0	G	19	0.21	0.26	0.38
Fathead Minnow Larval Survival & Growth	1000.0	S	16	0.11	0.22	0.32
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	R	33	0.17	0.27	0.45
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	S	25	0.11	0.23	0.41
Green Alga (*Selenastrum*) Growth	1003.0	G	6	0.25	0.26	0.39
Sheepshead Minnow Larval Survival & Growth	1004.0	G	5	0.09	0.13	0.14
Sheepshead Minnow Larval Survival & Growth	1004.0	S	2	0.15	0.16	0.17
Inland Silverside Larval Survival & Growth	1006.0	G	16	0.18	0.27	0.43
Inland Silverside Larval Survival & Growth	1006.0	S	13	0.22	0.35	0.42
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	F	4	0.30	0.38	0.41
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	G	10	0.24	0.28	0.32
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	S	7	0.17	0.21	0.28
Red Macroalga (*Champia parvula*) Reproduction	1009.0	R	2	0.58	0.58	0.59

[a] Cd = *Ceriodaphnia dubia*, Ab = *Americamysis* (*Mysidopsis*) *bahia*
[b] G = growth, S = survival, R = reproduction, F = fecundity

Table 3-3. Quartiles (25th and 75th) and Median (50th) of the Within-Laboratory Values of CV for LC50

Test Method[a]	Test Method No.	Endpoint[b]	No. of Labs	Percentiles of CV		
				25th	50th	75th
Freshwater Methods for Chronic Toxicity[c]						
Fathead Minnow Larval Survival & Growth	1000.0	S	19	0.15	0.23	0.31
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	S	33	0.10	0.16	0.29
Sheepshead Minnow Larval Survival & Growth	1004.0	S	5	0.07	0.08	0.12
Inland Silverside Larval Survival & Growth	1006.0	S	16	0.16	0.28	0.35
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	S	10	0.16	0.26	0.27
Methods for Acute Toxicity[d,e]						
Fathead Minnow Larval Survival	2000.0	S	21	0.10	0.16	0.19
Ceriodaphnia (Cd) Survival	2002.0	S	23	0.11	0.19	0.29
Sheepshead Minnow Survival	2004.0	S	5	0.12	0.14	0.21
Inland Silverside Larval Survival	2006.0	S	5	0.15	0.16	0.21
Mysid (Ab) Survival	2007.0	S	3	0.17	0.25	0.26
Mysid (Hc) Survival	2011.0	S	2	0.27	0.30	0.34
Rainbow Trout Survival	2019.0	S	1	0.23	0.23	0.23
Daphnia (Dm) Survival	2021.0	S	5	0.07	0.22	0.24
Daphnia (Dp) Survival	2022.0	S	6	0.19	0.21	0.27

[a] Cd = *Ceriodaphnia dubia*, Ab = *Americamysis* (*Mysidopsis*) *bahia*, Hc = *Holmesimysis costata*, Dm = *Daphnia magna*, Dp = *Daphnia pulex*
[b] S = survival
[c] See publications EPA/600/4-89-001 (USEPA 1989) and EPA/600/4-91-002 (USEPA 1994b).
[d] See publications EPA/600/4-85-013 (USEPA 1985 and EPA/600/4-90/027F (USEPA 1993).
[e] EPA did not assign method numbers for acute methods in EPA/600/4-90/027F. The numbers assigned here were created for use in this document and in related materials and data bases.

Table 3-4. Quartiles (25th and 75th) and Median (50th) of the Within-Laboratory Values of CV for NOEC

Test Method[a]	Test Method No.	Endpoint[b]	No. of Labs	Percentiles of CV		
				25th	50th	75th
Freshwater Methods for Chronic Toxicity[c]						
Fathead Minnow Larval Survival & Growth	1000.0	G	19	0.22	0.37	0.53
Fathead Minnow Larval Survival & Growth	1000.0	S	19	0.26	0.39	0.48
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	R	33	0.25	0.33	0.49
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	S	33	0.21	0.30	0.43
Green Alga (*Selenastrum*) Growth	1003.0	G	9	0.40	0.46	0.56
Marine & Estuarine Methods for Chronic Toxicity[d]						
Sheepshead Minnow Larval Survival & Growth	1004.0	G	5	0.34	0.40	0.44
Sheepshead Minnow Larval Survival & Growth	1004.0	S	5	0.14	0.18	0.24
Inland Silverside Larval Survival & Growth	1006.0	G	16	0.31	0.46	0.57
Inland Silverside Larval Survival & Growth	1006.0	S	16	0.30	0.42	0.55
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	F	4	0.17	0.36	0.40
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	G	10	0.35	0.39	0.43
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	S	10	0.28	0.33	0.38
Red Macroalga (*Champia parvula*) Reprod.	1009.0	R	2	0.85	0.10	0.12
Methods for Acute Toxicity[e,f]						
Fathead Minnow Larval Survival	2000.0	S	21	0.18	0.22	0.34
Ceriodaphnia (Cd) Survival	2002.0	S	23	0.18	0.35	0.41
Sheepshead Minnow Survival	2004.0	S	3	0	0.31	0.33
Inland Silverside Larval Survival	2006.0	S	5	0	0.33	0.35
Mysid (Ab) Survival	2007.0	S	3	0.29	0.38	0.43
Mysid (Hc) Survival	2011.0	S	2	0.21	0.26	0.31
Rainbow Trout Survival	2019.0	S	1	0.35	0.35	0.35
Daphnia magna (Dm) Survival	2021.0	S	5	0.09	0.36	0.47
Daphnia pulex (Dp) Survival	2022.0	S	6	0.21	0.38	0.61

[a] Cd = *Ceriodaphnia dubia*, Ab = *Americamysis* (*Mysidopsis*) *bahia*, Hc = *Holmesimysis costata*, Dm = *Daphnia magna*, Dp = *Daphnia pulex*

[b] G = growth, S = survival, R = reproduction, F = fecundity

[c] See publications EPA/600/4-89-001 (USEPA 1989) and EPA/600/4/4-91-002 (USEPA 1994b).

[d] See publication EPA/600/4-91-003 (USEPA 1994a) and EPA/600/4-87/028 (USEPA 1988).

[e] See publications EPA/600/4-85/013 (USEPA 1985) and EPA/600/4-90/027F (USEPA 1993).

[f] EPA did not assign method numbers for acute methods in EPA/600/4-90/027F. The numbers assigned here were created for use in this document and in related materials and data bases.

Effect concentrations having a p-percent effect are symbolized as ECp and may be calculated for sublethal and lethal (survival) endpoints (USEPA 1993,1994a,1994b). Effect concentrations commonly estimated for WET methods are LC50, EC50, IC25, and EC25. The symbol ECp is more general and may be used to represent an LCp, ECp, or ICp endpoint. To simplify presentation of results in this document, the term EC25 is used to represent the concentration at which a 25-percent effect has occurred for either lethal

or sublethal endpoints. The term LC50 is used to represent the concentration at which a 50-percent effect has occurred for lethal endpoints. The EC25 for survival is not routinely used in generating self-monitoring data and is presented here for comparison to the EC25 for sublethal endpoints (i.e., IC25). Estimates of EC25, LC50, and NOEC were calculated for this document as required in the EPA test methods (USEPA 1993, 1994a, 1994b). A CV is reported for NOEC measurements in this document. See Appendix A for further details.

The results in Tables 3-2 through 3-4 were obtained as follows, using as an example the EC25 of the growth endpoint in Method 1000.0 (fathead minnow larval chronic test) on the first row of Table 3-2. The CV of the EC25 estimates was calculated for each laboratory. This calculation resulted in 19 CVs (one per laboratory with each laboratory tested using one toxicant). The sample percentiles were calculated for this set of 19 CVs. In Table 3-2, the column headed "50th" shows the 50th percentile (median value) of CV found across these 19 laboratories; the 50th percentile value is 0.26. In the column headed "75th," the 75th percentile CV is reported as 0.38. When a method is represented by fewer than four laboratories, the minimum and maximum CVs are shown in the columns headed "25th" and "75th," respectively. Note that these CVs represent within-laboratory variability, and that Tables 3-2 through 3-4 show the quartiles and median of the within-laboratory CVs. These tables thus report the typical range of within-laboratory test method variation.

Variation across laboratories in the CV for effect concentration estimates (Tables 3-2 through 3-4) may be summarized as follows, ignoring methods represented by only one or two laboratories. [Refer to the column headed "75th" (the 75th percentile).]

For the EC25 of the growth and reproduction endpoints in chronic toxicity tests, 75 percent of laboratories have a CV no more than 0.14 to 0.45 depending on the method (Table 3-2). For the two most commonly used methods (1000.0, fathead minnow larval chronic test; and 1002.0, *Ceriodaphnia* chronic test), 75 percent of the laboratories have CVs no more than 0.38 and 0.45, respectively.

For the LC50 of the survival endpoint in chronic toxicity tests, 75 percent of laboratories have a CV no more than 0.12 to 0.35, depending on the method. For the two most commonly used methods (1000.0 and 1002.0), 75 percent of laboratories have CVs no more than 0.31 and 0.29, respectively (Table 3-3). For the LC50 in acute toxicity tests, 75 percent of laboratories have a CV no more than 0.19 to 0.29, depending on the method. For the two most commonly used methods (2000.0 and 2002.0), 75 percent of laboratories have CVs no more than 0.19 and 0.29, respectively.

For the NOEC of growth or reproduction endpoints in chronic toxicity tests, 75 percent of laboratories have a CV no more than 0.40 to 0.57, depending on the method. For the two most commonly used methods (1000.0 and 1002.0), 75 percent of laboratories have CVs no more than 0.53 and 0.49, respectively (Table 3-4). For the NOEC of survival in chronic toxicity tests, 75 percent of laboratories have a CV no more than 0.24 to 0.55, depending on the method. For the two most commonly used methods (1000.0 and 1002.0), 75 percent of laboratories have CVs no more than 0.48 and 0.43, respectively. For the NOEC of survival in acute toxicity tests, 75 percent of laboratories have a CV no more than 0.34 to 0.61, depending on the method. For the two most commonly used acute methods (2000.0 and 2002.0), 75 percent of laboratories have CVs no more than 0.34 and 0.41, respectively.

Appendix B discusses the range of toxicant concentrations reported as the NOEC. For chronic toxicity tests, most laboratories report the NOEC to within two to three concentration intervals, and half the laboratories report most NOECs within one to two concentration intervals for reference toxicants. For acute toxicity tests, most laboratories report NOECs at one or two concentrations. This outcome agrees with EPA's expected performance for these methods. The normal variation of the effect concentration estimate in reference toxicant tests has been reported for some EPA WET methods (USEPA 1994a, 1994b) to be plus or minus one dilution concentration for the NOEC and less for LC50.

3.2.2 Between-Laboratory Variability of EC25, LC50, and NOEC

The data set compiled for this document provided reasonable estimates of between-laboratory variability for only a few methods. For many methods and toxicants, there were too few laboratories in the data base. Additional summaries of between-laboratory variability of WET methods are included in the TSD (USEPA 1991a, Part 1.3.3) and the WET methods manuals (USEPA 1994a, 1994b). EPA also intends to provide new data in a forthcoming EPA between-laboratory study of promulgated methods.

Using the data set, credible estimates of between-laboratory variability could be made for a few toxicants and methods having data for six or more laboratories (Table 3-5). The statistical methods are described in Appendix B. Table 3-5 shows values of the square root of within-laboratory and between-laboratory variance components (i.e., standard deviations, σ). The standard deviations and mean are expressed in units of toxicant concentration (e.g., g/L or mg/L). Between-laboratory σ_b estimates the standard deviation for laboratory means of EC25, LC50, and NOEC. The "Mean" column in Table 3-5 shows the mean of the laboratory means, not the mean for all tests. Because the number of tests differed among laboratories, these two means are different. These data suggest that between-laboratory variability (σ_b) is comparable to within-laboratory variability (σ_w) for the methods listed in the table.

In Table 3-5, the ratio of σ_b to the mean is an estimate of the relative variability (CV_b) of laboratory means around their combined mean. The ratio of σ_w to the mean may approach the value of the average within-laboratory CV when the sample of laboratories is large, but to characterize within-laboratory CVs, readers should use Tables 3-2 through 3-4.

Table 3-5. Estimates of Within-Laboratory and Between-Laboratory Components of Variability[a]

Test Method[b]	Test EC Estimate	Toxicant	End-Point[c]	Tests	Labs	Within-lab σ_w	Between-lab σ_b	Mean	CV_w	CV_b
1000.0	EC25	NaCl	G	73	6	0.67	0.44	2.63	0.25	0.17
1000.0	LC50	NaCl	S	73	6	1.14	0.45	4.15	0.27	0.11
1000.0	NOEC	N Cl	G	73	6	0.72	0.35	2.18	0.33	0.16
1000.0	NOEC	NaCl	S	73	6	0.96	0.51	2.43	0.40	0.21
1002.0	EC25	NaCl	R	292	23	0.29	0.27	0.92	0.32	0.29
1002.0	LC50	NaCl	S	285	23	0.48	0.24	1.78	0.27	0.13
1002.0	NOEC	NaCl	G	292	23	0.28	0.18	0.74	0.38	0.24
1002.0	NOEC	NaCl	S	292	23	0.47	0.26	1.42	0.33	0.18
1006.0	EC25	Cu	G	130	9	45.1	52.4	97.4	0.46	0.54
1006.0	LC50	Cu	S	130	9	48.4	70.7	127.0	0.38	0.56
1006.0	NOEC	Cu	G	130	9	51.8	44.4	80.1	0.65	0.55
1006.0	NOEC	Cu	S	130	9	34.2	39.5	65.4	0.52	0.60
2000.0	LC50	NaCl	S	154	14	1.05	1.24	7.46	0.14	0.17
2002.0	LC50	NaCl	S	167	15	0.36	0.38	1.97	0.18	0.19

[a] σ_w = within-laboratory standard deviation, σ_b = between-laboratory standard deviation
CV_w = within-laboratory coefficient of variation, CV_b = between-laboratory coefficient of variation

[b] EPA did not assign method numbers for acute methods in EPA/600/4-90/027F. The numbers assigned here were created for use in this document and in related materials and data bases.

[c] G = growth, S = survival, R = reproduction

3.3 Variability of Endpoint Measurements

This section characterizes the within-laboratory precision of endpoint measurements (e.g., growth, reproduction, and survival). Endpoint variability in methods for chronic toxicity is characterized here using sublethal endpoints. The sublethal endpoint was designed to be more sensitive than the survival endpoint, and it incorporates the effect of mortality (i.e., it incorporates biomass). For example, for the chronic survival and growth fathead minnow larval test, the total dry weight at each replicate is divided by the original number of larvae, rather than the surviving number of larvae.

EPA reports measures of test precision based on the control CV [(control standard deviation)/(control mean)] and the "Percent MSD" [100×MSD/(control mean)], symbolized as PMSD. Recall that MSD, the "minimum significant difference," is calculated as [d $\sqrt{}$EMS $\sqrt{}$(2/r)], where "d" is the critical value of Dunnett's statistic when comparing "k" treatments to a control, EMS is the error mean square from the analysis of variance of the endpoint responses, and "r" is the number of replicates at each concentration (USEPA 1993, 1994a, 1994b). These measures of test precision quantify within-test variability, or the sensitivity of each test to toxic effects on the biological endpoint.

Measures of variability relative to the control mean are used for two reasons. First, a laboratory having consistently large mean endpoint values for the control will also tend to have larger values of MSD and control standard deviation. Second, PMSD is readily interpreted as the minimum percent difference between control and treatment that can be declared statistically significant in a WET test. A significant effect occurs when (control mean - treatment mean) exceeds the MSD. Dividing by the control mean and multiplying by 100 states this relationship in terms of the percent difference between control and treatment.

To characterize the distribution of values of PMSD, values from all laboratories and toxicants for a given method and endpoint were combined, and sample percentiles reported. Percentiles are also reported for the CV of the control, which also indicates variability among replicates under non-toxic conditions and may be a useful indicator of uniformity of the test organisms. The sample percentiles are reported in more detail in Appendix B; the 10th and 90th percentiles are shown in Table 3-6. Method 1009.0 (red macroalga) is omitted from Table 3-6 because it would be inadvisable to characterize method variability using only 23 tests from only two laboratories.

The 90th percentile may be used as an upper PMSD bound (i.e., a limit on the insensitivity of a test). The 10th percentile may be used as a lower PMSD bound for declaring a significant difference or a lower limit to test sensitivity. The 90th percentile has been used in other WET programs (Chapter 5). The 95th percentile is used as a practical upper limit for the variability of analytical results in well-controlled between-laboratory studies that use a standard protocol and specific quality assurance procedures (ASTM 1992, 1998; USEPA 1993, 1996a, 1996b). The tests summarized here have not been subjected to the rigorous standardization and quality assurance of collaborative studies, and the data have not been screened for outliers as specified by ASTM Practices D2777 and E691 (ATSM 1992, 1998). These considerations justify using the sample 90th percentile to set an upper bound. A lower bound is necessary to avoid creating a disincentive for improving test precision and to objectively specify a limit to the test sensitivity achieved in practice. If no more than ten percent of tests are more precise than this lower bound, then in practice, the analytical method rarely detects toxic effects of this small magnitude.

When comparing values in Table 3-6 to a test result, it is important that the test's MSD be calculated according to procedures described in the EPA method manuals (USEPA 1993, 1994a, 1994b) for Dunnett's test for multiple comparisons with a control (see Section 6.4.1). An analysis of variance (ANOVA) is conducted using several treatments, including the control. EPA methods require excluding from the ANOVA those concentrations for which no organisms survived in any replicate. For a sublethal endpoint, concentrations are excluded from the analysis if they exceed the NOEC for survival. The MSD is calculated

using the square root of the error mean square (rEMS) from the ANOVA, and using Dunnett's critical value (which depends on the number of replicates and concentrations used in the ANOVA).

Table 3-6. Range of Relative Variability for Endpoints of Promulgated WET Methods, Defined by the 10th and 90th Percentiles from the Data Set of Reference Toxicant Tests[a]

Test Method[b]	Endpoint[c]	No. of Labs	No. of Tests	PMSD		Control CV[d]	
				10th	90th	10th	90th
1000.0 Fathead Minnow	G	19	205	9.4	35	0.035	0.20
1002.0 *Ceriodaphnia dubia*	R	33	393	11	37	0.089	0.42
1003.0 Green Alga	G	9	85	9.3	23	0.034	0.17
1004.0 Sheepshead Minnow	G	5	57	6.3	23	0.034	0.13
1006.0 Inland Silverside	G	18	193	12	35	0.044	0.18
1007.0 Mysid	G	10	130	12	32	0.088	0.28
2000.0 Fathead Minnow	S	20	217	4.2	30	0	0.074
2002.0 *Ceriodaphnia*	S	23	241	5.0	21	0	0.11
2004.0 Sheepshead Minnow	S	5	65	0[e]	55	0	0
2006.0 Inland Silverside	S	5	48	7.0	41	0	0.079
2007.0 Mysid (*A. bahia*)	S	3	32	5.1	26	0	0.081
2011.0 Mysid (*H. costata*)	S	2	14	18	47	0	0.074
2021.0 Daphnia (*D. magna*)	S	5	48	5.3	23	0	0.11
2022.0 Daphnia (*D. pulex*)	S	6	57	5.8	23	0	0.11

[a] The precision of the data warrants only three significant figures. When determining agreement with these values, one may round off values to two significant figures (e.g., values >3.45000... and ≤3.5000... are rounded to 3.5). Method 1009.0 (red macroalga) is not reported because it is inadvisable to characterize method variability using only 23 tests from just two laboratories.

[b] EPA did not assign method numbers for acute methods in EPA/600/4-90/027F. The numbers assigned here were created for use in this document and in related materials and data bases.

[c] G = growth, R = reproduction, S = survival

[d] CVs were calculated using untransformed control means for each test.

[e] An MSD of zero will not occur when the EPA flow chart for statistical analysis is followed. In this report, MSD was calculated for every test, including those for which the flow chart would require a nonparametric hypothesis test. EPA recommends using the value 4.2 (the 10th percentile shown for the fathead minnow acute test) in place of zero as the 10th percentile PMSD (lower PMSD bound) for the sheepshead minnow acute test.

The MSD was calculated for all test results reported here, including those for which non-normality and heterogeneity of variance were indicated. Thus, this document presents MSD as an approximate index of test sensitivity. Estimates of power are also approximate. The MSD generally will be related to test sensitivity, even when the assumptions for ANOVA and Dunnett's test are not strictly satisfied.

Table 3-7 shows the number of laboratories in the WET variability data set having tests exceeding the upper PMSD bound reported in Table 3-6. One-half to two-thirds of the laboratories never or infrequently exceeded the bound, and roughly one in five exceeded it in at least 20 percent of their tests. By definition of the 90th percentile, about 10 percent of all the tests exceeded the bound.

Table 3-7. Number of Laboratories Having a Given Percent of Tests Exceeding the PMSD Upper Bound for the Sublethal Endpoint

Test Method	No. Labs	Endpoints[a]	Number of Labs with Various Percentages of Tests Exceeding the PMSD Upper Bound				
			0%	0%-10%	10%-20%	20%-50%	50%-100%
1000.0 Fathead Minnow	19	G	8	2	7	2	0
1002.0 *Ceriodaphnia dubia*	33	R	15	7	5	6	0
1003.0 Green Alga	9	G	6	1	0	2	0
1004.0 Sheepshead Minnow	5	G	3	1	0	1	0
1006.0 Inland Silverside	16	G	6	5	1	4	0
1007.0 Mysid (growth)	10	G	5	2	0	3	0

[a] G = growth, R = reproduction

3.4 Conclusions about Variability of WET Methods

3.4.1 Variability of EC25, LC50, NOEC

For EC25, the quartiles of the within-laboratory CVs ranged across the promulgated methods from 0.09 to 0.45, and the median CV ranged from 0.13 to 0.38. For LC50, the quartiles of the within-laboratory CVs ranged from 0.07 to 0.35, and the median CV ranged from 0.08 to 0.30. For NOEC, the quartiles of the within-laboratory CVs ranged from 0 to 0.61, and the median CV ranged from 0.10 to 0.46. This summary applies to those methods represented by at least 20 tests and three laboratories.

EPA concludes from Tables 3-2 through 3-4 that point estimates are substantially less variable than the NOEC for the same method and endpoint, and that the LC50 for an acute toxicity test usually is less variable than the LC50 for a chronic toxicity test. The estimated NOEC is more variable than ECp *using current experimental designs* because NOEC can take only those values equal to the concentrations tested, while ECp interpolates between tested concentrations (there may be other, more technical reasons as well). In principle, NOEC could be estimated more accurately and precisely by changing the experimental design to use more concentrations at narrower dilution ratios and by using more replicates. The greater variability of the NOEC underscores the desirability of using point estimates to characterize effluent toxicity.

Tables 3-2 through 3-4 may be used as benchmarks for variability, allowing comparison of one laboratory's CV for reference toxicant testing with CVs reported by experienced laboratories reporting tests that passed the TAC. However, CVs for methods represented by too few laboratories in the table may be atypical.

The CVs in Tables 3-2 through 3-4 may be used as an adjunct to the control chart. If the CV for reference toxicant tests is above the 75th percentile in Tables 3-2 through 3-4, variability likely can be reduced, even if the individual EC25 or LC50 values fall within the control limits. If a control chart is constructed using an unreasonably large standard deviation, the control limits will be unreasonable. If a high CV is not fully explained by an unusually small mean, the standard deviation of EC25 or LC50 should be reduced to bring the CV within the normal range. If the CV exceeds the 90th percentile (Appendix B), there is no question that variability is unacceptably large. Detailed guidance is provided in Chapter 5 (Section 5.3.1.1).

Tables 3-2 through 3-4 indicate the magnitude of the analytical variability that becomes part of the variability of effluent test results under certain conditions. This occurs when effluent test results (NOECs, LC50s, or EC25s) fall between the lowest and highest concentrations tested. Under other conditions, these

CVs may not accurately represent analytical variability. If tests give results consistently near or at the lowest or highest concentrations tested, or if the tests often produce "less than" or "greater than" results, Tables 3-2 through 3-4 will not accurately characterize the analytical CV for such tests. To measure the analytical CV under such conditions, reference toxicant tests would have to be designed to have the effect concentration at or near the lowest or highest concentration. The CV and standard deviation measured under such conditions are unknown, but are likely to differ from those for standard reference toxicant tests.

The data set did not contain information supporting an analysis of the causes of between-laboratory variability. Possible causes may include laboratory differences in concentration series, incorrect or ambiguous calculation or reporting of concentrations (e.g., concentration of the metal ion versus the salt), laboratory differences in dilution water (e.g., water hardness or pH), laboratory differences in foods and feeding regimes, and laboratory differences in cultures (genotypic and phenotypic differences in sensitivity to various toxicants).

The lack of a standard or common reference toxicant creates a problem for permittees and regulatory authorities attempting to evaluate and compare laboratories. Real or apparent differences occur between laboratories in the mean values of EC25, LC50, and NOEC. Some of this difference is random and reflects only the within-laboratory variance; some may be systematic. Systematic, between-laboratory differences can be inferred reliably only when laboratories use the same test method, use the same reference toxicants and dilution series, use similar dilution waters, and report a sufficient number of tests.

3.4.2 Variability of Endpoint Measurements

EPA has selected the PMSD to characterize endpoint variability for WET test methods because it integrates variability from several concentrations (always including the control), and it represents the MSD used in the WET hypothesis test. The control CV, by itself, does not fully represent the variability affecting a WET hypothesis test or point estimate. The PMSD also represents the variability affecting point estimates because it is calculated using the EMS for the endpoint measurement. (However, the standard error of a point estimate of an effect concentration may be a complicated function of the EMS.)

PMSD for sublethal endpoints ranged from 6 to 37 across the promulgated chronic methods. For the fathead minnow chronic method, PMSD ranged from 9 to 35; for the *Ceriodaphnia* chronic method, PMSD ranged from 11 to 37. Thus, most chronic tests were able to distinguish a reduction of 37 percent or smaller in the endpoint. Further analysis in Chapter 5 shows that most tests were unable to distinguish consistently a 25-percent reduction. For the survival endpoint of promulgated acute methods, PMSD ranged from 0 to 55. For the two most commonly used acute methods (fathead minnow and *Ceriodaphnia*), PMSD ranged from 4 to 30 and from 5 to 21, respectively. Thus, PMSD varied markedly for some acute methods and not for others.

As shown by the size of PMSD, test sensitivity to detect substantial toxic effects is occasionally insufficient at some laboratories and routinely insufficient at a few laboratories. Inadequate test sensitivity is not always signaled by control charts of EC25, LC50, and NOEC. Laboratories should consider maintaining control charts for MSD or PMSD, and should report MSD and the control mean with all WET tests.

Some portion of MSDs in the WET variability data set could be considered exceptionally large, if not outliers. This observation underscores the importance of a careful review for each WET test, including an examination of means and standard deviations for endpoint responses at each concentration; the plotting of replicate data (not just concentration means); and, when necessary, a search for possible causes of excessive variability. The tables and plots in the promulgated methods (USEPA 1994a, 1994b) provide good examples.

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4.0 VARIABILITY IN CONTEXT

EPA manages the regulation of WET in the same way it manages the regulation of chemical-specific pollutants in order to determine reasonable potential (RP), derive permit limits, determine data quality control, and evaluate self-monitoring data. Many similarities between chemical-specific toxicant and WET controls can be found in the TSD (USEPA 1991a). Determining RP in both cases uses many of the same strategies. Permit limit derivation makes similar exposure assumptions and relies on nearly identical toxicological data bases.

Considering a value other than the best analytical estimate as a measure for WET or for specific chemical analytes is inappropriate. All analytical results, in either chemical-specific analyses or WET tests, incorporate some estimated range of uncertainty. While infrequently discussed for chemical methods, uncertainty does play a role in the meaning of analytical results. One end of the confidence interval likely will be less protective of aquatic resources than the other. The derived limit and therefore final reported analytical results become the best estimate of the actual ecological need and assessment of the effect.

Significant debate has occurred over assertions that WET data have too much inherent variability for reliable use in the NPDES program. This debate has engendered considerable evaluation of WET precision. Groups of scientists and individual researchers have repeatedly concluded that currently promulgated WET methods are technically sound and that the observed precision is within the range of precision of other analyses frequently required in NPDES permits (Grothe et al. 1996). The findings of some of the significant sources of these conclusions are summarized below.

4.1 Society of Environmental Toxicology and Chemistry Pellston WET Workshop

The 1995 Society of Environmental Toxicology and Chemistry (SETAC) Pellston Workshop on Whole Effluent Toxicity convened 47 experts in the discipline to assess applied methods and their application in the regulatory process. Representation at the workshop was intentionally balanced among government, business, and academic participants. These scientists published consensus conclusions and recommendations, including the following.

4.1.1 General Conclusions and Recommendations

Grothe et al. (1996) state *"Existing WET testing methods (USEPA 1985, USEPA 1988, USEPA 1989) are technically sound, but certain modifications would improve endpoint interpretation. Such changes involve implementing improvements to currently used statistical procedures, establishing acceptable limits for MSD values, and adding confidence limits to WET test endpoints."*

"A number of problems with WET tests are caused by misapplication of the tests, misinterpretation of the data, lack of competence of the laboratories conducting WET testing, poor condition/health of test organisms, and lack of training of laboratory personnel, regulators, and permittees. More widespread use of WET related guidance provided in USEPA's TSD (1991a) would help alleviate some of these problems. In addition, an effective QA/QC program will improve data quality and reduce test variability."

"Increase training opportunities for regulators and permittees to improve the implementation of WET objectives and to promote national consistency in permitting and compliance issues."

"Implement a broadly based and standardized QA/QC program to improve WET testing performance and data quality."

"Quantify the 'confidence' around test endpoints to improve interpretation of WET test results. Specific statistical methods that could improve precision are presented in Chapter 3 of this document and processes to reduce variability are discussed in Chapter 5. In addition, WET tests should be performed using a dilution series of exposure concentrations to establish a dose-response relationship."

4.1.2 Conclusions about Data Precision

Ausley (1996) compared CVs of chemical analyses and aquatic toxicity tests conducted by North Carolina NPDES permittees. Ausley found that CVs of reported values for chemical analytes (including metals, organic analytes, and non-metal inorganic analytes) ranged from 11.8 percent to 291.7 percent. Coefficients of variation for toxicity parameters (acute and chronic *Ceriodaphnia dubia*, acute and chronic *Pimephales promelas*, acute *Daphnia pulex*, and acute *Mysidopsis bahia*) ranged from 14.8 percent to 67.6 percent. From this review, he concluded that "*the precision of toxicity analyses is within the range of that being reported for commonly analyzed and regulated chemical parameters.*" Ausley highlighted the difficulty in comparing precision estimates of chemical analytes and WET analyses (particularly NOECs), noting that while chemical precision is often determined well above analytical detection, WET precision is often based on the minimum detection level. An assumption that WET precision will vary among toxicants is also logical. To establish "inherent variability," considering toxicants that cause minimal variability in the analysis may be appropriate. The high coefficients of variation for some chemical parameters reported by Ausley reflect the fact that, in practice, analytical precision can vary widely in individual studies in which the effects of a single (or a few) poorly operating laboratory can adversely affect precision estimates. In practice, this kind of data must be screened for quality prior to use to evaluate self-monitoring data or estimates of overall method quality.

Ausley's results closely approximate analytical precision of chemical analytes referenced in the TSD (USEPA 1991a, Chapter 1.2). The CVs for metals (aluminum, cadmium, chromium, copper, iron, lead, manganese, mercury, silver, and zinc) ranged from 18 percent to 129 percent at the low end of the measurement detection range. Between-laboratory CVs for organic analytes ranged from greater than 12 percent to 91 percent. The CVs for non-metal analytes (alkalinity, residual chlorine, ammonia nitrogen, Kjeldahl nitrogen, nitrate nitrogen, total phosphorus, biological oxygen demand, chemical oxygen demand, and total organic carbon) ranged from 4.6 percent to 70 percent in between-laboratory studies of precision.

Burton et al. (1996) concluded that "*USEPA-published methods are functional and appropriate in the context of effluent toxicity control programs.*" They recommended developing limits on within-test variability, a quality assurance and audit program, and guidance for permittee procurement of WET analytical services.

Denton and Norberg-King (1996) cited various studies that favorably compare WET methods with chemical analytical methods (Grothe and Kimerle 1985, Rue et al. 1988, Morrison et al. 1989, Grothe et al. 1990). They proposed that improvements in test result consistency could be accomplished by limiting the range of within-test variability through controls of upper and lower statistical power (e.g., limits on test MSD). Three practices to control within-test variability most effectively are (1) controlling within-test sensitivity, (2) following well-defined test methods, and (3) maintaining communication within the regulatory community. For example, the permittee and regulatory authorities should discuss any facility-specific issues to fully characterize the appropriate permit conditions.

4.2 Water Environment Research Foundation Study

Another publication, "*Whole Effluent Toxicity Testing Program: Evaluation of Practices and Implementation*" (DeGraeve et al. 1998), presents the results of a survey of publicly owned treatment works and State regulatory programs about WET issues. The Water Environment Research Foundation (WERF) sponsored this study. Conclusions by DeGraeve et al. (1998) include the following:

> "*The project team believes that the results demonstrate that the test methods can be routinely completed successfully by well-trained, competent WET testing laboratories and that the results, considered collectively, suggest that the test methods that are being used to measure WET are technically sound."*
>
> "*There is a need for better training/guidance in WET-related issues for both the regulatory staff responsible for implementing WET requirements and for permittees responsible for meeting WET limits*."

DeGraeve et al. (1998) considered the conclusions of the SETAC Pellston WET publication concurring that between-laboratory CV values of toxicity test methods were low, training of regulatory and permittee staff is needed nationally, and strengthened quality assurance (QA)/quality control (QC) practices could improve performance of analyses. Unlike the SETAC Pellston WET conclusions, they found that there are enough laboratories to meet the current market demand for analyses. Like the SETAC effort, DeGraeve et al. (1998) concluded that a national center of expertise on WET issues would be beneficial to provide guidance to regulatory agencies, permittees, and laboratories.

WERF also funded a project entitled "*Whole Effluent Toxicity Testing Methods: Accounting for Variance*" (Warren-Hicks et al. 1999). This study compared within- and between-laboratory results of reference toxicant test variation as measures of reproducibility and comparability, respectively. The authors concluded that some laboratories could consistently reproduce test results, while others could not and inferred that test precision is a factor of laboratory experience and not inherent methodological weakness. The authors recommended that national studies be conducted to evaluate within- and between-laboratory precision of promulgated WET test methods. (EPA has already initiated this study.) They also recommended that additional test acceptability criteria (TAC), such as upper and lower bounds of MSD, be established and incorporated in the NPDES process. The latter recommendation corroborates other researchers' recommendations discussed above.

4.3 Minimizing Variability by Adhering to WET Toxicity Test Methods

Specific factors that affect variability in WET analyses have been described in several papers (Burton et al. 1996, Ausley 1996, Erickson et al. 1998, Davis et al. 1998). The most important initial consideration in developing precise data is a laboratory's experience and success in performing a specific analysis. Most critical reviews of WET data precision emphasize this initial consideration. Experienced professionals most likely will be able to develop the most consistent and reliable information and can interpret anomalous conditions in the testing or results.

An additional factor in considering WET test method variability is whether the prescribed methods (e.g., the EPA toxicity test methods promulgated in 40 CFR Part 136) are being followed appropriately (see Chapter 5). If tests are submitted that do not meet specified TAC or are produced when laboratory QA testing indicates analyses are beyond control limits, these results should not be used in the NPDES process. Tests performed on effluent samples that have not met required temperature maxima or holding times should not be considered for regulatory purposes. Rigorous QA practices are critical to the success of any analytical program. Both the regulatory authority and permittee should strive to ensure that such practices are in place

for any program developing WET data, whether by national laboratory accreditation, State regulatory certification, direct permittee oversight, or specific contractual agreement with the laboratory.

Comparisons of WET method precision with analytes commonly limited in NPDES permits clearly demonstrate that the promulgated WET methods are within the range of variability experienced in other analyses. Several researchers also noted clear indications that method performance improves when prescribed methods are followed closely by experienced analysts (Grothe et al. 1996, DeGraeve et al. 1998).

A review of WET test results confirms that imprecise WET data are being reported. As with any analytical technique, inexperienced individuals can perform analyses incorrectly or fail to follow appropriate methods and quality assurance practices. Using the training that is available for these methods and quality assurance techniques referenced by this document will help ensure that data of maximum reliability are used and that sound decisions are made based on those results. The Western Coalition of Arid States conducted a study in 1997 (Moore et al. 2000), which reported the results of 16 tests with a non-toxic test sample using the *Ceriodaphnia dubia* chronic test. These results indicated that 43 percent of the tests showed toxicity. EPA is in the process of reviewing the paper and the raw data.

Persons interested in WET issues may consult another source of information developed by the SETAC Whole Effluent Toxicity Expert Advisory Panels. This group, established under a cooperative agreement with EPA, provides scientific opinion and training on WET technical issues. This information is available on the Internet at the SETAC web site, **http://www.setac.org**. Appendix D contains frequently asked questions with answers prepared by the SETAC WET Expert Advisory Panels. The expert panels have identified and discussed various factors that affect WET variability.

4.4 Conclusion

When the variability of WET analyses is viewed in the context of the NPDES program, these techniques produce data that are as precise as those from chemical analyses. As with any other analytical system, lack of experience in performing the analyses, adherence to prescribed QA practices, or good laboratory practices will reduce the precision of the results. Studies of these factors by independent researchers from both the regulatory and regulated communities support these conclusions. While examples of poor-quality, highly variable results from chemical analyses have also been publicized, these results are frequently influenced by the shortcomings mentioned above. Permittees that must generate and use WET data should become well-educated in data quality interpretation, and permittees should require that QC practices be followed by laboratories generating the data. Various sources of information presented in this chapter should assist permittees, testing laboratories, and regulatory authorities with this education process. Examples of practices that can further reduce the imprecision of analyses are also discussed in Chapters 5 and 6 of this document. Additional refinements of TAC can likewise improve test power to detect effects (or the lack thereof) and increase the statistical confidence in results.

5.0 GUIDANCE TO REGULATORY AUTHORITIES, LABORATORIES AND PERMITTEES: GENERATING AND EVALUATING EFFECT CONCENTRATIONS

5.1 Steps for Minimizing Test Method Variability

This chapter provides the background and recommendations on WET test procedures related to sampling, conducting the toxicity test methods, and conducting the statistical methods. Implementing these recommendations should decrease or minimize WET test method variability, thereby increasing confidence to make regulatory decisions (see Figure 5-1). EPA stands behind the technical soundness of the current WET test methods. The critical steps in minimizing WET test method variability are (1) obtaining a representative effluent sample, (2) conducting the toxicity tests properly to generate the biological endpoints, and (3) conducting the appropriate statistical analysis to obtain powerful and technically defensible effect concentrations. Minimizing variability at each step increases the reliability of the WET test results. For example, factors that affect variability include sampling procedures; sample representativeness; deviations from standardized test conditions (e.g., temperature, test duration, feeding); test organisms; source of dilution water; and analyst experience and technique in conducting the toxicity tests properly (Burton et al. 1996).

5.2 Collecting Representative Effluent Samples

The goal of effluent sampling is to obtain a representative sample that reflects real-world biological responses. Factors affecting the representativeness of effluent samples may include the sampling location, frequency, and type (e.g., composite or grab), and sample volume, container, preservation methods, and holding time. Burton et al. (1996) concluded that the above factors considerably influence test result variability.

Effluent samples must be collected at a location that represents the entire regulated flow or discharge. Typically, the sampling site is designated in the discharge permit. As with sampling for any parameter, effluent samples should be collected from a location where the flow is turbulent and well-mixed. Additionally, effluent samples should be collected at a frequency that enables adequate characterization of the discharge over time (e.g., accounts for daily to seasonal changes and variations in effluent quality). Major facilities should conduct WET testing monthly or quarterly, while minor facilities should conduct WET testing semi-annually or annually.

Appropriate sample types should be collected to represent the effluent fully. When the effluent is variable, collecting composite samples may be necessary. When the effluent is less variable, grab samples may be sufficient (e.g., from long-term retention pond facilities).

Sample containers should be non-reactive so that they do not affect sample characteristics. Table II of 40 CFR Part 136 requires that toxicity test samples be collected in glass or plastic containers, as specified in the methods. Sufficient sample volume should be collected for the type of test being conducted, including the number of test dilutions. When samples are collected in Cubitainers®, headspace should be minimized.

Samples must be properly preserved. Part 136 of 40 CFR requires that samples for WET testing be cooled to 4°C when shipped off-site and between test sample renewals. Samples must be cooled during all phases of collection, transportation, and storage to minimize physicochemical changes. Samples must be tested within the specified maximum holding times before significant changes occur, such as volatilization or biological or chemical degradation. If samples are not tested within specified maximum holding times, the test is invalid and must be repeated by collecting a new effluent sample and conducting a new toxicity test to comply with the NPDES permit.

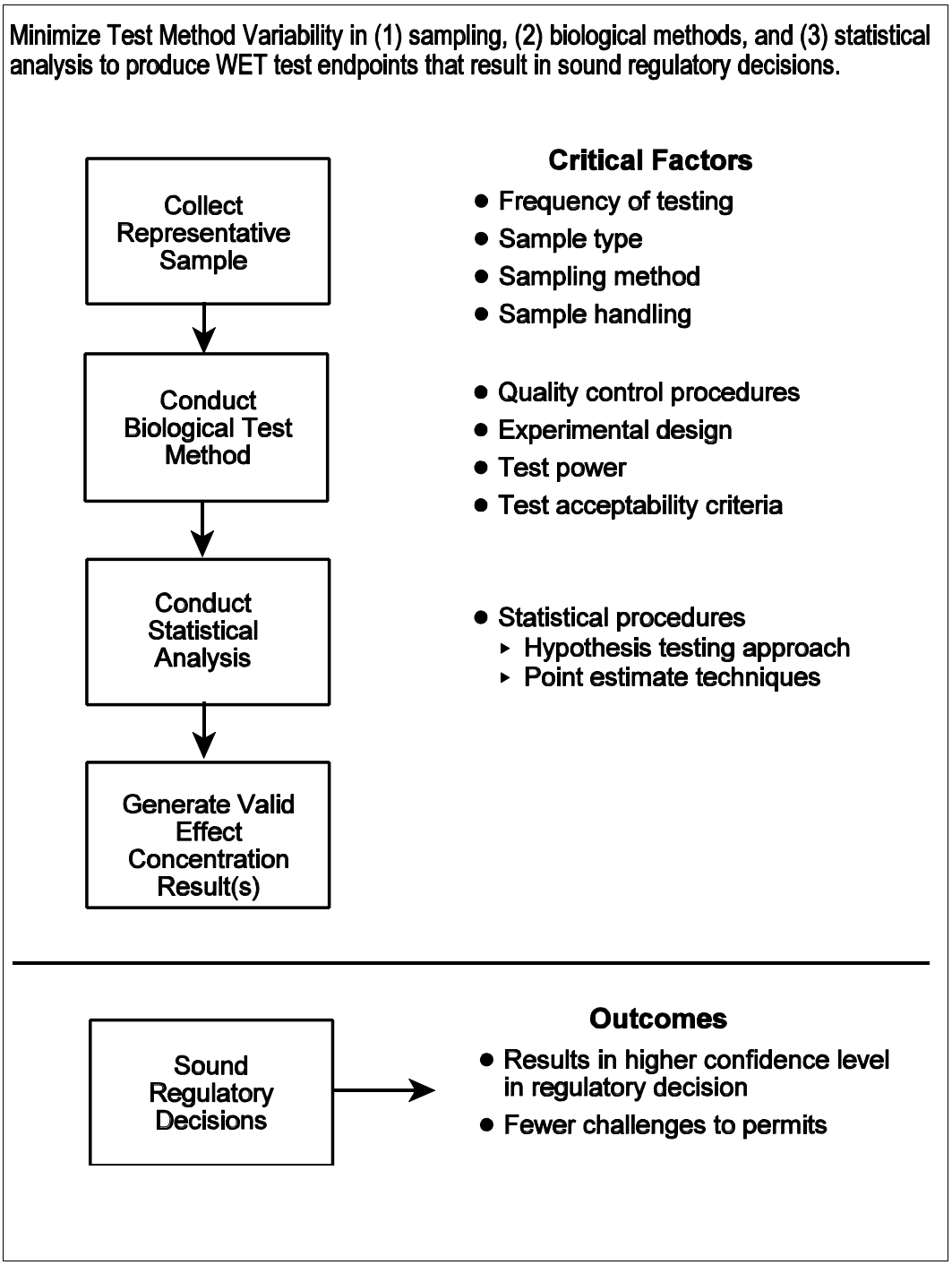

Figure 5-1. Steps to minimize WET test method variability.

5.3 Conducting the Biological Test Methods

Four main components of WET tests afford opportunities to control and minimize variability within tests and within and between laboratories: (1) quality control (QC) procedures; (2) experimental design; (3) test power; and (4) test acceptability criteria (TAC) beyond the minimum requirements specified in EPA's WET test methods.

5.3.1 Quality Control Procedures

Quality assurance (QA) practices for toxicity tests address all aspects of the tests that affect data quality. These practices include effluent sampling and handling, test organism source and condition, equipment condition, test conditions, instrument calibration, replication, use of reference toxicants, recordkeeping, and data evaluation. The EPA WET toxicity testing manuals specify the minimum requirements for each aspect. Regulatory authorities have the discretion to prepare and implement additional guidance beyond the minimum requirements specified in EPA's WET test methods.

An integral part of the QA program is quality control (QC). The QC procedures are the more focused and routine activities conducted under the overall QA program. An important QC component in WET testing is the requirement to conduct reference toxicant tests with effluent tests. The WET test methods outline when reference toxicant tests are to be conducted. (See sections on quality of test organisms in the manuals.) Reference toxicant testing serves two purposes: (1) determine the sensitivity of the test organisms over time; and (2) assess the comparability of within- and between-laboratory test results. Reference toxicant test results can be used to identify potential sources of variability, such as test organism health, differences among batches of organisms, changes in laboratory water or food quality, and performance by laboratory technicians. In the QA section of each promulgated test method (USEPA 1993, 1994a, 1994b), EPA recommends sodium chloride, potassium chloride, cadmium chloride, copper sulfate, copper chloride, sodium dodecyl sulfate, and potassium dichromate as suitable reference toxicants. The methods do not, however, specify a particular reference toxicant or the specific test concentrations for each test method.

The current characterization of WET test method variability is limited by the ability to quantify sources of within- and between-laboratory variability, because laboratories can use different reference toxicants and test concentrations for a particular method. Future evaluations of method variability would be greatly enhanced by having data to analyze from multiple laboratories for the same reference toxicant, the same dilution water at similar pH and hardness, and the same test concentrations. By standardizing reference toxicants, testing laboratories could compare test results, permittees and regulatory authorities could better compare and evaluate laboratories, and the data could be used to further quantify within- and between-laboratory test precision. Specification of the reference toxicant and test concentrations for a method across laboratories would provide a much larger and consistent data base to assess the comparability of within- and between-laboratory test results.

Standardizing reference toxicants and test concentrations has been discussed in the literature. For example, the chronic methods manual for West Coast species (USEPA 1995) specifies the reference toxicant and test concentrations for each test species. The Southern California Toxicity Assessment Group (SCTAG) is comprised of representatives from permittees, testing laboratories, regulatory authorities, and academic institutions that met to discuss technical aspects of WET testing (e.g., standardization of reference toxicants, control charts). The SCTAG (1996) prepared a report to standardize reference toxicants for the chronic freshwater test methods. This report evaluated an extensive data base of reference toxicant data. The report recommended specific reference toxicants and test concentrations for these methods. The SCTAG (1997) also prepared a QA/QC checklist to help toxicity testing laboratories establish and maintain appropriate data quality measures. Regulatory authorities should review these publications when standardizing reference toxicants.

The selection of reference toxicants and test concentrations should be based on specific criteria. The following criteria, recommended in the SCTAG report, provide an excellent basis for selecting standardized reference toxicants:

1. The toxicant should provide precise and reliable measures of toxicological sensitivity.
2. Toxicant disposal should not be legally or environmentally problematic.

3. The toxicant should produce a concentration-response effect for the test organism.
4. The toxicant should be quantifiable.
5. The toxicant should not pose an unacceptable health hazard to laboratory personnel.
6. The toxicant should be readily available.

Most recently, Warren-Hicks et al. (1999) recommended that national acceptance criteria be specified with upper and lower acceptance limits for reference toxicant test results, which all laboratories would need to achieve to obtain accreditation. Variability could decrease nationally if testing laboratories are provided with more detail on the evaluation and interpretation of reference toxicant control charts (APHA-AWWA-WEF 1998). For example, such guidance could describe how to evaluate test results within the warning limits. Both Environment Canada (1990, 2000) and APHA-AWWA-WEF (1998) have prepared guidance on evaluating control chart data. The Environment Canada (2000) report specifies using zinc as an inorganic reference toxicant and phenol as an organic reference toxicant for many aquatic tests. The report also specifies eight criteria for selecting specific reference toxicants.

1. Previous use
2. Availability in a pure form
3. Solubility
4. Stability in solution
5. Stability during storage
6. Ease of analysis
7. Stable toxicity with normal changes in qualities of laboratory water
8. Ability to detect abnormal organisms

Regulatory authorities may want to evaluate the above reports and the SCTAG reference toxicant recommendations for the chronic freshwater test methods. Regulatory authorities may also want to evaluate and recommend a standard reference toxicant and a specific concentration series for each acute and chronic test method each using data from this guidance document.

5.3.1.1 Guidance Related to Quality Control Charts and Laboratory Audits

Ausley (1996) recommends some oversight of data quality, such as evaluating tests in meeting QC criteria, using randomization procedures, and operating in allowed reference toxicant ranges to ensure that QC procedures are properly implemented. Another integral component of QC is the maintenance of control charts for reference toxicants and effluents. Laboratories should provide regular review of control charts. EPA suggests keeping a control chart for each combination of test material, test species, test conditions, and endpoints with a maximum of 20 test results. Modern software makes accumulating data and reviewing key test statistics possible with relatively little effort. Elementary methods can identify problems contributing to variability. Laboratories should practice regular control charting of test PMSDs and control performance for all tests along with control charting of effect concentrations such as NOEC and point estimates for reference toxicants tests. Successive tests should be compared occasionally to detect repeated patterns, such as one replicate's being consistently higher or aberrant, or a trend over time. Time sequence plots of concentration means and standard deviations would be useful in this regard. Occasionally, a set of 5 to 20 tests, in which block positions (see Appendix A in USEPA 1994b) have been recorded, should be subjected to ANOVA for block or position effects. If such effects are significant or large, the laboratory should seek advice on randomizing the replicates and concentrations.

If a laboratory's CV exceeds the 75th percentile CV from Tables 3-2 through 3-4, EPA recommends calculating warning and control limits based on the 75th and 90th percentiles, respectively, of CVs for the method and endpoint (Tables 3-2 and 3-3 and Appendix Tables B-1 and B-2). For example, suppose the mean EC25 for a series of *Ceriodaphnia* chronic tests (Method 1002.0 with reproduction as the endpoint) conducted at one laboratory with reference toxicant is 1.34 g/L NaCl. Also suppose that the standard deviation of the EC25s for these tests is 0.85. The CV for this set of EC25s is thus 0.63. In Table 3-2, the 75th percentile of CVs for this test's reproduction endpoint is 0.45. Calculate the standard deviation corresponding to the 75th percentile CV, $S_{A.75} = 1.34 \times 0.45 = 0.60$. In Appendix Table B-1, the 90th percentile of CVs is 0.62 for this method and endpoint. Calculate $S_{A.90} = 1.34 \times 0.62 = 0.83$. Because the CV for this series of EC25s exceeds the 90th percentile reported in Table B-1, EPA recommends the following:

- Set control limits using $S_{A.90} = 0.83$,
- Set warning limits using $S_{A.75} = 0.60$,
- Promptly take actions to bring results within the control limits, and
- Attempt to bring results within the warning limits in 3-12 months.

If the CV for the set of EC25s is less than the 90th percentile reported in Table B-1, use that CV to set control limits. If the CV for the set of EC25s is less than the 75th percentile in Table 3-2, do not set warning limits using the latter value.

In addition, Burton et al. (1996) encourage regulatory programs to have a laboratory audit component to document the existence and effectiveness of a QA/QC program directed at toxicity testing, including analyst training and experience. Regulatory authorities should use the National Environment Laboratory Accreditation Program (NELAP) (USEPA 1999a) and routine Performance Audit Inspections to evaluate individual laboratory performance. Inspections should evaluate the laboratory's performance with QC control charts based on reference toxicants, examine procedures for conducting the toxicity test procedures, and examine procedures for analyzing test results.

Regulatory authorities should develop a QC checklist to assist in evaluating and interpreting toxicity test results. Appendix E presents examples of State WET implementation procedures related to reviewing reference toxicant data and information on additional QA/QC criteria that have been developed and implemented. Regulatory authorities should also provide additional guidance related to the interpretation of QC control charts. This additional guidance could be that laboratories maintain control charts on within-test variability (e.g., PMSD) and use warning and control limits based on the 75th and 90th percentiles of CVs for the test method and endpoint.

5.3.2 Experimental Design

Experimental design includes randomizing the experimental units (i.e., treatments, organisms, replicates); establishing the statistical significance level (i.e., alpha level); and specifying the minimum numbers of replicates, test organisms, and treatments. Oris and Bailer (1993) recommend that test design and TAC be based, not only on a minimum level of control performance, but also on the ability to detect a particular level of effect (i.e., test power).

A Type I error (i.e., "false positive") results in the false conclusion that an effluent is toxic when it is not toxic. A Type II error (i.e., "false negative") results in the false conclusion that an effluent is not toxic when it actually is toxic. Power (1 - beta) is the probability of correctly detecting a true toxic effect (i.e., declaring an effluent toxic when it is in fact toxic). Acceptable values for alpha range from 0.01 to 0.10 (1 to 10 percent). The current EPA test methods recommend an alpha rate of 0.05 or 5 percent in the toxicity

testing manuals. Currently, EPA is preparing guidance on when an alpha rate of 0.01 or 1 percent would be considered acceptable (USEPA 2000a).

5.3.2.1 False Positives in WET Testing

The hypothesis test procedures prescribed in EPA's WET methods provide adequate protection against incorrectly concluding that an effluent is toxic when it is not. The expected *maximum* rate of such errors is the alpha level used in the hypothesis test. The hypothesis test procedure is designed to provide an error rate *no greater than* alpha when the default assumptions are met. The statistical flow chart provided with each EPA WET method identifies cases when default assumptions are not satisfied and, therefore, when data transformations or alternative statistical methods (e.g., a nonparametric test) should be used.

Alpha and beta are related (i.e., as alpha increases, beta decreases), assuming that the sample size (number of treatments, number of replicates), size of difference to be detected, and variance are held constant. The alpha and beta error rates depend on satisfying the assumptions of the hypothesis test. To ensure that statistical assumptions and methods are properly applied, testing laboratories should review the statistical procedures used to produce WET test results and other factors, such as biological and statistical quality assurance, and verify that test conditions and test acceptability criteria were achieved.

If a test is properly conducted and correctly interpreted, identifying any particular outcome as a "false positive" is impossible. An effluent that is deemed toxic test may require that the permittee conduct additional toxicity tests to determine if toxicity is re-occurring. Even if no toxicity is demonstrated in follow-up tests, that does not rule out that the original toxic event was a true toxic spike in the effluent. False negatives, however, impact the environment by allowing the discharge of harmful toxicants without identification. This may occur because the toxic effects are not identified as statistically significant due to lack of test sensitivity (see Sections 5.3.3 and 6.4).

Measurement error should not affect the protection against false positives provided by hypothesis tests and confidence intervals when they are appropriately applied. Measurement error, in the case of WET test treatment mean values, likely consists largely of sampling errors (e.g., variability among organisms or containers), although errors in counting, weighing, and other procedures may also occur. Such sources of imprecision are implicitly accounted for in WET test statistical inferences, because the sample variance among the replicates within each treatment (dilution) is used for inference. The test "size" 1 - alpha will protect adequately against false positives. A larger variance among replicates, however, could make detecting real toxicity (i.e., false negatives) more difficult unless the number of replicates is increased to provide more test sensitivity and power, which will reduce the rate of false negatives.

5.3.2.2 False Negatives in WET Testing

For a given alpha, beta decreases (power increases) as the sample size increases and the variance decreases. Decreasing alpha from 0.05 to 0.01 without otherwise changing the hypothesis test will reduce the ability of the test to detect toxicity, that is, will reduce the power of the test. Thus, as alpha for the hypothesis test is decreased, there is an inevitable trade-off between the rate of false positives when toxicity is not present and the ability to detect toxicity when it is present (i.e., statistical power).

To limit within-test variability and thus increase power, EPA developed a minimum significant difference (MSD) criterion that must be achieved in the chronic West Coast marine test methods (USEPA 1995). The MSD is a measure of the within-test variability and represents differences between treatments and the control that can be detected statistically. Distributions of the MSD values of multiple tests for a specific reference toxicant and test method can be used to determine the level of test sensitivity achievable by a certain percentage of tests. Denton and Norberg-King (1996) analyzed several chronic test methods to quantify the effect size based on the existing toxicity test method experimental design and MSD distributions.

Denton and Norberg-King found when setting the beta error rate at 0.20 (power = 0.80), the effect size detected varies from at least a 15-percent reduction from the control response for the chronic red abalone larval development test to a 40-percent reduction from the control response for the chronic *Ceriodaphnia dubia* test. In this document, EPA has calculated power for each test method (see Section 5.3.3).

5.3.3 Test Power To Detect Toxic Effects

This section describes the statistical power and ability to detect toxic effects achieved by the current WET methods, as inferred from the WET variability data set used to develop this document. These inferences are approximate, because assumptions of normality and homogeneity of variance were not always satisfied.

Power can be characterized only by repeated testing. Power is an attribute not of a single test, but of a sequence of many tests conducted under similar conditions and with the same test design. Therefore, in this document, EPA used the sample averages for each laboratory's data set to characterize each laboratory. The following two parameters were required: (1) the mean endpoint response in the control (growth, reproduction, survival); and (2) the mean value of the error mean square (EMS) for tests.

EPA evaluated the ability to detect toxic effects using three approaches for each test method: (1) number of replicates required to detect a 25-percent difference from the control with power of 0.80; (2) percent difference from the control that can be detected with power of 0.80; and (3) power to detect a 25-percent difference from the control. All calculations are based on a one-sided, two-sample t-test at a level of 0.95 (alpha of 0.05). The power for a multiple comparison (Dunnett's or Steel's test) will be less than the power for this two-sample t-test.

Table 5-1 summarizes the results for this evaluation. Depending on the method, between 30-percent and 80 percent of the laboratories were able to detect a 25-percent effect for the sublethal endpoint consistently. Between 60 percent and 100 percent of the laboratories were able to detect a 33-percent effect.

To examine whether the upper bounds presented in Table 3-6 provide adequate test precision, EPA calculated an estimate of the power to detect a 25-percent effect on a sublethal endpoint when the PMSD equals the upper bound reported in Table 3-6. The upper bounds of the PMSD are shown in Table 3-6 in Chapter 3. At the lower PMSD bound, the power always exceeded 0.98. Tests with PMSD equaling the upper bound are not often able to detect a 25-percent effect. This finding does not mean that the upper bound is ineffective. The PMSD varies between tests, and each laboratory has a distribution of PMSDs. To avoid exceeding this upper bound often, a laboratory would have to achieve substantially lower PMSDs in most tests.

5.3.3.1 Attainment of the PMSD Related to Power

The power of the current experimental design could be reevaluated by comparing it to alternative designs that use increased number of replicates or number of test concentrations (Chapman et al. 1996). In this document, EPA found that about half of the laboratories in the data set were able routinely to detect a 25-percent difference between control and treatment. About two-thirds of the laboratories could routinely detect a 33-percent difference (Table 5-2). For example, mere attainment of the 90th percentile PMSD values shown in Table 3-6 will not ensure the ability to detect a 25-percent effect (Table 5-2). If every acceptable test has a PMSD below that upper bound, however, the average PMSD will be lowered. Based on the within-laboratory variability of PMSD,[1] the average PMSD likely will be substantially lower than the upper bound in Table 3-6, if *most* tests conducted by a laboratory are to have acceptable PMSDs.

[1] The average CV for PMSD is one-third to one-half its mean in commonly used methods.

Table 5-1. Tests for Chronic Toxicity: Power and Ability To Detect a Toxic Effect on the Sublethal Endpoint

Test Method	No. Labs	No. Labs with Power		Power (Range)	No. Labs Having Power at Least 0.8 To Detect Effect of		Effect Detected with Power 0.8 as Percent of Control Mean (Range)
		≥ 0.8	≥ 0.5		≤ 25%	≤ 33%	
1000.0 Fathead Minnow	19	6	14	0.21 - 1.00	6	13	8.2 - 62
1002.0 *Ceriodaphnia*	33	10	29	0.38 - 1.00	10	19	14 - 45
1003.0 Green Alga	9	7	8	0.33 - 0.99	7	8	13 - 49
1004.0 Sheepshead Minnow	5	4	5	0.77 - 1.00	4	5	8.6 - 26
1006.0 Inland Silverside	16	7	13	0.23 - 0.97	7	12	17 - 59
1007.0 Mysid (growth)	10	5	8	0.21 - 0.91	5	8	21 - 70

Note: Power was calculated for a two-sample, one-sided t-test at alpha = 0.05, for a 25-percent difference from the control. Effect size detected was calculated for the same test using power 0.80. Calculations used the average EMS from all tests at each laboratory and the minimum number of replicates reported for those tests. Calculations assumed that the parametric mean and variance equal the corresponding sample estimates. They also assumed approximate normality of means and homogeneity of variance. Because these assumptions may be violated, the results here are approximate. By saying "detect a 25-percent difference from control," this alternative hypothesis is intended: (control mean - treatment mean) > 0.25 × control mean.

Table 5-2. Power To Detect a 25-Percent Difference from the Control at the 90th Percentile PMSD

Chronic Method	Replicates	90th Percentiles of PMSD	Three Treatments		Four Treatments		Five Treatments	
			alpha = 0.05	alpha = 0.05/3	alpha = 0.05	alpha = 0.05/4	alpha = 0.05	alpha = 0.05/5
1000.0 Fathead Minnow	3	35	0.39	0.25	0.39	0.19	0.39	0.15
	4	35	0.41	0.30	0.42	0.26	0.43	0.23
1002.0 *Ceriodaphnia*	10	37	0.39	0.31	0.41	0.30	0.43	0.30
1003.0 Green Alga	3	35	0.39	0.25	0.39	0.19	0.39	0.15
	4	35	0.41	0.30	0.42	0.26	0.43	0.23
1004.0 Sheepshead Minnow	3	23	0.72	0.69	0.72	0.62	0.73	0.55
	4	23	0.73	0.71	0.74	0.68	0.75	0.66
1006.0 Inland Silverside	3	23	0.72	0.69	0.72	0.62	0.73	0.55
	4	23	0.73	0.71	0.74	0.68	0.75	0.66
1007.0 Mysid	8	32	0.48	0.41	0.50	0.40	0.52	0.40

Notes: Values are rounded to two significant figures. Number of treatments is the number of concentrations compared with the control in the hypothesis test. The calculations assumed (1) the usual assumptions of the test are satisfied (approximate normality, homogeneity of variances); and (2) equal replication in treatments and control. Because these assumptions may be violated, the results here are approximate. Because the MSD/mean implies a value for [root (error mean square)/mean], the latter could be calculated from the MSD, Dunnett's critical value, and the number of replicates, and then used in a calculation of power. Calculations apply to a one-sided, two-sample t-test of equal means, assuming equal variances and equal replication, with hypotheses H_o:{control mean - treatment mean = 0} versus H_a:{control mean - treatment mean > 0.25 × control mean}. The power achieved by Dunnett's multiple comparison procedure will lie between the two-sample power at alpha = 0.05 and that for alpha = 0.05/(no. of treatments).

Testing laboratories and permittees can examine the EMS or MSD in Tables B-14 and B-15 (Appendix B) to estimate the ability of a WET test to detect toxic effects. Some regulatory authorities may require a comparison between the control and the receiving water concentration, which requires a two-sample, one-sided test. Others may require the multiple comparisons procedure described in the EPA WET methods (Dunnett's or Steel's tests, one-sided, with alpha of 0.05). The power of Dunnett's procedure falls between the power of the one-sided, two-sample t-test with alpha of 0.05 and alpha of 0.01, assuming that no more than five toxicant concentrations are compared to a control. The power of Steel's procedure will be related to, and should usually increase with, the power of Dunnett's procedure and the t-tests. Tables B-14 and B-15 in Appendix B also provide an appropriate guide to achieving power using a nonparametric test.

Recently, the State of Washington (1997) issued guidance specifying an acute and chronic statistical power standard to be achieved for compliance testing. EPA's sediment toxicity testing manuals (USEPA 1994c, USEPA 2000) include power curves for various numbers of experimental units, CV ranges, and associated alpha and beta levels. Sheppard (1999) is a good source to provide a simple explanation of how power helps determine how large a sample should be. Additional information on power may be obtained at: http://www.psychologie.uni-trier.de:8000/projects/gpower/literature.html.

EPA recommends that regulatory authorities specify in their State WET implementation procedures that individual test results achieve a level of within-test sensitivity by using the upper and lower PMSD test sensitivity bounds (see Section 6.4). To achieve the test sensitivity bounds, testing laboratories may need to minimize within-test variability (e.g., EMS) or increase the number of replicates tested, or both. If laboratories cannot achieve PMSD values of less than 25 percent for the toxicity test methods that require a minimum of only three replicates (Methods 1000.0, 1004.0, 1006.0), then the numbers of replicates may need to be increased. Appendix B (Section B.4) provides information related to the number of replicates needed and discusses the relationship between test power and effect size achieved. The magnitude of the effect size achieved relates to the test sensitivity.

5.4 Test Acceptability Criteria

EPA test methods have specific TAC that the effluent and reference toxicant tests must meet. A test is considered invalid if the TACs are not met. The recommended test conditions for each test method specify the minimum requirements and the TAC. For example, control survival must be 80 percent or greater and average control reproduction at least 15 young per surviving female in the chronic *Ceriodaphnia dubia* survival and reproduction test.

The chronic West Coast marine methods (USEPA 1995) require additional TAC. For example, to limit the degree of within-test variability, the methods specify a maximum allowable value for PMSD (see Section 5.3.3 on experimental design). Some States have additional TAC in their State WET implementation policies. North Carolina (1998) for example, requires that the chronic *Ceriodaphnia dubia* analyses meet an additional TAC of complete third brood neonate production by at least 80 percent of the control organisms and that the control reproduction CV be less than 40 percent.

Additional TAC might be specified to minimize variability among replicates. Variability of any toxicity test result is influenced by the number of replicates used, number of organisms tested, and variability among replicates at each test concentration and the control. Variability among replicates has been quantified by treatment CV, EMS, or MSD. The application of a maximum acceptable value for CV or MSD helps ensure adequate laboratory QA/QC and increases the reliability of submitted data. One benefit of requiring a maximum allowable within-test variability limit is that laboratories will improve culturing, test handling, and housekeeping, which are usually incorporated into the laboratories' standard operating procedures. For example, the CV requirement might be incorporated directly into the NPDES permit. Sample EPA Region 6 permit language reads:

1. *The coefficient of variation between replicates shall be less than or equal to 40 percent in the control.*

2. *The coefficient of variation between replicates shall be less than or equal to 40 percent at the instream waste concentration (IWC).*

3. *Test failure may not be construed or reported as invalid due to a CV of greater than 40 percent. A repeat test shall be conducted within the required reporting period if any test is determined to be invalid.*

Occasionally, statistical analyses indicate a test failure when as little as 15-percent mortality has occurred in a test dilution. Permit language has been developed to address this occurrence, as in the following example:

> *If all TAC conditions are met and the percent survival of the test organism is greater than or equal to 80 percent (in a chronic test) or 90 percent (in an acute test) in the critical dilution concentration and all lower dilution concentrations, the test shall be considered to be a valid test, and the PERMITTEES shall report an NOEC of not less than the critical dilution for the discharge monitoring report (DMR) reporting requirements.*

Regulatory authorities may consider providing guidance or imposing additional TAC, such as those implemented by EPA Region 6 or like some States have implemented (North Carolina 1998, Washington 1997). Appendix E provides additional examples of States that have implemented further guidance on WET QA/QC procedures and TAC. Warren-Hicks (1999) also recommended that additional national TAC be established for each test method (e.g., upper and lower bounds on the MSD). Therefore, EPA recommends that regulatory authorities require that additional TACs be implemented in permits to minimize within-test variability and increase test sensitivity (see Section 6.4 and Appendix C for sample permit language).

5.5 Conducting the Statistical Analysis To Determine the Effect Concentration

EPA test methods currently recommend two statistical approaches to estimate a chemical or effluent concentration for each biological effect endpoint (e.g., survival, growth, and reproduction). One approach is to derive the NOEC by hypothesis testing, which equates biological significance with statistical significance. The second approach is to estimate an effect concentration that reduces the control response by 25 percent for chronic methods. The expanded use of WET tests in the NPDES program has brought increased attention to the statistical analysis of toxicity test data. A common goal for both regulatory authorities and permittees is to confirm that the effect concentrations were derived correctly using the appropriate analysis approaches. Reliable effect concentrations lead to increased confidence in the data used for making regulatory decisions, such as determining reasonable potential, deriving a permit limit or monitoring trigger, and generating self-monitoring test results.

Another important consideration in conducting statistical analyses is the inconsistent use of statistical programs. The proliferation of statistical packages has been helpful in data analysis; however, these packages also can result in the misapplication of the statistical methods. APCA-AWWA-WEF (1998) cautions the user to confirm the results of each analysis with each package before accepting them. The data user is responsible for evaluating all data submitted to the regulatory authorities.

The 1995 SETAC Pellston Workshop discussed unresolved scientific issues and highlighted significant research needs associated with WET testing. The attendees recommended the following:

> *Immediately instigate studies to evaluate improvements in the statistical analysis of WET test data. These studies should include, but not necessarily be limited to, the following activities:*

(a) investigate the implications of concurrent application of NOEC/MSD, tests of bioequivalence, and ECp estimators (Chapman et al. 1996a).

In response to this recommendation, EPA began projects to evaluate the bioequivalence approach and additional point estimate models for the WET program. At present, two test methods are being used for this evaluation: (1) the chronic *Ceriodaphnia dubia* survival and reproduction tests and (2) the giant kelp germination and germ-tube length test with reference toxicants.

The bioequivalence approach poses the following question: Do the mean responses of the effluent concentration and the control differ by more than some amount? For example, the control response and the response at the critical effluent concentration (i.e., instream waste concentration) must differ by no more than a fixed value in order to accept the hypothesis of no significant difference (i.e., no toxicity). This approach could address the concern that an imprecise test might not detect toxicity when toxicity is present or that a small but statistically significant effect would detect toxicity that may not be biologically important. Some researchers have suggested that the bioequivalence approach could provide a positive incentive for dischargers to produce test results with lower within-test variability to demonstrate that no toxicity occurs at a level greater than a biologically (bioequivalence approach) significant amount (Shukla et al. 2000, Wang et al. 2000).

Bailer et al. (2000) evaluated the proposed regression-based estimators with the current EPA point estimate models. They found that it appears reasonable to incorporate parametric estimation models in the WET program. Bailer et al. (2000) concluded that these models are appropriate for all response scales (i.e., dichotomous, count, and continuous) and can incorporate monotonicity without bias. However, confidence intervals still need to be developed for these parametric models.

In this document, EPA has not recommended either the bioequivalence or additional point estimate models to supplement the current statistical approaches as described in the testing manuals. EPA, however, does encourage an independent, peer-reviewed workshop to evaluate the benefits of these alternative statistical approaches to enhance the statistical approaches currently applied.

5.6 Chapter Conclusions

In this chapter, EPA provides guidance to permittees and testing laboratories on collecting representative effluent samples, conducting the biological test methods, and evaluating the statistical analyses. EPA recommends that States implement the lower and upper PMSD test sensitivity bounds to achieve an acceptable level of test sensitivity and minimize within-test variability (see Section 6.4). EPA also provides guidance to permittees and testing laboratories on the number of replicates required to achieve the PMSD bounds. Testing laboratories should maintain and evaluate both effluent and reference toxicant data using a measure of within-test variability such as the PMSD.

Permittees and toxicity testing laboratories may need to increase replication in order to reduce PMSD below the upper bound. Table B-15 can be used for initial planning of replication, given knowledge of typical values of the error mean square (EMS) or MSD and the number of concentrations used in the multiple comparison hypothesis test. To ensure that all PMSD values fall below the upper bound in Table 3-6, a laboratory would select the largest EMS value experienced in its past testing.

EPA recommends that testing laboratories require a minimum of four replicates for the fathead minnow, sheepshead minnow, and inland silverside chronic test methods (Methods 1000.0, 1004.0, and 1006.0, respectively). Four replicates are needed to execute the statistical flow chart when a nonparametric test is needed. Three replicates are also sometimes insufficient to keep PMSD below the recommended upper bound. In addition, four replicates are needed to help achieve the upper PMSD bound.

6.0 GUIDANCE TO REGULATORY AUTHORITIES: DETERMINING REASONABLE POTENTIAL AND DERIVING WET PERMIT CONDITIONS

EPA developed the TSD (USEPA 1991a) to support implementation of national policy to control the discharge of toxic pollutants. The TSD presents a statistical approach for determining the need for and the method of deriving water quality-based effluent limits (WQBELs) based on aquatic life (including WET), human health, and wildlife criteria. This approach accounts for the uncertainty associated with small data sets and data variability by assuming a statistical distribution of effluent data (usually lognormal) and calculating a CV or using a default CV to describe data variability.

6.1 Analytical and Sampling Variability in Calculations for Reasonable Potential and Permit Limits

Section 6.1 discusses use of the CV of sample measurements of toxicity to make a reasonable potential determination and to calculate permit limits. Two points must be understood: (1) this CV is to be calculated using toxic unit (TU) values (USEPA 1991a) (see Section 6.2); and (2) EPA strongly recommends that point estimates (not NOEC or LOEC values) be used to calculate the TU values (USEPA 1994a, 1994b).

Water quality-based effluent limits are required when a discharge causes, has reasonable potential to cause, or contributes to an instream excursion above a water quality standard. Throughout this document, EPA uses the commonly understood, shorthand reference "reasonable potential" to refer to this standard for determining the need for a water quality-based effluent limit.

6.1.1 "Adjusting for Analytical Variability" in Calculations for Reasonable Potential and Permit Limits

Adjustment approaches (see Appendix G.3) have been suggested to "adjust for analytical variability" when deriving permit limits and determining the need for a WET limit in the first place. EPA does not recommend these adjustment approaches (Appendix G.3) and strongly reaffirms the statistical approach and methods for calculating permit limits provided in the TSD (USEPA 1991a). *EPA recommends that regulatory authorities use the statistical approach and calculation methods in the TSD.* The TSD methods were designed to provide a reasonable degree of protection for water quality (i.e., to avoid exceedances of water quality criteria), while providing a reasonable degree of protection from the variability of effluent toxicity and analytical variability. The various "adjustment" approaches would undermine these objectives.

The TSD limit calculation for a point source can be divided into two steps: first, convert the wasteload allocation (WLA) to a long-term average (LTA), and then convert the LTA to effluent limits (maximum daily, average weekly, and average monthly limits). WET limit calculations include an intermediate step in which the acute WLA is converted to a WLAa,c. These calculations employ a facility-specific CV based upon effluent sampling data. The TSD approach uses this CV in both steps.

Adjustment approaches intended to account for analytical variability, discussed in detail in Appendix G, would inappropriately use different CVs in these two steps. The first step would use an estimate of the CV of "true" effluent toxicity, which is smaller than the CV for measured toxicities. This approach would result in a larger calculated LTA. The second step would use the CV for the measured toxicities, which is the same CV used in both steps of the TSD approach.

Use of such adjustment approaches would frequently result in setting an average monthly permit limit (AML) that exceeds the chronic WLA. Appendix G demonstrates that such outcomes (i.e., the AML exceeds

the chronic WLA) generally can be expected to occur when various adjustment approaches are used. Appendix G, Table G-1, presents a numerical example of how an adjustment approach would allow calculation of an AML exceeding the chronic WLA (a four-day average value), even when sampling frequency for the calculation is set at the recommended minimum of four samples per month. [It is acceptable for the maximum daily limit (MDL), which applies to a single sample, to exceed the chronic WLA. It is also acceptable for the AML to exceed the chronic WLA, if the AML calculation is based on fewer than four samples per month. Note, however, that the TSD recommends always assuming at least four samples per month when calculating the AML.]

The TSD reasonable potential calculation first calculates the percentile represented by the maximum observed TU value. For example, the maximum of 10 reported TU values is identified with the 63rd percentile. Then the sample CV is used to project the 95th or 99th percentile TU value, using a table of reasonable potential multiplying factors. This value is combined with the appropriate mixing-zone dilution to project a maximum receiving water toxicity, which is compared with the applicable water-quality criterion. If an adjustment were applied to the reasonable potential calculation, the CV would be adjusted downward and the maximum projected receiving water toxicity would be smaller. This would make a determination of need for a permit limit less likely.

Because of these considerations, EPA strongly recommends that no adjustment be made to the CV or variance of toxicity, either for reasonable potential or permit limit calculations. The TSD statistical approaches already account for analytical variability appropriately. EPA continues to recommend the TSD approach, which ensures that effluent limits and, thereby, *measured* effluent toxicity or pollutant parameter concentrations are consistent with calculated WLAs.

6.1.2 Analytical Variability and Self-monitoring Data

EPA determines compliance with permit limits on the basis of self-monitoring data, and these data include some measure of analytical variability. The influence of analytical variability is accounted for in the TSD statistical procedures used to set water-quality limits and determine the potential for toxicity, as explained in Appendix G.

The permittee is responsible for ensuring that measured discharge toxicity never exceeds the permit limits. No special allowance is made for analytical variability in assessing compliance. The maximum discharge toxicity should incorporate a margin of safety, which will account for sampling and analytical variability. In other words, to avoid exceeding permit limits, the facility's treatment system should be designed so that the maximum toxicity is somewhat lower than its permit limits.

6.1.3 Precision of WET Measurements and Estimates of Effluent CV

Single measurements on effluent involve some uncertainties about the true concentration or toxicity related to representativeness of the sample, including sample holding time and conditions, and the analytical measurement system. Like all analytical measurements, WET measurements (NOEC, EC25, LC50) are inexact. That is, the exact toxicity of an analyte in a sample can be specified only within some range. This imprecision can be reduced by using a suitable number of organisms and replicates for each test (see Section 5.3.3 on experimental design).

The numbers of organisms and replicates required for EPA WET method test acceptability are specified as minimums. Test precision will be approximately proportional to the square root of the number of replicates. Thus, doubling the number of replicates may decrease the MSD to approximately 70 percent of its former value. Increased replication also tightens the confidence interval for a point estimate of the effect concentration (e.g., EC25 and LC50).

EPA strongly recommends that toxicity measurements of an effluent be obtained at least quarterly for three years to provide a good basis for determining the need for limits and for calculating limits. One year should be regarded as the minimum duration needed to characterize effluent variability (due to seasonal, stream flow, or process fluctuations), and ten the minimum number of measurements, unless scientific and technical knowledge supports a shorter period as representative of the distribution of pollutant types and concentrations of toxicity.

Estimates based on multiple measurements involve the same uncertainties that apply to single measurements. They also may involve larger uncertainties related to sampling error, that is, the chance that typical levels of toxicity or concentrations of pollutant may not be encountered during the sampling program. The sampling program may not fully characterize effluent variability if too few samples are taken, the sampling times and dates are not representative, or the duration of the sampling program is not long enough to represent the full range of effluent variability. When determining the need for limits and calculating limits, the variance or the CV of toxicity measurements is key. The larger the number of samples, the more precise is the estimate. Confidence intervals for the variance and CV can be calculated and carried through the calculations for reasonable potential and effluent limits (Appendix G). Even when assumptions are not strictly met, confidence intervals provide a useful perspective on the uncertainty of the results and the need for more samples. The *minimum* number of measurements recommended for calculating estimates of the CV for effluent toxicity is 10.

6.1.4 Between-Laboratory Variability

Between-laboratory variability may increase the CV as discussed in Section 6.1.1, if the toxicity tests were conducted by more than one laboratory for a specific facility. A concern to permittees is that this may increase the likelihood of making a finding of reasonable potential.

Within-laboratory variability is the component of analytical variability that should be reflected in regulatory calculations. If the data used for reasonable potential or permit limit calculations are effluent measurement data reported by at least two laboratories, there are ways to appropriately estimate the variance to be used in TSD statistical calculations.

For example:

- If the same laboratories continue to be used in the same proportion or frequency and the measurements from the individual laboratories represent different sampling dates, the measurement data can be treated as if they were generated by a single laboratory. This approach may increase the estimated variance and the AML, which is not in the interest of the permittee. Selecting one laboratory for future monitoring, based on the variance of its reported reference toxicant test results, should mitigate this problem.

- If only one laboratory has reported data on each sampling date, and the other laboratories report over different time spans or over the same time span on alternating dates, EPA recommends forming a pooled estimate of variance. Calculate the sample variance (S^2) of log(TU) for each laboratory separately, and combine these using the formula:

  $$\text{pooled variance of } \log(X) = [(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2] / [(N_1 - 1) + (N_2 - 1)]$$

  An analogous formula is used for more than two laboratories. The same result can be obtained by conducting a one-way analysis of variance on log(TU) (with laboratories treated as the groups or classes) and using the reported EMS.

Changing a laboratory may change analytical (within-laboratory) variability of measurements and test sensitivity (i.e., PMSD values). That is, the average effect concentration may change (e.g., Warren-Hicks et al. 1999). Ideally, the permittee will anticipate and plan for a change of testing laboratory. Permittees should compare reference toxicant test data for current and candidate replacement laboratories, selecting one with acceptable variability and a similar average effect concentration.

6.2 Determining Reasonable Potential and Establishing Effluent Limits

Effluent characterization is an essential step in determining the need for an NPDES permit limit. NPDES regulations under 40 CFR Part 122.44(d)(1)(ii) specify that reasonable potential include "*whether a discharges causes, has the reasonable potential to cause, or contributes to an instream excursion above a State water quality standard*." Calculations for reasonable potential determination and for permit limits should follow EPA guidance in the TSD (USEPA 1991a). In particular, the TSD statistical methods should be used. Such calculations should use TUs for WET data, not effect concentrations (percent whole effluent). Toxic units are defined (USEPA 1991a, Chapter 1.3.1, page 6) as the reciprocal of the effect concentration times 100, where the effect concentration is expressed as a percentage of whole effluent, thus TUa = 100/ LC50 and TUc = 100/ECp.

When characterizing an effluent to determine whether a permit limit is necessary, permit writers can use the available effluent WET data and a water-quality model to perform a reasonable potential analysis. The TSD outlines the statistical approach. This approach uses existing effluent data to project a maximum pollutant concentration or a maximum toxicity in the effluent (USEPA 1991a). The projected maximum concentration or toxicity is used as an input in the water quality model to determine whether the effluent has the reasonable potential to cause or contribute to an excursion of ambient water quality criteria. If reasonable potential exists, the permit writer must derive a WET permit limit for that facility.[1]

The variability of the existing effluent data, as measured by the CV, has a significant effect on the projected maximum pollutant concentration or toxicity. The higher the CV, the higher the projected maximum, and the more likely that there is reasonable potential and a limit is needed. EPA recommends that regulatory authorities use all valid, relevant, and representative data in making reasonable potential determinations. EPA is developing a national policy clarifying use of the TSD procedures for determining reasonable potential for WET. Important components of this policy include specifying the minimum number of valid WET tests necessary to calculate facility-specific CVs,[2] as well as recommending a step-wise approach to determining the need for WET permit limits. This approach reflects a strong preference by EPA and its stakeholders to rely on facility-specific WET testing, based on adequate frequency and duration of effluent sampling, for making reasonable potential determinations for toxicity.

EPA recommends that point estimates be used to estimate effluent variability, to determine the need for limits, and to set permit limits. This is recommended whether the self-monitoring test results will be determined using hypothesis tests or point estimates. Point estimates have less analytical variability than NOECs using current experimental designs, as shown in Chapter 3. Point estimates make the best use of the WET test data for purposes of estimating the CV, LTA, and RP factor and calculating the permit limit.

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[1] When the State has narrative criteria for toxicity and the TIE/TRE identifies a specific chemical that is the source of toxicity, the permit writer may include a chemical-specific limit for that parameter instead of a WET permit limit in accordance with 40 CFR Part 122.44(d)(v).

[2] If fewer than ten data points are available, the regulatory authority must use a default CV. As a result, the need for a WET permit limit may be based on a default value rather than actual data.

6.3 Development of a Total Maximum Daily Load for WET

Total maximum daily loads (TMDLs) may be indicated when there is acute or chronic toxicity in a waterbody, leading to the listing of the waterbody as impaired under CWA Section 303(d), and when there are multiple sources of the toxicity. EPA believes that TMDL calculations should be performed on the pollutants causing toxicity whenever possible. In these situations, EPA suggests that the first step of the analysis is to conduct ambient toxicity identification evaluations to identify the pollutant(s) and the source(s) causing the toxicity. Once the pollutant(s) and source(s) causing toxicity have been identified for the waterbody, then a TMDL should be developed for the individual pollutant(s).

6.4 Accounting for and Minimizing Variability In the Regulatory Decision Process

A common goal for the permittee and the regulatory authority is to have confidence in the test results from the biological and statistical procedures. Both permittees and regulatory authorities would then have more confidence in taking regulatory actions, such as evaluating multiple effluent samples to determine reasonable potential and derive permit conditions (e.g., permit limits, monitoring triggers). If steps such as collecting a representative effluent sample to conducting the toxicity tests properly, as discussed in Sections 5.2 through 5.4, and requiring additional TACs (Section 6.4) are used to reduce or minimize within-test variability, then the reliability of the WET test results increases.

6.4.1 Recommended Additional TACs: Lower and Upper Bounds for PMSD

Reference toxicant data from a large number of tests and laboratories were used to generate PMSD values; percentiles of these values are reported in Table 3-6. The MSD represents the smallest difference between the control mean and a treatment mean that leads to the statistical rejection of the null hypothesis (i.e., no toxicity) using Dunnett's multiple comparison test. MSD values are divided by the control mean and multiplied by 100 to produce a "percent MSD" (PMSD) value. The PMSD allows comparison of different tests and represents the smallest significant difference from the control as a percentage of the control mean. Thus, it represents the smallest significant value of the relative difference [100 (control mean - treatment mean)/control mean]. The MSD is often expressed as a percentage of the biological endpoint in the control response.

The following formula is used to calculate MSD (as recommended by USEPA 1995):

$$MSD = d s_w \sqrt{(1/n_1) + (1/n)}$$

where

- d = critical value for the Dunnett's procedure
- s_w = the square root of the error mean square (EMS)
- n_1 = number of experimental units in the control treatment
- n = the number of experimental units per treatment, assuming an equal number at all other treatments

Percent MSD is calculated as follows:

$$PMSD = \frac{MSD}{\text{control mean}} \times 100$$

EPA recommends that regulatory authorities implement both the lower and upper PMSD bound approach to minimize within-test variability when using hypothesis testing approaches to report an NOEC. The implementation of the upper PMSD bound should also apply when using point estimate techniques. There are five possible outcomes for regulatory decisions (see Figure 6-1). Two outcomes imply unqualified acceptance of the WET test statistical result:

1. **Unqualified Pass**–The test's PMSD is within bounds and there is no significant difference between the means for the control and the instream waste concentration (IWC) treatment. The regulatory authority would conclude that there *is no toxicity at the IWC concentration.*

2. **Unqualified Fail**–The test's PMSD is larger than the lower bound (but not greater than the upper bound) in Table 3-6 and there is a significant difference between the means for the control and the IWC treatment. The regulatory authority would conclude that there *is toxicity at the IWC concentration.*

3. **Lacks Test Sensitivity**–The test's PMSD exceeds the upper bound in Table 3-6 and there is no significant difference between the means for the control and the IWC treatment. The test is considered invalid. A new effluent sample must be collected and another toxicity test must be conducted.

4. **Lacks Test Sensitivity**–The test's PMSD exceeds the upper bound in Table 3-6 and there is a significant difference between the means for the control and the IWC treatment. The test is considered valid. The regulatory authority would conclude that there *is toxicity at the IWC concentration.*

5. **Very Small but Significant Difference**–The relative difference (see Section 6.4.2, below) between the means for the control and the IWC treatment is smaller than the lower bound in Table 3-6 and this difference is statistically significant. The test is acceptable. The NOEC is determined as described in Section 6.4.2 below.

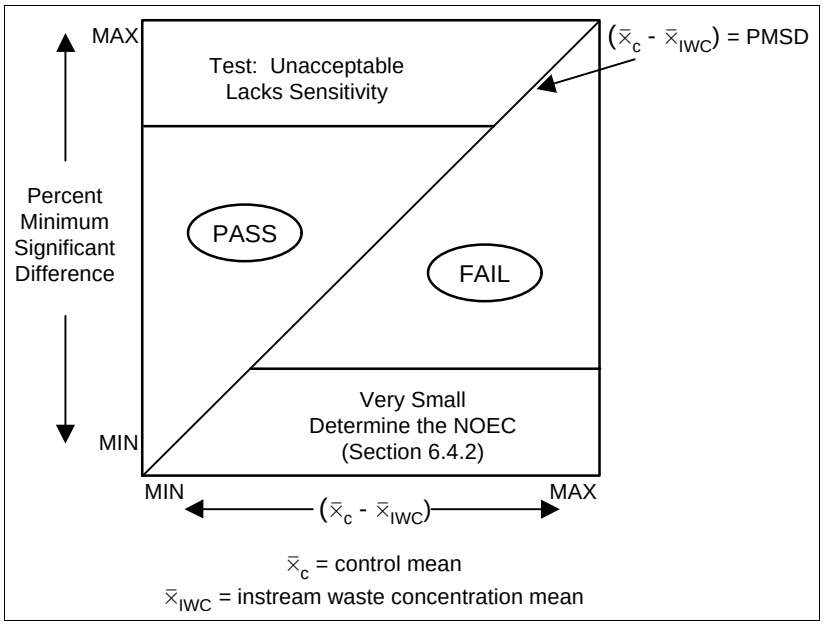

Figure 6-1. Paradigm that incorporates the lower and upper percent minimum significant difference.

Regulatory authorities should examine the sample permit language as provided in Appendix C, for incorporation of the PMSD bound language in a NPDES permit.

Note that "unqualified acceptance" of a WET test result requires that all of the following must be achieved: (1) collect the effluent sample properly; (2) conduct the toxicity test methods as specified in the toxicity manuals; (3) meet the required TACs; (4) meet the proper water quality parameters (e.g., such as temperature, pH); and (5) conduct the proper statistical calculations. All these conditions must be reviewed and deemed acceptable before a test is evaluated for self-monitoring data and reporting.

Figure 6-2 provides a decision tree that regulatory authorities can use when implementing the lower and upper PMSD bounds.

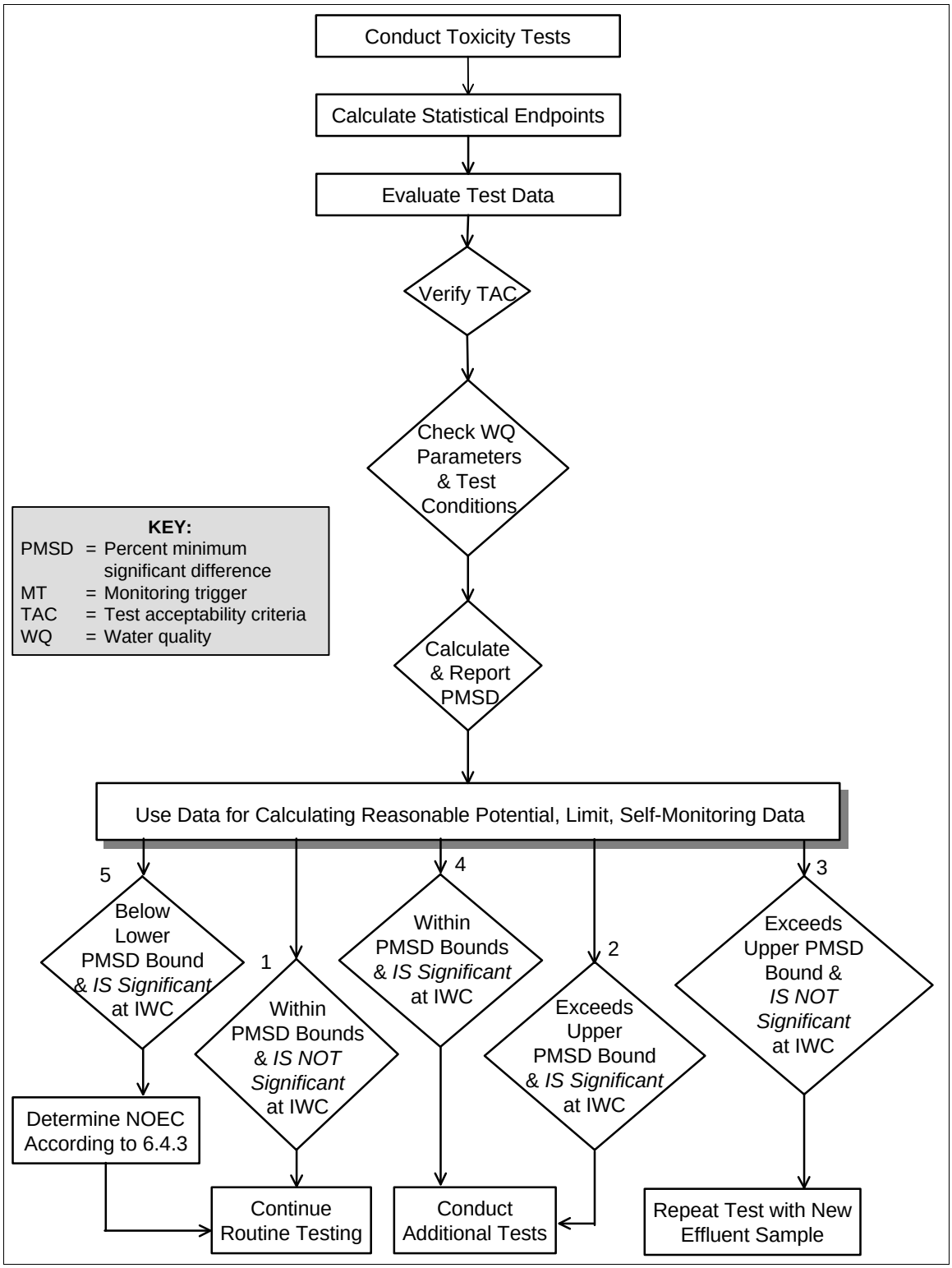

Figure 6-2. Implementing applications of upper and lower PMSD bounds for effluent toxicity testing requirements.

6.4.2 How to Determine the NOEC Using the Lower PMSD Bound

If the permit specifies that self-monitoring data are to be generated using hypothesis testing approaches, then the analyst should report the NOEC as the following. Find the smallest concentration for which (a) the treatment mean differs significantly from the control mean and (b) the relative difference (see example below) is not smaller than the 10th percentile in Table 3-6. Therefore, the NOEC is the next smaller test concentration.

In other words, concentrations having a very small relative difference with control (smaller than the lower PMSD bound) would be treated as if they do not differ significantly from control (even if they do so), for the purpose of determining the NOEC.

Table 6-1 illustrates the application of the lower PMSD bound for the reproduction endpoint of a *Ceriodaphnia* chronic test. In this example, the test's PMSD was 9.9, smaller than the 10th percentile value of 11 found in Table 3-6. The IWC concentration differed significantly from the control. The test falls under outcome number 5, a significant but very small difference at the IWC. The first step is to calculate the relative differences from control (Table 6-1) as [(control mean - treatment mean) divided by (control mean)] × 100. The next step is to determine which relative differences exceed the PMSD lower bound, 11 in this case (see the last column of Table 6-1). Finally, the NOEC is determined as described above. The NOEC is 12.5 percent effluent for this example.

Table 6-1. Example of Applying the Lower PMSD Bound for the Chronic *Ceriodaphnia* Test with the Reproduction Endpoint

Concentration (percent effluent)	Reproduction (mean of ten replicates)	Relative Difference from Control	Does Relative Difference Exceed 11?
100%	5.08 *	82	Yes
50%	12.4 *	56	Yes
25%	23.4 *	17	Yes
IWC = 12.5%	25.3 *	10	No
6.25%	26.1	7.4	No
Control	28.2	0	No

NOTE: The lower PMSD bound for this method and endpoint is 11 (Table 3-6). In this example, the NOEC is 6.25 percent effluent using the test's (very small) PMSD. Therefore, the reported NOEC should be 12.5 percent effluent after applying the lower PMSD bound.

* Differs statistically from the control as determined by MSD = 2.8 neonates. Thus, treatment means that are less than 28.2 - 2.8 = 25.4 would be statistically significant. These correspond to relative differences greater than 100 (2.8 / 28.2) = 9.9 percent.

6.4.3 Justification for Implementing the Test Sensitivity Bounds

A lower bound is needed to avoid penalizing laboratories that achieve unusually high precision. The 10th percentile PMSD represents a practical limit to the sensitivity of the test method because few laboratories are able to achieve such precision on a regular basis and most do not achieve it even occasionally. Several independent researchers have evaluated and provide support for using the MSD approach as additional TAC for the toxicity test methods. Thursby et al. (1997) advocate and provide reasons for using an empirical data base of minimum significant differences to provide TAC using statistical performance assessment. The State of California (Hunt et al. 1996, Starrett et al. 1993) and the West Coast marine toxicity test methods (USEPA 1995) have implemented an upper PMSD bound to minimize insensitive tests. Also the State of North Carolina has implemented additional requirements for the *Ceriodaphnia* chronic tests that reduced method

variability. North Carolina's evaluation of these additional TACs and subsequent improvements in test sensitivity appears in Appendix F.

The North Carolina data base affords the opportunity to evaluate the effectiveness of additional TAC and changes to the toxicity test procedures as they relate to the variability of WET test results (see Appendix F). For example, for PMSD, the median value decreased from 21 percent to 16 percent, while the 90th percentile decreased from 39 percent to 31 percent, indicating an overall increase in test sensitivity. The range in median values across all laboratories before adopting additional TACs was 12 percent to 36 percent. After adopting additional TACs, the range in median values was 10 percent to 27 percent, indicating a decrease in the overall spread between laboratories. The range in control CVs within a laboratory was from 21 percent to 79 percent before adopting TACs, compared to the range in control CVs within a laboratory after adopting TACs, which was narrowed to 17 percent to 36 percent. Overall, laboratories are generating data with more consistency within and between laboratories, after implementation of the additional TACs and additional method guidance provided by the State for the chronic *Ceriodaphnia dubia* test method.

6.4.4 Guidance to Testing Laboratories on How to Achieve the Range of Performance for PMSD

EPA recommends that regulatory authorities use the upper bounds (90th percentiles for PMSD in Table 3-6) to identify tests that are insufficiently sensitive. If PMSD exceeds this upper bound more often than occasionally, the laboratory should thoroughly investigate ways to reduce variability. There are three principal ways to reduce PMSD: (1) decrease within-test variability (that is, decrease the error mean square and therefore the standard deviation at each concentration); (2) increase the control mean; and (3) increase the number of replicates. The number of replicates required could be determined by trial-and-error calculations using the error mean square values obtained from a series of WET tests. At least 20 tests are recommended. The number "n" in the formula for MSD (number or replicates) would be increased and MSD re-calculated for each error mean square value. This approach uses a sample of tests specific to a particular laboratory and reveals the variation among tests. This approach would demonstrate how many replicates would be needed to achieve the upper PMSD bound, as required in Table 3-6.

6.5 Additional Guidance That Regulatory Authorities Should Implement to Further Support the WET Program

As discussed in Section 5.3, regulatory authorities have the discretion to develop and implement additional WET program requirements and guidance to ensure that WET test method variability is reduced by specifying additional guidance beyond the minimum requirements of EPA's WET test method's QA/QC and TACs. Appendix E provides a snapshot of State approaches to implementing NPDES WET programs to minimize WET test variability.

These State approaches include WET information to assist the regulated community with the following:

- Guidance regarding the evaluation of reference toxicant and effluent test results
- Guidance regarding how the State reviews reference toxicant data for laboratory performance
- Guidance regarding additional QA/QC criteria the State has developed and implemented
- Guidance regarding efforts the State has made to minimize test method variability
- Description of how the State reviews or conducts performance laboratory audits
- Description of specific implementation guidance that the State has developed to assist permit writers
- Description of how the State provides or uses toxicity test training

States contemplating such changes should consult with EPA to ensure the changes will be appropriate in the context of the State's overall NPDES WET program. In addition, States should implement a step-wise approach to address toxicity when the permit limit or monitoring trigger is exceeded in their State WET implementation plans.

For example, when an effluent is deemed toxic, then the permittee should take appropriate steps to demonstrate the magnitude, frequency, and potential source(s) of the toxicity. The components of the step-wise approach could include increased frequency of toxicity testing to characterize the magnitude and frequency of toxicity. If continued toxicity is demonstrated, then the permittee could conduct a Toxicity Reduction Evaluation/Toxicity Identification Evaluation (TRE/TIE) with toxic effluent sample(s) (USEPA 1991b, 1992). For example, EPA Regions 9 and 10 have prepared WET implementation guidance to assist their States (Denton and Narvaez 1996). This guidance provides sample permit language for a step-wise approach to address toxic samples (see Appendix C).

6.6 Chapter Conclusions

The TSD statistical approach to reasonable potential determination and permit limit derivation considers combined effluent and analytical variability through the CV of measured effluent values. Because determination of effluent variability is based on empirical measurements, the variability estimated for effluent measurements includes the variability of pollutant levels, sampling variability, and a smaller component owed to method variability. Steps should be taken to reduce these sources of variability. EPA believes that the TSD statistical procedures are appropriately protective in considering both effluent and analytical variability in reasonable potential and effluent limit calculations.

EPA recommends that regulatory authorities use a sampling program that conducts at least ten representative WET tests over a period of three years to represent the full range of effluent variability. Regulatory authorities should use recommended procedures in the TSD to determine when numeric WET limits or WET monitoring triggers are needed. Other permit conditions may include monitoring triggers, such as increased toxicity testing, TREs/TIEs, and follow-up actions initiated because a permit limit is exceeded or a monitoring trigger is not met. Regulatory authorities should implement the additional test sensitivity requirements by requiring that each test result not exceed the upper PMSD bound. In addition, regulatory authorities should determine the appropriate NOEC for test results below the lower PMSD bound as described in Section 6.4.2. These efforts should lead to increased confidence in the effect concentrations that are generated to evaluate self-monitoring data.

7.0 CONCLUSIONS AND GUIDANCE TO LABORATORIES, PERMITTEES, AND REGULATORY AUTHORITIES

This document was prepared to address whole effluent toxicity (WET) test variability. The document has three goals: (1) quantify the variability of promulgated test methods and report a coefficient of variation (CV) as a measure of test method variability; (2) evaluate the statistical methods described in the TSD for determining the need for and deriving WET permit conditions; and (3) suggest guidance for regulatory authorities on approaches to address and minimize test method variability. This document quantified the variability of toxicity test methods based on the end use of the data, that is, the effect concentrations (e.g., NOEC, LC50, EC25). The within-laboratory variability of these effect concentrations was quantified by obtaining multiple test results under similar test conditions using the same reference toxicant. The major conclusions of this document are discussed below.

7.1 General Conclusions

- EPA's *Technical Support Document for Water Quality-based Toxics Control* (referred to as the TSD) presents guidance for developing effluent limits based on three key components: (1) water quality criteria; (2) a calculated dilution factor used to derive a waste load allocation (WLA) from the criteria; and (3) a statistical calculation procedure that uses a CV based on effluent data to calculate effluent limits from the WLA. EPA's TSD statistical approach is appropriately protective, regarding both effluent and analytical variability, provided that the criteria and WLA are derived correctly. It is inappropriate to adjust the TSD statistical methodology for determining when water quality-based effluent limits are needed and for calculating such limits (Section 6 and Appendix G).
- EPA's analysis indicates that the TSD approach appropriately accounts for both effluent variability and method variability. EPA does not believe a reasonable alternative approach is available to determine a factor that would discount the effects of method variability using the TSD procedures (Section 6.1.1 and Appendix G).
- Interim CVs are identified for promulgated WET test methods [Appendix A, Table A-1 (acute methods) and Table A-2 (chronic methods)], pending completion of between-laboratory studies, which may affect these interim CV estimates.
- Comparisons of WET method precision with method precision for analytes commonly limited in the National Pollutant Discharge Elimination System (NPDES) permits clearly demonstrate that the variability of the promulgated WET methods is within the range of variability experienced in other types of analyses. Several researchers also noted that method performance improves when prescribed methods are followed closely by experienced analysts (Section 4.1.2).
- The hypothesis test procedures prescribed in EPA's WET methods will provide adequate protection against false conclusions that an effluent is toxic. However, the incidence of false negatives can be high because of high within-test variability, making it difficult to detect toxicity when toxicity is truly present. Therefore, evaluating the power of current experimental designs is desirable. EPA expects that regulatory authorities will make prompt and measurable progress toward the goal of requiring all WET tests to detect a toxic effect of 25 percent to 33 percent with power of 0.80 (Section 5.3.3 and Appendix B.4).
- Quality assurance problems became apparent when evaluating the data for this study, especially for the metal reference toxicants and sodium dodecyl sulfate (SDS). Standardizing the choice of reference toxicant and the concentrations to be tested may be appropriate, as well as establishing bounds on the range of acceptable effect concentrations for each test method. As a result,

quantifying between-laboratory variability will be difficult unless these issues can be resolved (Appendix G.2.6).

- The data analysis did not reveal the potential sources and causes of variability, such as using different sources of test organisms, dilution water, and food. To assess the sources of variability fully, experimenters must carefully design new studies (Section 3.4.1).

7.2 Recommendations for Minimizing Variability and Its Effects

Three critical areas are identified to minimize WET test method variability:

- Obtaining a representative effluent sample,
- Conducting the toxicity tests properly to generate biological endpoints, and
- Calculating the appropriate statistical endpoints to have confidence in the effect concentration.

This document provides guidance to toxicity testing laboratories, permittees, and regulatory authorities in conducting biological and statistical methods and evaluating test effect concentrations. It also develops guidance for regulatory authorities on approaches to address and minimize test method variability. The principal aspects of the guidance are presented in Table 3-6 and re-presented here.

Range of Relative Variability for Endpoints of Promulgated WET Methods, Defined by the 10th and 90th Percentiles from the Data Set of Reference Toxicant Tests[a]

Test Method[b]	Endpoint[c]	No. of Labs	No. of Tests	PMSD		Control CV[d]	
				10th	90th	10th	90th
1000.0 Fathead Minnow	G	19	205	9.4	35	0.035	0.20
1002.0 *Ceriodaphnia dubia*	R	33	393	11	37	0.089	0.42
1003.0 Green Alga	G	9	85	9.3	23	0.034	0.17
1004.0 Sheepshead Minnow	G	5	57	6.3	23	0.034	0.13
1006.0 Inland Silverside	G	18	193	12	35	0.044	0.18
1007.0 Mysid	G	10	130	12	32	0.088	0.28
2000.0 Fathead Minnow	S	20	217	4.2	30	0	0.074
2002.0 *Ceriodaphnia*	S	23	241	5.0	21	0	0.11
2004.0 Sheepshead Minnow	S	5	65	0[e]	55	0	0
2006.0 Inland Silverside	S	5	48	7.0	41	0	0.079
2007.0 Mysid (*A. bahia*)	S	3	32	5.1	26	0	0.081
2011.0 Mysid (*H. costata*)	S	2	14	18	47	0	0.074
2021.0 Daphnia (*D. magna*)	S	5	48	5.3	23	0	0.11
2022.0 Daphnia (*D. pulex*)	S	6	57	5.8	23	0	0.11

[a] The precision of the data warrants only three significant figures. When determining agreement with these values, one may round off values to two significant figures (e.g., values >3.45000... and ≤3.5000... are rounded to 3.5). Method 1009.0 (red macroalga) is not reported because it is inadvisable to characterize method variability using only 23 tests from just two laboratories.

[b] Method numbers from 2000.0 through 2022.0 are acute toxicity methods.

[c] G = growth, R = reproduction, S = survival

[d] CVs were calculated using untransformed control means for each test.

[e] An MSD of zero will not occur when the EPA flow chart for statistical analysis is followed. In this report, MSD was calculated for every test, including those for which the flow chart would require a nonparametric hypothesis test. EPA recommends using the value 4.2 (the 10th percentile shown for the fathead minnow acute test) in place of zero as the 10th percentile PMSD (lower PMSD bound) for the sheepshead minnow acute test.

7.2.1 Guidance to Toxicity Testing Laboratories

- Testing laboratories should maintain quality assurance/quality control (QA/QC) control charts for percent minimum significant difference (PMSD) along with the statistical endpoints such as NOEC, LC50, and EC25. Testing laboratories should regularly plot the individual raw test data and the average treatment responses to examine possible causes of excessive variability (Section 5.3.1.1).

- The minimum number of replicates for the chronic toxicity tests should be four for the chronic fathead minnow, sheepshead minnow, and inland silverside test methods (Section 5.3.3.1).

- Testing laboratories should take steps to ensure that the test PMSD does not exceed the upper bound provided in the table above (Sections 3.3, 5.3.3, and 6.4 and Table 3-6). This may require ensuring more uniformity among test organisms and/or using more replicates. Tables are provided to aid in choosing the number of replicates (Tables B-14 and B-15).

- Testing laboratories should examine the power tables to ensure that test results will meet the recommended test sensitivity criteria. These tables can be used to make decisions about replication, given the knowledge of typical values for error mean square (EMS) and number of tested concentrations (Section 5.3.3 and Tables B-9 through B-15).

7.2.2 Guidance to NPDES Permittees

- Permittees should select and conduct all data analyses with one qualified toxicity testing laboratory to determine reasonable potential, derive permit limits, and generate self-monitoring test results. Conducting all effluent testing consistently using one reference toxicant is also prudent (Section 6.1.4 and Appendix G.2.5).

- Permittees should generate WET data (n = 10) that have been accumulated over a year or more to fully characterize effluent variability over time. The sampling dates and times should span a sufficient duration to represent the full range of effluent variability (Sections 6.1.3 and 6.2 and Appendix G.2.4).

- Permittees should examine testing laboratories' QA/QC control charts. If the CV for reference toxicant tests is greater than the 75th percentile in Tables 3-2 through 3-4, variability can likely be reduced, even if the individual EC25 and LC50 values fall within the control limits (Section 5.3.1.1).

- Permittees should examine toxicity test data to ensure that data being submitted to regulatory authorities meet specified effluent holding times, temperature, laboratory control limits, and test acceptability criteria, such as requirements for test sensitivity lower and upper PMSD bounds (Sections 5.2 through 5.4).

- Permittees should anticipate and plan for a change if switching to a different testing laboratory. The permittee should compare reference toxicant test data from the current laboratory with data from the candidate replacement laboratory in order to ensure acceptable variability and a similar average effect (Section 6.1.4).

7.3 Guidance to Regulatory Authorities

Guidance to Regulatory Authorities Related to Determining Reasonable Potential and Deriving Permit Limits:

- Regulatory authorities should use EPA's recommended statistical approach in deriving permit limitations. The statistical approach outlined in the TSD represents an effective and appropriately protective approach to effluent limit development (Section 6.1 and Appendix G.1).

- Regulatory authorities should calculate the facility-specific CV using point estimate techniques for determining RP and for setting a permit limit, even if the self-monitoring test results will be determined using hypothesis test procedures (Sections 3.4.1 and 6.2).

- Regulatory authorities that need to cite a characteristic CV for a promulgated method may use Tables A-1 and A-2 in Appendix A, which show the median CV from Tables 3-2 through 3-4.

- EPA recommends that regulatory authorities evaluate the merits of a step-wise approach to address toxicity. This approach can determine the magnitude and frequency of toxicity and appropriate follow-up actions for test results that indicate exceedance of a monitoring trigger or a permit limit (Section 6.5).

Guidance to Regulatory Authorities Related to Collecting Effluent Samples, Conducting the Toxicity Test, and Evaluating the Effect Concentrations:

- Regulatory authorities should design a sampling program that collects representative effluent samples to fully characterize effluent variability for a specific facility over time. At least 10 samples are needed to estimate a variance or CV with acceptable precision for a specific facility (Sections 6.1.3 and 6.2).

- Regulatory authorities should ensure that statistical procedures and test methods have been properly applied to produce WET test results. Evaluating other factors and data, such as biological and statistical quality assurance, and ensuring that test conditions and test acceptability criteria (TAC) have been met would be prudent (Sections 5.2 through 5.5).

- Regulatory authorities should apply both the upper and lower bounds using the PMSD as an additional TAC (Section 6.4 and Table 3-6). The State of North Carolina implemented an effective WET program that required additional TAC and guidance for test methods that served to minimize test method variability (Appendix F).

- Regulatory authorities should develop a QC checklist to assist in evaluating and interpreting toxicity test results (Section 5.3.1.1). See Appendix E for examples of State WET implementation procedures.

- Regulatory authorities should consider participation in the National Environment Laboratory Accreditation Program and should conduct routine performance audit inspections to evaluate individual laboratory performance. Inspections should determine compliance with minimum acceptable criteria for collecting appropriate and representative effluent samples, conducting the toxicity test procedures, and analyzing test results (Section 5.3.1.1).

- Regulatory authorities should incorporate revised technical guidance recently published by EPA captioned "Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing" (40 CFR Part 136) (USEPA 2000a). The guidance addresses: (1) error rate assumption

adjustments; (2) concentration-response relationships; (3) incorporation of confidence intervals; (4) acceptable dilution waters for testing; (5) guidance on blocking by parentage for the chronic *C. dubia* test method; and (6) procedures for controlling pH drift.

7.4 Future Directions

- An independent peer-reviewed workshop should be convened to evaluate alternatives to the statistical approaches currently used in EPA's WET test methods. Such a workshop might suggest alternatives regarding (1) WET statistical flowcharts, (2) WET statistical methods used to estimate effect concentrations, and (3) test data interpretation and review guidelines (Section 5.5).

- Such a workshop might also evaluate additional QC requirements and recommendations regarding the specification of a reference toxicant and the concentrations to be tested for each test method (Section 5.3.1).

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APPENDIX A

INTERIM COEFFICIENTS OF VARIATION OBSERVED WITHIN LABORATORIES FOR REFERENCE TOXICANT SAMPLES ANALYZED USING EPA'S PROMULGATED WHOLE EFFLUENT TOXICITY METHODS

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INTERIM COEFFICIENTS OF VARIATION OBSERVED WITHIN LABORATORIES FOR REFERENCE TOXICANT SAMPLES ANALYZED USING EPA'S PROMULGATED WHOLE EFFLUENT TOXICITY METHODS

Tables A-1 and A-2 identify interim coefficients of variation for each promulgated WET method. The Agency identifies these as "interim" because EPA may revise some or all of these estimates based on between-laboratory studies currently underway to evaluate some of the test methods. For the acute toxicity methods, only "primary" organisms identified in the EPA method manuals (USEPA 1994a, 1994b) are reported in the tables. The primary data used to calculate these CVs were estimated effect concentrations (EC25, LC50, and NOEC) in units of concentration (e.g., mg/L of toxicant). Most CVs in Tables A-1 and A-2 come directly from Tables 3-2 through 3-4. Those data were supplemented as necessary with data from EPA publications (USEPA 1991, 1994a, 1994b). In Table 3-2, the NOEC values are reported separately for each test endpoint. In Tables A-1 and A-2, however, the NOEC values are reported as the most sensitive test endpoint. The data for a given method represent a variety of toxicants. In general, laboratories reported data for only one toxicant for a given method. Some of the data taken from EPA publications involved tests using different toxicants but conducted at one laboratory. In such cases, CVs were calculated separately for each toxicant.

Tables A-1 and A-2 report a default value when results were available from fewer than three laboratories and a similar species could be used as a basis for the default value of the CV. The sources of default values are identified in the footnotes to Tables A-1 and A-2. For methods and endpoints represented by fewer than three laboratories, the interim CV should be regarded as highly speculative.

Coefficients of variation are used as descriptive statistics for NOECs in this document. Because NOECs can take on only values that correspond to concentrations tested, the distribution (and CV) of NOECs can be influenced by the selection of experimental concentrations, as well as additional factors (e.g., within-test variability) that affect both NOECs and point estimates. This makes CVs for NOECs more uncertain than those of point estimates, and the direction of this uncertainty is not uniformly toward larger or smaller CVs. Despite these confounding issues, CVs are used herein as the best available means of expressing the variability of interest in this document and for general comparisons among methods. Readers should be cautioned, however, that small differences in CVs between NOECs and point estimates may be artifactual; large differences are more likely to reflect real differences in variability (a definition of what is "small" or "large" would require a detailed statistical analysis and would depend upon the experimental and statistical details surrounding each comparison).

These results are based on tests conducted using reference toxicants. These CVs may not apply to tests conducted on effluents and receiving waters unless the effect concentration (i.e., the EC25, LC50, or NOEC) happens to fall in the middle of the range of concentrations tested. More often, tests of effluents and receiving waters show smaller effects at the middle concentrations. Many effluent tests also demonstrate that the effect concentration equals or exceeds the highest concentration tested. In such cases, the sample standard deviation and CV tend to be smaller than reference toxicant CVs.

Table A-1. Interim Coefficients of Variation for EPA's Promulgated Whole Effluent Toxicity Methods for Acute Toxicity

Test Method No. [a]	Test Organism	Estimate	CV	No. of Laboratories
2002.0	*Ceriodaphnia dubia*	LC50	0.19[b]	23
2021.0	*Daphnia magna*	LC50	0.22[b]	5
2022.0	*Daphnia pulex*	LC50	0.21[b]	6
2000.0	*Pimephales promelas*	LC50	0.16[b]	21
2019.0	*Oncorhynchus mykiss*	LC50	0.16[c]	na[c]
NA	*Salvelinus fontinalis*	LC50	0.16[c]	na[c]
2004.0	*Cyprinodon variegatus*	LC50	0.14[b]	5
2006.0	*Menidia beryllina*	LC50	0.16[b]	5
2007.0	*Mysidopsis bahia*	LC50	0.25[b]	3

[a] These codes for acute methods were developed specifically for this document.

[b] From Table 3-3.

[c] Default values. These values are identified for methods represented by fewer than three laboratories. Default values for the trout (*Salvelinus fontinalis*) are based on Method 2000.0. Default values for *Menidia menidia* and *M. penisulae* (not shown) are based on the median for *M. beryllina*.

NOTE: CVs represent the median coefficient of variation observed within laboratories for WET tests conducted on reference toxicant samples. The test endpoint is survival.

Table A-2. Interim Coefficients of Variation for EPA's Promulgated Whole Effluent Toxicity Methods for Short-Term Chronic Toxicity

Test Method No.	Test Organism	Endpoint	Estimate	CV	No. of Laboratories
1000.0	*Pimephales promelas*	Growth Survival Most sensitive	EC25 LC50 NOEC	0.26[a] 0.23[a] 0.31[a]	19 19 19
1001.0	*Pimephales promelas* Embryo-larval	Mortality + Teratogenicity Mortality + Teratogenicity Mortality + Teratogenicity	EC01 LC50 NOEC	0.52[b] 0.07[c] 0.22[c]	1 na na
1002.0	*Ceriodaphnia dubia*	Reproduction Survival Most sensitive	EC25 LC50 NOEC	0.27[a] 0.16[a] 0.35[a]	33 33 33
1003.0	*Selenastrum capricornutum*[d]	Cell count Cell count	EC25 NOEC	0.26[a] 0.46[a]	6 9 9
1004.0	*Cyprinodon variegatus*	Growth Survival Most sensitive	EC25 LC50 NOEC	0.13 0.08 0.38[c]	5 5 5
1005.0	*Cyprinodon variegatus* Embryo-larval	Mortality + Teratogenicity Mortality + Teratogenicity Mortality + Teratogenicity	EC10 LC50 NOEC	0.19[e] 0.07[e] 0.22[e]	1 1 1
1006.0	*Menidia beryllina*	Growth Survival Most sensitive	EC25 LC50 NOEC	0.27[a] 0.28[a] 0.46[a]	16 16 16
1007.0	*Mysidopsis bahia*	Growth Survival Most sensitive	EC25 LC50 NOEC	0.28[a] 0.26[a] 0.40[a]	10 10 10
1008.0	*Arbacia punctulata*	Fertilization Fertilization	EC25 NOEC	0.36[e] 0.50[c]	2 na
1009.0	*Champia parvula*	Cystocarp production Cystocarp production	EC25 NOEC	0.59[a, e] 0.85[a, e]	3 3

[a] Tables 3-2 through 3-4.
[b] USEPA 1994b, USEPA 1991.
[c] Default values. These values are identified, when possible, for methods represented by fewer than three laboratories. The default value for *Cyprinodon* is based on *Pimephales*. Default values for *Menidia menidia* and *M. penisulae* (not shown) are based on the median for *Menidia beryllina*. Default values for Method 1001.0 were based on Method 1005.0. The default value for Method 1008.0 was based on Method 1016.0 of Table B-3 in Appendix B.
[d] Genus and species recently changed to *Raphidiopsis subcapitata*.
[e] USEPA 1994a, USEPA 1991.

NOTE: CVs represent the median coefficient of variation observed within laboratories for WET tests conducted on reference toxicant samples. NOEC estimates are reported for the most sensitive endpoint. This means that, for each test, the NOEC value was recorded for the endpoint that produced the lowest NOEC test result.

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APPENDIX B

SUPPLEMENTARY INFORMATION FOR REFERENCE TOXICITY DATA

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SUPPLEMENTARY INFORMATION FOR REFERENCE TOXICITY DATA

Appendix B contains technical and explanatory notes, and supplementary tables pertaining to the statistical analyses of reference toxicant test results presented in Chapters 3 and 5.

B.1 Acquisition, Selection, and Quality Assurance of Data

Details of data quality assurance and test acceptance are provided in a separate document, available from the EPA Office of Water's Office of Science and Technology ("Whole Effluent Toxicity (WET) Data Test Acceptance and Quality Assurance Protocol"). On request, EPA will also make available a list by laboratory of quality assurance (QA) flags, test dates, toxicant concentration, and summary statistics for the NOEC, EC25, and EC50 estimates and the test endpoints (survival, growth, reproduction, etc.). Laboratories are not named. Data were obtained as data sets from the data base and statistical software packages TOXIS® and TOXCALC® (see Chapter 8 for citations).

TOXIS® software produces an acceptability criterion field code based on the TAC specified by the EPA WET test methods. The tests having "I" (Incomplete) or "F" (Failed) values in this field were eliminated from consideration. TOXCALC® data were examined at the individual test level. The first step, before data entry, consisted of examining the test for TAC from bench sheets. The data were then imported into TOXCALC® for analysis. However, TOXCALC®, unlike TOXIS®, does not generate error codes but issues a warning on the screen. These messages were examined and decisions were made case-by-case following EPA test methods. In the second step, a QA program code was written in SAS® to check the TAC listed in the WET test methods for acute and chronic toxicity tests.

The effect concentration values produced using TOXCALC® or TOXIS®, along with related test information, were exported to spreadsheets and then imported into a SAS® data set. All statistical analyses, other than calculations of effect concentration estimates, were conducted using SAS®. Various data QA tests were conducted. Checks were made to ensure that data were within acceptable concentration-response ranges. Also, the frequency of tests, laboratories, and toxicants were compared for initial and final data sets to ensure that the data were properly imported and exported. Furthermore, TOXIS® effect concentrations having unacceptable error codes such as 905 (i.e., exposure concentrations for LC/EC values unrealistically high due to small slope and estimates well beyond the highest concentration used) and 904 (i.e., non-homogeneity of variance for a Probit estimate) were rejected. The TAC were not verified independently of TOXIS®, although the data used passed the required TAC. Because TOXIS® does not export the qualifier for censored endpoint values (i.e., ">" for greater than and "<" for less than), these qualifiers were later added to cases in which the point estimate equaled the maximum or minimum concentration in the dilution series. The methods having two biological endpoints per test method (e.g., survival and reproduction) had to pass both endpoint TACs to be included in the data analysis.

Non-standard laboratory codes were investigated by follow-up with the data provider; such cases were resolved either by reconfirming the laboratory identity or in a few cases by flagging the data as unusable. Duplicate data sets were identified and eliminated; this involved comparing the test methods, organisms, laboratory codes, test dates, test codes, concentration series, and replicate endpoint means. Concentration units were standardized for each toxicant. Errors in concentration units (e.g., µg versus mg) were identified and resolved. The number of organisms and number of replicates were not used to select or reject tests. For example, the minimum number of replicates was three for Method 1000.0 (which applied to only a few tests, since most tests used four replicates, but some used three) and seven for Method 1002.0 (which was exceptional since most tests used ten replicates).

Only the 20 most recent tests were used if more were submitted. Only laboratories having at least six data points were reported for the toxicants potassium chloride (KCl) and sodium chloride (NaCl) for two common methods: Method 1000.0 (fathead minnow larval survival and growth) and Method 1002.0

(*Ceriodaphnia* survival and reproduction). For other toxicants and methods, the minimum number of data points per laboratory was set at four. The within-laboratory statistics based on only four tests can be imprecise and should be regarded with caution.

In past protocols, the growth and reproduction effect values for the fathead minnow test (Method 1000.0), inland silverside test (Method 1006.0), and mysid test (Method 1007.0) were determined by dividing the weight or reproduction by the number of survivors. In contrast, the currently promulgated methods require that the weight or reproduction values be divided by the original (starting) number of organisms. All such results herein were calculated as currently required, using the weight or reproduction divided by the original number of organisms.

Note that data for Method 1016.0 (purple urchin fertilization test) and Method 1017.0 (sand dollar fertilization) included three different test methods with primary method differences including different sperm-egg ratios, sperm collection procedures, and sperm exposure time. This method has since been standardized and included in the West Coast chronic marine test methods manual (USEPA 1995).

A large percentage of data from a few laboratories was censored (i.e., recorded as "<" or ">") because the effect concentration was outside the range of the concentration series. In some cases, the data were censored because of the number or range of toxicant concentrations tested. When many data are censored, a reversal in the most sensitive endpoint can occur. For example, in the data for Method 1006.0 (*Menidia beryllina* larval survival and growth test), the NOEC for the survival endpoint indicated a more sensitive response than the sublethal endpoint for some tests.

B.2 Summary Statistics for IC25, LC50, and NOEC

B.2.1 Within-Laboratory Variability of EC25, EC50, and NOEC

Test data were not screened for outliers as provided for in ASTM Practices D2777 and E691 (ASTM 1992, 1998). Thus, maximum and minimum values for the laboratory statistics summarized in Tables B-1 through B-6 may be distorted by outliers. Therefore, EPA concluded that the maximum and minimum values are not necessarily reliable and has not reported them in these tables. EPA recommends that the 10^{th} and 90^{th} percentiles reported in Tables B-1 through B-6 be used to characterize the range of test variability.

Tables B-1 through B-3 show percentiles of the within-laboratory coefficients of variation (CVs) for EC25, EC50, and NOEC for all methods in the variability data set. However, when a method is represented by few laboratories, this summary cannot be considered typical or representative. When there were fewer than ten laboratories for a method, the 10^{th} and 90^{th} percentiles could not be estimated in an unbiased manner. Columns P10 and P90 show the minimum and maximum in such cases. Similarly, when there were fewer than four laboratories, columns P10 and P25 show the minimum and columns P75 and P90 show the maximum. An unbiased estimate of the median is always shown.

These percentiles are found by interpolation between two sample order statistics. The k^{th} sample order statistic has an expected probability estimated by $P_k = (k - 0.375)/(N + 0.25)$. Linear interpolation between two order statistics (X_k and X_{k+1}) having expected probabilities $P_k < P < P_{k+1}$ provides the estimate of the P^{th} quantile.

Tables B-4 through B-6 summarize variation across laboratories for the within-laboratory normal ratio of extremes for the EC25, EC50, and NOEC estimates. Instead of using the ratio of largest-to-smallest observations, which is vulnerable to outliers, the ratio of the 90^{th} to the 10^{th} percentiles (symbolized P90:P10) was used to provide some robustness to outliers. This ratio is a measure of variability in terms of concentration ratio. About 80 percent of observations are expected to fall between these percentiles. Thus, if P90:P10 equals 4, about 80 percent of observations are expected to fall within a dilution ratio of 4 (e.g., 0.25 mg/L to 1.00 mg/L).

The ratio is dimensionless and a more useful measure of the "range" of test results than the concentration range. For example, NOECs may vary at one laboratory between 0.5 mg/L and 2.0 mg/L (giving a range of 1.5 mg/L) and at another laboratory between 0.25 mg/L and 1.0 mg/L (giving a range of 0.75 mg/L), yet both NOECs span two standard concentrations having a ratio of 1:4. Also, using a ratio allows direct comparison among different toxicants having different concentration units. Further, toxicity tests often require a log scale (that is, a ratio scale) of concentration to provide an approximately linear curve of endpoint response (Collett 1991). Environment Canada (2000) expects that plotting and statistical estimation for WET tests will employ a logarithmic scale. In EPA publications, logarithmic (constant-ratio) graphical scales are used for concentrations (USEPA 1994a,1994b).

Tables B-4 through B-6 provide an easy way to quantify the ratio among effect concentrations expected for 80 percent of tests. For example, in Table B-6 under the NOEC for the growth endpoint of Method 1000.0, the median laboratory has a ratio of 2.0. This means that for half of the laboratories, repeated reference toxicant tests gave NOECs, 80 percent of which differed by no more than one standard dilution. That is, most NOECs occurred at only one concentration or at two adjacent concentrations at half of the laboratories. Note that most tests used 1:2 dilutions, so for the NOEC, the only exact ratios possible for each test are 1:1, 1:2, 1:4, 1:8, and 1:16. Thus, for NOECs, the results presented in the tables may be interpreted by rounding to these ratios.

The ratios P90:P10 in Tables B-4 through B-6 can be summarized as follows. For the NOEC in most of the promulgated WET methods, 75 percent of laboratories achieve a ratio of no more than 1:4, and half of the laboratories routinely achieve ratios of 1:1 or 1:2. For the LC50 (survival endpoint) for most methods, 75 percent of laboratories have ratios no more than 1:3, and half the laboratories have ratios no more than 1:2. For the IC25 (growth and reproduction endpoints), 75 percent of laboratories have ratios no more than 1:4, and half of laboratories have ratios no more than 1:2.5. The ratio for acute methods is usually somewhat less than that for chronic methods.

Note that two laboratories having the same ratio P90:P10 do not necessarily have similar NOECs; between-laboratory variation also occurs. For example, consider three laboratories that reported data for the growth endpoint of Method 1000.0 tested with NaCl. Each has a ratio P90:P10 of 2.0. One laboratory reported 11 tests, with the NOEC ranging from 0.4 mg/L to 3.2 mg/L. The 10^{th} and 90^{th} percentile estimates were 1.6 and 3.2. A second laboratory reported 8 tests, with the NOEC ranging from 1.0 mg/L to 2.0 mg/L. The 10^{th} and 90^{th} percentile estimates were 1.0 and 2.0. A third laboratory reported 12 tests, with the NOEC ranging from 1.0 mg/L to 4.0 mg/L. The 10^{th} and 90^{th} percentile estimates were 1.0 and 2.0.

B.2.2 Between-Laboratory Variability of EC25, EC50, and NOEC

The estimates of within- and between-laboratory variability for WET tests in Table 3-5 (Chapter 3) are based on Type-I analysis of variance and expected mean squares for random effects. Within-laboratory variability is estimated as the square root of the error mean square (column "Within-lab σ_w"), that is, the pooled standard deviation for all tests and all laboratories available for a given method, toxicant, and endpoint. Column "Between-lab σ_b" is the square root of the between-laboratory variance term, calculated as shown below. The column headed "Mean" shows the mean of the (unweighted) laboratory means. Sample sizes (numbers of laboratories) are insufficient for credible estimates of between-laboratory variability for most methods. The expected mean squares assume that the population of laboratories is large. Finite population estimates would be more accurate for some combinations of method and toxicant.

Table B-1. Percentiles of the Within-Laboratory Values of CV for EC25

Test Method[a]	Test Method No.[b]	End-point[c]	No. of Labs	CV				
				P10	P25	P50	P75	P90
Chronic, Promulgated								
Fathead Minnow Larval Survival & Growth	1000.0	G	19	0.12	0.21	0.26	0.38	0.45
Fathead Minnow Larval Survival & Growth	1000.0	S	16	0.03	0.11	0.22	0.32	0.52
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	R	33	0.08	0.17	0.27	0.45	0.62
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	S	25	0.07	0.11	0.23	0.41	0.81
Green Alga (*Selenastrum*)[d] Growth	1003.0	G	6	0.02	0.25	0.26	0.39	0.51
Sheepshead Minnow Larval Survival & Growth	1004.0	G	5	0.03	0.09	0.13	0.14	0.18
Sheepshead Minnow Larval Survival & Growth	1004.0	S	2	0.15	0.15	0.16	0.17	0.17
Inland Silverside Larval Survival & Growth	1006.0	G	16	0.05	0.18	0.27	0.43	0.55
Inland Silverside Larval Survival & Growth	1006.0	S	13	0.15	0.22	0.35	0.42	0.62
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	R	4	0.22	0.03	0.38	0.41	0.42
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	G	10	0.21	0.24	0.28	0.32	0.04
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	S	7	0.17	0.17	0.21	0.28	0.32
Red Macroalga *(Champia parvula)* Reprod	1009.0	R	2	0.58	0.58	0.58	0.59	0.59
West Coast								
Topsmelt Larval Survival & Growth	1010.0	G	1	0.25	0.25	0.25	0.25	0.25
Topsmelt Larval Survival & Growth	1010.0	S	1	0.20	0.20	0.20	0.20	0.20
Pacific Oyster Embryo-Larval Survival & Dev.	1012.0	D	1	0.25	0.25	0.25	0.25	0.25
Mussel Embryo-Larval Survival & Dev.	1013.0	D	3	0.14	0.14	0.27	0.42	0.42
Red Abalone Larval Development	1014.0	D	10	0.13	0.15	0.25	0.35	0.36
Sea Urchin Fertilization	1016.0	F	12	0.18	0.26	0.41	0.58	0.68
Sand Dollar Fertilization	1017.0	F	7	0.25	0.35	0.43	0.51	0.60
Giant Kelp Germination & Germ-Tube Length	1018.0	G_e	11	0.33	0.34	0.40	0.43	0.60
Giant Kelp Germination & Germ-Tube Length	1018.0	L	11	0.22	0.25	0.31	0.36	0.36
Acute								
Fathead Minnow Larval Survival	2000.0	S	7	0.05	0.09	0.15	0.21	0.44
Ceriodaphnia (Cd) Survival	2002.0	S	8	0.04	0.09	0.10	0.19	0.33
Sheepshead Minnow Survival	2004.0	S	3	0.08	0.08	0.13	0.46	0.46
Inland Silverside Larval Survival	2006.0	S	4	0.03	0.09	0.20	0.40	0.55
Mysid (Ab) Survival	2007.0	S	1	0.26	0.26	0.26	0.26	0.26
Mysid (Hc) Survival	2011.0	S	1	0.20	0.20	0.20	0.20	0.20
Rainbow Trout Survival	2019.0	S	1	0.11	0.11	0.11	0.11	0.11
Daphnia (Dm) Survival	2021.0	S	1	0.19	0.19	0.19	0.19	0.19
Daphnia (Dp) Survival	2022.0	S	3	0.06	0.06	0.41	0.48	0.48

[a] Cd = *Ceriodaphnia dubia*, Ab = *Americamysis (Mysidopsis) bahia*, Hc = *Holmesimysis costata*, Dm = *Daphnia magna*, Dp = *Daphnia pulex*

[b] EPA did not assign method numbers for acute methods in EPA/600/4-90/027F. The numbers assigned here were created for use in this document and in related materials and data bases.

[c] D = development, F = fertilization, G = growth, G_e = Germination, L = length, R = reproduction or fecundity, S = survival

[d] Genus and species recently changed to *Raphidocelis subcapitata*.

Table B-2. Percentiles of the Within-Laboratory Values of CV for EC50[a]

Test Method[b]	Test Method No.[c]	End-point[d]	No. of Labs	CV				
				P10	P25	P50	P75	P90
Chronic, Promulgated								
Fathead Minnow Larval Survival & Growth	1000.0	G	19	0.10	0.15	0.24	0.26	0.46
Fathead Minnow Larval Survival & Growth	1000.0	S	19	0.12	0.15	0.23	0.31	0.44
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	R	33	0.06	0.12	0.23	0.29	0.46
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	S	33	0.04	0.10	0.16	0.29	0.46
Green Alga (*Selenastrum*)[e] Growth	1003.0	G	9	0.16	0.19	0.27	0.30	0.63
Sheepshead Minnow Larval Survival & Growth	1004.0	G	5	0.02	0.04	0.06	0.11	0.13
Sheepshead Minnow Larval Survival & Growth	1004.0	S	5	0.02	0.07	0.08	0.12	0.13
Inland Silverside Larval Survival & Growth	1006.0	G	16	0.03	0.16	0.26	0.37	0.50
Inland Silverside Larval Survival & Growth	1006.0	S	16	0.05	0.16	0.28	0.35	0.49
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	R	4	0.06	0.17	0.30	0.37	0.43
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	G	10	0.15	0.19	0.22	0.27	0.31
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	S	10	0.12	0.16	0.26	0.27	0.28
Red Macroalga *(Champia parvula)* Reprod	1009.0	R	2	0.35	0.35	0.36	0.38	0.38
West Coast Methods								
Topsmelt Larval Survival & Growth	1010.0	G	1	0.25	0.25	0.25	0.25	0.25
Topsmelt Larval Survival & Growth	1010.0	S	1	0.17	0.17	0.17	0.17	0.17
Pacific Oyster Embryo-Larval Survival & Dev.	1012.0	D	1	0.21	0.21	0.21	0.21	0.21
Mussel Embryo-Larval Survival & Dev.	1013.0	D	3	0.25	0.25	0.35	0.35	0.35
Red Abalone Larval Development	1014.0	D	10	0.13	0.16	0.21	0.28	0.33
Sea Urchin Fertilization	1016.0	F	12	0.24	0.30	0.35	0.52	0.61
Sand Dollar Fertilization	1017.0	F	7	0.28	0.33	0.34	0.50	0.79
Giant Kelp Germination & Germ-Tube Length	1018.0	G_e	11	0.18	0.20	0.30	0.37	0.40
Giant Kelp Germination & Germ-Tube Length	1018.0	L	11	0.17	0.18	0.25	0.32	0.32
Acute								
Fathead Minnow Larval Survival	2000.0	S	21	0.08	0.10	0.16	0.19	0.33
Ceriodaphnia (Cd) Survival	2002.0	S	23	0.06	0.11	0.19	0.29	0.34
Sheepshead Minnow Survival	2004.0	S	5	0.11	0.12	0.14	0.21	0.37
Inland Silverside Larval Survival	2006.0	S	5	0.07	0.15	0.16	0.21	0.44
Mysid (Ab) Survival	2007.0	S	3	0.17	0.17	0.25	0.26	0.26
Mysid (Hc) Survival	2011.0	S	2	0.27	0.27	0.30	0.34	0.34
Rainbow Trout Survival	2019.0	S	1	0.23	0.23	0.23	0.23	0.23
Daphnia (Dm) Survival	2021.0	S	5	0.05	0.07	0.22	0.24	0.46
Daphnia (Dp) Survival	2022.0	S	6	0.15	0.19	0.21	0.27	0.48

[a] EC50 is a more general term than LC50 and may be used to represent an LC50 endpoint (such as survival).
[b] Cd = *Ceriodaphnia dubia*, Ab = *Americamysis (Mysidopsis) bahia*, Hc = *Holmesimysis costata*, Dm = *Daphnia magna*, Dp = *Daphnia pulex*
[c] See footnote b on Table B-1.
[d] D = development, F = fertilization, G = growth, G_e = Germination, L = length, R = reproduction or fecundity, S = survival
[e] Genus and species recently changed to *Raphidocelis subcapitata*.

Table B-3. Percentiles of the Within-Laboratory Values of CV for NOEC

Test Method[a]	Test Method No.[b]	End-point[c]	No. of Labs	CV				
				P10	P25	P50	P75	P90
Chronic, Promulgated								
Fathead Minnow Larval Survival & Growth	1000.0	G	19	0	0.22	0.37	0.53	0.65
Fathead Minnow Larval Survival & Growth	1000.0	S	19	0.13	0.26	0.39	0.48	0.59
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	R	33	0.20	0.25	0.33	0.49	0.60
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	S	33	0.09	0.21	0.30	0.43	0.55
Green Alga (*Selenastrum*)[d] Growth	1003.0	G	9	0.30	0.40	0.46	0.56	0.82
Sheepshead Minnow Larval Survival & Growth	1004.0	G	5	0.20	0.34	0.40	0.44	0.52
Sheepshead Minnow Larval Survival & Growth	1004.0	S	5	0	0.14	0.18	0.24	0.38
Inland Silverside Larval Survival & Growth	1006.0	G	16	0.14	0.31	0.46	0.57	0.63
Inland Silverside Larval Survival & Growth	1006.0	S	16	0.19	0.30	0.42	0.55	0.66
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	R	4	0	0.17	0.36	0.40	0.41
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	G	10	0.22	0.35	0.39	0.43	0.67
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	S	10	0.13	0.28	0.33	0.38	0.41
Red Macroalga *(Champia parvula)* Reprod	1009.0	R	2	0.85	0.85	1.00	1.16	1.16
West Coast Methods								
Topsmelt Larval Survival & Growth	1010.0	G	1	0.31	0.31	0.31	0.31	0.31
Topsmelt Larval Survival & Growth	1010.0	S	1	0.42	0.42	0.42	0.42	0.42
Pacific Oyster Embryo-Larval Survival & Dev.	1012.0	D	1	0.45	0.45	0.45	0.45	0.45
Mussel Embryo-Larval Survival & Dev.	1013.0	D	3	0	0	0.39	0.43	0.43
Red Abalone Larval Development	1014.0	D	10	0.24	0.25	0.29	0.31	0.38
Sea Urchin Fertilization	1016.0	F	12	0.31	0.40	0.50	0.69	0.76
Sand Dollar Fertilization	1017.0	F	7	0.40	0.41	0.53	0.75	0.81
Giant Kelp Germination & Germ-Tube Length	1018.0	G_e	11	0.36	0.40	0.54	0.65	0.81
Giant Kelp Germination & Germ-Tube Length	1018.0	L	11	0.39	0.48	0.59	0.68	0.76
Acute								
Fathead Minnow Larval Survival	2000.0	S	21	0.15	0.18	0.22	0.34	0.61
Ceriodaphnia (Cd) Survival	2002.0	S	23	0.07	0.18	0.35	0.41	0.57
Sheepshead Minnow Survival	2004.0	S	3	0.0	0	0.31	0.33	0.33
Inland Silverside Larval Survival	2006.0	S	5	0.0	0	0.33	0.35	0.72
Mysid (Ab) Survival	2007.0	S	3	0.29	0.29	0.38	0.43	0.43
Mysid (Hc) Survival	2011.0	S	2	0.21	0.21	0.26	0.31	0.31
Rainbow Trout Survival	2019.0	S	1	0.35	0.35	0.35	0.35	0.35
Daphnia (Dm) Survival	2021.0	S	5	0	0.09	0.36	0.47	0.83
Daphnia (Dp) Survival	2022.0	S	6	0.20	0.21	0.38	0.61	0.67

[a] Cd = *Ceriodaphnia dubia*, Ab = *Americamysis (Mysidopsis) bahia*, Hc = *Holmesimysis costata*, Dm = *Daphnia magna*, Dp = *Daphnia pulex*

[b] See footnote b on Table B-1.

[c] D = development, F = fertilization, G = growth, G_e = germination, L = length, R = reproduction or fecundity, S = survival

[d] Genus and species recently changed to *Raphidocelis subcapitata*.

Table B-4. Variation Across Laboratories in the Within-Laboratory Value of P90:P10 for EC25

Test Method[a]	Test Method No.[b]	End-point[c]	No. of Labs	CV				
				P10	P25	P50	P75	P90
Chronic, Promulgated								
Fathead Minnow Larval Survival & Growth	1000.0	G	19	1.3	1.7	2.1	3.6	4.1
Fathead Minnow Larval Survival & Growth	1000.0	S	16	1.0	1.3	1.7	2.3	3.5
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	R	33	1.2	1.4	2.2	3.6	6.3
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	S	25	1.1	1.3	1.6	2.6	4.8
Green Alga (*Selenastrum*)[d] Growth	1003.0	G	6	1.7	1.8	2.0	2.5	3.8
Sheepshead Minnow Larval Survival & Growth	1004.0	G	5	1.1	1.1	1.4	1.4	1.4
Sheepshead Minnow Larval Survival & Growth	1004.0	S	2	1.3	1.3	1.3	1.3	1.3
Inland Silverside Larval Survival & Growth	1006.0	G	16	1.1	1.5	2.0	2.5	4.2
Inland Silverside Larval Survival & Growth	1006.0	S	13	1.3	1.7	2.2	3.2	4.3
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	R	4	1.7	2.1	2.4	2.7	2.9
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	G	10	1.4	1.8	2.2	2.6	3.0
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	S	7	1.5	1.5	1.8	2.4	2.5
Red Macroalga *(Champia parvula)* Reprod	1009.0	R	2	6.7	6.7	10.2	13.7	13.7
West Coast								
Topsmelt Larval Survival & Growth	1010.0	G	1	1.7	1.7	1.7	1.7	1.7
Topsmelt Larval Survival & Growth	1010.0	S	1	1.8	1.8	1.8	1.8	1.8
Pacific Oyster Embryo-Larval Survival & Dev.	1012.0	D	1	2.0	2.0	2.0	2.0	2.0
Mussel Embryo-Larval Survival & Dev.	1013.0	D	3	1.4	1.4	2.2	4.0	4.0
Red Abalone Larval Development	1014.0	D	10	1.3	1.5	2.0	2.9	3.1
Sea Urchin Fertilization	1016.0	F	12	1.6	1.8	3.0	6.7	14.9
Sand Dollar Fertilization	1017.0	F	7	2.4	3.1	3.8	3.9	6.1
Giant Kelp Germination & Germ-Tube Length	1018.0	G_e	11	2.1	2.1	3.3	4.1	5.9
Giant Kelp Germination & Germ-Tube Length	1018.0	L	11	1.7	1.8	2.3	2.5	3.1
Acute								
Fathead Minnow Larval Survival	2000.0	S	7	1.1	1.2	1.4	1.5	3.7
Ceriodaphnia (Cd) Survival	2002.0	S	8	1.1	1.1	1.3	1.4	1.6
Sheepshead Minnow Survival	2004.0	S	3	1.2	1.2	1.3	5.2	5.2
Inland Silverside Larval Survival	2006.0	S	4	1.0	1.3	1.7	2.6	3.4
Mysid (Ab) Survival	2007.0	S	1	1.7	1.7	1.7	1.7	1.7
Mysid (Hc) Survival	2011.0	S	1	1.5	1.5	1.5	1.5	1.5
Rainbow Trout Survival	2019.0	S	1	1.2	1.2	1.2	1.2	1.2
Daphnia (Dm) Survival	2021.0	S	1	1.9	1.9	1.9	1.9	1.9
Daphnia (Dp) Survival	2022.0	S	3	1.1	1.1	2.5	2.8	2.8

[a] Cd = *Ceriodaphnia dubia*, Ab = *Americamysis (Mysidopsis) bahia*, Hc = *Holmesimysis costata*, Dm = *Daphnia magna*, Dp = *Daphnia pulex*

[b] See footnote b on Table B-1.

[c] D = development, F = fertilization, G = growth, G_e = germination, L = length, R = reproduction or fecundity, S = survival

[d] Genus and species recently changed to *Raphidocelis subcapitata*.

Table B-5. Variation Across Laboratories in the Within-Laboratory Value of P90:P10 for EC50[a]

Test Method[b]	Test Method No.[c]	End-point[d]	No. of Labs	CV				
				P10	P25	P50	P75	P90
Chronic, Promulgated								
Fathead Minnow Larval Survival & Growth	1000.0	G	19	1.3	1.5	1.8	2.4	3.3
Fathead Minnow Larval Survival & Growth	1000.0	S	19	1.4	1.5	1.8	2.3	3.0
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	R	33	1.2	1.3	1.7	2.3	3.7
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	S	33	1.1	1.3	1.5	2.2	3.5
Green Alga (*Selenastrum*)[e] Growth	1003.0	G	9	1.2	1.5	1.7	2.4	9.4
Sheepshead Minnow Larval Survival & Growth	1004.0	G	5	1.0	1.1	1.1	1.2	1.3
Sheepshead Minnow Larval Survival & Growth	1004.0	S	5	1.0	1.1	1.1	1.2	1.3
Inland Silverside Larval Survival & Growth	1006.0	G	16	1.1	1.5	1.8	2.7	3.5
Inland Silverside Larval Survival & Growth	1006.0	S	16	1.2	1.5	1.9	2.8	2.9
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	R	4	1.2	1.5	1.9	2.4	2.9
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	G	10	1.4	1.5	1.8	2.2	2.4
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	S	10	1.4	1.6	1.9	2.0	2.3
Red Macroalga *(Champia parvula)* Reprod	1009.0	R	2	2.3	2.3	4.9	7.6	7.6
West Coast								
Topsmelt Larval Survival & Growth	1010.0	G	1	1.7	1.7	1.7	1.7	1.7
Topsmelt Larval Survival & Growth	1010.0	S	1	1.5	1.5	1.5	1.5	1.5
Pacific Oyster Embryo-Larval Survival & Dev.	1012.0	D	1	2.0	2.0	2.0	2.0	2.0
Mussel Embryo-Larval Survival & Dev.	1013.0	D	3	2.0	2.0	2.0	2.8	2.8
Red Abalone Larval Development	1014.0	D	10	1.4	1.4	1.8	2.4	2.6
Sea Urchin Fertilization	1016.0	F	12	1.8	2.0	2.9	4.2	6.5
Sand Dollar Fertilization	1017.0	F	7	2.4	2.6	2.8	4.4	6.0
Giant Kelp Germination & Germ-Tube Length	1018.0	G_e	11	1.7	1.8	2.1	3.3	3.6
Giant Kelp Germination & Germ-Tube Length	1018.0	L	11	1.6	1.6	1.8	2.5	2.7
Acute								
Fathead Minnow Larval Survival	2000.0	S	21	1.2	1.3	1.5	1.7	2.6
Ceriodaphnia (Cd) Survival	2002.0	S	23	1.1	1.2	1.7	2.0	2.4
Sheepshead Minnow Survival	2004.0	S	5	1.1	1.2	1.4	1.7	2.8
Inland Silverside Larval Survival	2006.0	S	5	1.2	1.4	1.6	1.7	2.7
Mysid (Ab) Survival	2007.0	S	3	1.7	1.7	2.1	2.1	2.1
Mysid (Hc) Survival	2011.0	S	2	1.8	1.8	2.5	3.1	3.1
Rainbow Trout Survival	2019.0	S	1	1.8	1.8	1.8	1.8	1.8
Daphnia (Dm) Survival	2021.0	S	5	1.2	1.2	1.8	2.2	4.1
Daphnia (Dp) Survival	2022.0	S	6	1.4	1.5	1.9	2.1	2.2

[a] EC50 is a more general term than LC50 and may be used to represent an LC50 endpoint (such as survival).
[b] Cd = *Ceriodaphnia dubia*, Ab = *Americamysis (Mysidopsis) bahia*, Hc = *Holmesimysis costata*, Dm = *Daphnia magna*, Dp = *Daphnia pulex*
[c] See footnote b on Table B-1.
[d] D = development, F = fertilization, G = growth, G_e = germination, L = length, R = reproduction or fecundity, S = survival
[e] Genus and species recently changed to *Raphidocelis subcapitata*.

Table B-6. Variation Across Laboratories in the Within-Laboratory Value of P90:P10 for NOEC

Test Method[a]	Test Method No.[b]	End-point[c]	No. of Labs	CV				
				P10	P25	P50	P75	P90
Chronic, Promulgated								
Fathead Minnow Larval Survival & Growth	1000.0	G	19	1.0	1.5	2.0	4.2	8.0
Fathead Minnow Larval Survival & Growth	1000.0	S	19	1.0	1.7	2.0	4.0	5.0
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	R	33	1.3	1.9	2.2	4.0	4.0
Ceriodaphnia (Cd) Survival & Reproduction	1002.0	S	33	1.0	1.5	2.0	3.0	5.3
Green Alga (*Selenastrum*)[d] Growth	1003.0	G	9	1.8	2.0	2.7	4.0	10.0
Sheepshead Minnow Larval Survival & Growth	1004.0	G	5	1.3	2.0	2.0	4.0	4.0
Sheepshead Minnow Larval Survival & Growth	1004.0	S	5	1.0	1.0	1.3	2.0	2.0
Inland Silverside Larval Survival & Growth	1006.0	G	16	1.3	2.0	4.0	4.2	7.8
Inland Silverside Larval Survival & Growth	1006.0	S	16	1.8	2.0	2.9	4.0	4.1
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	R	4	1.0	1.5	2.0	2.0	2.0
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	G	10	1.9	2.0	2.0	4.0	7.6
Mysid (Ab) Survival, Growth, & Fecundity	1007.0	S	10	1.4	2.0	2.0	2.0	3.4
Red Macroalga *(Champia parvula)* Reprod	1009.0	R	2	5.6	5.6	12.8	20.0	20.0
West Coast								
Topsmelt Larval Survival & Growth	1010.0	G	1	1.8	1.8	1.8	1.8	1.8
Topsmelt Larval Survival & Growth	1010.0	S	1	3.2	3.2	3.2	3.2	3.2
Pacific Oyster Embryo-Larval Survival & Dev.	1012.0	D	1	4.0	4.0	4.0	4.0	4.0
Mussel Embryo-Larval Survival & Dev.	1013.0	D	3	1.0	1.0	3.2	4.0	4.0
Red Abalone Larval Development	1014.0	D	10	1.2	1.8	1.8	1.8	3.2
Sea Urchin Fertilization	1016.0	F	12	1.8	2.0	4.0	6.9	9.4
Sand Dollar Fertilization	1017.0	F	7	2.1	3.1	4.0	6.0	17.8
Giant Kelp Germination & Germ-Tube Length	1018.0	G_e	11	1.8	2.3	3.2	5.7	5.7
Giant Kelp Germination & Germ-Tube Length	1018.0	L	11	3.1	3.1	5.6	5.7	10.0
Acute								
Fathead Minnow Larval Survival	2000.0	S	21	1.3	1.5	1.6	2.0	4.0
Ceriodaphnia (Cd) Survival	2002.0	S	23	1.0	1.3	2.0	3.3	5.0
Sheepshead Minnow Survival	2004.0	S	3	1.0	1.0	2.0	2.0	2.0
Inland Silverside Larval Survival	2006.0	S	5	1.0	1.0	1.8	2.0	4.0
Mysid (Ab) Survival	2007.0	S	3	2.7	2.7	3.2	5.0	5.0
Mysid (Hc) Survival	2011.0	S	2	1.8	1.8	1.9	2.1	2.1
Rainbow Trout Survival	2019.0	S	1	2.0	2.0	2.0	2.0	2.0
Daphnia (Dm) Survival	2021.0	S	5	1.0	1.3	2.0	4.0	6.1
Daphnia (Dp) Survival	2022.0	S	6	1.3	1.7	2.0	2.0	10.0

[a] Cd = *Ceriodaphnia dubia*, Ab = *Americamysis (Mysidopsis) bahia*, Hc = *Holmesimysis costata*, Dm = *Daphnia magna*, Dp = *Daphnia pulex*

[b] See footnote b on Table B-1.

[c] D = development, F = fertilization, G = growth, G_e = germination, L = length, R = reproduction or fecundity, S = survival

[d] Genus and species recently changed to *Raphidocelis subcapitata*.

Estimation formulas were:

Expected mean square for error (within-laboratory): σ_w^2

Expected mean square between-laboratories: $\sigma_w^2 + U\,\sigma_b^2$

$U = [\sum n_i - (\sum n_i^2 / \sum n_i)] / (L-1)$

L is the number of laboratories and n_i the number of tests within the i^{th} laboratory (i = 1, ... L).

B.3 Variability of Endpoint Measurements

Dunnett's critical value, needed for the minimum significant difference (MSD), was computed using the SAS function "PROBMC," for a one-sided test at the 0.95 level ($\alpha = 0.05$). Note that Dunnett's test can be applied when the number of replicates differs among treatments (Dunnett 1964), and that the SAS function "PROBMC" can calculate an appropriate critical value for the case of unequal replication.

The MSD was calculated for sublethal endpoints using untransformed values of "growth" (larval biomass) and "reproduction" (number of offspring in the *Ceriodaphnia* test, or cells per mL in the *Selenastrum* test), and for lethal endpoints using the arc sine transform (arc sine ($\sqrt{p}$)) of the proportion surviving. The CV was calculated for all endpoints using the untransformed mean control response.

Tables B-7 and B-8 show percentiles of CV and of the percent minimum significant difference (PMSD), which is [100×MSD/(control mean)]. These are the sample percentiles for all tests in the data set (see row "No. of tests"). Data for all laboratories and toxicants for a given method and endpoint were combined.

Methods in Tables B-1 through B-3 that are represented by fewer than three laboratories or fewer than 20 tests are not shown in Tables B-7 and B-8, because characterizing method variability using so few tests and laboratories would be inadvisable.[1]

B.4 Test Power to Detect Toxic Effects

Power can be characterized only by repeated testing. It is an attribute, not of a single test, but of a sequence of many tests conducted under similar conditions and the same test design. Therefore, the sample averages for each laboratory's data set are used in this analysis to characterize each laboratory. The key parameters required were the (a) mean endpoint response in the control (growth, reproduction, survival) and (b) the mean value of the error mean square (EMS) for tests.

Power is reported in this section for single two-sample, one-sided t-tests at $1-\alpha = 0.95$, and for a set of k such tests (comparing k treatments to a control) at level $1 - \alpha/k = 1 - 0.05/k$. Some permitting authorities may require a comparison between control and the receiving water concentration, which requires a two-sample, one-sided test. Others may require the multiple comparisons procedure described in the EPA WET methods (Dunnett's or Steel's tests, one-sided, with $\alpha = 0.05$). The power of Dunnett's procedure (using $\alpha = 0.05$ as recommended in EPA effluent test methods) will fall between the power of the one-sided, two-sample t-test with $\alpha = 0.05$ and that with $\alpha = 0.05/k$, when k toxicant concentrations are compared to a control. The power of Steel's procedure will be related to and should usually increase with the power of Dunnett's procedure and the t-tests, so the following tables will also provide an inexact guide to power achieved by the nonparametric test.

Tables B-9 through B-13 illustrate the ability of the sublethal endpoint for the chronic toxicity promulgated methods to detect toxic effects using a two-sample, one-sided hypothesis test (t-test) at two

[1] Tables B-7 through B-18 begin on page B-14.

significance levels, $\alpha = 0.05$ and $\alpha = 0.01$. Data for Method 1009.0 (red macroalga) are not presented, because characterizing method performance using data from only two laboratories and 23 tests is inadvisable.

Table B-14 shows the power and PMSD to be expected for various combinations of (1) number of replicates; (2) k, number of treatments compared with a control; and (3) value of the square root of the error mean square (rEMS) divided by the control mean, when the t-test can be used.

Table B-15 shows the value of PMSD for various combinations of number of replicates, number of treatments compared with a control, and rEMS/(Control Mean). (For definitions and explanations of the terms used here, see Chapters 2 and 3.) This table can be used as a guide to planning the number of replicates needed to achieve a given PMSD. The number of replicates needed can be determined by calculating MSD using the average EMS for a series of tests (at least 20 tests are recommended) and experimenting with various choices of number of replicates (the same number for each concentration and test). This approach is recommended because it uses a sample of test EMSs specific to a particular laboratory. This approach also reveals variation by test, showing how frequently PMSD exceeds the upper bound in Table 3-6 if the number of replicates is increased.

The number of replicates needed to achieve a given value of PMSD will depend on the variability among replicates (rEMS). Table B-16 shows percentiles of the rEMS divided by the control mean, for each promulgated method for chronic toxicity, pooling all tests available in the WET variability data set. The data for Method 1009.0 (red macroalga, *Champia parvula*) are based on only two laboratories and 23 tests and therefore cannot be considered representative.

Table B-15 can be used to infer the number of replicates needed to make the MSD a certain percentage of the control mean (25 percent and 33 percent are used here) for any particular value of rEMS. Table B-17 shows the number of replicates needed to do the same for the 90^{th} and 85^{th} percentiles of rEMS found in Table B-16, in which three or four treatments are compared to a control. These percentiles represent rather extreme examples of imprecision. The precision achieved in most tests and by most laboratories is within the bounds set by these percentiles. The exact number of replicates was not determined beyond ">15" (*Ceriodaphnia* chronic test).

Table B-17 agrees with conclusions drawn from Table 5-1: For most methods, most laboratories can detect a 33 percent effect most of the time, but many laboratories are unable to detect a 25 percent difference between treatment and control in many tests.

B.5 NOEC for Chronic Toxicity Test Methods (Calculated Using the Most Sensitive Endpoint)

NOEC for chronic toxicity methods is calculated using the most sensitive endpoint in each test (meaning the smallest NOEC among those for the two or three endpoints). Table B-18 shows percentiles of within-laboratory CVs in a format like that for Tables B-1 through B-6, and similar calculations were used.

Table B-7a. Percentiles of Control CV for Sublethal Endpoints of Chronic WET Tests, Using Data Pooled Across All Laboratories and Toxicants[a]

	Test Method					
	1000.0 Fathead Minnow	**1002.0 *Cerio-daphnia***	**1003.0 Green Alga**	**1004.0 Sheepshead Minnow**	**1006.0 Inland Silverside**	**1007.0 Mysid (*A. bahia*)**
No. of tests	205	393	85	57	193	130
No. of labs	19	33	9	5	16	10
Endpoint[b]	G	R	G	G	G	G
Percentile	**Control CV**					
5%	0.03	0.08	0.03	0.03	0.03	0.07
10%	0.04	0.09	0.03	0.03	0.04	0.09
15%	0.05	0.10	0.04	0.04	0.05	0.09
20%	0.06	0.11	0.05	0.04	0.06	0.10
25%	0.06	0.12	0.05	0.04	0.06	0.11
50%	0.10	0.20	0.08	0.07	0.10	0.15
75%	0.14	0.33	0.12	0.09	0.14	0.20
80%	0.16	0.36	0.14	0.09	0.14	0.22
85%	0.17	0.39	0.16	0.10	0.16	0.25
90%	0.20	0.42	0.17	0.13	0.18	0.28
95%	0.23	0.52	0.18	0.17	0.23	0.37

[a] Methods in Table B-1 having fewer than three laboratories or fewer than 20 tests are not shown here because so few results may not be representative of method performance.

[b] G = growth, R = reproduction

Table B-7b. Percentiles of Control CV for Endpoints of Chronic WET Tests, Using Data Pooled Across All Laboratories and Toxicants (West Coast Methods)[a]

	Test Method					
	1013.0 Mussel Embryo-Larval Survival & Development	**1014.0 Red Abalone Larval Development**	**1016.0 Sea Urchin Fertilization**	**1017.0 Sand Dollar Fertilization**	**1018.0 Giant Kelp Germination & Germ-Tube Length**	**1018.0 Giant Kelp Germination & Germ-Tube Length**
No. of tests	34	137	159	67	159	159
No. of labs	3	10	11	7	11	11
Endpoint[b]	S	L	F	F	G_e	L
Percentile	**Control CV**					
5%	0.01	0.01	0.01	0.01	0.01	0.02
10%	0.01	0.01	0.01	0.01	0.02	0.03
15%	0.01	0.01	0.01	0.02	0.02	0.03
20%	0.01	0.01	0.02	0.02	0.02	0.04
25%	0.01	0.02	0.02	0.03	0.02	0.05
50%	0.02	0.03	0.04	0.04	0.04	0.07
75%	0.04	0.05	0.07	0.06	0.06	0.09
80%	0.05	0.05	0.08	0.07	0.06	0.11
85%	0.06	0.05	0.10	0.08	0.07	0.11
90%	0.07	0.06	0.12	0.08	0.08	0.12
95%	0.07	0.08	0.18	0.12	0.10	0.14

[a] Methods in Table B-1 having fewer than three laboratories or fewer than 20 tests are not shown here because so few results may not be representative of method performance.

[b] G_e = germination, F = fertilization, L = length, S = survival

Table B-7c. Percentiles of Control CV for Survival Endpoint of Acute WET Tests, Using Data Pooled Across All Laboratories and Toxicants

	Test Method							
	2000.0 Fathead Minnow	**2002.0 *Cerio-daphnia***	**2004.0 Sheepshead Minnow**	**2006.0 Inland Silverside**	**2007.0 Mysid (*A. bahia*)**	**2011.0 Mysid (*H. costata*)**	**2021.0 Daphnia (*D. magna*)**	**2022.0 Daphnia (*D. pulex*)**
No. of tests	217	241	65	48	32	14	48	57
No. of labs	20	23	5	5	3	2	5	6
Percentile	**Control CV**							
5%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
25%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
50%	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
75%	0.00	0.00	0.00	0.07	0.07	0.07	0.00	0.00
80%	0.00	0.00	0.00	0.07	0.07	0.07	0.00	0.00
85%	0.07	0.00	0.00	0.07	0.07	0.07	0.10	0.07
90%	0.07	0.11	0.00	0.08	0.08	0.07	0.11	0.11
95%	0.11	0.11	0.06	0.10	0.11	0.10	0.12	0.11

Table B-8a. Percentiles of PMSD for Sublethal Endpoints of Chronic WET Tests, Using Data Pooled Across All Laboratories and Toxicants[a,b]

	Test Method					
	1000.0 Fathead Minnow	**1002.0 *Cerio-daphnia***	**1003.0 Green Alga**	**1004.0 Sheepshead Minnow**	**1006.0 Inland Silverside**	**1007.0 Mysid (*A. bahia*)**
No. of tests	205	393	85	57	193	130
No. of labs	19	33	9	5	16	10
Endpoint[c]	G	R	G	G	G	G
Percentile	**PMSD**					
5%	6.8	10	8.2	5.5	10	10
10%	9	11	9.3	6.3	12	12
15%	11	13	10	6.8	12	14
20%	13	15	11	7.9	13	16
25%	14	16	11	8.4	14	16
50%	20	23	14	13	18	20
75%	25	30	19	18	25	25
80%	28	31	20	19	27	26
85%	29	33	21	21	31	28
90%	35	37	23	23	35	32
95%	44	43	27	26	41	34

[a] PMSD = Percent MSD [100×MSD/(Control Mean)]

[b] Methods in Table B-1 having fewer than three laboratories or fewer than 20 tests are not shown here because so few results may not be representative of method performance.

[c] G = growth, R = reproduction

Table B-8b. Percentiles of PMSD for Endpoints of Chronic WET Tests, Using Data Pooled Across All Laboratories and Toxicants (West Coast Methods)[a, b]

	Test Method					
	1013.0 Mussel Embryo-Larval Survival & Development	**1014.0 Red Abalone Larval Development**	**1016.0 Sea Urchin Fertilization**	**1017.0 Sand Dollar Fertilization**	**1018.0 Giant Kelp Germination & Germ-Tube Length**	**1018.0 Giant Kelp Germination & Germ-Tube Length**
No. of tests	34	137	159	67	159	159
No. of labs	3	10	11	7	11	11
Endpoint[c]	S	L	F	F	G_e	L
Percentile	**PMSD**					
5%	3.9	3.1	3.7	6.5	5.7	6.6
10%	5.5	3.8	5.1	6.9	6.5	7.9
15%	6.2	4.6	6.5	8.0	7.0	8.8
20%	7.1	5.0	7.3	8.5	7.4	9.2
25%	8.5	5.3	8.1	9.0	8.2	9.6
50%	11	7.9	12	12	10	11
75%	16	12	18	17	14	15
80%	19	13	19	19	15	16
85%	20	15	21	21	17	18
90%	42	16	25	26	18	21
95%	49	20	29	30	20	24

[a] PMSD = Percent MSD [100×MSD/(Control Mean)]

[b] Methods in Table B-1 having fewer than three laboratories or fewer than 20 tests are not shown here because so few results may not be representative of method performance.

[c] G_e = germination, F = fertilization, L = length, S = survival

Table B-8c. Percentiles of PMSD for Survival Endpoint of Acute WET Tests, Using Data Pooled Across All Laboratories and Toxicants[a]

	Test Method							
	2000.0 Fathead Minnow	**2002.0 *Cerio-daphnia***	**2004.0 Sheepshead Minnow**	**2006.0 Inland Silverside**	**2007.0 Mysid (*A. bahia*)**	**2011.0 Mysid (*H. costata*)**	**2021.0 *Daphnia* (*D. magna*)**	**2022.0 *Daphnia* (*D. pulex*)**
No. of tests	217	241	65	48	32	14	48	57
No. of labs	20	23	5	5	3	2	5	6
Percentile	**PMSD**							
5%	0	4.6	0	4.5	3.9	14	4.5	4.3
10%	4.2	5.0	0	7.0	5.1	18	5.3	5.8
15%	5.0	5.6	0	8.9	6.9	21	6.4	6.8
20%	6.6	5.9	0	10	8.4	22	6.9	7.5
25%	7.4	7.1	6.1	12	8.9	23	8.4	8.3
50%	13	11	16	20	15	30	13	14
75%	21	16	32	26	23	38	19	20
80%	23	18	36	29	24	40	20	21
85%	26	19	49	36	24	42	20	22
90%	30	21	55	41	26	47	23	23
95%	51	25	67	46	33	58	27	27

[a] PMSD = Percent MSD [100×MSD/(Control Mean)]

Table B-9. Test Method 1000.0, Fathead Minnow Chronic Toxicity Test, Growth Endpoint: Power and Effect Size Achieved

Lab	No. of Tests	No. of Reps Per Test	Average Control Mean	Average Control Std Dev	Square Root of Variance of Control Mean	Square Root of Average EMS	Average PMSD	Power of Hypothesis Test (2-sample, 1-sided t-test)							
								α = 0.05				α = 0.01			
								N (Reps)	Delta	100×Delta/ Mean	Power	N (Reps)	Delta	100×Delta/ Mean	Power
1	9	4	0.38	0.040	0.081	0.043	19	4	0.09	23	0.85	6	0.12	33	0.48
2	13	4	0.32	0.013	0.028	0.013	6	2	0.03	8	1.00	3	0.04	12	1.00
3	11	3	0.55	0.066	0.117	0.069	25	5	0.17	31	0.62	7	0.26	48	0.13
4	18	4	0.45	0.051	0.107	0.066	21	6	0.13	30	0.67	9	0.19	42	0.25
5	8	4	0.41	0.041	0.115	0.064	26	6	0.13	31	0.63	10	0.18	44	0.21
6	10	3	0.60	0.081	0.189	0.082	28	5	0.20	34	0.54	8	0.31	52	0.10
7	7	4	0.39	0.063	0.064	0.073	31	9	0.15	38	0.47	14	0.21	54	0.12
8	20	4	0.55	0.053	0.109	0.065	17	4	0.13	24	0.82	7	0.19	34	0.43
9	5	4	0.46	0.054	0.217	0.044	17	3	0.09	20	0.93	5	0.13	28	0.68
10	11	3 to 4	0.34	0.047	0.042	0.043	20	5	0.11	32	0.60	7	0.16	49	0.13
11	11	3 to 4	0.54	0.074	0.101	0.084	21	6	0.21	39	0.44	10	0.32	59	0.08
12	11	4	0.59	0.083	0.142	0.076	20	5	0.15	26	0.77	7	0.22	37	0.35
13	10	4	0.42	0.046	0.080	0.044	16	4	0.09	21	0.90	6	0.13	30	0.58
14	11	3 to 4	0.39	0.055	0.063	0.063	26	7	0.16	41	0.40	11	0.24	63	0.07
15	8	3 to 4	0.48	0.048	0.108	0.051	18	4	0.13	27	0.76	6	0.19	41	0.22
16	11	3 to 4	0.35	0.041	0.056	0.052	23	6	0.13	37	0.48	9	0.20	57	0.08
17	6	3	0.40	0.050	0.055	0.098	31	13	0.25	62	0.21	22	0.38	95	0.03
18	20	4	0.40	0.061	0.095	0.064	27	6	0.13	32	0.60	10	0.18	46	0.19
19	6	4	0.54	0.061	0.177	0.060	19	4	0.12	22	0.87	6	0.17	32	0.51

NOTE: Column "N (Reps)" shows the number of replicates needed to detect a 25 percent difference from control with power 0.8, given the observed averages for EMS and control mean. Column "Delta" gives the effect size of the endpoint in milligrams that can be detected with power 0.8, given the observed averages for EMS and control mean. Column "100×Delta/Mean" gives the effect size as a percent of the control mean. Column "Power" gives the power to detect a 25 percent difference from control, given the observed averages for EMS and control mean. PMSD = 100 × MSD / (Control Mean); EMS = error mean square.

Table B-10. Test Method 1002.0, *Ceriodaphnia* Chronic Toxicity Test, Reproduction Endpoint: Power and Effect Size Achieved

Lab	No. of Tests	No. of Reps Per Test	Average Control Mean	Average Control Std Dev	Square Root of Variance of Control Mean	Square Root of Average EMS	Average PMSD	Power of Hypothesis Test (2-sample, 1-sided t-test)							
								α = 0.05				α = 0.01			
								N (Reps)	Delta	100×Delta/ Mean	Power	N (Reps)	Delta	100×Delta/ Mean	Power
1	11	10	34	3.3	2.9	4.6	13	5	5.3	16	0.99	8	7.0	21	0.94
2	9	10	25	7.2	2.6	7.1	29	18	8.2	33	0.59	28	10.8	44	0.28
3	13	10	17	2.6	1.4	3.6	18	10	4.1	24	0.82	16	5.4	32	0.55
4	20	7 to 10	28	8.8	9.5	7.2	25	15	10.2	37	0.51	24	13.6	49	0.20
5	15	10 to 15	19	6.1	4.0	6.6	32	24	7.7	40	0.46	39	10.1	52	0.19
6	20	9 to 10	22	8.5	3.4	7.8	32	26	9.5	44	0.40	42	12.6	58	0.15
7	20	9 to 10	34	11.8	9.7	10.3	31	19	12.7	37	0.50	31	16.8	49	0.21
8	18	10	22	8.6	6.3	7.4	31	23	8.6	39	0.48	37	11.3	51	0.20
9	13	10	25	4.9	3.0	4.8	17	8	5.6	22	0.88	13	7.3	29	0.66
10	12	10	20	2.1	0.8	2.4	12	4	2.8	14	1.00	6	3.6	18	0.98
11	13	10	17	1.5	0.5	3.2	15	8	3.7	21	0.90	13	4.8	28	0.68
12	12	10	31	4.8	2.8	5.0	15	6	5.8	19	0.95	10	7.6	24	0.82
13	8	10	24	5.1	2.5	5.3	22	11	6.2	25	0.79	17	8.1	33	0.51
14	8	10	24	9.2	5.0	6.7	27	17	7.8	33	0.59	28	10.2	43	0.28
15	12	10	18	5.2	2.7	4.8	24	15	5.6	31	0.65	24	7.4	40	0.34
16	20	10	21	5.4	4.6	4.9	22	12	5.7	27	0.74	19	7.5	36	0.44
17	10	9 to 10	24	6.1	4.5	6.9	29	18	8.5	35	0.54	29	11.2	47	0.23
18	10	10	20	5.8	3.7	5.5	24	15	6.4	31	0.64	25	8.4	41	0.32
19	6	9 to 10	23	10.9	3.9	8.4	36	28	10.3	45	0.38	45	13.6	60	0.13
20	12	10	23	3.3	4.7	4.9	21	10	5.7	24	0.81	16	7.5	32	0.54
21	9	10	28	5.3	3.0	6.0	20	11	6.9	25	0.79	17	9.1	33	0.51
22	10	10	17	4.5	2.2	4.9	26	17	5.7	33	0.59	28	7.6	43	0.28
23	9	9 to 10	27	6.9	3.6	7.4	27	16	9.1	33	0.58	25	12.0	44	0.27
24	10	10	18	4.4	1.4	4.5	23	13	5.3	29	0.70	21	6.9	38	0.39
25	12	10	20	6.4	3.6	6.0	30	19	7.0	35	0.55	30	9.2	46	0.25
26	12	10	27	4.4	3.2	4.2	14	6	4.9	18	0.96	10	6.5	24	0.84
27	10	10	21	6.0	4.0	6.1	27	19	7.0	34	0.56	30	9.3	45	0.26
28	6	10	20	6.1	5.2	4.7	23	12	5.5	27	0.74	20	7.3	36	0.43
29	14	10	31	5.6	3.0	5.9	19	9	6.8	22	0.87	14	9.0	29	0.64
30	5	10	16	4.7	0.3	4.9	28	20	5.7	36	0.53	32	7.4	47	0.24
31	12	10	24	5.4	5.9	6.1	25	14	7.1	30	0.67	23	9.3	39	0.35
32	4	10	32	5.9	6.3	5.6	17	8	6.5	21	0.91	12	8.6	27	0.72
33	18	10	24	6.9	5.6	6.8	28	17	7.9	32	0.61	27	10.3	42	0.30

NOTE: See note at bottom of Table B-9.

Table B-11. Test Method 1004.0, Sheepshead Minnow Chronic Toxicity Test, Growth Endpoint: Power and Effect Size Achieved

Lab	No. of Tests	No. of Reps Per Test	Average Control Mean	Average Control Std Dev	Square Root of Variance of Control Mean	Square Root of Average EMS	Average PMSD	Power of Hypothesis Test (2-sample, 1-sided t-test)							
								α = 0.05				α = 0.01			
								N (Reps)	Delta	100×Delta/ Mean	Power	N (Reps)	Delta	100×Delta/ Mean	Power
1	12	4	0.88	0.040	0.11	0.037	6.6	2	0.08	8.6	1.00	3	0.11	12	1.00
2	11	4	0.68	0.051	0.11	0.071	16	4	0.14	21	0.90	6	0.20	30	0.59
3	16	4	0.65	0.088	0.091	0.084	20	5	0.17	26	0.77	7	0.24	37	0.34
4	14	4	1.00	0.074	0.13	0.076	12	3	0.15	15	0.98	4	0.22	22	0.91
5	4	4	0.86	0.048	0.12	0.066	11	3	0.13	16	0.98	4	0.19	22	0.90

NOTE: See note at bottom of Table B-9.

Table B-12. Test Method 1006.0, Inland Silverside Chronic Toxicity Test: Power and Effect Size Achieved

Lab	No. of Tests	No. of Reps Per Test	Average Control Mean	Average Control Std Dev	Square Root of Variance of Control Mean	Square Root of Average EMS	Average PMSD	Power of Hypothesis Test (2-sample, 1-sided t-test)							
								α = 0.05				α = 0.01			
								N (Reps)	Delta	100×Delta/ Mean	Power	N (Reps)	Delta	100×Delta/ Mean	Power
1	10	4	2.3	0.18	0.58	0.26	18	4	0.53	23	0.86	6	0.75	32	0.50
2	15	4	0.94	0.10	0.24	0.17	20	8	0.34	36	0.52	12	0.48	51	0.15
3	19	4	2.1	0.24	0.86	0.27	19	5	0.54	25	0.79	7	0.76	36	0.38
4	12	3	1.4	0.20	0.56	0.22	32	7	0.56	42	0.40	11	0.86	63	0.07
5	6	3 to 4	1.8	0.25	0.57	0.43	31	12	1.07	59	0.23	20	1.6	90	0.04
6	19	4	0.85	0.11	0.23	0.10	20	4	0.20	24	0.83	7	0.29	34	0.43
7	20	3 to 4	1.4	0.15	0.53	0.31	31	11	0.79	56	0.24	18	1.2	86	0.04
8	4	4 to 5	1.1	0.10	0.20	0.11	15	4	0.23	21	0.91	5	0.33	29	0.62
9	20	4	2.4	0.23	0.47	0.25	17	4	0.51	22	0.89	6	0.73	31	0.56
10	20	3 to 4	0.91	0.088	0.35	0.11	22	4	0.27	30	0.65	7	0.42	46	0.15
11	9	4	1.2	0.13	0.19	0.11	14	3	0.22	18	0.96	5	0.31	25	0.79
12	7	4	2.1	0.22	0.38	0.25	17	4	0.50	24	0.84	6	0.72	34	0.45
13	14	4	0.76	0.095	0.12	0.11	22	5	0.22	28	0.70	8	0.31	40	0.27
14	5	4	1.5	0.12	0.33	0.12	13	3	0.25	17	0.97	4	0.35	24	0.84
15	8	4	0.77	0.10	0.22	0.12	25	6	0.24	31	0.64	9	0.34	44	0.22
16	5	3	1.2	0.11	0.20	0.14	20	4	0.35	30	0.67	6	0.53	45	0.16

NOTE: See note at bottom of Table B-9.

Table B-13. Test Method 1007.0, Mysid Chronic Toxicity Test, Growth Endpoint: Power and Effect Size Achieved

Lab	No. of Tests	No. of Reps Per Test	Average Control Mean	Average Control Std Dev	Square Root of Variance of Control Mean	Square Root of Average EMS	Aver-age PMSD	Power of Hypothesis Test (2-sample, 1-sided t-test)							
								α = 0.05				α = 0.01			
								N (Reps)	Delta	100×Delta/ Mean	Power	N (Reps)	Delta	100×Delta/ Mean	Power
1	18	8	0.25	0.040	0.042	0.041	17	7	0.054	22	0.89	11	0.072	29	0.66
2	19	8	0.37	0.15	0.13	0.11	25	20	0.15	41	0.44	33	0.20	54	0.16
3	7	4	0.36	0.042	0.065	0.047	21	5	0.094	26	0.77	7	0.13	37	0.35
4	12	8	0.25	0.044	0.035	0.13	37	58	0.18	70	0.21	94	0.23	94	0.06
5	10	8	0.37	0.073	0.049	0.075	22	9	0.098	26	0.76	15	0.13	35	0.45
6	14	8	0.23	0.034	0.059	0.040	20	7	0.053	22	0.87	11	0.070	30	0.62
7	18	8	0.28	0.075	0.056	0.067	26	13	0.089	32	0.62	20	0.12	42	0.30
8	12	8	0.30	0.048	0.070	0.053	19	8	0.070	23	0.85	12	0.093	31	0.58
9	16	8	0.38	0.041	0.048	0.060	16	7	0.079	21	0.90	10	0.11	28	0.68
10	4	8	0.30	0.041	0.018	0.047	14	6	0.061	21	0.91	10	0.081	27	0.71

NOTE: See note at bottom of Table B-9.

Table B-14. Power to Detect a 25% Difference Between Two Means in a Two-sample, One-sided Test (continued)

N (Reps)	k	df	rEMS / Control Mean = 0.10			rEMS / Control Mean = 0.20			rEMS / Control Mean =0.30			rEMS / Control Mean = 0.40		
				Power With			Power With			Power With			Power With	
			PMSD	α= 0.05	α= 0.05/k	PMSD	α= 0.05	α= 0.05/k	PMSD	α= 0.05	α= 0.05/k	PMSD	α= 0.05	α= 0.05/k
3	2	4	21	0.80	0.66	43	0.29	0.17	64	0.16	0.09	85	0.12	0.07
3	3	6	21	0.80	0.68	42	0.29	0.18	63	0.16	0.10	84	0.12	0.07
3	4	8	21	0.80	0.68	42	0.29	0.18	63	0.16	0.10	83	0.12	0.07
3	5	10	21	0.80	0.68	42	0.29	0.18	63	0.16	0.10	84	0.12	0.07
4	2	6	17	0.92	0.86	33	0.43	0.29	50	0.24	0.15	66	0.17	0.10
4	3	9	17	0.92	0.86	34	0.43	0.28	50	0.24	0.14	67	0.17	0.09
4	4	12	17	0.92	0.85	34	0.43	0.27	51	0.24	0.13	68	0.17	0.09
4	5	15	17	0.92	0.84	35	0.43	0.26	52	0.24	0.13	69	0.17	0.08
5	2	8	14	0.97	0.94	28	0.55	0.41	42	0.30	0.20	56	0.20	0.13
5	3	12	14	0.97	0.93	29	0.55	0.38	43	0.30	0.18	58	0.20	0.12
5	4	16	15	0.97	0.93	30	0.55	0.36	44	0.30	0.17	59	0.20	0.11
5	5	20	15	0.97	0.92	30	0.55	0.35	45	0.30	0.16	60	0.20	0.10
6	2	10	12	0.98	0.97	25	0.63	0.51	37	0.36	0.25	50	0.24	0.16
6	3	15	13	0.98	0.97	26	0.63	0.47	39	0.36	0.22	52	0.24	0.14
6	4	20	13	0.98	0.96	27	0.63	0.45	40	0.36	0.20	53	0.24	0.12
6	5	25	14	0.98	0.96	27	0.63	0.43	41	0.36	0.19	54	0.24	0.12

Table B-14. Power to Detect a 25% Difference Between Two Means in a Two-sample, One-sided Test

			rEMS / Control Mean = 0.10			rEMS / Control Mean = 0.20			rEMS / Control Mean =0.30			rEMS / Control Mean = 0.40		
				Power With			Power With			Power With			Power With	
N (Reps)	k	df	PMSD	α= 0.05	α= 0.05/k	PMSD	α= 0.05	α= 0.05/k	PMSD	α= 0.05	α= 0.05/k	PMSD	α= 0.05	α= 0.05/k
7	5	30	12	0.99	0.98	25	0.71	0.50	37	0.41	0.23	50	0.28	0.13
8	2	14	10	1.00	0.99	21	0.76	0.66	31	0.46	0.34	42	0.31	0.21
8	3	21	11	1.00	0.99	22	0.76	0.62	33	0.46	0.31	44	0.31	0.18
8	4	28	11	1.00	0.99	23	0.76	0.59	34	0.46	0.28	45	0.31	0.16
8	5	35	12	1.00	0.99	23	0.76	0.57	35	0.46	0.26	46	0.31	0.15
9	2	16	10	1.00	1.00	19	0.81	0.72	29	0.51	0.39	39	0.34	0.24
9	3	24	10	1.00	1.00	20	0.81	0.68	31	0.51	0.35	41	0.34	0.21
9	4	32	11	1.00	1.00	21	0.81	0.65	32	0.51	0.32	42	0.34	0.18
9	5	40	11	1.00	1.00	22	0.81	0.63	33	0.51	0.30	44	0.34	0.17
10	2	18	9	1.00	1.00	18	0.85	0.77	27	0.55	0.43	36	0.37	0.26
10	3	27	10	1.00	1.00	19	0.85	0.73	29	0.55	0.39	39	0.37	0.23
10	4	36	10	1.00	1.00	20	0.85	0.71	30	0.55	0.36	40	0.37	0.21
10	5	45	10	1.00	1.00	21	0.85	0.69	31	0.55	0.33	41	0.37	0.19
11	2	20	9	1.00	1.00	17	0.88	0.81	26	0.59	0.47	35	0.40	0.29
11	3	30	9	1.00	1.00	18	0.88	0.78	27	0.59	0.42	37	0.40	0.25
11	4	40	10	1.00	1.00	19	0.88	0.75	29	0.59	0.39	38	0.40	0.23
11	5	50	10	1.00	1.00	20	0.88	0.73	29	0.59	0.37	39	0.40	0.21
12	2	22	8	1.00	1.00	16	0.90	0.85	25	0.63	0.51	33	0.43	0.32
12	3	33	9	1.00	1.00	17	0.90	0.82	26	0.63	0.46	35	0.43	0.27
12	4	44	9	1.00	1.00	18	0.90	0.79	27	0.63	0.43	36	0.43	0.25
12	5	55	9	1.00	1.00	19	0.90	0.78	28	0.63	0.40	37	0.43	0.23
13	2	24	8	1.00	1.00	16	0.92	0.87	24	0.66	0.55	32	0.45	0.34
13	3	36	8	1.00	1.00	17	0.92	0.85	25	0.66	0.50	33	0.45	0.30
13	4	48	9	1.00	1.00	17	0.92	0.83	26	0.66	0.46	35	0.45	0.27
13	5	60	9	1.00	1.00	18	0.92	0.81	27	0.66	0.44	36	0.45	0.25
14	2	26	8	1.00	1.00	15	0.94	0.90	23	0.69	0.58	30	0.48	0.37
14	3	39	8	1.00	1.00	16	0.94	0.88	24	0.69	0.53	32	0.48	0.32
14	4	52	8	1.00	1.00	17	0.94	0.86	25	0.69	0.50	33	0.48	0.29
14	5	65	9	1.00	1.00	17	0.94	0.84	26	0.69	0.47	34	0.48	0.27
15	2	28	7	1.00	1.00	15	0.95	0.92	22	0.72	0.61	29	0.50	0.39
15	3	42	8	1.00	1.00	15	0.95	0.90	23	0.72	0.56	31	0.50	0.34
15	4	56	8	1.00	1.00	16	0.95	0.88	24	0.72	0.53	32	0.50	0.31
15	5	70	8	1.00	1.00	17	0.95	0.87	25	0.72	0.50	33	0.50	0.29

NOTE: Power is reported for tests with two values of α, 0.05 and 0.05/k. Power for Dunnett's multiple comparison test will fall between these two values. All numbers have been rounded to two significant figures. The number of treatments tested (k) and used to calculate EMS and MSD for a sublethal endpoint will vary depending on the NOEC for survival. k = number of treatments in Dunnett's test; df = degrees of freedom; PMSD = 100 × MSD / (Control Mean); EMS = error mean square; rEMS = square root of the error mean square.

Table B-15. Values of PMSD in Dunnett's Test in Relation to the Square Root of the Error Mean Square (rEMS) for the Test

Reps	k	df	d	Value of PMSD When rEMS / (Control Mean) Equals These Values			
				0.1	0.2	0.3	0.4
3	2	4	2.61	21	43	64	85
4	2	6	2.34	17	33	50	66
5	2	8	2.22	14	28	42	56
6	2	10	2.15	12	25	37	50
7	2	12	2.11	11	23	34	45
8	2	14	2.08	10	21	31	42
9	2	16	2.06	10	19	29	39
10	2	18	2.04	9	18	27	37
11	2	20	2.03	9	17	26	35
12	2	22	2.02	8	16	25	33
13	2	24	2.01	8	16	24	32
14	2	26	2.00	8	15	23	30
15	2	28	1.99	7	15	22	29
3	3	6	2.56	21	42	63	84
4	3	9	2.37	17	34	50	67
5	3	12	2.29	14	29	43	58
6	3	15	2.24	13	26	39	52
7	3	18	2.21	12	24	35	47
8	3	21	2.19	11	22	33	44
9	3	24	2.17	10	20	31	41
10	3	27	2.16	10	19	29	39
11	3	30	2.15	9	18	27	37
12	3	33	2.14	9	17	26	35
13	3	36	2.13	8	17	25	33
14	3	39	2.13	8	16	24	32
15	3	42	2.12	8	15	23	31
3	4	8	2.55	21	42	63	83
4	4	12	2.41	17	34	51	68
5	4	16	2.34	15	30	44	59
6	4	20	2.30	13	27	40	53
7	4	24	2.28	12	24	37	49
8	4	28	2.26	11	23	34	45
9	4	32	2.25	11	21	32	42
10	4	36	2.24	10	20	30	40
11	4	40	2.23	10	19	29	38
12	4	44	2.22	9	18	27	36

Table B-15. Values of PMSD in Dunnett's Test in Relation to the Square Root of the Error Mean Square (rEMS) for the Test

Reps	k	df	d	Value of PMSD When rEMS / (Control Mean) Equals These Values			
				0.1	0.2	0.3	0.4
13	4	48	2.22	9	17	26	35
14	4	52	2.21	8	17	25	33
15	4	56	2.21	8	16	24	32
3	5	10	2.56	21	42	63	84
4	5	15	2.44	17	35	52	69
5	5	20	2.39	15	30	45	60
6	5	25	2.36	14	27	41	54
7	5	30	2.34	12	25	37	50
8	5	35	2.32	12	23	35	46
9	5	40	2.31	11	22	33	44
10	5	45	2.30	10	21	31	41
11	5	50	2.29	10	20	29	39
12	5	55	2.29	9	19	28	37
13	5	60	2.28	9	18	27	36
14	5	65	2.28	9	17	26	34
15	5	70	2.28	8	17	25	33

NOTE: The number of treatments tested (k) and used to calculate EMS and MSD for a sublethal endpoint will vary depending on the NOEC for survival. k = number of treatments in Dunnett's test; df = degrees of freedom; d = Dunnett's statistic ($\alpha = 0.05$); PMSD = 100 × MSD / (Control Mean); EMS = error mean square; rEMS = square root of the error mean square.

Table B-16. Percentiles of the rEMS/Control Mean, for the Growth or Reproduction Endpoint of Chronic WET Tests, Using Data Pooled Across All Laboratories and Toxicants[a]

	Test Method						
	1000.0 Fathead Minnow	**1002.0 *Cerio-daphnia***	**1003.0 Green Alga**	**1004.0 Sheepshead Minnow**	**1006.0 Inland Silverside**	**1007.0 Mysid (*A. bahia*)**	**1009.0 Red Macroalga**
No. of tests	206	393	85	57	193	130	23
No. of labs	19	33	9	5	16	10	2
Endpoint	G	R	G	G	G	G	R
Percentile	**rEMS/Control Mean**						
25%	0.09	0.17	0.06	0.05	0.09	0.15	0.11
50%	0.12	0.24	0.08	0.08	0.11	0.18	0.18
75%	0.16	0.31	0.10	0.11	0.15	0.23	0.25
80%	0.17	0.32	0.11	0.12	0.16	0.24	0.26
85%	0.18	0.34	0.12	0.13	0.18	0.27	0.27
90%	0.21	0.39	0.13	0.14	0.21	0.29	0.27
95%	0.26	0.44	0.16	0.15	0.26	0.33	0.34

[a] rEMS = square root of the error mean square
[b] G = growth, R = reproduction

Table B-17. Number of Replicates Needed to Provide PMSD of 25% and 33% for Some Less Precise Tests in Each Chronic Test Method (that is, for 85th and 90th Percentiles from Table B-17) for the Sublethal Endpoints in Table B-16

		rEMS / Control Mean		Number of Replicates to Make PMSD = 25		Number of Replicates to Make PMSD = 33	
Test Method	**Required No. of Replicates**	**85th Percentile**	**90th Percentile**	**For 85th Percentile**	**For 90th Percentile**	**For 85th Percentile**	**For 90th Percentile**
1000.0 Fathead Minnow	4 (3)	0.18	0.21	6	8 (7)	4	5
1002.0 *Ceriodaphnia*	10	0.34	0.39	19 (17)	24 (22)	11	14 (13)
1003.0 Green Alga	4 (3)	0.12	0.13	4	4	3	3
1004.0 Sheepshead Minnow	4 (3)	0.13	0.14	4	4	3	3
1006.0 Inland Silverside	4 (3)	0.18	0.21	6	8 (7)	4	5
1007.0 Mysid	8	0.27	0.29	12 (11)	14 (13)	7	9 (8)
1009.0 Red Macroalga	4 (3)	0.27	0.27	12 (11)	12 (11)	7	7

NOTE: The number for k = 3 treatments appears in parentheses if it differs from the number needed when four treatments are compared with the control; rEMS = square root of the error mean square; PMSD = percent minimum significant difference.

Table B-18. Percentiles of the Within-Laboratory Values of CV for NOEC (using NOEC for the Most Sensitive Endpoint in Each Test)

Method No.	Method	No. Labs	P10	P25	P50	P75	P90
1000.0	Fathead Minnow Larval Survival & Growth	19	0	0.22	0.31	0.52	0.65
1002.0	*Ceriodaphnia* Survival & Reproduction	33	0.20	0.25	0.35	0.49	0.60
1003.0	Green Alga Growth	9	0.30	0.40	0.46	0.56	0.82
1004.0	Sheepshead Minnow Larval Survival & Growth	5	0.20	0.36	0.38	0.44	0.52
1006.0	Inland Silverside Larval Survival & Growth	16	0.19	0.35	0.46	0.59	0.66
1007.0	Mysid Survival, Growth, & Fecundity	10	0.28	0.32	0.40	0.50	0.60
1009.0	Red Macroalga Reprod	2	0.85	0.85	1.00	1.16	1.16
1010.0	Topsmelt Larval Survival & Growth	1	0.22	0.22	0.22	0.22	0.22
1012.0	Pacific Oyster Embryo-Larval Survival & Dev.	1	0.45	0.45	0.45	0.45	0.45
1013.0	Mussel Embryo-Larval Survival & Dev.	3	0	0	0.39	0.43	0.43
1014.0	Red Abalone Larval Development	10	0.24	0.25	0.29	0.31	0.38
1016.0	Sea Urchin Fertilization[a]	12	0.31	0.40	0.50	0.69	0.76
1017.0	Sand Dollar Fertilization[a]	7	0.40	0.41	0.53	0.75	0.81
1018.0	Giant Kelp Germination & Germ-Tube Length	11	0.33	0.36	0.59	0.68	0.72

[a] These two test species include previous test method procedures (Dinnel 1987, Chapman 1992). However, EPA (USEPA 1995) has standardized these two methods to provide further guidance and therefore minimize within-test variability.

APPENDIX C

SAMPLE CALCULATION OF PERMIT LIMITS USING EPA'S STATISTICALLY-BASED METHODOLOGY AND SAMPLE PERMIT LANGUAGE

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SAMPLE CALCULATIONS OF PERMIT LIMITS USING EPA'S STATISTICALLY-BASED METHODOLOGY AND SAMPLE PERMIT LANGUAGE

The NPDES regulation (40 CFR Part 122.44(d)(1)) implementing section 301 (b)(1)(C) of the CWA requires that permits include limits for all pollutants or parameters that "*are or may be discharged at a level which will cause, have the reasonable potential to cause, or contribute to an excursion above any State water quality standard, including State narrative criteria for water quality*." Once it has been established that a permit limit is needed, Federal regulations at 40 CFR Part 122.45(d) require that limits be expressed as maximum daily discharge limits (MDL) and average monthly discharge limits (AML) for all dischargers other than publicly owned treatment works (POTWs), and as average weekly and average monthly discharge limits for POTWs, unless impracticable. EPA does not believe that it is impracticable to express WET permit limits as MDLs and AMLs.

C.1 Sample Calculations

To set MDLs and AMLs based on acute and chronic wasteload allocations (WLAs), use the following four steps.

1. Convert the acute wasteload allocation to chronic toxic units.

2. Calculate the long-term average wasteload that will satisfy the acute and chronic wasteload allocations.

3. Determine the lower (more limiting) of the two long-term averages.

4. Calculate the maximum daily and average monthly permit limits using the lower (more limiting) long-term average.

Step 1 - Determine the Wasteload Allocation

The acute and chronic aquatic life criteria are converted to acute and chronic wasteload allocations (WLAa or WLAc) for the receiving waters based on the following mass balance equation:

$$Q_d C_d = Q_e C_e + Q_u C_u \qquad \textbf{(Eq. 1)}$$

where

Q_d = downstream flow = $Q_u + Q_e$
C_d = aquatic life criteria that cannot be exceeded downstream
Q_e = effluent flow
C_e = concentration of pollutant in effluent = WLAa or WLAc
Q_u = upstream flow
C_u = upstream background concentration of pollutant.

Rearranging Equation 1 to determine the effluent concentration (C_e) or the wasteload allocation (WLA) results in the following:

$$C_e = WLA = \frac{Q_d C_d - Q_u C_u}{Q_e} \qquad \textbf{(Eq. 2)}$$

When a mixing zone[1] is allowed, this equation becomes:

$$C_e = WLA = \left[\frac{C_d\left(Q_u \times \%MZ\right) + C_d Q_e}{Q_e}\right] - \left[\frac{Q_u C_u\left(\%MZ\right)}{Q_e}\right] \qquad \textbf{(Eq. 2a)}$$

where %MZ is the mixing zone allowable by State standards. In this example, the State authorized a mixing zone of 50 percent of river volume for WET. The effluent limits were derived using the State's guidelines. Establishing a mixing zone, however, is a discretionary function of the State. If the State does not certify a mixing zone in the 401 certification process, the effluent limits must be recalculated without a mixing zone.

There is an additional step for WET. The WLAa needs to be converted from acute toxic units (TUa) to chronic toxic units (TUc). The acute WLA is converted into an equivalent chronic WLA by multiplying the acute WLA by an acute-to-chronic ratio (ACR). Optimally, this ratio is based on effluent data. A default value of 10, however, can be used based on the information presented in Chapter 1 and Appendix A of the TSD.

WLAa,c = WLAa × ACR, where
ACR = acute-to-chronic ratio

For this example, the following information applies:

	C_d	Q_e	Q_u	%MZ	Q_{umix} [a]	Q_d	C_u	CV[b]
Acute	0.3 TUa	15.5 cfs	109 cfs	50	54.5 cfs	70 cfs	0 TU_a	0.6
Chronic	1.0 TUc	15.5 cfs	170 cfs	50	85 cfs	100.5 cfs	0 TU_c	0.6

[a] Q_{umix} is the upstream flow in the mixing zone ($Q_{umix} = Q_u \times \%MZ$)
[b] Only 7 valid data points were available, so a default coefficient of variation was used in the calculations.

$$WET\ WLAa = \left[\frac{\left(0.3\,TUa\right)\times\left(109\times 0.50\right)+\left(0.3\times 15.5\right)}{15.5}\right] - \left[\frac{109\times 0\times 0.25}{15.5}\right] = 1.35\,TUa$$

$$WET\ WLAa,c = 10\times 1.35\,TUa = 13.5\,TUa,c$$

$$WET\ WLAc = \left[\frac{1.0\,TUc\times\left(170\times 0.50\right)+\left(1.0\times 15.5\right)}{15.5}\right] - \left[\frac{170\times 0\times 0.50}{15.5}\right] = 6.5\,TUc$$

Step 2 - Determine the Long-Term Average (LTA)

The acute WLA is converted to a long-term average concentration (LTAa,c) using the following equation:

$$LTAa,c = WLAa,c \times e^{\left[0.5s^2 - zs\right]} \qquad \textbf{(Eq. 3)}$$

where,

σ^2 = $\ln(CV^2 + 1) = \ln(0.6^2 + 1) = 0.307$; $\sigma = 0.555$
z = 2.326 for 99th percentile probability basis
CV = coefficient of variation = standard deviation/mean = 0.6
Acute multiplier = $e^{(0.5 \times 0.307 - (2.326 \times 0.555)} = 0.321$.

$$LTAa,c = 13.5\,TUa,c \times 0.321 = 4.33\,TUa,c$$

[1] A mixing zone is an allocated impact zone where water quality criteria can be exceeded if acutely toxic conditions are prevented. Only the State has the regulatory authority to grant the establishment of a mixing zone.

The chronic WLA is converted to a long-term average concentration (LTAc) using the following equation:

$$LTAc = WLAc \times e^{\left[0.5s^2 - zs\right]} \quad \textbf{(Eq. 4)}$$

where,

σ^2 = $\ln(CV^2/4 + 1) = \ln(0.6^2/4+1)$ 0.086; $\sigma = 0.294$
z = 2.326 for 99th percentile probability basis
CV = coefficient of variation = standard deviation/mean = 0.6
Chronic multiplier = $e^{(0.5 \times 0.086 - 2.326 \times 0.294)} = 0.542$.

$$LTAc = 6.5\,TUc \times 0.542 = 3.43\,TUc$$

Step 3 - Determine the More Limiting Long-Term Average

To protect a waterbody from both acute and chronic effects, the more limiting of the calculated LTAa and LTAc is used to derive the effluent limits. The TSD recommends using the 95th percentile for the AML and the 99th percentile for the MDL. As shown above, the LTAc value was less than the LTAa value.

Step 4 - Determine the Permit Limits

The MDL and the AML are calculated as follows.

$$MDL = LTAc \times e^{\left[zs - 0.5s^2\right]} \quad \textbf{(Eq. 5)}$$

where,

σ^2 = $\ln(CV^2 + 1) = 0.307$; $\sigma = 0.555$
z = 2.326 for 99th percentile probability basis
CV = coefficient of variation = 0.6

$$AML = LTAc \times e^{\left[z\sigma - 0.5\sigma^2\right]} \quad \textbf{(Eq. 6)}$$

where,

σ^2 = $\ln(CV^2/n + 1) = 0.086$; $\sigma = 0.294$
z = 1.645 for 95th percentile probability basis
CV = coefficient of variation = 0.6
n = number of sampling events required per month for WET = 1
n = 4 for calculations[2]

The following table lists the effluent limits for this example:

Parameter	CV	LTA_c	$e^{[z\sigma-0.5\sigma^2]}$ (for MDL)	$e^{[z\sigma-0.5\sigma^2]}$ (for AML)	MDL	AML
WET	0.6	3.43	3.11	2.13	10.7 TU_c	7.3 TU_c

[2] When the sample frequency is monthly or less than monthly, the TSD recommends that "n" be set equal to 4.

C.2 Sample Chronic Toxicity Permit Language

Sample chronic toxicity permit language is provided in the following paragraphs. Alternative wording, as appropriate for a specific permit, is provided in redline typeface for the regulatory authority to decide.

The permittee shall conduct monthly/quarterly/semi-annual/annual toxicity tests on grab/24-hour composite effluent samples. Samples shall be taken at the NPDES sampling location. In addition, a split of each sample collected must be analyzed for the chemical and physical parameters required in Part 1.A below. When the timing of sample collection coincides with timing of the sampling required in Part I.A, analysis of the split sample will fulfill the requirements of Part I.A. as well.

1. Test Species and Methods

NOTE: CHOOSE EITHER FRESHWATER OR MARINE LANGUAGE

Freshwater

a. The permittee shall conduct short-term tests with the cladoceran, water flea, *Ceriodaphnia dubia* (survival and reproduction test), the fathead minnow, *Pimephales promelas* (larval survival and growth test), and the green alga, *Selanastrum capricornutum* (growth test) for the first three suites of tests. After this screening period, monitoring shall be conducted using the most sensitive species.

b. Every year, the permittee shall re-screen once with the three species listed above and continue to monitor with the most sensitive species. Re-screening shall be conducted at a different time of year from the previous year's re-screening. Note to permit writers: If testing is annual or less than annual, omit this step.

c. The presence of chronic toxicity shall be estimated as specified in EPA's methods (USEPA 1994b).

Marine and Estuarine

a. The permittee shall conduct tests as follows with a vertebrate, an invertebrate, and a plant for the first three suites of tests. After the screening period, monitoring shall be conducted using the most sensitive species.

b. Every year, the permittee shall re-screen once with the three species listed above and continue to monitor with the most sensitive species. Re-screening shall be conducted at a different time of year from the previous year's re-screening. Note to permit writers: If testing is annual or less, omit this step.

For West Coast only:

c. The presence of chronic toxicity shall be estimated as specified using West Coast marine organisms according to EPA's methods (USEPA 1995).

or

For East Coast only:

c. The presence of chronic toxicity shall be estimated as specified using East Coast marine organisms according to EPA's methods (USEPA 1994c).

2. Toxicity Limits/Toxicity Monitoring Trigger

a. Chronic toxicity measures a sublethal effect (e.g., reduced growth, reproduction) to experimental test organisms exposed to an effluent or ambient waters compared to that of the control organisms. When a permit limit is appropriate, the chronic toxicity limitation is written based on State Water Quality Standards. If a permit limit is not appropriate, then this section should be called "Toxicity Monitoring Trigger."

b. Results shall be reported in TUc, where TUc = 100/NOEC or 100/ICp or ECp (in percent effluent). The no observed effect concentration (NOEC) is the highest concentration of toxicant to which organisms are exposed in a chronic test that causes no observable adverse effect on the test organisms (e.g., the highest concentration of toxicant to which the values for the observed responses are not statistically significantly different from the controls). The inhibition concentration, IC, is a point estimate of the toxicant concentration that causes a given percent reduction (p) in a non-quantal biological measurement (e.g., reproduction or growth) calculated from a continuous model (the EPA Interpolation Method). The effective concentration, EC, is a point estimate of the toxicant concentration that would cause a given percent reduction (p) in quantal biological measurement (e.g., larval development, survival) calculated from a continuous model (e.g., Probit).

3. Quality Assurance

a. A series of at least five dilutions and a control will be tested. The series shall include the instream waste concentration (IWC) (permit writer should insert the actual value of the IWC), two dilutions above the IWC, and two dilutions below the IWC. The IWC is the concentration of effluent at the edge of the mixing zone. If there is no mixing zone, then the dilution series would be the following concentrations: 12.5, 25, 50, 75, and 100 percent effluent.

b. If organisms are not cultured in-house, concurrent testing with a reference toxicant shall be conducted. Where organisms are cultured in-house, monthly reference toxicant testing is sufficient. Reference toxicant tests also shall be conducted using the same test conditions as the effluent toxicity tests (e.g., same test duration, etc).

c. If either the reference toxicant test or effluent test does not meet all test acceptability criteria (TAC) as specified in the manual, then the permittee must re-sample and re-test within 14 days or as soon as possible.

d. The reference toxicant and effluent tests must meet the upper and lower bounds on test sensitivity as determined by calculating the percent minimum significant difference (PMSD) for each test result. The test sensitivity bound is specified for each test method (see variability document EPA/833-R-00-003, Table 3-6). There are five possible outcomes based on the PMSD result:

1. **Unqualified Pass**–The test's PMSD is within bounds and there is no significant difference between the means for the control and the IWC treatment. The regulatory authority would conclude that there *is no toxicity at the IWC concentration*.

2. **Unqualified Fail**–The test's PMSD is larger than the lower bound (but not greater than the upper bound) in Table 3-6 and there is a significant difference between the means for the control and the IWC treatment. The regulatory authority would conclude that there *is toxicity at the IWC concentration*.

3. **Lacks Test Sensitivity**–The test's PMSD exceeds the upper bound in Table 3-6 and there is no significant difference between the means for the control and the IWC treatment. The test

is considered invalid. An effluent sample must be collected and another toxicity test must be conducted. The permittee must re-sample and retest within fourteen (14) days or as soon as possible.

4. **Lacks Test Sensitivity**–The test's PMSD exceeds the upper bound in Table 3-6 and there is a significant difference between the means for the control and the IWC treatment. The test is considered valid. The regulatory authority will conclude that the *is toxicity at the IWC concentration*.

5. **Very Small but Significant Difference**–The relative difference (see Section 6.4.2, below) between the means for the control and the IWC treatment is smaller than the lower bound in Table 3-6 and this difference is statistically significant. The test is acceptable. The NOEC is determined as described in Sections 6.4.2 and 6.4.3 (below).

e. Control and dilution water should be receiving water or laboratory water, as appropriate, as described in the manual. If the dilution water used is different from the culture water, a second control using culture water shall be used.

4. Preparing the Initial Investigation of the TRE Workplan

The permittee shall submit to EPA a copy of the permittee's initial investigation Toxicity Reduction Evaluation (TRE) workplan (1-2 pages) within 90 days of the effective date of this permit. This plan shall describe the steps the permittee intends to follow if toxicity is detected, and should include, at least the following items:

a. A description of the investigation and evaluation techniques that would be used to identify potential causes and sources of toxicity, effluent variability, and treatment system efficiency.

b. A description of the facility's methods of maximizing in-house treatment efficiency and good housekeeping practices.

c. If a toxicity identification evaluation (TIE) is necessary, an indication of the person who would conduct the TIEs (i.e., an in-house expert or an outside contractor).

5. Accelerated Testing

a. If the initial investigation indicates the source of toxicity (for instance, a temporary plant upset), then only one additional test is necessary. If toxicity is detected in this test as specified in Section 2a, then Section 6 shall apply.

b. If chronic toxicity/the chronic toxicity monitoring requirements as defined in Section 2a are triggered, then the permittee shall conduct six more tests, approximately every two weeks, over a twelve-week period. Testing shall commence within two weeks of receipt of the sample results of the exceedance of the WET monitoring trigger.

c. If none of the six tests indicate toxicity as specified in Section 2a, then the permittee may return to the normal testing frequency.

6. Toxicity Reduction Evaluation (TRE) and Toxicity Identification Evaluation (TIE)

a. If chronic toxicity (defined as either the toxicity permit limit or monitoring trigger specified in Section 2a) is detected in any of the six additional tests, then, in accordance with the facility's initial investigation according to the TRE workplan, the permittee shall initiate a TRE within

fifteen (15) days of the exceedance to reduce the cause(s) of toxicity. At a minimum, the permittee shall use EPA manuals EPA/600/2-88/070 (industrial) or EPA/833B-99/002 (municipal) as guidance. The permittee will expeditiously develop a more detailed TRE workplan, which includes:

(1) Further actions to investigate and identify the cause of toxicity
(2) Actions the permittee will take to mitigate the impact of the discharge and prevent the recurrence of toxicity
(3) A schedule for these actions

b. The permittee may initiate a TIE as part of the TRE process to identify the cause(s) of toxicity. The permittee shall use the EPA acute and chronic manuals, EPA/600/6-91/005F (Phase I)/EPA/600/R-96-054 (for marine), EPA/600/R-92/080 (Phase II), and EPA-600/R-92/081 (Phase III) as guidance.

7. Reporting

a. The permittee shall submit the results of the toxicity tests, including any accelerated testing conducted during the month, in TUs with the discharge monitoring reports (DMR) for the month in which the test is conducted. If an initial investigation indicates the source of toxicity and accelerated testing is unnecessary, pursuant to Section 5, then those results also shall be submitted with the DMR for the quarter in which the investigation occurred.

b. The full report shall be submitted by the end of the month in which the DMR is submitted.

c. The full report shall consist of (1) the results; (2) the dates of sample collection and initiation of each toxicity test; (3) the monthly average limit or trigger and daily maximum limit or trigger as described in Section 2a.

d. Test results for chronic tests also shall be reported according to the chronic manual chapter on Report Preparation and shall be attached to the DMR.

e. The permittee shall notify EPA in writing 15 days after the receipt of the results of a monitoring limit or trigger. The notification will describe actions the permittee has taken or will take to investigate and correct the cause(s) of toxicity. It may also include a status report on any actions required by the permit, with a schedule for actions not yet completed. If no actions have been taken, the reasons shall be given.

8. Reopener

a. This permit may be modified in accordance with the requirements set forth at 40 CFR Parts 122 and 124 to include appropriate conditions or limits to address demonstrated effluent toxicity based on newly available information.

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APPENDIX D

FREQUENTLY ASKED QUESTIONS (FAQS)

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FREQUENTLY ASKED QUESTIONS (FAQS)

Appendix D contains some of the frequently asked questions regarding WET and WET testing. These questions and answers were prepared by and appear on a web site maintained by the Society of Environmental Toxicology and Chemistry (SETAC) (http://www.setac.org). The SETAC WET Expert Advisory Panels provide scientific opinion and training on WET technical issues under a cooperative agreement with EPA (WET Cooperative Agreement No. CX 824845-01-0). EPA's inclusion of these questions and answers in this document is not an endorsement of the Panels' opinions or responses to the FAQs, but rather provides readers with an additional source of information in issues commonly raised with regard to WET and WET testing. This information was prepared in response to questions received by SETAC about WET. It was generated by the WET Expert Advisory Panels (EAP) Steering Committee (SC), all volunteers and all member of the Society of Environmental Toxicology and Chemistry. Each person is considered an expert in some aspect of WET, and the information provide in these FAQs represents the consensus of the Committee's collective expertise at the time this summary was written (Feb., 1999).

This information is intended to stimulate further discussion about WET, WET-related research, and the science underlying WET. The information is not to be construed as representing an official position of SETAC, the SETAC Foundation for Environmental Education, or the U.S. Environmental Protection Agency. Any questions, comments, and requests should be sent to: Society of Environmental Toxicology and Chemistry (SETAC), 1010 North 12th Avenue, Pensacola, FL 32501-3367, Telephone: 850-469-1500, Facsimile: 850-469-9778, e-mail: setac@setac.org. [1,2]

Whole effluent toxicity tests rely on the assumption that test organisms used are representative of a normal and healthy population. What indicators of test organism health are utilized in testing programs?

Both subjective and objective (e.g., test acceptability criteria) indicators of organism health are available, some described within the methods manuals. Some national indicators exist which allow comparison of analytical results between laboratories (i.e., the DMRQA program for major NPDES facilities) or regional activities such as State WET certification programs which provide round-robin validation of test practice including organism health (e.g., North Carolina's Biological Laboratory Certification program). Other national programs like the National Environmental Laboratory Accreditation Program (NELAP) are being followed by the WET EAP SC. Commonly used indicators of organism health are the required reference toxicity analyses and individual test acceptability criteria. Tests properly utilizing randomization procedures along with required and suggested quality control standards retain many built-in checks of typical organism response.

What are the definitions of acceptability criteria for reference toxicant tests?

Reference toxicant tests should meet the same test acceptability criteria as those of compliance test. With regard to assessment of organism health and the overall test practice, USEPA has recommended that routine reference toxicant tests be performed to establish a CUSUM or cumulative summation chart of testing results. Normal results should lie within plus or minus two standard deviations of the cumulative mean value

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1 Reprinted with permission of SETAC.

2 Note that the terms, abbreviations, and acronyms used in this appendix may differ from their usage throughout the rest of this document. EPA consciously chose not to edit this SETAC-supplied information so that the actual nomenclature and terminology as used by SETAC on their web site would be reflected here.

of point estimate endpoints. Values falling outside of those ranges should result in careful scrutiny of the data and testing systems. Data produced during these "out of control" conditions should be considered suspect.

How does increasing the difference in test concentration dilutions affect the prediction of response?

Better resolution around threshold effect concentrations provide better input to mathematical models to predict point estimations of effect and reduce uncertainty in hypothesis tests of effect. Reducing the distance between effluent dilutions should be encouraged. There may be some confusion about USEPA's specification of dilution series in these cases. The methods specify a minimum set of dilutions, i.e., no wider than 0.5 dilution between concentrations. No limitations on added concentrations within that range exist. Experimental design should account for concentrations of concern and should attempt to maximize resolution in that range. Test design should maximize test concentrations around the effect concentration of concern, i.e., the instream waste concentration or limited concentration of a discharging facility, in order to minimize the need for interpolation of effects between tested concentrations.

What are the different types of variability in whole effluent toxicity tests?

Variability is inherent in any analytical procedure. The precision of a method describes the closeness of agreement between test results obtained from repeated testing of a prescribed method. WET test precision can be categorized by: 1) intratest (within-test) variability, 2) intralaboratory (within-laboratory) variability, and 3) interlaboratory (between-laboratory) variability. Intratest variability can be attributed to variables such as the number of treatment replicates, the number of test organisms exposed per replicate, and the sensitivity differences between individual organisms (i.e., genetic variability). Intralaboratory variability is that which is measured when tests are conducted under reasonably constant conditions in the same laboratory (e.g., reference toxicant or effluent sample tested over time). Sources of intralaboratory variability include those factors described for intratest variability, as well as differences: 1) in test conditions (e.g., seasonal differences in dilution water quality, differences in environmental conditions), 2) from test to test in organism condition/health, and 3) in analyst performance from test to test. Interlaboratory variability reflects the degree of precision that is measured when the same sample or reference toxicant is analyzed by multiple laboratories using the same methods. Variability measured between laboratories is a consequence of variability associated with both intratest and intralaboratory variability factors, as well as differences allowed within the test methods themselves (e.g., source of dilution water), technician training programs, sample and organism culturing/shipping effects, testing protocols, food quality, and testing facilities.

Two general categories of variability are of greatest concern: 1) analyst experience, and 2) test organism condition/health. The experience and qualifications of the analyst who actually performs the toxicity test in the laboratory will dictate how well the culture and test methods are followed and the extent to which good judgment is exercised when difficulties/issues arise in the process of conducting the test, analyzing the data, and interpreting the results. Improper utilization of WET methods can have a substantial impact on test result variability. Guidance for specific test conditions and standard methods to control many causes of variability are found in the USEPA (U.S. Environmental Protection Agency) methods manuals (USEPA 1993, USEPA 1994a, USEPA 1994b, USEPA 1995). Strict adherence to these methods can greatly reduce variability.

USEPA. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. 4th ed. Weber C.I., editor. Cincinnati: U.S. Environmental Protection Agency (USEPA) Office of Research and Development. EPA/600/4-90/027F. 293 p.

USEPA. 1994a. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. 2nd ed. Klemm, D.J., Morrison, G.E., Norberg-King, T.J., Peltier, W.H. and Heber, M.A., editors.

Cincinnati: U.S. Environmental Protection Agency (USEPA) Office of Research and Development. EPA/600/4-91/003. 341 p.

USEPA. 1994b. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 3rd ed. Lewis, P.A., Klemm, D.J., Lazorchak, J.M., Norberg-King, T.J., Peltier, W.H. and Heber, M.A., editors. Cincinnati: U.S. Environmental Protection Agency (USEPA) Office of Research and Development. EPA/600/4-91/002. 341 p.

USEPA. 1995. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. Chapman, G.A., Denton, D.I., Lazorchak, J.M., editors. Cincinnati: U.S. Environmental Protection Agency (USEPA) Office of Research and Development. EPA/600/R-95-136. 661 p.

What specific factors influence WET test variability?

There are a number of factors that can meaningfully influence the variability of test results. These factors include, but are not limited to, those listed below.

Sample Characteristics

The nature of the sample collected can have a significant influence on the outcome of a WET test. Care must be exercised to collect the most representative sample possible during the time frame of interest. Sample volume can influence the outcome of a toxicity test. For example, if the sample-to-container-wall ratio is small, or if the sample-container contact time is especially long before the sample is refrigerated; certain particulate-active constituents such as zinc (Chapter 5 in Grothe et al. 1996), polymeric substances, charged materials, or hydrophobic chemicals in a sample can interact with the container. Samples too small in volume may also increase the potential of collecting a non-representative fraction of a non-homogenous sample stream. The type of sample (i.e., grab or composite) may influence the outcome of a WET test and contribute to variability. Grab samples may hit or miss toxicity spikes thus possibly increasing the variability between samples taken at different times at the same outfall. Composite samples will average concentrations over the entire collection period, possibly smoothing peaks and valleys of toxicity in variable water media. The various USEPA method manuals review the importance of using appropriate sample types for different types of effluents. Storage and handling can affect the toxicity and variability of samples. The general assumption is that the toxicity of a sample is most likely to decrease with holding time due to factors such as biodegradation, hydrolysis, and adsorption. These factors are minimized by "cold" storage and shipment on ice as well as test initiation within the specified USEPA guidelines. Water samples for WET testing may be manipulated in a variety of ways to comply with special requirements or circumstances. This applies, for example, when freshwater effluents are discharged to a saline receiving stream and marine or estuarine organisms are used for testing. Care must be taken, in this case, that ionic strength and composition are within levels tolerated by the specific test organisms or results may not be representative of actual toxicity or comparable between labs.

Abiotic Conditions

Abiotic conditions can strongly influence the variability of WET test results. For that reason, most of the abiotic conditions that should be standardized during WET testing (DO, light, hardness, alkalinity, etc.) are specified in protocols contained in the USEPA methods manuals. While these factors may not be problematic sources of variability within tests, they may be of major concern across tests (both within and among laboratories). Very small ranges of temperatures are specified for WET testing. Test solution pH can influence the bioavailability and toxicity of chemical constituents, such as some metals (e.g., Cu, Zn) and ammonia. Careful use of dilution waters, salinity adjustments, aeration, feeding, and other factors causing shifts in pH will help to reduce variability.

Exposure

In WET testing, we seek a balance between realistically mimicking exposure scenarios and evaluating effluents with sufficient testing while controlling testing costs. Variability in test results can be greatly influenced by the method of exposure chosen (i.e., static, static renewal, and flow-through). For example, tests of samples with nonpersistent toxicants or with chambers with high loading rates will be influenced to a greater degree using a static design rather than a flow-through design. As the number of variables which influence test results increases, overall test variability increases unless those variables are controlled. However, flow-through tests are much more costly than static tests. The number of concentrations and dilution series may influence variability of the test results. Point estimate models will more precisely estimate the statistical endpoint if the test concentrations are near the actual LCx (concentration that is lethal to x percent of organisms), ECx (concentration that affects x percent of organisms), or ICx (concentration that inhibits response by x percent). In contrast, as the NOEC approaches the concentration at which effects begin to be observed (i.e., LOEC), estimates may show greater variation. Many NPDES permits include a test dilution that is consistent with the Instream Waste Concentration (IWC) based upon dilution in the receiving system. The minimum number of tested dilutions recommended can be increased, particularly in the range of expected effects (if known), in order to improve resolution of the acute or chronic endpoint. Costs of increased dilutions testing are incremental to the cost of a typical test, but such testing is cost effective in cases where small changes in organism responses may affect compliance.

The WET endpoint is a function of test duration, in most cases (percent mortality after a period of time, for example). Test duration can be a function of the endpoint that is to be assessed. In at least one situation, the *C. dubia* survival and reproduction test, exposure duration is governed by the amount of time needed for 60 percent of the control organisms to produce a third brood (up to 8 days), at which time the test is repeated if the control performance is not acceptable (USEPA 1994b). The timing for test termination can therefore vary between 6 and 8 days. This introduces the possibility of intertest variability in terms of both number of young produced and test sensitivity due to exposure duration. The cost of reducing test duration variability is small; the corresponding reduction in test results variability could, however, be significant.

Sample Toxicity

The exposure-response relationship can be affected by the sensitivity of the test species to the individual and combined chemicals of a sample as well as the concentrations of those chemicals in that sample. Testing of samples which exhibit high slopes in their concentration-response curves at the test statistical endpoint (LCx, ECx, and ICx) tends to provide less variable (intratest and inter-test) results than tests of samples exhibiting low slopes in their concentration-response curves. The sensitivity of different species to any single chemical or mixture of chemicals can also be quite different, even when all variables are held constant. For example, rainbow trout are approximately an order of magnitude more acutely sensitive to cadmium than daphnids (USEPA 1985a) while daphnids are approximately 2.5 times more acutely sensitive to chlorine than rainbow trout (USEPA 1985b). Herbicides (e.g., atrazine) are more acutely toxic to plants than fish (Solomon et al. 1996). This is why vertebrates, invertebrates, and plants are recommended for testing effluents in the NPDES program.

Food

Food quality can vary in a number of ways. Organisms whose diets vary in nutritional quality and size, before and during testing, may respond differently to the same sample under identical test conditions. For example, brine shrimp nauplli that are less than 24 hours old are required in all tests using these organisms as food to maintain the nutritional quality of the nauplii and to keep their size at the optimum for consumption by test organisms. The YCT and algal diet for *C. dubia* should contain specific concentrations of solids and algal cells as outlined in the manual. The quantity of food available can affect dissolved oxygen and pH levels within a test chamber and act as a substrate for the absorption and adsorption of toxic chemicals from the tested sample, thus reducing bioavailability.

Dilution Water

Optimally, the dilution water should replicate the quality of the receiving water. However, if the objective of the test is to estimate the absolute toxicity of the sample (effluent), which is the primary objective of NPDES permit-related toxicity testing, then a synthetic (standard) dilution water is used (USEPA 1993, USEPA 1994a, USEPA 1994b). If the objective is to estimate the toxicity of the sample in uncontaminated receiving water, then the test may be conducted using non-toxic receiving water. Dilution water quality can affect the toxicity of effluent, surface water, and stormwater dilutions by modifying the bioavailability of toxic chemicals in the sample. In addition, parameters such as TDS (hardness, salinity, conductivity), turbidity, DO, pH, micronutrients, and bacteria counts can impact test organism physiology, sensitivity, and biological response. Therefore, test variability at all levels can be affected by variability in dilution water quality. Synthetic dilution water quality can also vary with the age of the prepared water in relation to the exposure of test organisms and with the source and quality of the base water.

Organism History and Handling

Perhaps one of the most important considerations in controlling WET variability is an organism's pretest history of health and maintenance, which consists of four factors: collection, culture, acclimation, and handling specific to the test. Organism history can be evaluated through charting performance of laboratory controls with a reference toxicant over time. All practical attempts should be made to avoid use of field-collected animals for WET testing. The most common sources of test organisms for WET tests are in-house cultures and/or organism suppliers. Organisms to be tested, whether field-collected or cultured, may require acclimation to test conditions. Variation in acclimation practices between tests can result in the use of organisms of varying sensitivity between tests. The importance of analyst technique is most pronounced when the analyst handles organisms before and during the test.

Randomization

Results will be variable in all analytical techniques, not just WET, despite all efforts to eliminate and reduce sources of variability. The randomization approach used to assign test replicates within an incubator or water bath and the approach used to assign test organisms to test replicates are attempts to evenly distribute this variability within the testing environment and between organisms. All test methods include procedures for randomization which must be followed.

Organism Numbers

The number of organisms exposed in a toxicity test has a direct and calculable bearing on the ability of that test to detect and estimate effects resulting from that exposure. Generally, as the total number of organisms increases in a test, the ability to detect effects (i.e., statistical power in a hypothesis test) and the certainty in point estimates increases. Differences in number of organisms per replicate and treatment can be due to the loss of individuals or replicates through analyst errors or to the death or lack of response of all organisms in one or more replicates. The former reduces power or effect-estimate certainty (point estimate confidence intervals) by reducing sample size. The latter may reduce power or effect-estimate certainty by increasing variation in response relative to other replicates and treatments. Intra- and interlaboratory variability can include the factors discussed above, as well as possible differences in study design (total number of organisms and total number of replicates).

Organism Age and Quality

The recommended ages of test organisms for established protocols have two general considerations: (1) relative physical sensitivity of different life stages to the test conditions, independent of the challenges of a toxicant and, (2) relative sensitivity of different life stages to toxic constituents. Young organisms are often considered more sensitive to toxic and physical stressors than their older counterparts. For this reason, the use of early life stages, such as first instars of daphnids and juvenile mysids and fish, is recommended for all tests.

The effects of organism age on WET variability are potentially greatest between tests and between laboratories where age differences may be greater. As examples, all *C. dubia* used in a reproduction test must be within 8 hours of age but can be up to 24 h old; and fathead minnow larvae used in the growth test must be within 24 hours of age in a single test but could range between 1 to 2 days depending on whether the organisms are cultured in-house or shipped from an off-site culture facility. In the acute tests with fathead and sheepshead minnows, the age difference between tests can range from <24 h to 14 d.

Grothe, D. R., K. L. Dickson, and D. K. Reed-Judkins, eds.1996. Whole Effluent Toxicity Testing: An Evaluation of Methods and Prediction of Receiving System Impacts, SETAC Press, Pensacola, FL, USA. 340 p.

Solomon, K.R., D.B. Baker, R.P. Richards, K.R. Dixon, S.J. Klaine, T.W. LaPoint, R.J. Kendall, J.M. Giddings, J.P. Giesy, L.W. Hall, Jr. and W.M. Williams. 1996. Ecological risk assessment of atrazine in North America surface waters. Environ. Toxicol. Chem. 15:31-76.USEPA. 1985a. Ambient water quality criteria for cadmium - 1984. EPA 440/5-84-032. Office of Regulations and Standards, Washington, DC.

USEPA. 1985b. Ambient water quality criteria for chlorine - 1984. EPA 440/5-84-030. Office of Regulations and Standards, Washington, DC.

USEPA. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. 4th ed. Weber C.I., editor. Cincinnati: U.S. Environmental Protection Agency (USEPA) Office of Research and Development. EPA/600/4-90/027F. 293 p.

USEPA. 1994a. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. 2nd ed. Klemm, D.J., Morrison, G.E., Norberg-King, T.J., Peltier, W.H. and Heber, M.A., editors. Cincinnati: U.S. Environmental Protection Agency (USEPA) Office of Research and Development. EPA/600/4-91/003. 341 p.

USEPA. 1994b. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 3rd ed. Lewis, P.A., Klemm, D.J., Lazorchak, J.M., Norberg-King, T.J., Peltier, W.H. and Heber, M.A., editors. Cincinnati: U.S. Environmental Protection Agency (USEPA) Office of Research and Development. EPA/600/4-91/002. 341 p.

How can WET variability be quantified?

Intratest Variability

Intratest variability is the variability of the responses (survival, growth, or reproduction), both among and between concentrations of the test material for a given test. Hypothesis test intratest variability is derived for an individual test by pooling the variability at each concentration including the control to obtain an estimate of the random error for the test. The intratest variability is used to determine the amount of difference from the control that can be detected statistically. When adjusted for the control mean, the minimum significant difference (MSD) represents the amount of difference expressed as a percentage of the control response (MSD%). Intratest variability for the point estimate approach is also represented by an estimate of the random error for the test, the mean square error (MSE). The MSE is one component in the calculation of confidence intervals for a point estimate, thus the width of a 95 percent confidence interval provides an indication of the magnitude of the intratest variability.

The intratest variability is the foremost single measure used to indicate the statistical sensitivity of a WET test analyzed with the hypothesis test approach. Statistical sensitivity, in this case, equates to a test's ability to distinguish a difference between an exposure concentration and the control. Controlling or reducing the amount of variability within a single test will increase the power of the test and therefore the ability of the test to detect responses that differ from the control response (decrease MSD). Increased power will also increase certainty in the determination of a difference from controls, which is important to regulators and the regulated community. However, minimal variability in all treatments of a test may lead

to such high statistical power that detected differences may not be biologically significant. Such tests should be interpreted with caution. Although there is no specific guidance from the USEPA on statistical versus biological significance, various States and USEPA Regions have developed some guidelines (e.g., see SETAC FAQ on addressing variability). Close attention to the factors described under the FAQ on factors affecting variability will tend to decrease heterogeneity among replicates and decrease intratest variability. In addition, increasing the number of replicates will also lead to an increase in the sensitivity of the test by decreasing the MSD.

Intratest variability is also important in representing the uncertainty associated with point estimates of toxicity. As the 95 percent confidence intervals of the point estimate increases, the uncertainty in that estimate of the statistical endpoint increases. The confidence intervals for chronic endpoints are directly influenced by the variability of response between replicates in each treatment and the model used to interpolate the point estimate. The confidence intervals for acute test results using a point estimate approach, however, are not influenced by variability between replicates but by the characteristics of the dose-response relationship. As discussed before, the certainty in point estimates is also a function of the dilutions tested and their proximity to the actual statistical endpoint being calculated. One will get a better estimate of the LC50 (tighter confidence intervals) if dilutions are tested near the concentration which actually results in 50 percent mortality.

Evaluation of a number of existing data sets by members of the Pellston workgroup (Sessions 3 and 4) (Grothe, et al, 1996) seemed to indicate that, for most WET test methods, MSDs of <40 percent were achievable. MSD's for most methods examined ranged from 18 percent to 40 percent. The consensus of the workgroup is that an additional study is necessary to determine the acceptable level of intratest variability for each USEPA recommended toxicity method, although some participants proposed that sufficient data exists to select MSD criteria. In the proposed study, data would be used to establish variability limits from laboratories that document data quality and adhere to USEPA method guidelines. Study data from each assay evaluation would include expected CVs, MSD, MSD%, MSE, and American Society for Testing and Materials (ASTM, 1992) "h" and "k" statistics. The "h" statistic represents a measure of the reproducibility between laboratories while the "k" statistic represents the repeatability within laboratories. Distributions of these values would be examined to determine criterion levels for intratest variability, and probabilities of laboratories exceeding the criterion levels would be calculated. The direct advantages of an acceptability criterion for intratest variability are 1) establishing a minimum protection level, 2) setting the power of a test to detect a toxic sample for each method, and 3) decreasing intra- and interlaboratory variability. Acceptability criteria will also allow users of WET data to better evaluate test acceptability, laboratory performance, and program effectiveness.

Intertest and Interlaboratory Variability

The scientific community familiar with analytical procedures, not just WET, recognizes that tests performed on presumably identical materials in presumably identical circumstances do not typically yield identical results. An indication of a test method's consistency is its repeatability and its reproducibility with repeatability defined as the variability between independent test results obtained from the same laboratory in a short period of time and reproducibility defined as the variability between test results obtained from different laboratories.

Several measures of repeatability and reproducibility have been proposed. The simplest of these is the intra- and interlaboratory CV (standard deviation (s) of repeated test results, divided by the mean (m) of the repeated test results, multiplied by 100 (CV = (s/m) x 100). The intralaboratory CV is generated by test results from repeated tests performed in the same laboratory, while the interlaboratory CV is obtained from test results from several different laboratories. The use of the CV removes from consideration the units of the measurement and allows the analyst to compare variability of different types of test methods (i.e., WET tests with analytical chemistry tests). It also allows analysts to compare tests that use different scales of measurement.

However, CVs alone cannot be used as diagnostic tools to help identify unusual test values or outliers. Since the CV is a function of the standard deviation of a set of test results, the measure suffers from the same problems associated with standard deviations, and there is no common agreement on what is an acceptable standard deviation. For instance, the range of test values is an easier descriptive statistic to understand. In addition, the value of the standard deviation is affected by extreme values in the data set; single large or small test values inflate the standard deviation. The CV also ignores the 95 percent confidence intervals (uncertainty) associated with each point estimate and can only be calculated for point estimates. CVs are not appropriate for hypothesis test endpoint comparisons since the effect levels are fixed by the choice of test concentrations.

Quality Management Considerations. Reference toxicant tests are typically used to monitor a laboratory's performance. Charting the performance of a laboratory's controls relative to its reference toxicant test results is a good way to track the laboratory's performance and to identify when the laboratory's performance is not acceptable. The width of a control chart's limits is an indication of a laboratory's capability to reproduce the desired endpoints of a reference toxicant test. However, control chart limits are a function of the reference toxicant, test species, test type (acute or chronic) and biological endpoint (survival, growth, etc.). These factors must be considered before drawing conclusions regarding laboratory performance. Performance on reference toxicant tests as recorded by control charts should be a criterion that is used by permittees in selecting which laboratories to use for WET tests.

Laboratories with very wide control limits, and/or many points outside of the control limits, should investigate problems related to the quality of the data being produced. Laboratories should monitor at a minimum, using control charts, the calculated endpoints for each test type/species combination. Laboratories can also monitor the control treatment mean response for survival, growth, and reproduction. In addition, laboratories can chart the control treatment replicate variance, or standard deviation. Reference toxicant tests are very important to track analyst technique and the health and condition of the test organisms. It is particularly important when performing these tests (as with all compliance toxicity tests) that the analysts precisely follow the published test methods, without deviation between tests.

ASTM-American Society for Testing and Materials. 1992. Standard practice for conducting an interlaboratory study to determine precision of a test method, E691-92. In: *Annual Book of ASTM Standards*, Vol. 14.02. Philadelphia, PA.

Grothe, D. R., K. L. Dickson, and D. K. Reed-Judkins, eds.1996. Whole Effluent Toxicity Testing: An Evaluation of Methods and Prediction of Receiving System Impacts, SETAC Press, Pensacola, FL, USA. 340 p.

APPENDIX E

EXAMPLES OF SELECTED STATE WET IMPLEMENTATION PROGRAMS

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EXAMPLES OF SELECTED STATE WET IMPLEMENTATION PROGRAMS

Appendix E contains summaries of approaches that States have taken in implementing their NPDES whole effluent toxicity (WET) programs and efforts instituted to reduce or ensure minimal test variability when conducting WET tests. Preceding the State responses is a matrix (Table E-1) that briefly summarizes the common approaches or program themes for the States that responded. The respondent States are a geographic sampling across the United States. EPA's inclusion of the various State approaches in this document is not an endorsement of their approaches, but a snapshot of additional steps that a permitting authority could consider taking beyond the minimum requirements (i.e., test acceptability criteria) outlined in EPA guidance. This sample of State approaches also responds to recommendations EPA received on the initial draft document to consider and provide reference to other State approaches.[1]

[1] Note that the terms, abbreviations, and acronyms used in this appendix may differ from their usage throughout the rest of this document. EPA consciously chose not to edit the State-supplied information so that the actual States' nomenclature and terminology as used in their NPDES programs would be reflected here.

Table E-1. Overview of Selected State WET Implementation Programs

ST	How do you evaluate reference toxicant & effluent test results?	How do you review reference toxicant test data for laboratory performance?	Describe additional QA/QC criteria developed & implemented.
KY	Acute—point-estimation Chronic—linear interpolation	Labs submit annual summary of RTT data, used to determine consistency & conformance with expected values.	1. Monthly acute/chronic RTT within 30 days of each WET test. 2. RTT conducted on each batch of purchased test organisms unless supplier provides information. 3. Culturing & testing in different incubators. 4. Chronic toxicity tests with CV > 40% evaluated on case-by-case basis.
NJ	Acute—point-estimation Chronic—linear interpolation	RTT results reported on standardized form, with UCL & LCL. Control charts submitted annually. RTT data reviewed in on-site audit.	1. C. *dubia* test: Number of males in surviving organisms over all concentrations $\leq$10%; number of males in controls $\leq$20%. 2. For all tests, no sporadic moralities present; $\leq$10% variation per concentration in start count. 3. For tests indicating permit violation, review raw data & test results (data trend, MSD, chain-of-custody, sample handling/holding time).
NC	Acute—point-estimation Chronic—linear interpolation Acute pass/fail, chronic pass/fail, & chronic multi-concentration effluent tests—hypothesis tests	RTT data reviewed during annual lab inspection. Lab provides bench sheets, water quality data, calculations, control charts, etc. Information reviewed for test frequency, test conditions, test result validity, & responses to out-of-control events.	1. Dilution water pH 6.5 -8.5, total hardness 30 - 50 ppm. 2. Biweekly acute RTT or within 7 days of any NPDES test. 3.Test organisms identified to species once/quarter. 4. Culturing & testing in different incubators. 5.Chronic *C. dubia* test: 3rd brood neonate production $\geq$ 80% of control; neonate reproduction from 1st - 3rd broods only; % male control organisms $\leq$ 20%; control reproduction CV $\leq$ 40%; solution DO $\geq$ 5.0 mg/l; exposure duration at least 7 d ± 2 h. 6. Acute tests terminated w/i 1 h of stated length.
WA	Acute—point estimation Chronic pass/fail, & chronic multi-concentration effluent tests—hypothesis tests	1. Review data in conjunction with effluent tests. 2. Lab provides bench sheets, water quality data, calculations, control charts, etc. Information reviewed for test frequency, test conditions, test result validity, & responses to out-of-control events. 3. If reference CV does not meet certain criteria, test is rejected.	1. Minimum % difference in survival between IWC & control (or NOEC for chronic) that is statistically significant: acute–30%, chronic–40%. 2. Tests failing must be repeated with more replicates. 3. Specific requirements for *Ceriodaphnia* & bivalve chronic tests.
WI	Acute & chronic—point-estimation Certified labs perform monthly reference toxicant tests	RTT data reviewed by auditor before on-site lab inspections. Lab provides bench sheets, water quality data, control charts, etc. Information reviewed for test frequency, proper test conditions, test result validity, & proper responses to out-of-control events.	1. Testing & culturing in separate rooms or incubators 2. Additional or revised TAC for Static Renewal Acute & Chronic Tests will be included in revised methods.

Abbreviations: CV = coefficient of variation; DO = dissolved oxygen; IWC = instream waste concentration; LCL = lower control limit; MSD = minimum significant difference; NOEC = no observed effect concentration; NPDES = National Pollutant Discharge Elimination System; ppm = parts per million; RTT = reference toxicity test; SETAC = Society of Environmental Toxicology and Chemistry; SOP = standard operating procedures; TAC = technical acceptability criteria; UCL = upper control limit; WET = whole effluent toxicity

Table E-1. Overview of Selected State WET Implementation Programs (continued)

ST	Describe efforts to minimize test method variability.	Explain how you review or conduct lab performance audits.	Describe specific implementation guidance developed for permit writers. How is the guidance available to the public?	Describe how you provide or use toxicity test training.
KY	1. Reporting on standardized form. 2. Labs submit culturing/testing SOP for State review. 3. Tests must comply with all EPA & State test manuals. 4. Dilution water moderately hard-reconstituted water or dilute mineral water. 5. Follow written protocol for splits. 6. Lab audits by State or EPA Region.	EPA Region or State conducts lab audits, following procedures in EPA inspection manual.	1. Several guidance documents developed by State. 2. Face-to-face training as needed, also available to the public. 3. Some documents available on web or through newsletters.	1. State communicates program changes & guidance on culturing & testing issues through newsletter & web page. 2. Training sessions for State personnel. 3. Participate in Wastewater Operator's Conference to discuss issues with regulated community & consultants. 4.Teach in SETAC's WET training & statistical analysis courses.
NJ	1. Standardized checklist for screening submitted tests. 2. Lab certification program, on-site audits. 3. Labs report premature cancellation of test & reason. 4. Quarterly meetings of State/lab representatives to discuss current test developments.	1. Inspections announced or unannounced. Lab SOPs reviewed for adherence to NJ & EPA protocols. Subsets of data reviewed, technician summarizes problems with test reports. 2. Inspections consist of opening conference, lab walk-through, closing conference. SOP review, problematic test results review. Auditor examines equipment, documentation, cultures, lab procedures, chain-of-custody, sample handing. Review of inspection results during closing conference.	1. Training sessions to permit writers & public. 2. Written guidance is copies of past training sessions on shared drive for permit writers. Generally not available to public.	Staff attend USEPA or SETAC-sponsored training.

Abbreviations: CV = coefficient of variation; DO = dissolved oxygen; IWC = instream waste concentration; LCL = lower control limit; MSD = minimum significant difference; NOEC = no observed effect concentration; NPDES = National Pollutant Discharge Elimination System; ppm = parts per million; RTT = reference toxicity test; SETAC = Society of Environmental Toxicology and Chemistry; SOP = standard operating procedures; TAC = technical acceptability criteria; UCL = upper control limit; WET = whole effluent toxicity

Table E-1. Overview of Selected State WET Implementation Programs (continued)

ST	Describe efforts to minimize test method variability.	Explain how you review or conduct lab performance audits.	Describe specific implementation guidance developed for permit writers. How is the guidance available to the public?	Describe how you provide or use toxicity test training.
NC	1. Review submitted test results against TAC. 2. Implement lab certification program. 3. Document investigations of differing test results from splits of effluent samples. 4. Test protocol modifications.	1. Inspections announced or unannounced. Lab SOPs reviewed for adherence to NJ & EPA protocols. Subsets of data reviewed, technician summarizes problems with test reports. 2. Inspections consist of opening conference, lab walk-through, closing conference. SOP review, problematic test results review. Auditor examines equipment, documentation, cultures, lab procedures, chain-of-custody, sample handing. Review of inspection results during closing conference.	1. Written guidance established by memo. Face-to-face training sessions as needed. 2. Written guidance available to public upon request, also sent to permit holders with permit & subsequent renewals. Also available on the web.	1. Participate in aquatic toxicologists group. Communicate program changes & guidance on culturing & testing issues through meetings. 2. Workshops held for Division's regional office personnel 3. Attend SETAC's WET training & statistical analysis courses.
WA	1. Develop, distribute *Laboratory Guidance and Whole Effluent Toxicity Test Review Criteria*. 2. Review tests for compliance with method & canary book. 3. Fish/mysid growth tests with SD of proportion alive > 0.25 in effluent concentration analyzed for original growth endpoint, not combined endpoint. 4. Permit requirements will lower alpha level for hypothesis testing when differences in test organism response are small. 5. Anomalous test identification criteria established to make WET test results fair & enforceable.	1. Inspections announced or unannounced. Lab SOPs reviewed for adherence to NJ & EPA protocols. Subsets of data reviewed, technician summarizes problems with test reports. 2. Inspections consist of opening conference, lab walk-through, closing conference. SOP review, problematic test results review. Auditor examines equipment, documentation, cultures, lab procedures, chain-of-custody, sample handing. Review of inspection results during closing conference. 3. Audit report prepared within 30 days of audit 4. Performance audits required twice/year, system audits every three years.	Develop & update language for use in NPDES permits & fact sheets for POTWs & industry. Language is part of templates for permits & fact sheets that permit writers use as they draft permits. Manual available to the public for cost of printing & also on Web.	Extensive training in all offices early in 1990s. WET test review & technical assistance are centralized functions, permit writing guidance available in *Permit Writer's Manual* & suggested permit language.

Abbreviations: CV = coefficient of variation; DO = dissolved oxygen; IWC = instream waste concentration; LCL = lower control limit; MSD = minimum significant difference; NOEC = no observed effect concentration; NPDES = National Pollutant Discharge Elimination System; ppm = parts per million; RTT = reference toxicity test; SETAC = Society of Environmental Toxicology and Chemistry; SOP = standard operating procedures; TAC = technical acceptability criteria; UCL = upper control limit; WET = whole effluent toxicity

Table E-1. Overview of Selected State WET Implementation Programs (continued)

ST	Describe efforts to minimize test method variability.	Explain how you review or conduct lab performance audits.	Describe specific implementation guidance developed for permit writers. How is the guidance available to the public?	Describe how you provide or use toxicity test training.
WI	1. Review submitted test results against TAC. 2. Lab certification program 3. Document investigations of differing test results from splits of effluent samples. 4. Strict adherence to clearly specified methods, such as sampling procedures, holding times, test duration. 5. Revising methods to require that labs verify staff training & qualifications.	1. Inspections announced or unannounced. Auditor reviews laboratory SOPs & recent RTT results for adherence to WDNR protocols. 2. Inspections consist of opening conference, lab walk-through, closing conference. SOP review, problematic test results review. Auditor examines equipment, documentation, cultures, lab procedures, chain-of-custody, sample handing. Review of inspection results during closing conference. 3. Auditor reviews reference toxicant data after inspection, generates inspection letter. Lab has 60 days to respond. Significant deficiencies may result in decertification.	Written guidance & clarification on existing rules & methods for State staff, permittees, labs, consultants, others.	1. One-on-one training for State staff & permittees. 2. University lab provides hands-on WET training to State staff, permittees, labs on request. 3. Attend SETAC's WET training & statistical analysis courses.

Abbreviations: **CV** = coefficient of variation; **DO** = dissolved oxygen; **IWC** = instream waste concentration; **LCL** = lower control limit; **MSD** = minimum significant difference; **NOEC** = no observed effect concentration; **NPDES** = National Pollutant Discharge Elimination System; **ppm** = parts per million; **RTT** = reference toxicity test; **SETAC** = Society of Environmental Toxicology and Chemistry; **SOP** = standard operating procedures; **TAC** = technical acceptability criteria; **UCL** = upper control limit; **WET** = whole effluent toxicity

E.1 RESPONSES FROM KENTUCKY DEPARTMENT FOR ENVIRONMENTAL PROTECTION

E.1.1 Describe How Your State Evaluates Reference Toxicant and Effluent Test Results

Acute reference toxicant test and multi-concentration effluent test results are evaluated using the point-estimate (LC50) technique described in the EPA acute testing manual.

Chronic reference toxicant and multi-concentration effluent test results are evaluated using the linear interpolation method (IC25) as described in the EPA chronic manual and using the TOXCALC statistical program software.

E.1.2 Explain How Your State Reviews Reference Toxicant Data for Laboratory Performance

Consulting laboratories that service permittees are required to annually submit to the Bioassay Section a summary of their reference toxicant test data. This information is used to determine consistency and conformance to the expected values. This serves as a review and audit of all consulting laboratories, measures consistency within a laboratory, and provides a level of reliability and accuracy between laboratories.

A letter of request is sent to each laboratory with a standardized response form. The labs provide the requested information, including test date, dilution series, type of control water, organism age, LC50/IC25, 95 percent confidence interval, and average control reproduction/weight. This information is entered into a laboratory QA data base where it is statistically analyzed.

This information is then compiled into an annual summary report. The compiled information includes the lab name, reference toxicant, test species, test type, test duration, number of tests performed, mean, standard deviation (SD), % coefficient of variation (CV), average reproduction, or growth with SD and % CV.

The results are mailed to each participating laboratory. In addition, the summary results are printed in the Kentucky Biomonitoring Newsletter and are presented on the Bioassay Section's web page (http://water.nr.state.ky.us/wq/bioassay/index.html).

A control chart is prepared for each reference toxicant and organism combination, and successive toxicity values are plotted and examined to determine if the results are within prescribed limits. A minimum of 30 test results are needed for a reliable mean and upper/lower control chart. If the toxicity value from a given test with the reference toxicant does not fall within the expected range for the test organism when using the standard dilution water, then the sensitivity of the organisms and the overall credibility of the test systems are suspect. In this case the test procedure, control water, and reference toxicant are examined.

Missing and/or out-of-range data must be explained and can result in the invalidation of Kentucky Pollution Discharge Elimination System (KPDES) WET test results.

E.1.3 Describe Any Additional QA/QC Criteria Your State Has Developed and Implemented Within Your State

1. Acute and chronic reference toxicant tests are to be conducted monthly. A reference toxicant test must be conducted within 30 days of each KPDES WET test.

2. If test organisms are purchased from a commercial supplier, a reference toxicant test must be conducted on each batch unless the supplier can provide this information.

3. Culturing and testing activities may not be contained within the same incubator.

4. Chronic toxicity tests where the coefficient of variation (CV) is greater than 40 percent will be evaluated on a case-by-case basis to determine if the results will be considered acceptable.

5. All other QA/QC criteria for culturing and testing, as set forth in the most current editions of the EPA manuals, must be followed.

E.1.4 Describe Any Efforts Your State Has Made to Minimize Test Method Variability

1. All KPDES WET test results are submitted using a standardized report form. Each report is closely reviewed by a member of the Bioassay Section to determine if proper test protocols have been followed.

2. Prior to conducting toxicity test for Kentucky permittees, each laboratory must submit its culturing/testing SOP for review by the Bioassay Section. This insures that proper methods and procedures are being followed.

3. Toxicity tests must comply with all conditions as stated in the EPA testing manuals and in the Kentucky Methods for Culturing and Conducting Toxicity Tests with *Pimephales promelas* and *Ceriodaphnia dubia*. (Fourth Edition, 1996). Special attention is paid to sample holding times and temperatures.

4. Dilution water is to be moderately hard-reconstituted water or moderately hard dilute mineral water.

5. If split samples are going to be used, the Biomonitoring Split-Sample Protocol must be followed. This protocol details sample collection and holding procedures as well as test conditions that must be followed.

6. Laboratories must submit all reference toxicant data for the annual summary. This information assists in determining the quality of information being received from these facilities.

7. Laboratories are audited by Kentucky or EPA Region IV to review testing and culturing procedures.

E.1.5 Explain How Your State Reviews or Conducts Performance Lab Audits

Kentucky has been fortunate in having the expertise of EPA Region IV in performing WET laboratory audits. Their experience has proven beneficial in keeping laboratories compliant with the testing requirements. When the services of EPA are not available, the State will conduct its own lab audits. In either case, the procedures are the same and follow those outlined in the EPA inspection manual.

Inspections are usually announced. If EPA is performing the inspection, a representative from the Bioassay Section will accompany the inspectors. Prior to the inspection, the auditor will review the laboratory's SOP for adherence to Kentucky and EPA protocols. Bioassay Section staff will review test reports to document any problems with the subject lab. In addition, the qualifications of the staff will be reviewed at this time. Generally, three test reports will be chosen for which the laboratory will be required to produce supporting documentation.

The inspection consists of an opening conference, a walk-through of the laboratory, and a closing conference. During the opening conference, the auditor discusses the SOP review and general procedures in the laboratory. In addition, information including culturing records, test data, chain of custody records, reference toxicant data, etc., supporting the three test reports selected prior to the inspection will be reviewed. During the walk-through, the auditor examines equipment, log books, written documentation and laboratory procedures. The closing conference serves as a review of observations and comments during the inspection.

The auditor will generate an inspection response letter detailing any deficiencies noted during the audit. All correspondence is addressed to the permittee, whose test results were used for the inspection. The permittee will have usually 60 days to respond to the deficiencies, noting what actions have been taken by the laboratory to correct them. If significant deficiencies are not addressed, then future data from this laboratory may not be accepted by the State.

E.1.6 Describe Any Specific Implementation Guidance That Your State Has Developed to Assist Permit Writers. How Is the Guidance Available to the Public?

Guidance is provided through several documents developed by the Bioassay Section. This section has developed standardized biomonitoring language, which is provided to the KPDES Permitting Branch. This language is incorporated into each permit with a WET limit or monitoring upon permit issuance or reissuance. In addition, a Standard Test Result Report form is provided to each permit holder with WET. The section has another document: Aquatic Toxicity Testing: Questions and Answers, which is available upon request.

The Bioassay Section provides face-to-face training to the KPDES Branch on an as-needed basis. This training is also available to the public if requested.

Some documents are available on the Bioassay Section's web page or through the Biomonitoring newsletter.

E.1.7 Describe How Your State Provides or Utilizes Any Toxicity Testing Training

The Bioassay Section communicates program changes and specific guidance on culturing and testing issues through the newsletter and the web page. The section has held several training sessions for State personnel since the inception of the program. In addition, the section participates in the State's annual Wastewater Operator's Conference to discuss issues with the regulated community and consultants.

Section members have attended and participated as instructors in the Society for Environmental Toxicology and Chemistry's two-day WET training course and statistical analysis course.

E.2 RESPONSES FROM NEW JERSEY DEPARTMENT OF ENVIRONMENTAL PROTECTION

E.2.1 Describe How Your State Evaluates Reference Toxicant and Effluent Test Results

Acute effluent tests are evaluated using the point estimate techniques described in the USEPA acute methods document. New Jersey also uses the NOAEC endpoint set equal to 100 percent effluent when an evaluation of no acute toxicity is required. The hypothesis testing techniques contained in the USEPA manual are used in that case.

Requests have been received from certified laboratories and from permittees that the point estimate techniques be further standardized. Using one version of Probit versus another can result in a different value, sometimes making a difference whether a facility passes or fails.

Chronic effluent and reference toxicant test results are evaluated using the linear interpolation method originally provided by Teresa Norberg King (July 1993). A p value of 25 is selected for all permits and for reference toxicant recording.

E.2.2 Explain How Your State Reviews Reference Toxicant Data For Laboratory Performance

New Jersey Pollution Discharge Elimination System (NJPDES) permits require that in order for chronic toxicity test results to be considered acceptable, there must be an acceptable Standard Reference Toxicant (SRT) result conducted within 30 days of the compliance test result, for the test species and reference toxicant in question. The States standardized report form requires the reporting of the applicable SRT result directly on the compliance test report, along with the applicable upper and lower control limits. Missing or out of range data can result in the invalidation of test results.

Control charts are forwarded to the Department on an annual basis, on the anniversary of the approval for the test species. Many labs have chosen to include copies of applicable control charts with the submittal of compliance test results. SRT data is also reviewed as part of an on-site audit, including a review of procedures, raw data, and data analysis any excluded results.

State methods governing laboratories also require that if a lab produces any SRT test result which is outside the established upper and lower control limits for a test species at a frequency greater than one test in any ten tests, a report shall be forwarded to the Department. That report shall include any identified problem which caused the values to fall outside the expected range and the corresponding actions that have been taken by the laboratory. If a laboratory produces two consecutive SRT test results or three out of any ten test results, which are outside the established upper and lower control limits for a specific test species, the laboratory shall be unapproved to conduct testing. Reapproval is contingent upon the laboratory producing SRT test results within the established upper and lower limits.

The laboratory selects the reference toxicant used. However, the Department recommends using KCl.

E.2.3 Describe Any Additional QA/QC Criteria Your State Has Developed and Implemented With Your State

For Ceriodaphnia testing:

- Number of males in surviving organisms overall concentration $\leq$10 percent [(no. males / total no. surv) x 100].
- Number of males in controls $\leq$20 percent (no. males / total no. organisms in controls).

All test species

- No sporadic mortalities present (Deaths that are not related to sample toxicity, confined to a few test chambers and scattered throughout the test).
- Variation in start count must be $\leq$10 percent per concentration (animals lost or killed by accident).

These items are specifically included on standardized review sheets.

For any tests that would result in the collection of penalties based on violation of an effective toxicity limit, a detailed review of the raw data and test results are conducted, including review of the data trend, minimum significant difference, chain-of-custody, sampling handling, and holding times.

E.2.4 Describe Any Efforts Your State Has Made To Minimize Test Method Variability

Each test that is submitted receives at least a screening using a standardized check list, anywhere from 30 to 40 questions depending upon the test species, dealing will all aspects of the test.

New Jersey maintains a laboratory certification program for toxicity testing, including on-site audits.

A laboratory who cancels a test prior to the scheduled ending time/date must report that cancelled test, including the reason for the cancellation, to the Department. This allows the Department to track a laboratory's ability to run a test to completion. Tests that do not meet USEPA's test acceptability criteria are not submitted to the Department since they are not valid. This way the frequency that this is occurring at a laboratory can be tracked. Frequent test cancellations are addressed during an on-site audit.

New Jersey has a Bioassay Subcommittee that is a subset of the State's Laboratory Advisory Committee. This committee meets quarterly and consists of State and laboratory representatives. The committee discusses problems with the tests, certification, updates from USEPA, SETAC, NELAC, or anything else applicable to toxicity testing. This gives the laboratories and the State an opportunity to discuss either deficiencies that are occurring at laboratories and are showing up in the test data, problems the laboratories are having with regard to any of the methods, and any improvements to the program that should be easily implemented.

E.2.5 Explain How Your State Reviews Or Conducts Performance Lab Audits

Inspections can be announced or unannounced, although generally time is not adequate to perform unannounced inspections. Prior to the inspection, the auditor will review the laboratory's SOPs for adherence to New Jersey and EPA protocols. Subsets of data will also be reviewed and the technician responsible for day to day screening using the standardized check list is asked to summarize any problems with the review of toxicity test reports.

The actual inspections consist of an opening conference, a walk-through of the lab facility, and a closing conference. During the opening conference, the auditor discusses the SOP review and general procedures in the lab. In addition she will request and review-supporting information associated with the any test reports identified prior to the inspection as a concern. During the walk-through, the auditor examines equipment, written documentation, cultures, laboratory procedures, chain-of-custody, and sample handing. The closing conference serves as a review of observations and comments during the inspection.

E.2.6 Describe Any Specific Implementation Guidance That Your State Has Developed To Assist Permit Writers. How Is The Guidance Available To The Public?

The Office of Quality assurance provides training sessions to the permit writer and the public upon request. Written guidance consists of copies of past training sessions, located on the share drive for permit writers. This guidance is not generally available to the public.

E.2.7 Describe How Your State Provides Or Utilizes Any Toxicity Testing Training

When possible, staff will attend any USEPA- or SETAC-sponsored training on the topic.

E.3 RESPONSES FROM NORTH CAROLINA DEPARTMENT OF ENVIRONMENT AND NATURAL RESOURCES

E.3.1 Describe How Your State Evaluates Reference Toxicant and Effluent Test Results

Acute reference toxicant test and multi-concentration effluent test results are evaluated using the point-estimation techniques described in the EPA manual.

Acute pass/fail, chronic pass/fail, and chronic multi-concentration effluent test results are evaluated using hypothesis tests as described in the EPA manuals.

Chronic reference toxicant test results are evaluated using the linear interpolation method (ICp, where p=25) described in the EPA manual.

For both types of chronic *Ceriodaphnia* effluent tests, a reproductive effect is defined by both a statistically significant difference between the treatment and the control and a 20 percent reduction in neonate reproduction of the treatment organisms as compared to the controls. Hypothesis tests for both acute and chronic pass/fail tests are performed at an alpha level of 0.01.

E.3.2 Explain How Your State Reviews Reference Toxicant Data for Laboratory Performance

The data is reviewed in conjunction with the laboratory's annual laboratory inspection. The laboratory provides copies of bench sheets, water quality data, and calculations or printouts from the data analysis for each reference toxicant test performed since the last laboratory inspection:

In addition, the lab submits the current control chart (with data listing) and any explanations of out-of-range test results for each test type and organism combination.

The materials are reviewed for appropriate test frequency, proper test conditions, test result validity, and proper responses to out-of-range events.

Missing or out-of-range data can result in the invalidation of NPDES test results.

E.3.3 Describe Any Additional QA/QC Criteria Your State Has Developed and Implemented Within Your State

— Laboratories must use dilution water in whole effluent toxicity testing with chemical characteristics such that the pH is between 6.5 and 8.5 and total hardness as calcium carbonate is between 30 and 50 µg/l as calcium carbonate.

— Acute and chronic reference toxicant tests must be performed once every two weeks or within one week of any NPDES tests.

— A representative of each test organism cultured shall be taxonomically identified to the species level at a minimum frequency of once per quarter.

— If closed incubators (refrigerator-sized) are utilized for toxicity testing and/or test organism culturing purposes, culturing and testing activities may not be contained within the same incubator.

— Chronic *Ceriodaphnia dubia* analyses will have an additional test acceptability criterion of complete third brood neonate production by at least 80 percent of the control organisms.

— *Ceriodaphnia dubia* neonate reproduction totals from chronic tests shall include only organisms produced in the first through third broods.

— The percentage of male *Ceriodaphnia* control organisms may not exceed 20 percent in chronic *Ceriodaphnia* tests.

— The *Ceriodaphnia* control organism reproduction coefficient of variation (CV) must be less than 40 percent for a chronic *Ceriodaphnia* test to be considered acceptable.

— *Ceriodaphnia* chronic test solutions must maintain dissolved oxygen levels greater than or equal to 5.0 mg/l.

— *Ceriodaphnia* chronic test exposure duration will be no greater than seven days ± 2 hours regardless of control organism reproductive success.

— Acute tests will be terminated within one hour of their stated length.

E.3.4 Describe Any Efforts Your State Has Made to Minimize Test Method Variability

1. Close review of each test result submitted with consistent adherence to test protocol test acceptability criteria.

2. Implementation of a biological laboratory certification program.

3. Paper trail investigations of test results from disagreeing “split” effluent sample analyses.

4. Test protocol modifications.

EPA methods allow for a relatively wide window for termination of the chronic *Ceriodaphnia* test. Tests may be terminated as soon as 60 percent of the control organisms produce three broods of young or as late as eight days after test initiation. Logically, narrowing the termination window will reduce variability and improve precision of test results. The North Carolina Division of Water Quality (NC DWQ) has narrowed the window available for the termination of the chronic *Ceriodaphnia* test by:

— Placing a shorter limit on the exposure period (seven days + two hours)

— Requiring that at least 80 percent of the control organisms produce a third brood prior to test termination

Analysis of a data base of NC chronic *Ceriodaphnia* test results has shown that reducing control organism reproduction variability improves the sensitivity of the reproduction analysis. Logically, holding all labs to a common precision standard with respect to control organism reproduction should reduce between-lab test result variability. The Division has reduced variability of control organism reproduction by:

— Implementing a test acceptability criterion limiting the control organism reproduction coefficient of variation to less than 40 percent

— Requiring that at least 80 percent of the control organisms produce a third brood prior to test termination

— Excluding fourth and subsequent brood neonates from the reproduction effects analysis

DWQ's experience has shown that high quality laboratories can produce extremely sensitive tests that can detect quite small differences between treatment and control reproduction. Unfortunately, this can be a disincentive for laboratories to produce high quality tests, since experience has shown that some clients gravitate toward laboratories that produce compliant test results. Less sensitive tests will be more likely to produce compliant results. Analysis of reproduction data from the same data base described above indicated that tests performed by NC certified labs could routinely detect a difference between the control and a treatment when there was a 20 percent reduction in neonate reproduction by the treatment organisms compared to the controls. Based on this data, NC DWQ has placed a second data evaluation criterion on the *Ceriodaphnia* chronic reproduction analysis. Specifically, for an effluent treatment to be considered producing an effect, the reproduction mean must be both statistically significantly lower than the control mean **and** represent at least a 20 percent reduction from that mean. In effect, this sets a lower limit on test sensitivity and also reduces within-laboratory and between-laboratory test result variability.

E.3.5 Explain How Your State Reviews or Conducts Performance Lab Audits

Inspections may be announced or unannounced. Prior to the inspection, the auditor will review the laboratory's SOP for adherence to North Carolina and EPA protocols. The Aquatic Toxicology Unit member responsible for reviewing test report submittals will be requested to summarize any recurring problems with the target laboratory regarding data submission. Three test reports will be chosen for which laboratory personnel will be asked to produce supporting documentation.

The actual inspection consists of an opening conference, a walk-through of the laboratory facilities, and a closing conference. During the opening conference, the auditor discusses the SOP review and general procedures in the laboratory. In addition he/she will request and review supporting information associated with the three test reports selected prior to the inspection. During the walk-through, the auditor examines equipment, written documentation, cultures, and laboratory procedures. The closing conference serves as a review of observations and comments during the inspection.

The auditor will review reference toxicant data (see question 2 above) after the inspection. Within two weeks, the auditor will generate an inspection response letter, to which the laboratory will be given 60 days to respond. If there are significant deficiencies discovered during the inspection, a laboratory or categorical decertification may occur.

E.3.6 Describe Any Specific Implementation Guidance That Your State Has Developed to Assist Permit Writers. How Is the Guidance Available to the Public?

Written guidance is established by memo from the Water Quality Section Chief to the NPDES Permitting Unit and other affected Water Quality Section Units. The Aquatic Toxicology Unit provides face-to-face training sessions to the NPDES Unit on an as-needed basis.

The written guidance in memo form is available to the public upon request. Parts of the guidance are included in a document called "Aquatic Toxicity Testing: Understanding and Implementing Your Testing Requirement," that is disseminated to each permit holder with a WET limit or monitoring requirement upon

permit issuance and subsequent renewals. The document is also available at the Aquatic Toxicology Unit web page, http://www.esb.enr.state.nc.us/ATUwww.default.html.

E.3.7 Describe How Your State Provides or Utilizes Any Toxicity Testing Training

NC DWQ actively participates in the Carolinas Area Aquatic Toxicologists group (CAAT). The Aquatic Toxicology Unit utilizes the meetings of this group to communicate program changes and specific guidance on culturing and testing issues. Additionally, the Unit has held two workshops for the Division's regional office personnel since the inception of the aquatic toxicity testing program. Unit members have attended The Society of Environmental Toxicology and Chemistry's two-day WET course and statistical analysis course.

E.4 RESPONSES FROM WASHINGTON DEPARTMENT OF ECOLOGY

E.4.1 Describe How Your State Evaluates Reference Toxicant and Effluent Test Results

The State of Washington Department of Ecology reviews every WET test report for compliance with the test method and instructions in the permit. Permit instructions include reference to a document called "Laboratory Guidance and Whole Effluent Toxicity Test Review Criteria" that provides the lab with standard testing instructions and provides the basis for test report review. Reference toxicant tests are not evaluated separately but are evaluated as a part of the review of WET test reports. The Department of Ecology also maintains a data base of WET test raw data and statistical results in order to have comprehensive records for each discharger and to enhance our ability to learn from experience and improve our WET program.

E.4.2 Explain How Your State Reviews Reference Toxicant Data for Laboratory Performance

The minimum reference toxicant testing needed to meet our interpretation of the requirements in the EPA manuals (both sections 4.7 and 4.16) is one per month for every acute and 7-day (short-term) chronic test species used routinely (more than once per month). Because an acute test result can be determined during a 7-day chronic test, acute and chronic reference toxicant testing for a fish or mysid can be combined. If a lab has difficulty establishing a concentration series that produces good results for both a lethal and sublethal endpoint, the lab may focus on lethality, as long as the sublethal endpoint is not completely abandoned in the conduct and analysis of the test.

In addition to the nonroutine tests (test performed once per month or less), all tests conducted with plants are required to have concurrent reference toxicant testing. In addition, brood stock can vary in condition, and the concurrent check on test organism sensitivity is a good precaution. Algal toxicity tests must have concurrent reference toxicant tests for similar reasons. Concurrent reference toxicant testing is also required when test organisms (or the brood stock used to produce the test organisms) have been collected from the wild. Increases in test costs, especially the cost of 7-day chronic tests, are to be avoided if possible. The alternative to concurrent reference toxicant testing in section 4.7 for labs getting test organisms from an outside supplier is reference toxicant testing by the organism supplier, and this alternative seems to be generally believed by testing labs as well as the Department of Ecology to be inferior to monthly reference toxicant testing by the testing lab. We do not accept the use by labs of reference toxicant tests performed by organism suppliers, and apparently labs agree because the vast majority have, to their credit, continued to conduct their own reference toxicant testing. Labs, however, should use organism suppliers that routinely conduct reference toxicant testing and control charting because, as noted in the table below, this information can be useful when deciding the consequences of lab conducted reference toxicant testing.

All labs must conduct ongoing control charting based on reference toxicant testing and report the results, acceptable or unacceptable, of the control charting in the report for each effluent or ambient water

test. Acceptability is based on the standard test acceptability criteria for the test and on control charting with the upper and lower control limits set at twice the standard deviation (95 percent confidence) of the point estimates (LC_{50}, EC_{50}, IC_{25}, etc.) accumulated from the last 20 reference toxicant tests. At least five reference toxicant tests are needed to establish a minimally effective control chart for new tests. The reference toxicant test data must be presented with the report for each associated test.

Any reference toxicant test determined to be unacceptable must be repeated either until an acceptable result is obtained or until there have been three consecutive unacceptable test results (the initial unacceptable test plus two repeats). Because about 1/20 reference toxicant test results will fall outside of control limits due to chance alone, it is necessary to repeat unacceptable reference toxicant tests in order to reduce the role of chance. Assuming no unusual problems with test organisms or lab performance, there is only a 1/400 chance of two unacceptable reference toxicant test results in a row and only a 1/8,000 chance of three unacceptable results in a row. If a lab has no unusual problems, repeating an unacceptable reference toxicant test should quickly produce an acceptable result. If a lab repeatedly produces unacceptable reference toxicant test results, it will give confidence to the conclusion that the lab has problems with test organisms or testing technique.

When the reference toxicant test result is within the 95 percent confidence limits, then the test report must state this fact and present the reference toxicant data at the end of the report. When the reference toxicant test result is outside the 95 percent confidence limits, then the test report must state this fact and present the reference toxicant data at the end of the report. The lab should not delay test reports while waiting for the results of reference toxicant test repeats. The results from the first repeated test might be available in time for inclusion in the test report. If begun promptly, the results of all of the reference toxicant testing in response to an unacceptable reference toxicant test result will be available in time for the review of the test report. The WET Coordinator will contact the lab during the test review for any additional reference toxicant test data not contained in the test report.

When a reference toxicant test result falls outside of the 95 percent confidence limits, a lab must qualify the associated test result for an effluent or ambient water sample by a statement in the test report that the reference toxicant test result was outside control limits. The Department of Ecology WET Coordinator will decide whether these tests are acceptable based on the degree of departure from control limits and the frequency of occurrence. Because it is expected that an average of one out of 20 tests will fall outside of the control limits due to chance alone, the degree of departure from the control limits and frequency of occurrence will be considered before rejecting toxicity tests. Because control limits narrow as laboratory performance improves, the width of the control limits will also be considered before rejecting toxicity test results when the associated reference toxicant test results are just outside the limits.

The Biomonitoring Science Advisory Board (BSAB) criteria for acceptable intralaboratory variability provide values that are useful for considering the width of control limits while deciding whether to reject toxicity tests on the basis of reference toxicant test results. If the coefficient of variation (standard deviation mean toxicity value) from the reference toxicant test data used in control charting falls into the excellent (< 0.35) or good (0.35 to 0.60) range established by the BSAB, then a higher confidence in the test results is justified. If the reference toxicant test data coefficient of variation for the lab falls into the acceptable range (0.61 to 0.85), then a smaller amount of confidence should be applied. If the reference toxicant test data coefficient of variation for the lab falls into the unacceptable range (> 0.85), then none of the lab's test results are acceptable. Labs must report the coefficient of variation for the last 20 reference toxicant tests in every report for the same test conducted on an effluent or environmental sample. (Reference: Biomonitoring Science Advisory Board. BSAB Report #1, ***Criteria for Acceptable Variability of Marine Chronic Toxicity Test Methods.*** Washington Dept. of Ecology. February 1994.) Effluent or ambient water toxicity test results will be accepted or rejected based on the following table. Rejection will occur when any condition in the appropriate "Test Accepted" box was not met or when any condition in the appropriate "Test Rejected" box was met.

Effluent tests and their associated (initial) reference toxicant tests must have start dates separated in time by no more than 18 days. Labs typically take about two weeks to produce a test report. From the point of view of practicality and the most meaningful control charting, it makes sense for a reference toxicant test result to be used retroactively about two weeks. The reference toxicant test result will then be used for control charting for the balance of the monthly time period. A grace period of 7 days will be added to the 18 days for tests begun from December 1st to the following January 10th. Acute tests will be allowed a grace period of 4 days over the 18 day maximum.

Table for Determining Test Rejection Based on Reference Toxicant Test Results

Unacceptable Reftox Tests	Test Accepted	Test Rejected
Only the original reftox test result was outside of control limits (the first repeat reftox test result fell within control limits)	If the organism supplier reftox results were within control limits, and the coefficient of variation for the last 20 reftox tests is ≤0.85	If there are notable reporting errors or deviations from test protocol, or if the reftox test result fell outside of control limits to the more sensitive side (point estimate was too low) by 3 or more standard deviations and the effluent test showed toxicity at levels of regulatory concern
Both the original and the first repeat reftox test results were outside of control limits (the second repeat reftox test result fell within control limits)	If the 95 percent confidence interval for the point estimate used in control charting can be calculated and in both failing reftox tests overlapped the control limits in the control chart, organism supplier reftox results were within control limits, and the coefficient of variation for the last 20 reftox tests is ≤0.60	If there are notable reporting errors or deviations from test protocol, or if any reftox test result fell outside of control limits to the more sensitive side (point estimate was too low) and the effluent test showed toxicity at levels of regulatory concern
All three reftox tests were outside of control limits	Never	Always
Coefficient of variation for the last 20 reftox tests > 0.85	Never	Always

Because point estimates provide the best basis for control charting, all labs must control chart using point estimates. Point estimates require fewer replicates than NOECs and reference toxicant testing may be done using the minimum number of replicates allowed by the test method.

Another Ecology staff person with primary responsibility for reference toxicant testing requirements is the Advisory Laboratorian in the Quality Assurance Section, who reviews standard operating procedures (SOPs) for toxicity tests and accredits labs. For bioassay labs to maintain Department of Ecology laboratory accreditation, the QA section has begun to require participation in a round-robin test (such as the DMR-QA) or the performance of one reference toxicant test at least once every six months. In the event that a lab does not conduct any tests on environmental samples using a particular species/method within a six-month period, it must perform a reference toxicant or round-robin test. In the event that a lab does not conduct any tests by a particular method within a one-year period, it must do two reference toxicant or round-robin tests for that year. Further, these tests must be done at least four months apart. This is to assure that the labs maintain proficiency with the species and methods for which they are accredited. The Quality Assurance Section can efficiently enforce good reference toxicant testing requirements because it has direct authority over labs to approve SOPs and conduct routine onsite audits.

E.4.3 Describe Any Additional QA/QC Criteria Your State Has Developed and Implemented Within Your State

— Sometimes variability across replicates will prevent a large difference in response (in other words, a toxic effluent) from being detected as statistically significant. False negatives can happen when the number of replicates is low. The acute statistical power standard says that acute toxicity tests must be able to detect a minimum of a 30 percent difference in survival between the IWC and a control as statistically significant. The chronic statistical power standard says that chronic toxicity tests must be able to detect a minimum of a 40 percent difference in response between the IWC (the NOEC if the IWC is unknown) and a control as statistically significant. Tests which fail to meet the power standard must be repeated with an increased number of replicates.

Ceriodaphnia Chronic Test

— ≤ 10 percent males in the surviving test organisms over all test concentrations.

— ≤ 20 percent males in the surviving test organisms in the IWC or LOEC.

— All surviving *Ceriodaphnia* producing no neonates in the test must be examined to determine gender, and the results of the determination reported. It is not necessary to identify gender when reproduction has been nearly eliminated in any test concentration when this fits an expected concentration-response relationship. It is understood that very young *Ceriodaphnia* can be difficult to sex, and any *Ceriodaphnia* that dies in the first two days of the test may be excluded from calculations for reproduction if gender is difficult to determine and it is one of no more than two mortalities in a concentration. Otherwise, difficult to sex young *Ceriodaphnia* must be considered to be female and included in all calculations.

E.4.4 Describe Any Efforts Your State Has Made to Minimize Test Method Variability

1. Development and distribution to all labs of a document called "Laboratory Guidance and Whole Effluent Toxicity Test Review Criteria" (*canary book*) that lets them know our expectations for an acceptable toxicity test. The *canary book* also narrows testing choices and provides for more consistent testing between labs.

2. Test reviews for compliance with the test method and canary book.

3. Fish or mysid growth tests that have a standard deviation for proportion alive above 0.25 in any effluent concentration (unless the partial mortality occurs at the threshold of toxicity in a good concentration-response relationship) are analyzed for the original growth endpoint instead of the combined ("biomass") endpoint.

4. To reduce the opportunity for WET limit violations due to statistically significant differences in response that are type I errors, permit requirements will lower the *alpha* level for hypothesis testing when differences in test organism response are small. To prevent excessive type I errors, eliminate some interrupted concentration-response relationships**,** and have more fair and enforceable test results, we will set *alpha* = 0.01 for small differences in response. If the difference in survival between the control and the IWC in an acute test is less than 10 percent, the level of significance will be lowered from 0.05 to 0.01. If the difference in test organism response between the control and the IWC in a chronic test is less than 20 percent, the level of significance will be lowered from 0.05 to 0.01.

5. The identification of anomalous tests is a valuable tool for reducing false positives. A concentration-response relationship where response increases with concentration is a good identifier of toxicity as opposed to other sources of organism stress such as disease. Test method variability or lab error will also very rarely produce a good concentration-response relationship.

Identifying a test as anomalous does not necessarily mean rejection of the test and a requirement to repeat. If a test result meets one of the criteria for anomalous test identification but has no statistically significant toxicity at concentrations of regulatory concern (IWC), then the test need not be repeated unless other factors contribute to a decision to reject the test.

The anomalous test definitions below must be considered in light of the expectations for the different toxicity tests and endpoints.

Criteria for Identifying Anomalous Test Results

— A WET test result is anomalous if it shows a statistically significant difference in response between the control and the IWC, but no statistically significant difference in response at one or more higher effluent concentrations. The lack of statistical significance must be associated with a lower toxic effect at the higher effluent concentration. Any higher effluent concentration used in this determination must be a part of a dilution series. Labs should not cluster test concentrations just above the IWC in order to increase the opportunity for an anomalous test result.

— A WET test is anomalous if there is a statistically significant difference in response between the control and the IWC which together with other nearby concentrations of effluent, have a zero slope and appear to be nontoxic (performance is typical of healthy test organisms). Another description of this criterion is a test with a control that seems not to belong to the concentration-response relationship because of exceptionally good performance.

— A WET test is anomalous if the overall slope of the line fitted to the concentration-response plot is opposite of normal expectations and there is a statistically significant difference in response at the IWC. A test might be considered acceptable if the slope is opposite over only part of the concentration series.

— A WET test is anomalous if the standard deviation for proportion alive equals or exceeds 0.3 in any test concentration unless the partial mortality fits a good concentration-response relationship. A WET test is anomalous if mortalities occur in any test concentration in excess of the control performance criterion for survival when the concentration-response relationship indicates that the effluent concentration is nontoxic (sporadic mortalities).

E.4.5 Explain How Your State Reviews or Conducts Performance Lab Audits

The Department of Ecology manages an Environmental Laboratory Accreditation Program designed to assure that accredited labs have the capability to provide reliable and accurate environmental data to the department. Applicant labs apply for accreditation for specific parameters and methods. An applicable parameter/method pair for WET testing would be "*Pimephales promelas* by EPA Method 1001.0."

Concurrent with submission of the initial application, the lab submits a quality assurance manual that is given a thorough review by Ecology staff. If there are reasonably-available performance evaluation (also known as "proficiency testing") samples available for the requested tests, the lab is required to submit one set of such PE results for initial accreditation. This is referred to in our program as a "performance audit." There are no PE samples we consider to be "reasonably available" for WET testing.

Following review of the lab's QA manual and PE study results and successful resolution of any noted problems, Ecology and the lab schedule a mutually agreeable date for an on-site, or system, audit. (Although this survey asks about "performance" audits, which could be construed as being synonymous with our required PE studies, we think it rather is synonymous with what we call the on-site, or system, audit). For initial system audits, depending on the scope of tests done by the lab, checksheets may be sent to the lab to

be completed and returned to the auditor prior to the audit. The auditor studies the checksheet responses and verifies accuracy of the response during the audit. For subsequent audits, which are routinely scheduled every three years but may be conducted at any time there is a need, the auditor may choose to send checksheets in time for them to be completed by the lab or take them to be filled in during the audit.

The actual audit, if for WET testing only, would involve one auditor and last one or two days depending on the scope of tests done in the lab. If the lab does other testing, the audit team may involve as many as five, and the audit may last as many as three days (or longer if required, but none have to date). The audit consists of an in-briefing, a thorough audit of personnel qualifications and equipment/supplies status (which were reported as part of the application), facility adequacy, sample management, records keeping/data management, performance evaluation study data (if applicable), the overall quality assurance program, status of quality control testing results (to see if the lab is meeting data quality objectives which were approved in the QA manual), and a check to see that current methods/SOPs are readily available and being followed. An out-briefing follows the audit during which the audit team informally summarizes major findings, both good and bad.

Following the audit, our program allows us 30 calendar days to prepare a written report. Depending on the scope of testing, this report, which addresses each of the factors discussed above, may be only 3 or 4 pages, or many more, and might include several attachments providing guidance or assistance to the lab. The secondary objective of our program as specified in the code is to assist labs in achieving the ability to meet required standards of performance, a perhaps novel but very effective approach to achieving desired capability in accredited labs. Historically, we have been deficient in meeting the 30-day report requirement, which has caused us to change our accreditation strategy. Using a fixed-price contract to encourage prompt reporting, we now contract out the audit task to a highly-qualified auditor whose last audit report was delivered within 10 days of the audit.

Performance audits (PE studies) are required in our program twice each year, and system audits are preferably conducted every three years with the code allowing four years for documented cause. At this time, we see no need to exceed three years for future audits of WET testing labs.

E.4.6 Describe Any Specific Implementation Guidance That Your State Has Developed to Assist Permit Writers. How Is the Guidance Available to the Public?

We have developed and kept updated suggested language for use in NPDES permits and fact sheets for POTWs and industries. The suggested language is a part of templates ("shells") for permits and fact sheets that permit writers use as they draft a permit. We also have a "Permit Writer's Manual" (USEPA 1996a) which addresses species choice, WET monitoring frequency, recommendations for number of test concentrations, etc. The "Permit Writer's Manual" was developed with public input/review and is available to the public for the cost of printing.

E.4.7 Describe How Your State Provides or Utilizes Any Toxicity Testing Training

We had extensive training in all of our offices at the beginning of our use of WET testing in water quality-based permitting early in the 1990s. Because of budget constraints, because WET test review and technical assistance are centralized functions, and because of the availability of permit writing guidance in the "Permit Writer's Manual" and suggested permit language, we no longer hold WET training sessions.

E.5 RESPONSES FROM WISCONSIN DEPARTMENT OF NATURAL RESOURCES

E.5.1 Describe How Your State Evaluates Reference Toxicant and Effluent Test Results

Reference toxicant and effluent test data is sent directly to the Biomonitoring Coordinator in Madison (central office). Certified labs are required to perform reference toxicant tests (using NaCl, specified dilutions and dilution water) on a monthly basis. Acute and chronic reference toxicant results are evaluated using the point-estimation techniques described in the EPA manual (LC_{50}, IC_{25}). Control charts (graphical and tabular) representing the mean LC_{50} or IC_{25} and upper and lower control limits (mean $\pm$ 2 standard deviations) are established for each species, using data from the previous 20 months. Any exceedance of either the upper or lower control limit after establishment of the control chart requires a review of the culture and test systems. Missing or out-of-range data must be explained (if possible) and may result in invalidation of Washington Pollution Discharge Elimination System (WPDES) test results conducted during the same period.

Each test report for all effluent tests is reviewed by the Biomonitoring Coordinator for completeness, adherence to QA and test acceptability requirements, and for compliance with the WPDES permit. Deviations from permit requirements, test acceptability criteria, or other factors may cause tests to be repeated.

E.5.2 Explain How Your State Reviews Reference Toxicant Data for Laboratory Performance

(See above.)

In addition to the regular review by the Biomonitoring Coordinator, reference toxicant data is reviewed by the Department's WET Laboratory Auditor prior to on-site laboratory inspections. The laboratory provides copies of bench sheets, water quality data, current control chart data, and any explanations of out-of-range test results for each test type and organism combination. The materials are reviewed for appropriate test frequency, proper test conditions, test result validity, and proper responses to out-of-range events.

E.5.3 Describe Any Additional QA/QC Criteria Your State Has Developed and Implemented Within Your State

Test acceptability requirements, based on current “State of Wisconsin Aquatic Life Toxicity Testing Methods Manual, Edition 1”:

Testing must be separated from culturing activities (separate rooms with separate ventilation systems; if closed incubators are used, culturing & testing may not be contained within the same incubator)

For Static Renewal Acute Tests:

Pretest Requirements (Requirements For Culture Acceptability)

- *C. dubia*:
  - Average Number Of Neonates In 3 Broods $\geq$ 15
  - Mean Survival $\geq$ 80 percent
  - Number Of Neonates In Each Brood $\geq$ 8
  - Age Of Organism $\leq$ 24-H
- Fathead Minnows:
  - Age Of Organism 1- 14 Days
  - Sample Requirements
  - Holding Time $\leq$ 36-H
  - Temperature During Collection & Prior To Shipping $\leq$ 4 °C
  - Temperature Upon Arrival At The Laboratory $\leq$ 10 °C

Test Requirements (Requirements For Test Acceptability)

- Temperature 20 ° $\pm$ 1 °C
- Dissolved Oxygen > 40 percent and < 100 percent saturation

— Effluent - pH ≥ 6.0 and ≤ 9.0.
— Control Survival ≥ 90 percent

For Static Renewal Chronic Tests:

Pretest Requirements (Requirements For Culture Acceptability)

— *C. dubia:*
  – Average Number Of Neonates ≥ 20
  – Mean Survival ≥ 80 percent
  – Neonates Used In Test Must Be From 3rd Or Subsequent Brood
  – Number Of Neonates In 3rd Or Subsequent Brood ≥ 8
  – Age Of Organism ≤ 24-H; Released Within Same 8-H Window
— Fathead Minnows:
  – Age Of Larvae ≤ 24-H
  – Sample Requirements
  – Holding Time ≤ 36-H
  – Temperature During Collection & Prior To Shipping ≤ 4 °C
  – Temperature Upon Arrival At The Laboratory ≤ 10 °C

Test Requirements (Requirements For Test Acceptability)

— Temperature 25 ° ± 1 °C
— Dissolved Oxygen > 40 percent and < 100 percent saturation
— Effluent - pH ≥ 6.0 and ≤ 9.0
— Control Survival ≥ 80 percent
— *C. dubia* Mean Control Reproduction ≥ 15 Neo./Adult; > 60 percent produce 3 broods
— Fathead Minnow Mean Control Biomass ≥ 0.25 mg/individual

The Wisconsin Department of Natural Resources (WDNR) is in the process of updating it's WET Methods Manual. Future methods (2nd Edition expected in 2001) will include *additional* or *revised* test acceptability criteria:

For Static Renewal Acute Tests:

Pretest Requirements (Requirements For Culture Acceptability)

— Fathead Minnows:
  – Age Of Organism 4 - 14 Days
  – Sample Requirements
  – Temperature Upon Arrival At The Laboratory ≤ 6 °C

Test Requirements (Requirements For Test Acceptability)

— Control Variability CV < 40 percent

For Static and Static Renewal Chronic Tests:

Sample Requirements

— Temperature Upon Arrival At The Laboratory ≤ 6 °C

Test Requirements (Requirements For Test Acceptability)

— Control Variability - Fathead Minnow & *C. dubia* CV < 40 percent
— Control Variability - *R. subcapitata* CV < 20 percent
— *C. dubia* Male Production < 20 percent in controls & < 20 percent all concentrations
— *C. dubia* Mean Control Reproduction >80 percent produce 3 broods
— *R. subcapitata* Control Performance Cell Density > 1 X 10^6 cells/ml at end of test

E.5.4 Describe Any Efforts Your State Has Made to Minimize Test Method Variability

1. Close review of each test result submitted with consistent adherence to test protocol test acceptability criteria.

2. Investigations of test results from disagreeing "split" effluent sample analyses.

3. State specific methods: In order to limit the variability that may occur when different procedures are used by different labs, WDNR requires strict adherence to clearly specified methods, regarding: (a) sampling procedures (volume, type, storage conditions, etc.); (b) holding times; (c) test duration; (d) deviations in feeding & environmental conditions (light, pH, temperature, DO, etc.); (e) dilution water; (f) number of concentrations and replicates tested; and (g) number of organisms per replicate.

   Each of these is addressed in EPA methods, but flexibility is allowed so labs can make tests fit in specific situations. The more flexibility allowed in test methods, the higher the chance that tests will be done differently between labs or between tests, resulting in increased WET variability. In order to control WET variability and improve the consistency of methods used by Wisconsin labs and permittees, WDNR created the "State of Wisconsin Aquatic Life Toxicity Testing Methods Manual," Edition 1 (PUBL-WW-033-96) (Methods Manual) and incorporated it by reference into NR 149.22 and NR 219.04, Wis. Adm. Code, in 1996. The Methods Manual contains specific procedures regarding testing and sampling procedures, types of tests, quality control/quality assurance procedures, test acceptability criteria (see above), etc., that labs must follow when performing WET tests for permit compliance.

4. Implementation of a WET Laboratory Certification program. In order to insure labs are of the highest quality and are able to demonstrate a serious commitment to a quality assurance/control program, WDNR, under State statutes, certifies labs to perform WET tests. In order for a lab to apply for certification for WET testing, the lab must submit a completed application and a quality assurance plan to the lab certification program and pass an on-site evaluation. WET labs must have an ongoing reference toxicant program, a review process for all test data and reporting, a good sample custody system, proper equipment maintenance, dilution water quality monitoring, facility maintenance, and attention to test organism health, and make other demonstrations of good lab practices in order to pass an audit.

5. The WDNR's WET Team strives to continually improve the WET program. The WET Team is now revising the Methods Manual to require that labs verify the training and qualifications of their staff, to include test acceptability criteria related to variability, and other changes to further improve WET test quality and reduce variability (see above).

E.5.5 Explain How Your State Reviews or Conducts Performance Lab Audits

Inspections may be announced or unannounced. Prior to the inspection, the auditor reviews laboratory SOPs and recent reference toxicant results for adherence to WDNR protocols. The actual inspection consists of an opening conference, a walk-through of the laboratory facilities, and a closing conference. During the opening conference, the auditor discusses the SOP review and general procedures in the laboratory. During the walk-through, the auditor examines equipment, written documentation, cultures, and laboratory procedures. He/she will also interview lab personnel to insure that they understand lab quality assurance and methods requirements. The closing conference serves as a review of observations and comments during the inspection. After the inspection, the auditor generates an inspection report, to which the laboratory will be given 60 days to respond. If there are significant deficiencies discovered during the inspection, and the laboratory fails to fix those deficiencies satisfactorily within the allotted time, the laboratory's certification may be revoked.

E.5.6 Describe Any Specific Implementation Guidance That Your State Has Developed to Assist Permit Writers. How Is the Guidance Available to the Public?

The WDNR created the "WET Program Guidance Document" in 1996, as a companion document to the "State of Wisconsin Aquatic Life Toxicity Testing Methods Manual," in order to provide guidance and clarification of existing rules, for WDNR staff, permittees, labs, consultants, and others. The WET Guidance Document is updated as program needs dictate, at least once yearly, and can be obtained by contacting the Biomonitoring Coordinator at: WDNR, Bureau of Watershed Management, P.O. Box 7921, 101 S. Webster St., Madison, WI, 53707-7921; email: flemik@dnr.state.wi.us; or at http://www.dnr.state.wi.us/org/water/wm/ww/biomon/biomon.htm.

E.5.7 Describe How Your State Provides or Utilizes Any Toxicity Testing Training

The Biomonitoring Coordinator provides one-on-one training, as needed, for WDNR staff and permittees (usually as permits are reissued with new WET requirements). The University of Wisconsin-Madison State Lab of Hygiene (who provides WET testing and research services to WDNR) can provide hands-on WET training to WDNR staff, permittees, and/or new staff at contract laboratories, at their request. WDNR staff, permittees, and contract lab staff have also attended The Society of Environmental Toxicology and Chemistry's two-day WET course and statistical analysis course.

APPENDIX F

IMPROVEMENTS IN MINIMIZING WET TEST VARIABILITY BY THE STATE OF NORTH CAROLINA

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IMPROVEMENTS IN MINIMIZING WET TEST VARIABILITY BY THE STATE OF NORTH CAROLINA

F.1 Background

The North Carolina Division of Water Quality (NC DWQ) began in-house WET testing in the late 1970s. Data collected through the mid-1980s indicate that one in four NC NPDES facility effluents tested had the potential to cause acute toxicity instream during low stream flow/high effluent flow conditions (Eagleson et al. 1986). The Division began to require WET self-monitoring by individual facilities in 1985 through administrative letters. DWQ first implemented WET limits in NPDES permits in 1987. As of March 29, 2000, 554 facilities are required to perform some type of WET monitoring; 453 of these have limits. North Carolina permittees have demonstrated compliance rates consistently above 90 percent since the additional TAC were implemented. Chronic *Ceriodaphnia dubia*, acute *C. dubia*, and acute fathead minnow are the primary test types used.

The Division uses two primary strategies to enhance data quality: (1) individual report review and (2) laboratory certification.

Division personnel review each analysis report for the following test acceptability criteria:

- Sample type (specified by permit)
- Sample hold time
- Sample temperature upon receipt at lab
- Control treatment water pH and dissolved oxygen
- Control water hardness*
- Effluent treatment dissolved oxygen
- Test type (specified by permit)
- Replication
- Effluent dilution (specified by permit)
- Control survival and/or reproduction
- Percentage of control organisms producing three broods (*Ceriodaphnia* chronic)
- Control organism reproduction coefficient of variation (*Ceriodaphnia* chronic)*
- Test duration

*NC State criteria

The reviewer may also statistically analyze data sets when the result is unclear based on a cursory review of the data.

The Division's Water Quality Rules specify that WET analyses associated with NPDES permits must be performed by certified laboratories. The Division implemented the laboratory certification program in 1988. Key requirements of that program are specific qualifications for laboratory supervisors, a reference toxicant testing program, annual inspections and audits, and performance evaluation analyses.

Laboratory Supervisor Qualifications

Laboratory supervisors must have either a Bachelor of Science degree in biology or a closely related field and three years of experience in aquatic toxicity testing, or a Master of Science degree in biology or a closely related field and one year of experience in aquatic toxicity testing.

Reference Toxicant Testing Program

The laboratory must maintain a reference toxicant testing program for each organism and test type category (chronic and acute). A reference toxicant test should be performed every two weeks for each organism used in acute WET testing. Alternatively, acute reference toxicant tests may be performed such that NC NPDES acute tests are performed within one week of an acute reference toxicant test for the organism in question. Similarly, a reference toxicant test should be performed once per month for each organism used in chronic WET testing. Alternatively, tests may be performed such that NC NPDES chronic tests are performed within two weeks of a chronic reference toxicant test. To maintain certification for an organism, reference toxicant tests must be performed at least quarterly.

Annual Inspection and Audit

The Division conducts at least one inspection per year at each laboratory. Most inspections are announced, but may be performed without notice. Inspections include the following activities:

- Inspect facilities, equipment, and QA procedures according to the laboratory's standard operating procedures
- Examine living and preserved test organisms
- Review reference toxicant testing program documentation
- Inspect meters and meter calibration records
- Trace randomly selected test records

Performance Evaluation Analyses

The Division may distribute unknown samples to laboratories up to three times per year for analysis. The Division constructs acceptability criteria using the pooled results of the analyses. Laboratories generating results outside of the acceptable range must repeat the analysis. Two consecutive out-of-range results result in decertification. A decertified laboratory regains certification by generating acceptable results on two follow-up analyses.

F.2 Data Evaluation (1992-94) Summary

In January 1992, NC DWQ began recording reproduction data from *Ceriodaphnia* chronic pass/fail tests performed by NC DWQ-certified laboratories in association with NPDES permit requirements. The majority of NC facilities with WET limits use this test. NC pass/fail tests consist of two treatments: a control and a critical concentration, each with 12 replicates. The purposes of the data base were to evaluate the sensitivity of the analysis, assess performance characteristics of the analyses, and evaluate performance of individual laboratories. Analysis was limited to test results with normally-distributed reproduction data.

In 1994, NC DWQ investigators reviewed the PMSD and MSD as a percentage of the control mean for each test (Rosebrock et al. 1994). Evaluation of the data indicated a correlation between PMSD and timing of test termination. EPA methods allow the test to be terminated once 60 percent of the control organisms produce three broods. Therefore, the percentage of adults producing a third brood at test termination may

vary from 60 to 100 percent. Plotting PMSD versus percent of control organisms producing three broods clearly showed that higher percentages of control organisms producing three broods were associated with lower PMSDs (Figure F-1).

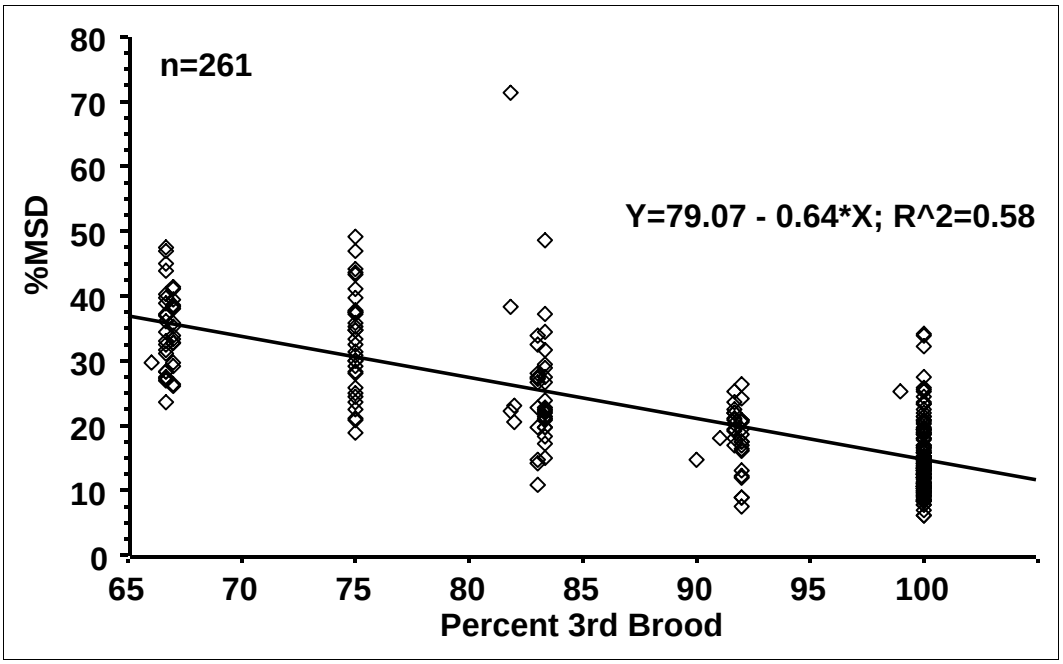

Figure F-1. PMSD versus percent control organisms producing three broods (1994).

Percentile analysis of the PMSD data produced a median PMSD of 20. This means that the "average" analysis, defined as the median, can statistically detect as small as a 20 percent difference between the treatment and control organism reproduction.

Percentile analysis of the CV data for control organism reproduction produced a median of 17 percent and a 95th percentile of 40 percent. This means that 95 percent of the control data sets produced CVs at or below 40 percent.

F.3 North Carolina Chronic Protocol Modifications

Using results from the data evaluations described above and empirical knowledge gained from experience with the test, NC DWQ made several changes to its chronic *Ceriodaphnia* protocol to improve sensitivity, precision, and practical application of test results in its compliance program. These changes were implemented in two stages in late 1994 and early 1996.

December 1994 Changes

- Exclusion of 4th brood and higher neonates from the reproduction analysis
- Addition of a TAC requiring that at least 80 percent of the control organisms produce three broods
- Addition of a TAC requiring that the test be terminated no later than seven days after initiation

January 1996

- Addition of a TAC requiring that the control organism reproduction CV be less than 40 percent
- Specification that for an effluent treatment to be considered as producing an effect, the reproduction mean must be statistically significantly lower than the control mean **and** represent at least a 20 percent reduction from the mean

Reducing the CV of the control reproduction can be shown mathematically to result in reductions in the MSD and PMSD, producing a more sensitive test. Placing an upper limit on the CV will eliminate less sensitive tests. Excluding 4th brood neonates from the reproduction analysis and requiring that at least 80 percent of the control organisms produce a 3rd brood will reduce the control organism reproduction CV.

The specification of at least a 20-percent reduction in reproduction from the control effectively sets a lower limit on test sensitivity. DWQ's experience has shown that high-quality laboratories can produce extremely sensitive tests that can detect very small differences between treatment and control reproduction. Unfortunately, this can be a disincentive for laboratories to produce high quality tests because some clients will gravitate toward laboratories that produce compliant test results. Less sensitive tests will more likely produce such results.

F.4 Evaluation of Program Modifications

The North Carolina data base affords the opportunity to evaluate the effectiveness of additional TAC and changes to the test protocol as they relate to the variability of WET test results. Effluent data for individual laboratories, and across all tests and laboratories, were examined to discern the impact of program changes on laboratory performance. Data were partitioned into two data bases, one for effluent tests completed before December 1994 (termed Pre-1995) and one for effluent tests completed after January 1996 (termed Post-1995). Pass/Fail tests were included in the evaluation. Only tests that did not have a significant mortality effect were considered. Two measures of laboratory performance were calculated using the reproductive data from the tests: PMSD and control CV. The PMSD data set contains all tests reported for compliance. The control CV data set contains all unique controls that were reported by the laboratories and used in compliance calculations. Conclusions reflect the cumulative impact of all changes made to the program from late 1994 to early 1996.

F.5 Overall Test Performance

Pre-1995 and Post-1995 percentile values were generated for the PMSD and the control CV combined across all tests and laboratories (Table F-1). For the PMSD, the median value decreased from 21 percent to 16 percent and the 90th percentile from 39 percent to 31 percent, indicating an overall increase in test sensitivity. The narrower interquartile range of Post-1995 PMSD values (IQR=12 percent), compared with the interquartile range of Pre-1995 PMSD (IQR=16 percent), implies an improvement in the ability of laboratories to achieve similar levels of test sensitivity. (The interquartile range is the difference between the 75th and 25th percentiles of the cumulative distribution function and is a measure of spread of the distribution.) For the control CV, the median value was reduced from 15 percent to 13 percent and the 90th percentile from 34 percent to 28 percent. The overall decrease in the control CV reflects the capacity of laboratories to improve their performance as measured by a decrease in control variability relative to the control mean. Changes in test acceptability criteria and in test protocols improved the consistency of control performance quantified by the reduction in the interquartile range of the control CV Pre-1995 (IQR=15 percent) and Post-1995 (IQR=10 percent).

Table F-1. PMSD and Control Organism CV

	PMSD		CV	
	Pre 1995	Post 1995	Pre 1995	Post 1995
# Tests	4110	5471	2478	3401
Min	0.055	0.049	0.033	0.034
Max	0.839	0.676	0.835	0.400
Median	0.212	0.160	0.155	0.133
IQR	0.164	0.118	0.150	0.103
10^{th} Percentile	0.105	0.095	0.078	0.077
25^{th} Percentile	0.142	0.116	0.103	0.097
50^{th} Percentile	0.212	0.160	0.155	0.133
75^{th} Percentile	0.306	0.233	0.253	0.200
90^{th} Percentile	0.391	0.307	0.343	0.285

F.6 Individual Laboratory Performance

Comparison of effluent data across multiple laboratories provides information about the influence of program changes on individual laboratory performance. Data for a laboratory (Lab 1) with low sensitivity were compared to data from a laboratory (Lab 2) with high sensitivity. Pre-1995 and Post-1995 percentile values were generated for the PMSD combined across all tests for each of the two laboratories (Table F-2). The performance of Lab 2, represented by the distribution of PMSD, was essentially the same Pre-1995 and Post-1995. However, the performance of Lab 1 improved, as evidenced by the changes in medians (33 percent to 18 percent), changes in the 90^{th} percentile (46 percent to 32 percent), and the slight decrease in the width of the interquartile range (13 percent to 12 percent). Additionally, the Post-1995 medians for the two laboratories were relatively close (18 percent and 12 percent) percent for Lab 1 and Lab 2, respectively. A comparison of the cumulative distribution functions for each laboratory indicates that performance was more consistent across laboratories after implementing program changes (Figures F-2 and F-3).

Table F-2. Lab 1 versus Lab 2 PMSD

	Pre-1995		Post-1995	
	Lab 1	Lab 2	Lab 1	Lab 2
# Tests	921	545	1424	466
Min	8.8	5.5	6.8	5.5
Max	67.3	48.9	67.6	39.9
Median	33.5	11.7	18.2	12.5
IQR	13.3	5.5	11.9	4.4

The distribution of PMSD values within a laboratory compared to distributions in other laboratories was examined Pre-1995 and Post-1995 (Figures F-4 and F-5). The range in median values across all laboratories Pre-1995 was 12 percent to 36 percent. Post-1995, the range in median values was 10 percent to 27 percent, indicating a decrease in the overall spread among laboratories. The range in PMSD values within a laboratory was 22 percent to 78 percent Pre-1995. The Post-1995 range in PMSD values within a laboratory compared across laboratories was 17 percent to 61 percent, indicating a narrowing of the range of values within a laboratory (Table F-3). A similar comparison was made using the control CV as an indicator of laboratory ability (Figures F-6 and F-7). The median control CV varied across laboratories from 9 percent to 30 percent Pre-1995. Post-1995, the median control CV ranged across laboratories from 9 percent to 26 percent, a slight improvement in the comparability of control CV. The range in control CVs within a laboratory was 21 percent to 79 percent Pre-1995, while the range in control CVs within a laboratory Post-1995 was 17 percent to 36 percent. Overall, laboratories are generating data with more consistency across, as well as within, laboratories after implementing additional TAC and modifications to testing protocols.

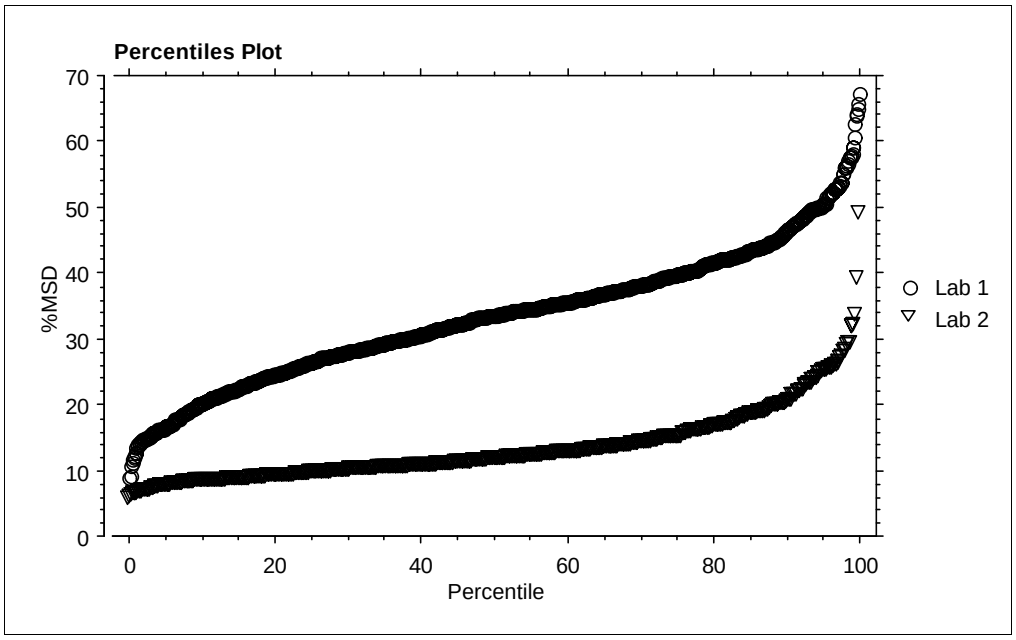

Figure F-2. Laboratory 1 versus Laboratory 2 Pre-1995 PMSD (species: *Ceriodaphnia dubia*).

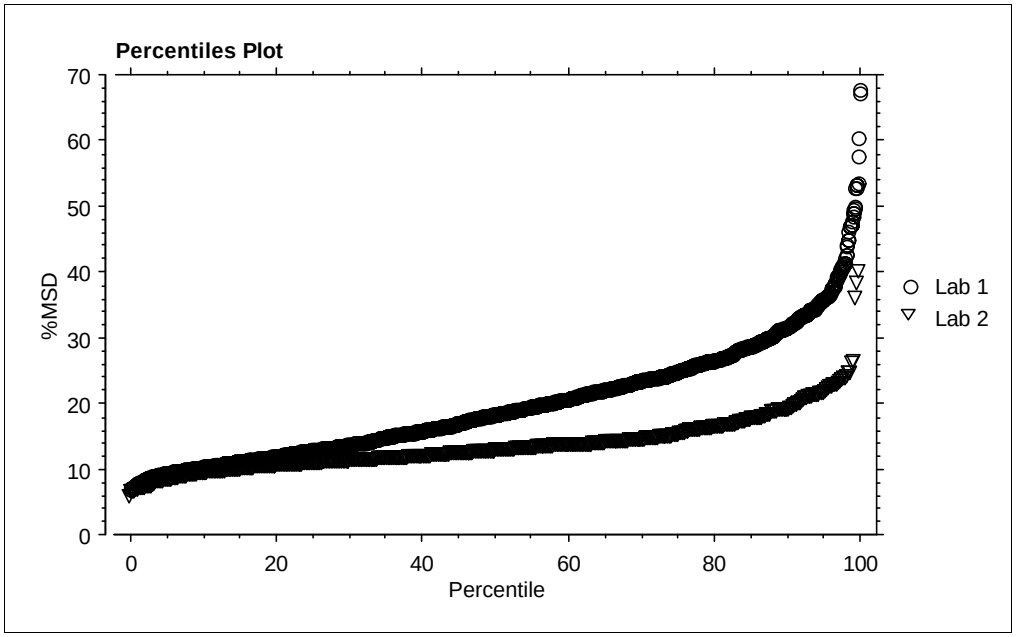

Figure F-3. Laboratory 1 versus Laboratory 2 Post-1995 PMSD (species: *Ceriodaphnia dubia*).

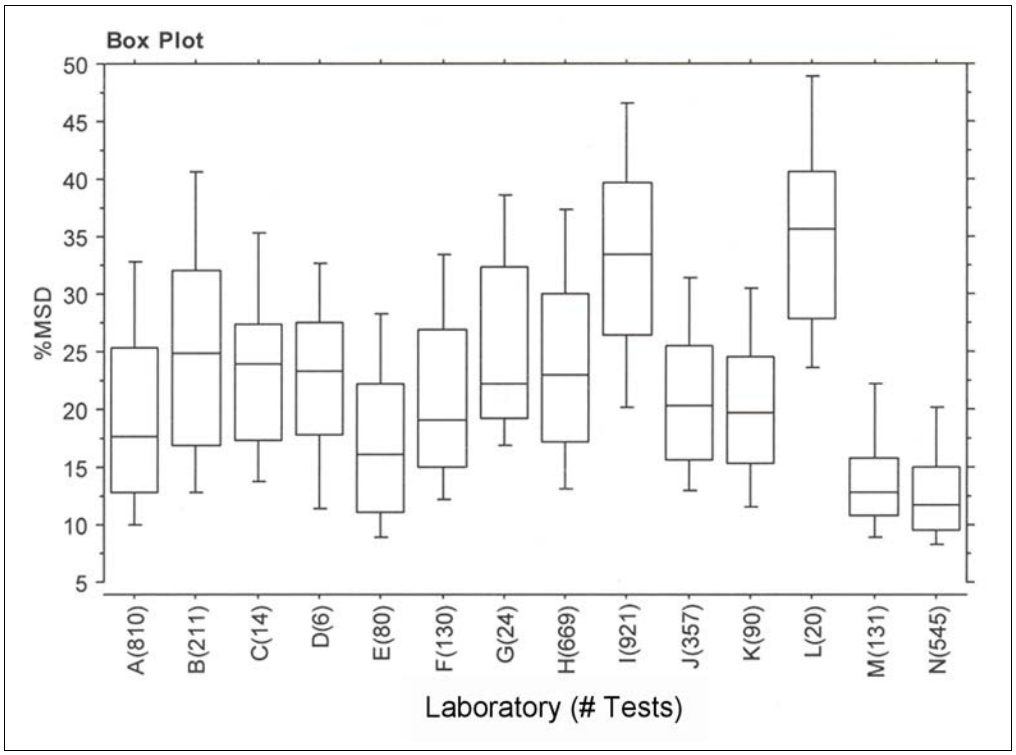

Figure F-4. Individual laboratory performance—Pre-1995 PMSD (species: *Ceriodaphnia dubia*).

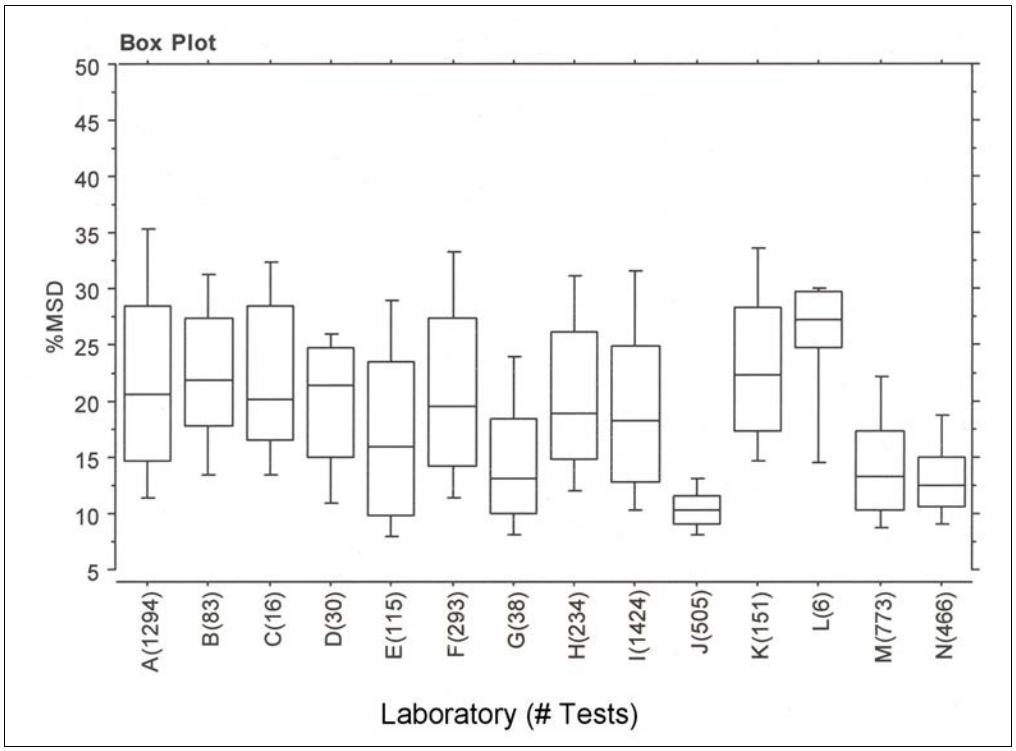

Figure F-5. Individual laboratory performance—Post-1995 PMSD (species: *Ceriodaphnia dubia*).

Table F-3. Descriptive Statistics—PMSD

	Pre-1995						Post-1995					
Lab	**N**	**Min**	**Max**	**Range**	**Median**	**IQR**	**N**	**Min**	**Max**	**Range**	**Median**	**IQR**
A	810	6.0	83.9	77.9	17.6	12.6	1294	6.4	58.9	52.5	20.6	13.7
B	211	8.6	59.7	51.1	24.8	15.0	83	10.2	39.9	29.7	21.9	9.6
C	14	13.7	35.6	21.9	23.9	10.0	16	12.5	34.5	22.1	20.1	11.9
D	6	10.6	33.2	22.6	23.3	9.7	30	9.6	33.9	24.3	21.5	9.6
E	80	6.5	43.5	37.0	16.1	11.1	115	5.6	43.8	38.3	15.9	13.6
F	130	6.9	69.4	62.5	19.1	11.8	293	6.8	55.0	48.2	19.5	13.0
G	24	13.9	45.0	31.1	22.2	13.2	38	6.6	33.1	26.5	13.1	8.4
H	669	6.2	71.5	65.3	23.0	12.8	234	8.4	38.9	30.5	19.0	11.4
I	921	8.8	67.3	58.4	33.5	13.3	1424	6.8	67.6	60.8	18.2	11.9
J	357	8.7	69.8	61.1	20.4	9.7	505	6.4	26.0	19.5	10.2	2.5
K	90	9.7	55.5	45.8	19.7	9.1	151	8.3	47.6	39.3	22.4	10.9
L	20	22.0	59.0	37.0	35.7	12.9	6	13.4	30.1	16.7	27.2	5.0
M	131	6.4	49.9	43.5	12.9	5.0	773	4.9	40.3	35.3	13.3	6.9
N	545	5.5	48.9	43.4	11.7	5.5	466	5.5	39.9	34.4	12.5	4.4

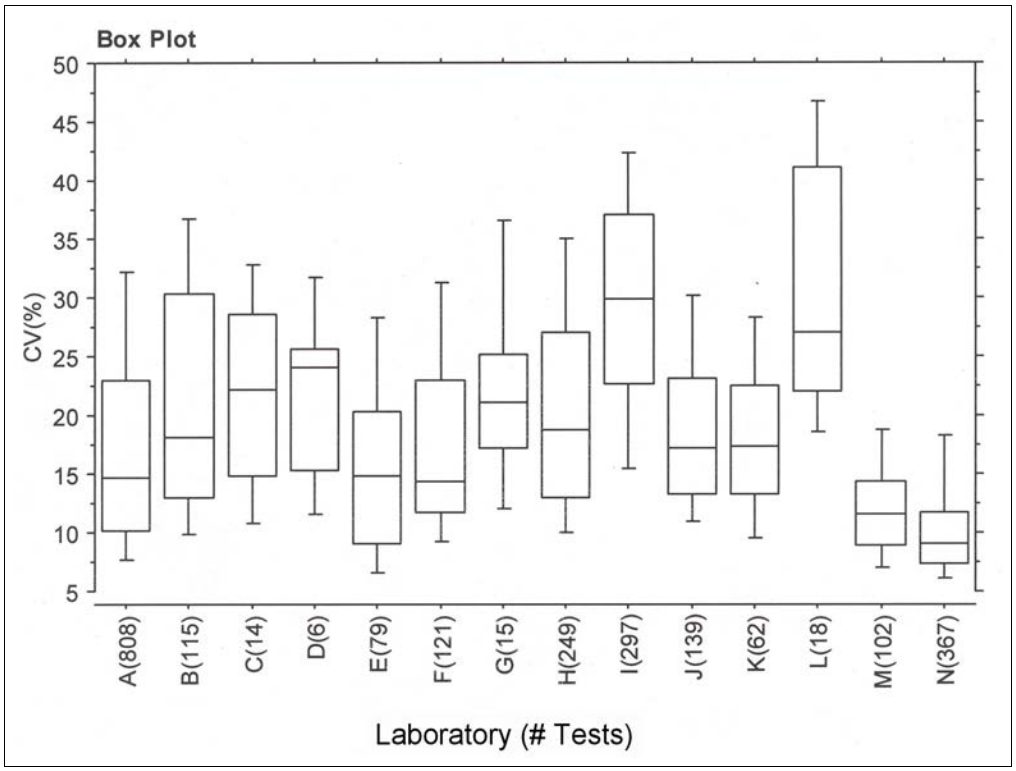

Figure F-6. Individual laboratory performance—Pre-1995 CV (species: *Ceriodaphnia duba*).

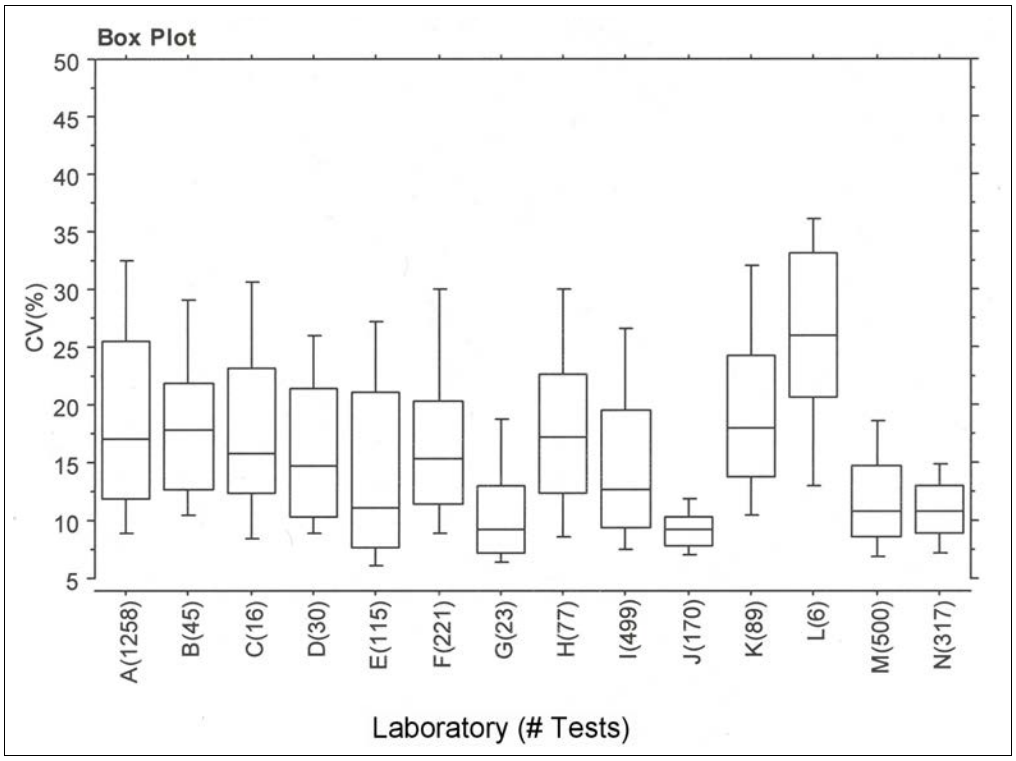

Figure F-7. Individual laboratory performance—Post-1995 CV (species: *Ceriodaphnia dubia*).

Table F-4. Descriptive Statistics—Coefficient of Variation (CV)

	Pre-1995						Post-1995					
Lab	**N**	**Min**	**Max**	**Range**	**Median**	**IQR**	**N**	**Min**	**Max**	**Range**	**Median**	**IQR**
A	808	0.041	0.835	0.794	0.146	0.129	1258	0.043	0.399	0.356	0.171	0.136
B	115	0.062	0.511	0.450	0.182	0.173	45	0.059	0.361	0.302	0.178	0.092
C	14	0.092	0.334	0.242	0.222	0.137	16	0.066	0.378	0.311	0.158	0.109
D	6	0.112	0.324	0.212	0.241	0.102	30	0.074	0.332	0.258	0.147	0.111
E	79	0.041	0.374	0.333	0.148	0.112	115	0.038	0.400	0.362	0.111	0.134
F	121	0.051	0.516	0.464	0.143	0.113	221	0.062	0.384	0.322	0.152	0.090
G	15	0.113	0.404	0.291	0.211	0.080	23	0.050	0.343	0.293	0.092	0.059
H	249	0.055	0.610	0.555	0.188	0.140	77	0.061	0.379	0.318	0.171	0.103
I	297	0.068	0.672	0.604	0.299	0.144	499	0.047	0.399	0.352	0.127	0.101
J	139	0.071	0.596	0.525	0.172	0.098	170	0.054	0.222	0.168	0.092	0.025
K	62	0.046	0.564	0.517	0.173	0.093	89	0.047	0.392	0.345	0.180	0.104
L	18	0.138	0.571	0.433	0.271	0.190	6	0.121	0.365	0.245	0.259	0.124
M	102	0.053	0.398	0.345	0.115	0.056	500	0.034	0.341	0.307	0.107	0.062
N	367	0.033	0.472	0.439	0.091	0.043	317	0.038	0.333	0.296	0.108	0.040

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Eagleson, K.W., S.W. Tedder, and L.W. Ausley. 1986. Strategy for whole effluent toxicity evaluations in North Carolina. In *Aquatic Toxicology and Environmental Fate: Ninth Volume, ASTM STP 921*. T.M. Poston, R Purdy, eds. American Society for Testing and Materials. Philadelphia, PA. 154-160.

Rosebrock, M.M., N.W. Bedwell, and L.W. Ausley. 1994. Indicators of *Ceriodaphnia dubia* chronic toxicity test performance and sensitivity. Poster presentation, Society of Environmental Toxicology and Chemistry 15th Annual Meeting, Denver, CO.

APPENDIX G

ANALYTICAL VARIABILITY IN REASONABLE POTENTIAL AND PERMIT LIMIT CALCULATIONS

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ANALYTICAL VARIABILITY IN REASONABLE POTENTIAL AND PERMIT LIMIT CALCULATIONS

Appendix G explains how analytical variability affects calculations used to determine reasonable potential and permit limits, and how such variability affects WET measurements. The appendix also considers suggested approaches to adjusting the reasonable potential and permit limit calculations to account for analytical variability. Only water quality-based effluent limitations are addressed because different considerations apply to technology-based limitations. While Appendix G addresses WET variability, its discussion and conclusions apply, with obvious modifications in terminology, to concentrations of chemical pollutants.

EPA has evaluated methodologies to adjust for analytical variability in setting permit limits. These methodologies would allow permit limits to exceed acute and chronic wasteload allocations (WLAs), sometimes two-fold or more. EPA believes that such approaches contradict the intent and practice of current guidance and regulations directed at preventing toxicity. The TSD calculations were carefully designed to avoid setting limits that allow a discharge to routinely exceed WLAs. Attempts to use an "adjusted," smaller estimate of variability in the first step of the effluent limit calculation (calculating the long-term average from the WLA) while using the variability of measured toxicity in the second step (calculating limits from the LTA), as done in the "adjustment approaches," will risk setting limits that exceed WLAs because the second variability factor is larger than the first. EPA also believes that the TSD statistical approach is adequately protective. On average, it achieves the desired level of protectiveness that is described in the NPDES regulations (40 CFR 122.44(d)) and EPA guidance.

This review did not evaluate the "conservativeness" of other components of WET limits, such as the acute-to-chronic ratio (ACR) for WET, the suggested WET criterion values (TUa = 0.3 and TUc = 1.0), and the methods of calculating the WLA using models of effluent dilution. Instead, this review took the WLAa (or WLAa,c) and WLAc as given and considered the TSD statistical method per se.

G.1 TSD Statistical Approach to Reasonable Potential And Limit Calculations

This appendix provides a simplified description of the TSD approach. That approach is more completely described in the *Technical Support Document for Water Quality-Based Toxics Control* (USEPA 1991a). Reasonable potential calculations are described in Section 3.3 of that document. The calculation is only one component of a reasonable potential determination. Permit limit calculations are described in Section 5.4 and Appendix E of the TSD.

To evaluate reasonable potential or calculate permit limits, one needs a coefficient of variation (CV) representing the variability of toxicity or a pollutant in the effluent discharge. The TSD recommends that the CV of measured effluent data be used in all reasonable potential and effluent limit calculations without attempting to "factor out" analytical variability. The specification of this CV is at issue in the alternatives to the TSD statistical procedures discussed later in this appendix.

G.1.1 Reasonable Potential

The goal of the TSD reasonable potential calculation is to estimate the probable value of an upper bound (e.g., 99th percentile) of toxicity in an effluent discharge using limited data. For whole effluent toxicity (WET), data are expressed in toxic units (TU) before calculating the CV. TU = (100/effect concentration). For chronic toxicity, TUc = 100/NOEC or 100/IC25. For acute toxicity, TUa = 100/LC50. The TSD calculations assume that effluent toxicity values follow a lognormal distribution, at least approximately. There is abundant evidence supporting the lognormal distribution, but the TSD also

acknowledges that other distributions might be found more appropriate if sufficient data can support the finding.

The sample CV of effluent monitoring data is obtained in TU. If there are fewer than ten data points, the TSD recommends a default CV of 0.6. The TSD recommends basing a calculated CV on at least ten data points, collected at the same time intervals as intended for monitoring.

Even if there are fewer than ten data points, the maximum value for the data (e.g., TU_{max}) is used to calculate a projected maximum value. A nonparametric, upper tolerance bound is calculated to infer the population percentile represented by TU_{max} with probability P: $X_{P,n} = (1 - P)^{1/n}$. For example, with probability 0.99 the largest of five observations will exceed the 39.8^{th} population percentile: $(1 - 0.99)^{1/5} = 0.398$. Next, the ratio between this percentile ($X_{P,n}$) and the population 99^{th} percentile is estimated using moment estimators for a lognormal distribution:

Reasonable potential multiplier = $X_{0.99} / X_P = \exp(Z_{99}\,\sigma - 0.5\sigma^2) / \exp(Z_P\,\sigma - 0.5\sigma^2)$.

Here, σ^2 is estimated as $\log(1 + CV^2)$, using the default CV if necessary. The maximum projected value is the product of the observed TU_{max} and the reasonable potential multiplier. This value may be compared to the WLA, which is based upon the criteria continuous concentrations (CCC) or criteria maximum concentration (CMC) and the appropriate dilution factors (if applicable). The projected maximum value also may be multiplied by a dilution factor and compared directly to the CMC or CCC (TSD Section 3.3, Box 3-2). The TSD recommends using TUa = 0.3 and TUc = 1.0 either as numeric toxicity criteria or as a means of interpreting the narrative "no toxics in toxic amounts" criteria.

G.1.1.1 Permit limit calculation

The first step in determining the appropriate water quality-based effluent limits for an effluent discharge is to calculate wasteload allocations WLAa and WLAc that correspond to the water quality criteria for acute exposures and chronic exposures or the ambient values used in interpreting narrative criteria (e.g., no discharge of toxic pollutants in toxic amounts). This step is distinct and separate from the "statistical" steps for calculating permit limits or reasonable potential. The WLAs are "givens" in the statistical calculations.

WLAa and WLAc are found through either a direct steady-state calculation or a dynamic model simulation. In either case, any applicable mixing zone and critical stream flows are taken into account. For WET, WLAa is converted to WLAa,c using an ACR. WLAs must not be exceeded if the water quality standards of the receiving water are to be met.

The essential idea behind setting a permit limit using the TSD approach is to find the lognormal distribution (i.e., its mean value or LTA) that would allow no more than a specified percentage of single observations to exceed the WLAa and no more than a specified percentage of the 4-day averages of observations to exceed the WLAc. If this percentage is set at 1 percent, for example, then the 99^{th} percentile of single observations must not exceed the WLAa, and the 99^{th} percentile of 4-day averages must not exceed the WLAc. The 4-day averaging period comes from the typical definitions of chronic exposure and the CCC. The CV has already indirectly specified the distribution's standard deviation. Together, the CV and the LTA specify the appropriate distribution completely.

The calculations which lead to finding the LTAa,c and LTAc (corresponding to the WLAa and WLAc) work in the following manner. The ratio between the LTA and a percentile (X_P) is called a variability factor (VF_P). The VF is calculated from the CV, the percentile (95^{th} or 99^{th}), and the averaging period [1 day (no averaging) or 4 days].

Thus, $LTA = X_P / VF_P$

If we set X_P equal to the WLAa, we find:

$$LTAa,c = WLAa / VF_{99,\ 1\text{-day}}$$
and
$$LTAc = WLAc / VF_{99,\ 4\text{-day}}$$

The smaller of the two LTAs is selected as the LTA used to calculate a limit. This step assures that the limits will exceed neither the WLAa nor the WLAc.

Having selected the smaller LTA, the VF calculation is reversed. Following the TSD recommendations,

$$\text{“Maximum Daily Limit” (“MDL”)} = LTA * VF_{99,\ 1\text{-day}}$$

and

$$\text{“Average Monthly Limit” (“AML”)} = LTA * VF_{95,\ N\text{-day}}$$
(based on N observations)

Note that in calculating the average limit the TSD recommends using a 95th percentile (rather than a 99th percentile) and the number of observations N for averaging may be less than four (although the TSD recommends $N \geq 4$ for purposes of calculating average limits). Limits calculated using the TSD-recommended approach are always equal to or less than the WLAa and WLAc.

G.1.1.2 Analytical variability in the TSD procedures

Analytical variability is a part of the variability of measurements used to analyze reasonable potential and set water quality-based limits. All components of variability that will enter into the permit development process are included in the measurements and calculations used to evaluate reasonable potential and set limits. This insures that the WLA is not exceeded.

Some laboratories have suggested alternative statistical calculations to EPA. Sections G.3 and G.4 discuss these approaches. These alternative calculations, however, would allow limits to exceed the WLA. When a sample effluent toxicity equals the WLA exactly, analytical variability would be expected to cause tests to exceed the WLA about half the time. Limits set above the WLA could allow routine exceedances of the WLA. In contrast, limits set using the TSD approach will provide some margin of safety between the limit and the WLA, guarding to some extent against analytical variability. On average, the TSD approach, employing the CV of measurements, is expected to ensure that the WLA is not exceeded when measured toxicities remain within the limits.

G.2 Background on Analytical Variability and Variability of Measurements

This section describes how analytical variability may cause the variance (σ^2) of measured values to exceed the variance of toxicity. This discussion will assume that WET tests for one discharge are conducted by one laboratory. Thus, “analytical variability” here will refer to within-laboratory variability (repeatability) of WET test results.

G.2.1 Components of Measurement Variability

The variance of monthly or quarterly measurements of effluent toxicity depends on at least two components: the variance of the toxicity, which changes over time, and the variance owed to the analytical process (including calibration, if applicable). One could also distinguish a third component—sampling variance—if simultaneous samples differ in toxicity. Herein, this component will not be examined separately, but is combined with the variance in toxicity over time.

A direct way to estimate the analytical component of variability is to analyze the same sample of effluent on different occasions so that the analytical method is the only source of measurement variance. The sample must be measured on different days because real samples are measured at intervals of weeks to months and the analytical process can change subtly over time. Unfortunately, effluent samples may not retain the same toxicity for long. Therefore, saving a batch of sample and analyzing it once a month for several months may over-estimate analytical variability. Analyzing two or three subsamples on the same date may underestimate analytical variability because the measurement system changes between sampling dates. The organisms, laboratory technicians and procedures, and laboratory materials may all change subtly over time. It would be reasonable to design a study that measures analytical variability in both ways, using effluent subsamples on one occasion and using the same (stored) effluent sample on separate occasions, attempting to bracket the correct value of analytical variance. EPA is not aware of any such studies. Reference toxicant samples are expected to have the same potency on different occasions and are used routinely for laboratory quality assurance of WET test methods. This document summarizes the variability resulting from repeated (usually monthly) WET testing of reference toxicant samples in the same laboratory.

G.2.2 Effect of Analytical Variability on Measured Values

Because of analytical variability the probability distribution of measured values Y is "wider" than the distribution of true values X. Thus, the mean and high percentiles of measurements will exceed the percentiles of the true values.

One component of the variance of measurements is analytical variance. Simple but plausible assumptions lead to the equation $V_Y = V_X + V_A$. In other words, the variance of a measurement Y (toxicity) is the sum of the variances for toxicity (V_X) and the analytical variance (V_A). When this equation is approximately correct, then one suitable estimate of V_X is ($V_Y - V_A$), where the parameters V_Y and V_A are replaced by their sample estimates. This estimate may be biased (i.e., inaccurate) to some degree. Similar reasoning about the mean (EY) leads to EY = EX. Then $V_Y = V_X + V_A$ can be divided by EX^2 to give $CV_Y^2 = CV_X^2 + CV_A^2$. This reasoning requires two assumptions: variance is constant and unrelated to the mean, and there is little or no correlation between X and the magnitude of the analytical error. When X is distributed lognormally, these assumptions are not true, but may be suitable for transformed values like log(Y) and log(X).

G.2.3 Analytical Variability and Self-monitoring Data

EPA determines compliance with a limit on the basis of self-monitoring data. No special allowance is made for analytical variability. This is accounted for by the TSD statistical procedures used to determine the need for limits and calculate permit limits.

The permittee must ensure that the toxicity in the discharge is never great enough to result in a compliance measurement that exceeds the permit limit. The maximum discharge toxicity allowed by the treatment system must incorporate a margin of safety to account for the sampling and analytical variability that attends compliance measurements. In other words, to avoid exceedances of a limit, a treatment system will be designed so that the maximum discharge toxicity is somewhat lower than the permit limit. Most industrial and municipal treatment facilities should be able to implement such a design. When they are not, appeals based on fundamentally different factors and economic hardships may be feasible.

G.2.4 Imprecision in WET Estimates, Reasonable Potential Determinations and Limits

Although WET tests provide protection against false positives, the estimates (NOEC, EC25, LC50) from WET tests, like all estimates based on limited data, are imprecise. That is, the exact level of toxicity in a sample is estimated with "error" (imprecision). This imprecision can be reduced by providing a suitable number of organisms and replicates for each test. The numbers required for EPA WET method test

acceptability are *minimums*. Test precision will be approximately proportional to the square root of the number of replicates. Thus, a doubling of replication may increase the precision of a test endpoint response (survival, growth, reproduction) to roughly 70 percent of its former level. For example, consider these calculations for fathead minnow growth (USEPA 1994a, pp. 102-105): the standard error of the difference between a treatment and the control is $Sw\sqrt{(1/n_T + 1/n_C)}$, which in one test took the value $(0.0972)\sqrt{(1/4 + 1/4)} = (0.0972)(0.707) = 0.0687$. If the root mean squared error Sw had been the same but the number of replicates had been doubled, the standard error would have been 0.0486. Dunnett's critical value would have been 2.24 instead of 2.36, and the MSD 0.109 instead of 0.162. With a doubling of replication, the test would be able to detect a 16-percent reduction from the control rather than a 24-percent reduction.

For reasonable potential and limit calculations, WET data are accumulated over a year or more to characterize effluent variability over time. This sampling program may not fully characterize effluent variability if too few samples are taken, if the sampling times and dates are not representative, or if the duration of the sampling program is not long enough to represent the full range of effluent variability. For reasonable potential and limits, the key quantity being estimated is the variance (or CV). A large number of samples is required to estimate a variance or CV with much precision. Confidence intervals for the variance and CV can be calculated easily and carried through the calculations for reasonable potential and effluent limits (Section G.1). Even when assumptions are not strictly met, this information may provide a useful perspective on the uncertainty of the calculation.

G.2.5 Between-laboratory Variability in Reasonable Potential and Permit Limit Calculations

It is inappropriate to use estimates of between-laboratory variability in calculations of reasonable potential and permit limits. Such estimates do not represent the variability affecting measurements of effluent discharge toxicity. In most cases, only one laboratory will produce the data for one discharge. In some cases, there will be a change of laboratory over time, which needs to be handled case-by-case. Using estimates of between-laboratory variability to represent the analytical component of variance for one discharge is equivalent to assuming that each new sample is sent to a new laboratory selected at random from the population of laboratories conducting the test method. This approach does not occur in practice.

Between-laboratory differences in test sensitivity are important and need to be addressed. To some extent, apparent differences in sensitivity between laboratories (Warren-Hicks et al. 1999) may be owed to several factors, including use of unstable reference toxicants like SDS (Environment Canada 1990), errors in calculating and recording stock concentrations (Chapter 3 of the Variability Guidance, SCTAG 1996), differences in dissociation and bioavailability of metal ions, comparisons of non-comparable ionic forms (e.g., potassium chromate versus potassium dichromate, SCTAG 1996), use of different waters, health of organisms, and varying techniques.

Within-laboratory variability should be reflected in regulatory calculations. If the data being used for reasonable potential or permit limit calculations consist of effluent measurement data reported by two or more laboratories, there are ways to account for between-laboratory differences:

- If the same laboratories are used in the same proportion or frequency, and the measurements for different laboratories represent different sampling dates, the measurement data may be treated as if they come from one laboratory. This may increase the estimated variance and the average monthly limit, which is not in the interest of the permittee. It would be better to select one laboratory, based on the variance of its reported reference toxicant test results.

- If only one laboratory has reported data on each date, with the different laboratories either reporting over different time spans or over the same time span on alternate dates, EPA recommends a pooled

estimate of variance. Calculate the sample variance S^2 for log(TU) separately for each laboratory, and combine the data in the following formula:

$$\text{pooled variance of } \log(X) = [(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2] / [(N_1 - 1) + (N_2 - 1)]$$

(i.e., the analogous formula for more than two laboratories). The same result can be obtained by conducting a one-way analysis of variance on log(X) and using the mean squared error. This approach would be undesirable if the different laboratories sampled times or time spans that were known or expected to differ in the average or variance of TU. In that case, one would pool the data, treating it as if it had come from one laboratory (see above).

A change of testing laboratory by a permittee may result in a change in analytical (within-laboratory) variability of measurements and a change in "sensitivity." The average effect concentration may change. There may be between-laboratory differences in sensitivity to some toxicants, such as metals (Warren-Hicks et al. 1999).

Ideally, a permittee will anticipate a change of the testing laboratory. Permittees should compare reference toxicant test data from current and candidate replacement laboratories, selecting a laboratory with acceptable variability and a similar average effect concentration. Regulatory authorities should compare reference toxicant data for old and new laboratories when interpreting a series of WET test results that involves a change of laboratory.

Some areas may help reduce laboratory differences in average effect concentration for the same reference toxicant test protocol. These include standardization and reporting of stock culture conditions (such as loading, age structure, age-specific weight, and other conditions), standardization of dilution water for reference toxicant tests, and reporting to verify such practices. Other areas for consideration include test protocols, test acceptability criteria, and dilution water. Another approach that could be evaluated further is conducting a reference toxicant test with each effluent test, and normalizing the effluent response using the toxicant response.

G.3 Adjustment Approaches To Account For Analytical Variability in Setting Permit Limits

G.3.1 Adjustment Approaches To Account for Analytical Variability

Methods have been proposed for determining reasonable potential and calculating permit limits by adjusting the calculations based on analytical variability. The more general principles are discussed here, details of these methods are outlined in Section G.4. The focus of these discussions is the limit calculation, although similar principles apply to the reasonable potential calculation.

The idea behind the proposed "adjustment methods" for calculating water quality-based effluent limits is to estimate the distribution of toxicity values using data on measured effects concentrations and analytical variability, and then to factor out analytical variability from some steps in the process of calculating limits. In proposed adjustment methods for calculating effluent limitations one would (1) estimate the variance of effluent concentrations (this entails subtracting an estimate of the analytical variance from the variance of effluent measurements, e.g., $V_X = V_Y - V_A$, or an equivalent calculation using CVs); (2) calculate the LTAa and LTAc using the TSD approach and the adjusted variance V_X; and (3) calculate the limit (from the lower of the two LTAs) using the variance of measurements V_Y. Because the V_Y necessarily exceeds V_X, these methods would result in limits that would exceed calculated WLAs, depending on other assumptions made in the limit calculations. As a result, the discharge may allow instream WET to routinely exceed the criterion limits, a condition that should not occur.

G.3.2 Adjustment Equations

As noted above, the adjustment approaches are based on the TSD statistical approach, modified to subtract analytical variability from the LTA calculation. These approaches refer to V_X as the "true" variance. In what follows, the sample estimate of V_X is S^2_{True}. Thus, $S^2_{True} = S^2_{Meas} - S^2_{Analy}$ (where S^2 is the sample estimate of variance) is used to calculate the LTAs and S^2_{Meas} is used to calculate the limits from the smallest of the two LTAs. The TSD equations as applied to WET would be adjusted as follows:

When the LTAa,c is the smallest LTA,

$$MDL = WLAa,c * (VF_{99,\ 1\text{-}day,\ Meas} / VF_{99,\ 1\text{-}day,\ True})$$
$$AML = WLAa,c * (VF_{95,\ N\text{-}day,\ Meas} / VF_{99,\ 1\text{-}day,\ True})$$

When LTAc is the smallest LTA (and assuming that the chronic criterion is a 4-day average)

$$MDL = WLAc * (VF_{99,\ 1\text{-}day,\ Meas} / VF_{99,\ 4\text{-}day,\ True})$$
$$AML = WLAc * (VF_{95,\ N\text{-}day,\ Meas} / VF_{99,\ 4\text{-}day,\ True})$$
$$\text{where } N = \text{samples/month (for purposes of AML calculation)}$$

The VF (variance factor) is the ratio of a percentile to a mean, in this case for the lognormal distribution.

$$VF_{99,\ 1\text{-}day,\ Meas} = \exp(Z_{99} S_{Meas} - 0.5S^2_{Meas})$$
$$VF_{99,\ 1\text{-}day,\ True} = \exp(Z_{99} S_{True} - 0.5S^2_{True})$$
$$VF_{95,\ n\text{-}day,\ Meas} = \exp(Z_{95} S_{n\text{-}day,\ Meas} - 0.5S^2_{n\text{-}day,\ Meas})$$
$$VF_{99,\ 4\text{-}day,\ True} = \exp(Z_{99} S_{4\text{-}day,\ True} - 0.5S^2_{4\text{-}day,\ True})$$

while
$$S^2_{Meas} = \log(1 + CV^2_{Meas})$$
$$S^2_{True} = \log(1 + CV^2_{True})$$
$$S^2_{N\text{-}day,\ Meas} = \log(1 + CV^2_{Meas}/N)$$
or
$$S^2_{N\text{-}day,\ Meas} = S^2_{Meas}/N = \log(1 + CV^2_{Meas})/N$$
$$S^2_{4\text{-}day,\ True} = \log(1 + CV^2_{True}/4)$$
or
$$S^2_{4\text{-}day,\ True} = S^2_{True}/4 = \log(1 + CV^2_{True})/4$$

G.3.3 Consequences of Adjustment Approaches

As an example of the consequences of applying an adjustment methodology to water quality-based effluent limit calculations, one may consider the following scenario. In this scenario, such a methodology would allow calculation of an average monthly limit (AML) exceeding the chronic WLA (a four-day average value) even when sampling frequency for the calculation is set at the recommended minimum of four samples per month. It is acceptable for the MDL (a single sample) to exceed the chronic WLA or for the AML to exceed the chronic WLA if the AML calculation is based on less than four samples per month. Note, however, that the TSD recommends always assuming at least four samples per month when calculating the AML.

Table G-1 below offers an example of MDLs and AMLs calculated using the TSD approach and an approach that adjusts the CV for analytic variability. This adjustment would allow effluent limits that exceed the WLA on the premise that analytical variability tends to make measured values larger than actual effluent values. Thus, this approach assumes that the "true" monthly average would be below the WLAc even though the limit and the measured monthly average may be above the WLAc.

EPA believes that these assumptions are invalid. Therefore, EPA cannot recommend an approach that makes such assumptions as part of national guidance to regulatory authorities. EPA is not recommending national application of an "adjustment approach" to either reasonable potential or effluent limit calculation

procedures. EPA continues to recommend the TSD approach, which ensures that effluent limits and, thereby, *measured* effluent toxicity, are consistent with calculated WLAs.

Table G-1. Sample Effluent Limit Calculations Using EPA's TSD Approach and an Adjustment Approach (USEPA 1991a)

WLA_c	Probability Basis	Approach	LTA_c	MDL	AML
10	MDL = 99th percentile AML = 95th percentile	TSD	4.4	17.6	7.7
10	MDL = 99th percentile AML = 95th percentile	Adjustment approach	6.43	25.8	***11.2*** *
10	MDL = 99th percentile AML = 99th percentile	TSD	4.4	17.6	9.99
10	MDL = 99th percentile AML = 99th percentile	Adjustment approach	6.43	25.8	***14.6*** *

Assumptions: Chronic LTA/WLA controls calculations, WLA = 99th percentile probability basis, n = 4 (sampling frequency for AML calculation), Total CV = 0.8 and Adjusted CV = 0.4 are used in calculations.
(*) These numbers exceed the WLAc.

G.3.4 Related Concerns

In addition to addressing the differences between measured and "true" values in the reasonable potential and effluent limit calculations, related concerns regarding WET testing and the water quality-based effluent permits process have been raised as reasons for adjusting the TSD statistical procedures.

G.3.4.1 Compounding protective assumptions

Approaches to "account for analytical variability" by adjusting the calculations for reasonable potential and limits usually state that several conservative assumptions are employed. In the TSD approach, a water quality-based effluent limit is the result of three key components: (1) a criterion concentration; (2) a calculated dilution or mixing-zone factor; and (3) a statistical calculation procedure that employs a CV based on effluent data. The conservative assumptions cited may involve deriving the criterion concentration, and assuming dilution and low-flow conditions, in addition to the probability levels used in the TSD statistical calculations. Even if these assumptions were considered conservative, the TSD statistical procedure remains valid. As explained above, the TSD statistical approach is *appropriately* protective, provided that the WLA is accepted as given. It is inappropriate for regulatory authorities to modify the TSD's correctly conceived statistical approach in order to compensate for assumptions intrinsic to derivation of the WLA that are perceived as over protective. Therefore, EPA does not believe that it is appropriate to adjust the TSD statistical methodology for conducting reasonable potential and calculating permit limits to address concerns about how WLAs are calculated.

G.3.4.2 Test sensitivity and method detection limit

EPA does not employ method detection limits (MDLs: 40 CFR part 136 Appendix B) for WET methods. For effect concentrations derived by a hypothesis test (LOEC and NOEC), the alpha level of the test provides one means of providing a functional equivalent of an MDL. The hypothesis test prescribed in the method provides a high level of protection from "false positives." For point estimates (ECp, ICp, LCp), a valid confidence region provides the equivalent of a hypothesis test. EPA will provide clarification regarding when confidence intervals are not or cannot be generated for point estimation procedures, including the ICp procedure. This variability guidance cites recommendations (Chapman et al. 1996a, Baird et al. 1996, Bailer et al. 2000) regarding alternative point estimation methodologies.

While protecting against false positives, hypothesis tests and confidence intervals, will provide little protection from toxicity unless the test method is designed to detect a suitable effect size. The two most commonly used chronic tests are incapable of routinely detecting effects of 20 percent to 30 percent (Denton and Norberg-King 1996) when employed by many laboratories using the minimum recommended number of replicates and treatments. To provide suitable test sensitivity, regulatory authorities should consider requiring more replication, a suitable minimum significant difference (MSD), or suitable effect sizes and power, particularly for the control and IWC test concentrations (e.g., Denton and Norberg-King 1996; Washington State Department of Ecology 1997, Ch. 173-205 WAC). It may be desirable to specify that a statistically significant effect at the IWC must exceed some percentage difference from the control before it is deemed to have regulatory significance. Combining these approaches, an effective strategy would require that a test consistently be able to detect the smallest effect size (percent difference between the control and the IWC) that would compromise aquatic life protection, and to disregard very small, statistically significant effects. To further these ends, this guidance document sets an upper limit to the value of MSD/(Control Mean), defining the maximum acceptable value. This document also sets a lower limit to the effect size, defined by 100×(Control Mean - Treatment Mean)/(Control Mean), which can be regarded as "toxic" in a practical sense (see Section 6.4).

The alpha level of a hypothesis test or confidence interval cannot be decreased from that level (α = 0.05) recommended for WET methods without sacrificing test power and sensitivity of the method. Alpha should not be decreased without a corresponding increase in sample size that would preserve the power to detect biologically significant effects. EPA will issue guidance on when the nominal error rate (alpha level) may be adjusted in the hypothesis test for some promulgated WET methods (USEPA 2000a).

G.4 Technical Notes on Methods of Adjusting For Analytical Variability

This section describes and comments on several adjustment methodologies suggested to EPA as alternatives to the TSD statistical calculations.

G.4.1 Notation

Explanations may help clarify the notations in this section. The symbols VX, V[X], and σ^2_X all mean: the variance of X. Standard deviation (σ_X) is the square root of the variance. The mean (average) is symbolized as EX and also as μ_X.

When X is lognormally distributed, there is a potential for confusing the mean and variance of log(X) with the mean and variance of X. Typically (and in the TSD), when X is lognormally distributed, the parameters will be given for log(X) as follows: X ~ lnorm(μ, σ). This is read as "X is distributed lognormally with the mean of log X equal to μ (mu) and the standard deviation of log X equal to σ (sigma)." Better notation would be X ~ lnorm(μ_{logX}, σ_{logX}); recommended terms for the parameters are "mu-logX" and "sigma-logX." The mean and variance of X for this distribution are

$$\mu_X = EX = \exp(\mu_{logX} + 0.5*\sigma^2_{logX})$$
$$\sigma^2_X = VX = \exp(2*\mu_{logX} + \sigma^2_{logX}) * [\exp(\sigma^2_{logX}) - 1]$$

To avoid confusion, the symbols EX and VX are used in preference to μ_X and σ^2_X to signify the mean and variance of X. Usually, mu and sigma are used only as symbols for the mean and standard deviation of log(X), that is, μ_{logX} and σ_{logX}, in the context of lognormal distributions. Below, μ_{logX} and σ_{logX} are abbreviated to μ and σ, with the addition of subscripts like "Effl" and "Meas" to further distinguish the intended quantity.

CV may be used to symbolize parametric values or their sample estimates, with the meaning indicated in the text. Symbols S^2_{Effl} , S^2_{Meas} , and S^2_{Analy} will represent sample estimates of variances $\sigma^2_{logX, Effl}$, $\sigma^2_{logX, Meas}$, and $\sigma^2_{logX, Analy}$.

G.4.2 General Comments on Analytical Variance as a Component of the Variance of Measurements

Two simple models lead to the same equation. The first model assumes that each measurement Y is the sum of a concentration X and an analytical error ϵ, that is $Y = X + \epsilon$. The analytical error ϵ may be positive or negative and has mean zero and variance V_A. X and ϵ are uncorrelated. (This is a strong assumption; it may be approximately correct only for some transformation of the data.) Then $V_Y = V_X + V_A$. The second, hierarchical, model assumes that X follows a distribution P_X with mean and variance E_X and V_X. Each measurement Yt (t indexes the time of measurement) follows another distribution having mean Xt and variance V_A. V_A is assumed to be constant, independent of Xt. (This is a strong assumption which may be approximately correct only for some transformation of the data.) Then, it can be shown that $V_Y = V_X + V_A$. The same models and assumptions lead to EY = EX. These models and assumptions are not correct when X is lognormally distributed. In that case, the models might provide reasonable approximations to the behavior of log(X) and log(Y). If EY = EX and $V_Y = V_X + V_A$ are both correct, then $V_Y = V_X + V_A$ can be divided by EX^2 to give $CV_Y^2 = CV_X^2 + CV_A^2$. In this case, the parameters V_X and CV_X^2 might be estimated by using sample estimates in the expressions $(V_Y - V_A)$ and $(CV_X^2 - CV_A^2)$, respectively. Such estimates will be somewhat biased.

G.4.3 Commonwealth of Virginia Approach

The Commonwealth of Virginia Toxics Management Program Implementation Guidance (1993) (revised on August 25, 1994) prescribes a method of accounting for analytical variability of WET data. A synopsis of the method follows. Symbolic notation has been changed; the numbered "steps" below were created for this synopsis.

1. Obtain the CV of WET monitoring data. This will be 0.6 (default value) if fewer than ten data are available. If there are at least ten data, a computer program (*described in Guidance Memo 93-015*) is used. *"Only acute test data are considered here because the LC_{50} is a statistically derived point estimate from a continuous data set. Also, the LC_{50}s must be real numbers. Values reported as '> 100%' should not be used in the calculation. .... Enter either LC_{50}s or TU_as for the most sensitive species into the program."* [Comments on Step 1: LC50 and TU values are not equivalent; they will not have the same CV values. The exclusion of ">100%" values will tend to bias the CV of TUs toward larger values.]

2. Calculate $S^2_{logX,\ Effl} = S^2_{logX,\ Meas} + S^2_{logX,\ Analy}$, using $S^2_{logX,\ Analy} = 0.20$. If $CV_{X,\ Meas} < 0.47$ (implying that $S^2_{logX,\ Meas} < 0.20 = S^2_{logX,\ Analy}$), instead use $S^2_{logX,\ Effl} = S^2_{logX,\ Meas}$. (These subscripts are not used in the Guide.) The value for $S^2_{logX,\ Analy}$ is based on data provided by several laboratories conducting tests for Virginia permits for the five most common species, using cadmium chloride as the reference toxicant. The Guide states that these data yielded a geometric mean CV_X of 0.47, and $0.20 = \ln(1 + 0.47^2)$; the last formula is the relation between the parametic variance and CV of a lognormal variate. [Comments on Step 2: The calculations should employ sample variances of log(TU), not sample CVs, in the interest of accuracy and precision. The estimate $S^2_{logX,Effl}$ is a discontinuous function, decreasing toward zero as $S^2_{logX,\ Meas}$ decreases toward 0.2, then jumping to 0.2 and decreasing again toward zero as $S^2_{logX,\ Meas}$ decreases further. The default value of $S^2_{logX,\ Effl}$ becomes $\ln(1 + 0.60^2) - \ln(1 + 0.47^2) = 0.11$.]

3. Calculate LTAa,c and LTAc as in the TSD, using $S^2_{logX,\ Effl}$ instead of $S^2_{logX,\ Meas}$, and using Z_{97}, the 97th percentile Z-statistic, instead of Z_{99}. WLA and LTA values are in units of TUc. The smaller of LTAa,c and LTAc is selected as LTA_{min}.

4. Calculate the "MDL" limit from LTA_{min} as in the TSD, now using $S^2_{logX,\ Meas}$ rather than $S^2_{logX,\ Effl}$ and still using the 97th percentile Z-statistic. No procedure is described for a limit of averages ("AML").

   By using this procedure, the WLAa,c may be exceeded when the CV of measurements exceeds 0.47 (because then the estimate $S^2_{logX,\ Effl} < S^2_{logX,\ Meas}$). The maximum ratio of Limit to WLA occurs when the CV of observations is just over 0.47, when the ratio of Limit to WLA is just over 2. Numerical evaluations (Table G-2) show that the daily limit can exceed the WLAa,c. The daily limit (DL or MDL) should be compared to the WLAa,c. It is not unusual for the daily limit to exceed the WLAc when LTAc is smaller than LTAa,c. This outcome does not necessarily indicate a problem. Instead, the regulatory authority should compare the average limit to WLAc in this case (see "Modified TSD Approach" below).

Table G-2. Numerical Effect of State of Virginia WET Limit Calculation on Ratio of Daily Limit to WLA

CV_{Meas}	S^2_{Effl}	$S^2_{Effl,\ 4\text{-day average}}$	Ratio of Daily Limit to WLAa,c	Ratio of Daily Limit to WLAc
0.10	0.01	0.00	1.00	1.09
0.20	0.04	0.01	1.00	1.19
0.30	0.09	0.02	1.00	1.27
0.40	0.15	0.04	1.00	1.35
0.45	0.18	0.05	1.00	1.38
0.470	0.1996	0.0538	2.097	1.393
0.471	0.0004	0.0002	2.026	2.042
0.50	0.02	0.01	1.65	1.87
0.60	0.11	0.03	1.39	1.76
0.70	0.20	0.06	1.28	1.74
0.80	0.29	0.09	1.22	1.72
0.90	0.39	0.13	1.18	1.71
1.00	0.49	0.17	1.16	1.70

The State of Virginia Guide, Appendix D, also states: "*Because the statistical approach evaluates both acute and chronic toxicity of the effluent, only one limit is necessary to protect from both acute and chronic toxicity. The limit is expressed only as a maximum daily limit (MDL) because the frequency of monitoring will typically be less than once per month. If the testing is to be monthly, then the MDL can also be expressed as an average monthly limit (AML).*" [Comment: a single MDL limit is not as protective as the combination of limits, one for single observations (MDL) and another for averages (for example, the quarterly or annual average). Refer to the TSD (USEPA 1991a, Section 5.3).]

G.4.4 Rice Approach

James K. Rice's unpublished draft, "Laboratory QC and the Regulatory Environment: Relation Between Method Performance and Compliance" prescribes a method of accounting for analytical variability of WET data. The document was provided with a notation that the typescript was originally submitted to EPA as a comment on the draft "TSD," presumably in the period 1989 to 1991. A synopsis of the method follows. The numbered "steps" below were created for this synopsis. Calculations and symbols have been

simplified. This synopsis omits many detailed observations that provide context and guidelines for readers intending to apply Rice's method.

1. Obtain the CV of WET monitoring data (measured values), and the CV of the analytical method, in symbols $CV_{X, Meas}$ and $CV_{X, Analy}$. Sample size is not addressed, but the text indicates that "a large number" of measurements are needed to characterize variability and bias.

2. Solve for $CV_{X, Effl}^2$ in $CV_{X, Meas}^2 = CV_{X, Analy}^2 + CV_{X, Ttue}^2 + (CV_{X, Analy}^2 * CV_{X, Effl}^2)$, after substituting the sample estimates of $CV_{X, Meas}^2$ and $CV_{X, Analy}^2$. Thus, solve

   $$CV_{X, Effl}^2 = (CV_{X, Meas}^2 - CV_{X, Analy}^2) / (1 + CV_{X, Analy}^2).$$

   [Comment: This formula assumes a model such as Measurement = (Concentration * Recovery), with multiplicative errors for Concentration and Recovery. This is one plausible model, especially for data that are distributed lognormally. Another plausible model would lead to the formula $CV_{X, Meas}^2 = CV_{X, Analy}^2 + CV_{X, Ttue}^2$.]

3. Calculate LTA values as in the TSD, using $CV_{X, Effl}$ instead of $CV_{X, Meas}$, and use Z_{99}, the 99th percentile Z-statistic. First calculate $\sigma^2_{logX, Effl} = \ln(1 + CV_{X, Effl}^2)$ for the variance of log(TU), and $\sigma^2_{logX, Effl, n} = \ln(1 + (CV_{X, Effl}^2)/n)$ for an n-day average. Then $LTA_{Effl} = WLA * \exp(0.5\sigma^2_{logX, Effl, n} - Z_P \sigma_{logX, Effl, n})$. Rice then calculates $LTA_{meas} = (R/100) * LTA_{Effl}$, where R is the percent recovery of the analytical method. [Comments: Many chemical methods are now calibrated instrumentally so that E[R] = 100 percent. It will be assumed herein that R = 100 percent for WET methods. There is no discussion of, or accounting for, the sampling error (the uncertainty) that attends the estimates of R or σ^2, of the sample sizes required to estimate these well. The example does not encompass the derivation and comparison of acute versus chronic LTAs using estimates of the variance of single observations and averages and selection of the smaller one, as in the 1991 TSD. Rice's method could easily be modified for the current TSD approach (see for example, the State of Virginia method, above).

4. Calculate the MDL and AML limits from the LTA as in the TSD, now using $\sigma^2_{logX, Meas}$ rather than $\sigma^2_{logX, Effl}$, and using the 99th percentile Z-statistic. Thus,

   $$MDL = LTA_{meas} * \exp(-0.5\sigma^2_{logX, Meas, 1} + Z_P \sigma_{logX, Meas, 1})$$
   $$AML_n = LTA_{meas} * \exp(-0.5\sigma^2_{logX, Meas, n} + Z_P \sigma_{logX, Meas, n})$$

   Using this procedure, the limits exceed the WLAc.

   $$MDL = WLAc * (VF_{.99, 1, Meas} / VF_{.99, 4, Effl}) > WLAc$$
   $$AML_n = WLAc * (VF_{.99, n, Meas} / VF_{.99, 4, Effl}) > WLAc \text{ if } n \le 4$$

   The AML can exceed WLAc even if n >4, depending upon the variance values. Because the current TSD approach of comparing LTAa,c and the LTAc had not been developed by the time of Rice's report, he did not apply his procedure to the WLAa,c.

G.4.5 Amelia River Report

The Amelia River Report (USEPA 1987, Appendix G) describes a similar approach, estimating $S^2_{logX, Effl} = S^2_{logX, Meas} + S^2_{logX, Analy}$ (without any provision for the case $S^2_{logX, Meas} \le S^2_{logX, Analy}$), calculating LTA from WLA using $S^2_{logX, Effl}$, and calculating the limits using $S^2_{logX, Meas}$.

G.4.5.1 Modified TSD approach

The methods described above predate the current TSD statistical approach and differ from it. As noted in the previous section, one could consider how the current TSD statistical approach could be modified to account for analytical variability using the same principles. The LTAs would be calculated using a variance estimate $S^2_{Effl} = S^2_{Meas} - S^2_{Analy}$, the smallest would be selected, and limits would be calculated from the smaller LTA using S^2_{Meas}. Table G-3 compares the current and modified calculations for whole effluent toxicity. Numerical calculations appear in Tables G-4 and G-5.

Table G-3. A Comparison of the Current TSD Calculation of Limits with a Modification That Takes into Account the Analytical Variability

Method	Smallest LTA	Limits
TSD statistical approach	LTAa,c	MDL = WLAa,c ($VF_{.99, 1, Meas}$ / $VF_{.99, 1, Meas}$) = WLAa,c AML = WLAa,c ($VF_{.95, N, Meas}$ / $VF_{.99, 1, Meas}$) < WLAa,c
	LTAc	MDL = WLAc ($VF_{.99, 1, Meas}$ / $VF_{.99, 4, Meas}$) < or > WLAa,c AML = WLAc ($VF_{.95, N, Meas}$ / $VF_{.99, 4, Meas}$) < WLAc
TSD modified to use S^2_{Effl} to calculate LTA	LTAa,c	MDL = WLAa,c ($VF_{.99, 1, Meas}$ / $VF_{.99, 1, Effl}$) > WLAa,c AML = WLAa,c ($VF_{.95, N, Meas}$ / $VF_{.99, 1, Effl}$) < or > WLAa,c
	LTAc	MDL = WLAc ($VF_{.99, 1, Meas}$ / $VF_{.99, 4, Effl}$) < WLAc AML = WLAc ($VF_{.95, N, Meas}$ / $VF_{.99, 4, Effl}$) < or > WLAc

Symbols for estimates based on data (sample estimates):

S^2_{Meas} sample variance of natural logs of measured TUs
S^2_{Analy} sample variance of natural logs of measurements on the same or TU
S^2_{Effl} estimate of variance of natural logs of TUs
$S^2_{Effl} = S^2_{Meas} - S^2_{Analy}$

$VF_{P, N, xxxx} = \exp(Z_P S_{xxx, N} - 0.5 S^2_{xxx, N})$ estimates the ratio of the P-th percentile to the mean for a lognormal variate: the P-th percentile is $\exp(\mu + Z_P \sigma)$ and the mean is $\exp(\mu + 0.5\sigma^2)$. The mean of a 4-day average of lognormal observations is assumed to be lognormal (Kahn, H.D., and M.B. Rubin. 1989. Use of statistical methods in industrial water pollution control regulations in the United States. *Environmental Monitoring and Assessment* 12:129-148).

The variance estimates may change with and be a function of the TU.
"N" is the number of samples (measurements) intended for use in determining compliance with the average limit, not the number of data used to calculate the sample variances used in setting limits.
It can be shown that LTAc < LTAa,c implies that WLAc < WLAa,c

For WET, WLAa,c = WLAa * ACR. It is assumed that the variance of observations (S^2_{Meas}) equals or exceeds the analytical variance (S^2_{Analy}). Numerical comparisons appear in Tables G-2 to G-4.

Calculations in Tables G-4 and G-5 show the numerical effect of adjustment on permit limits in relation to the WLA. These tables show the ratio of the limit to the WLA. For these calculations, S^2_{Meas} was calculated as $\log(1 + CV^2_{Meas})$, while $S^2_{Meas, 4\text{-}day} = \log(1 + CV^2_{Meas}/4)$, giving slightly different numerical results than if $S^2_{Meas, 4\text{-}day} = S^2_{Meas}/4 = \log(1 + CV^2_{Meas})/4$. The first formula is prescribed in the TSD, Box 5-2 and Table 5-1. The tables show the combinations of CV values used for CV_{Meas} and CV_{Analy}. The variance of TUs was calculated as $S^2_{Effl} = S^2_{Meas} - S^2_{Analy}$ using $S^2_{Meas} = \log(1 + CV^2_{Meas})$ and $S^2_{Analy} = \log(1 + CV^2_{Analy})$.

Table G-4. Ratio of MDL to WLA-LTA from WLA and CV_{Effl} and Limit from LTA and CV_{meas}

	LTAac is Smallest Ratio is MDL:WLAa,c					LTAc is Smallest Ratio is MDL:WLAc				
	CV_{Analy}					CV_{Analy}				
CV_{Meas}	0.1	0.2	0.3	0.4	0.5	0.1	0.2	0.3	0.4	0.5
0.1	1.25	0.00	0.00	0.00	0.00	1.25	0.00	0.00	0.00	0.00
0.2	1.06	1.55	0.00	0.00	0.00	1.28	1.55	0.00	0.00	0.00
0.3	1.04	1.17	1.90	0.00	0.00	1.38	1.47	1.90	0.00	0.00
0.4	1.03	1.11	1.31	2.28	0.00	1.48	1.55	1.69	2.28	0.00
0.5	1.02	1.09	1.22	1.48	2.68	1.58	1.63	1.73	1.93	2.68
0.6	1.02	1.07	1.16	1.33	1.65	1.66	1.70	1.79	1.93	2.18
0.7	1.01	1.06	1.13	1.26	1.47	1.72	1.76	1.83	1.94	2.12
0.8	1.01	1.05	1.11	1.21	1.37	1.77	1.81	1.87	1.96	2.10
0.9	1.01	1.04	1.10	1.18	1.30	1.81	1.84	1.90	1.98	2.09
1.0	1.01	1.04	1.08	1.16	1.26	1.84	1.86	1.91	1.98	2.08

[a] The LTA was calculated using the WLA and $_{Cveffl}$. The limit was calculated using the LTA and $CV_{meas.}$

Table G-5. Ratio of AML to WLA

	LTAa,c is smallest ratio is AML:WLAa,c					LTAc is smallest ratio is AML:WLAc				
	CV_{Analy}					CV_{Analy}				
CV_{Meas}	0.1	0.2	0.3	0.4	0.5	0.1	0.2	0.3	0.4	0.5
0.1	1.08	0.00	0.00	0.00	0.00	1.08	0.00	0.00	0.00	0.00
0.2	0.80	1.17	0.00	0.00	0.00	0.96	1.17	0.00	0.00	0.00
0.3	0.69	0.78	1.26	0.00	0.00	0.92	0.98	1.26	0.00	0.00
0.4	0.61	0.66	0.78	1.36	0.00	0.89	0.93	1.01	1.36	0.00
0.5	0.55	0.59	0.66	0.80	1.45	0.85	0.88	0.94	1.05	1.45
0.6	0.51	0.53	0.58	0.66	0.82	0.83	0.85	0.89	0.96	1.08
0.7	0.47	0.49	0.53	0.58	0.68	0.80	0.82	0.85	0.90	0.98
0.8	0.44	0.46	0.49	0.53	0.60	0.77	0.79	0.82	0.86	0.92
0.9	0.42	0.43	0.45	0.49	0.54	0.75	0.76	0.79	0.82	0.87
1.0	0.40	0.41	0.43	0.46	0.50	0.73	0.74	0.76	0.79	0.83

NOTE: If the AML were set at a 99th percentile value, all ratios would exceed 1.00. It is not surprising that the ratio in the table for AML is less than 1, should not come close to one, because the 95th percentile was used in the second part of the equation. The ratio should be constantly less than one in order to protect water quality criteria.

[a] The LTA was calculated using the WLA and $_{Cveffl}$. The limit was calculated using the LTA and $CV_{meas.}$

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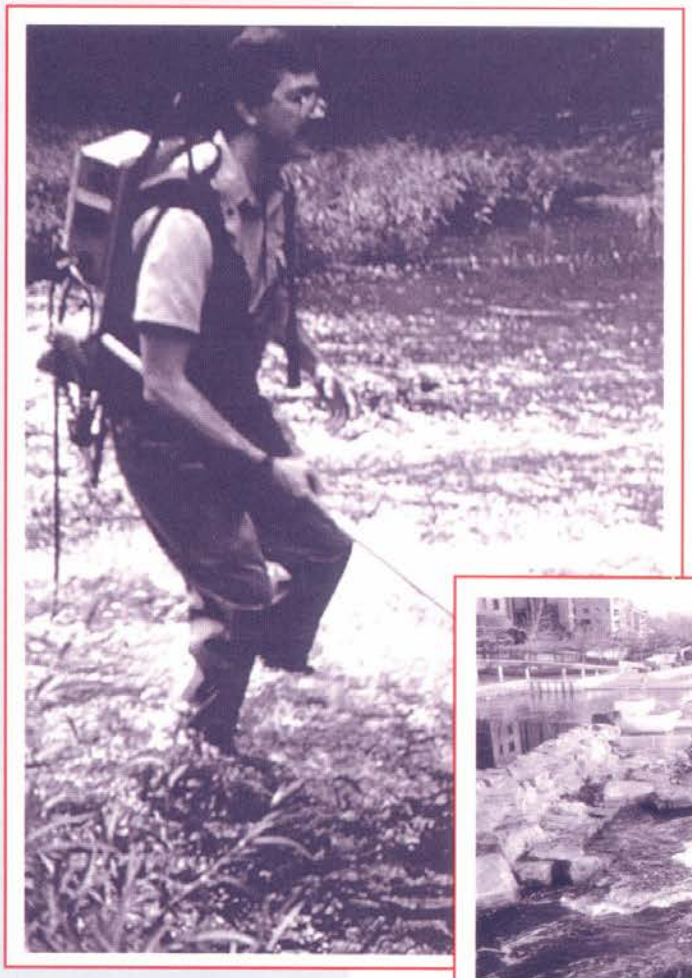

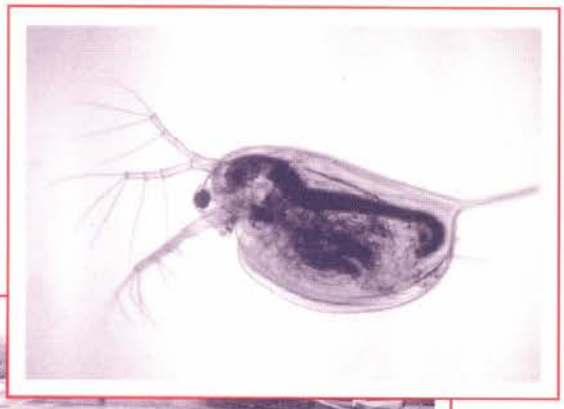

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LEWIS PUBLISHERS

A CRC Press Company
Boca Raton London New York Washington, D.C.

Library of Congress Cataloging-in-Publication Data

Burton, G. Allen
Stormwater effects handbook : a toolbox for watershed managers, scientists, and engineers / by G. Allen Burton, Jr. and Robert Pitt.
p. cm.
Includes bibliographical references and index.
ISBN 0-87371-924-7 (alk. paper)
1. Runoff—Management—Handbooks, manuals, etc. 2. Runoff—Environmental aspects—Handbooks, manuals, etc. 3. Water quality—Measurement—Handbooks, manuals, etc. 4. Water quality biological assessment—Handbooks, manuals, etc. I. Pitt, Robert. II. Title.

TD657 .B86 2001
628.1'68—dc21 2001029906
CIP

International Standard Book Number 0-87371-924-7
Library of Congress Card Number 2001029906
Printed in the United States of America 1 2 3 4 5 6 7 8 9 0
Printed on acid-free paper

Dedication

This book is dedicated to those who were instrumental in guiding and supporting our development as scientists and engineers and our appreciation of the outdoors.

Preface

This handbook is intended to be a working document which assists scientists, engineers, consultants, regulators, citizen groups, and environmental managers in determining if stormwater runoff is causing adverse effects and beneficial-use impairments in local receiving waters. This includes adverse effects on aquatic life and human health and considers exposures to multiple stressors such as pathogens, chemicals, and habitat alteration. Given the complicated nature of the problem, where diffuse inputs contain multiple stressors which vary in intensity with time (and often in areas which are simultaneously impacted by point source discharges or other development activities, e.g., channelization), it is difficult to define and separate stormwater effects from these other factors. To accomplish this task requires an integrated watershed-based assessment approach which focuses on sampling before, during, and after storm events.

This handbook provides a logical approach for an experimental design that can be tailored to address a wide range of environmental concerns, such as ecological and human health risk assessments, determining water quality or biological criteria exceedances, use impairment, source identification, trend analysis, determination of best management practice (BMP) effectiveness, stormwater quality monitoring for NPDES Phase I and II permits and applications, and total maximum daily load (TMDL) assessments. Despite the complexity of stormwater, successful and accurate assessments of its impact are possible by following the logical integrated approaches described in this handbook.

New methods and technologies are rapidly being developed, so this should be considered a "living" document which will be updated as the science warrants. We welcome your input on ways to improve future editions.

Allen Burton
Bob Pitt
May 2001

Disclaimer: The views presented within this document do not necessarily represent those of the U.S. Environmental Protection Agency.

Acknowledgments

We are indebted to our professional colleagues whose prior contributions enabled us to produce this book. In addition, the long productive hours of a host of graduate and undergraduate students at Wright State and the University of Alabama at Birmingham are acknowledged for their essential research contributions.

We greatly appreciate the word processing of Nancy Pestian and Amy Ray. We also thank the production staff and editors at Lewis Publishers/CRC Press for their hard work and patience.

The support of the U.S. EPA, especially Richard Field, is also appreciated, not only for help in the preparation of this current work, but also for the prior support given to many of the research projects described in this book.

Special thanks are also due to our families, who provided never-ending support during the preparation of this book.

About the Authors

G. Allen Burton, Jr., is the Brage Golding Distinguished Professor of Research and Director of the Institute for Environmental Quality at Wright State University. He obtained a Ph.D. degree in Environmental Science from the University of Texas at Dallas in 1984. From 1980 until 1985 he was a Life Scientist with the U.S. Environmental Protection Agency. He was a Postdoctoral Fellow at the National Oceanic and Atmospheric Administration's Cooperative Institute for Research in Environmental Sciences at the University of Colorado. Since then, he has had positions as a NATO Senior Research Fellow in Portugal and Visiting Senior Scientist in Italy and New Zealand.

Dr. Burton's research during the past 20 years has focused on developing effective methods for identifying significant effects and stressors in aquatic systems where sediment and stormwater contamination is a concern. His ecosystem risk assessments have evaluated multiple levels of biological organization, ranging from microbial to amphibian effects. He has been active in the development and standardization of toxicity methods for the U.S. EPA, American Society for Testing and Materials (ASTM), Environment Canada, and the Organization of Economic Cooperation and Development (OECD). Dr. Burton has served on numerous national and international scientific committees and review panels, and written more than 100 publications dealing with aquatic systems.

Robert Pitt is currently a Professor in the Department of Civil and Environmental Engineering at the University of Alabama. Bob had previously served on the School of Engineering faculty at the University of Alabama at Birmingham since 1987. Prior to that, he was a Senior Engineer for 16 years in industry and government, and continues to consult to many municipalities and engineering firms. He received his Ph.D. in Civil and Environmental Engineering from the University of Wisconsin–Madison, his M.S.C.E. in Environmental Engineering/Hydraulic Engineering from San Jose State University, CA, and his B.S. in Engineering Science, from Humboldt State University, Arcata, CA. He is a registered professional engineer (WI) and a Diplomate of the American Academy of Environmental Engineers.

During the past 30 years, Bob has been the project manager and principal investigator for many water resources research projects conducted for the U.S. EPA, Environment Canada, Ontario Ministry of the Environment, and state and local governments concerning the effects, sources, and control of urban runoff. Some are used as case studies in this book. His major area of interest is in stormwater management, especially the integration of drainage and water quality objectives. He currently teaches classes in water supply and drainage design, hydrology, hydraulics, experimental design, and field sampling, plus a series on stormwater management. Bob has published more than 100 chapters, books, journal articles, and major research reports. He is a member of the American Society of Civil Engineers, the Water Environment Federation, the North American Lake Management Society, the American Water Resources Association, and the Society for Environmental Toxicology and Chemistry.

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UNIT 1

The Problem of Stormwater Runoff

CHAPTER 1

Introduction

"A stench from its inky surface putrescent with the oxidizing processes to which the shadows of the over-reaching trees add stygian blackness and the suggestion of some mythological river of death. With this burden of filth the purifying agencies of the stream are prostrated; it lodges against obstructions in the stream and rots, becoming hatcheries of mosquitoes and malaria. A thing of beauty is thus transformed into one of hideous danger."

Texas Department of Health 1925

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OVERVIEW: THE PROBLEM OF STORMWATER RUNOFF

The vivid description, above, of the Trinity River as it flowed through Fort Worth and Dallas, TX, in 1925 is no longer appropriate. The acute pollution problems that occurred in the Trinity River and throughout the United States before the 1970s have been visibly and dramatically improved. The creation of the U.S. Environmental Protection Agency (EPA) and the passage of the Clean Water Act (CWA) in 1972 resulted in improved treatment of municipal and industrial wastewaters, new and more stringent water quality criteria and standards, and an increased public awareness of water quality issues. During the first 18 years of the CWA, regulatory efforts, aimed at pollution control, focused almost entirely on point source, end-of-pipe, wastewater discharges. However, during this same period, widespread water quality monitoring programs and special studies conducted by state and federal agencies and other institutions implicated nonpoint sources

(NPS) as a major pollutant category, affecting most degraded waters around the country. For example, in Ohio 51% of the streams assessed were thought to be adversely impacted by NPS pollution. Nonpoint source pollution presents a challenge from both a regulatory and an assessment perspective. Unlike many point source discharges, pollution inputs are not constant, do not reoccur in a consistent pattern (i.e., discharge volume and period), often occur over a diffuse area, and originate from watersheds whose characteristics and pollutant loadings vary through time. Given this extreme heterogeneity, simple solutions to NPS pollution control and the assessment of ecosystem degradation are unlikely. Fortunately, methods do exist to accomplish both control and accurate assessments quite effectively. To accomplish this, however, one must have a clear understanding of the nature of the problem, the pollutant sources, the receiving ecosystem, the strengths and weaknesses of the assessment tools, and proper quality assurance (QA) and quality control (QC) practices. This handbook will discuss these issues as they pertain to assessing stormwater runoff effects on freshwater ecosystems.

SOURCES OF NPS POLLUTION

A wide variety of activities and media comprise NPS pollution in waters of the United States (Table 1.1). The major categories of sources include agriculture, silviculture, resource extraction, hydro-modification, urban areas, land disposal, and contaminated sediments. The contribution of each category is, of course, a site-specific issue. In Ohio, as in many midwestern and southern states, agriculture is the principal source of NPS stressors, as shown in Table 1.2 (ODNR 1989).

These stressors include habitat destruction (e.g., channelization, removal of stream canopy and riparian zone, loss of sheltered areas, turbidity, siltation) and agrichemicals (e.g., pesticides and nutrients). In urban areas, stream and lake impairment is also due to habitat destruction; but, in addition, physical and chemical contaminant loadings come from runoff from impervious areas (e.g., parking lots, streets) of construction sites, and industrial, commercial, and residential areas. Numerous studies (such as May 1996) have examined the extent of urbanization in relation to decaying receiving water conditions (Figure 1.1). Other contaminant sources that have been doc-

Table 1.1 Nonpoint Source Pollution Categories and Subcategories

Category: Agriculture	**Category: Hydromodification**
General agriculture	General hydromodification
Crop production	Channelization
Livestock production	Dredging
Pasture	Dam construction
Specialty crop production	Stream bank modification
Category: Silviculture	Bridge construction
General silviculture	**Category: Urban**
Harvesting, reforestation	General urban
Residue management	Storm sewers
Road construction	Sanitary sewers
Forest management	Construction sites
Category: Resource Extraction	Surface runoff
General resource extraction	**Category: Land Disposal**
Surface coal mining	General land disposal
Subsurface coal mining	Sludge disposal
Oil/Gas production	Wastewater
Category: In-place (Sediment) Pollutants	Sanitary landfills
	Industrial land treatment
	On-site wastewater treatment

From EPA. *Results of the Nationwide Urban Runoff Program.* Water Planning Division, PB 84-185552, Washington, D.C. December 1983.

Table 1.2 Major Categories of Nonpoint Source Pollution Impacting Surface Water Quality in Ohio

Major Categories of Nonpoint Source Pollution	Stream Miles Affected	Percentage of Miles Affected
Agriculture	5300	44
Resource extraction	2000	17
Land disposal	1600	13
Hydromodification	1500	13
Urban	1100	9
Silviculture	400	3
In-place pollutants	100	1
Total stream miles affected	12,000	

From ODNR (Ohio Department of Natural Resources). Ohio Nonpoint Source Management Program. Ohio Department of Natural Resources, Columbus, OH. 1989.

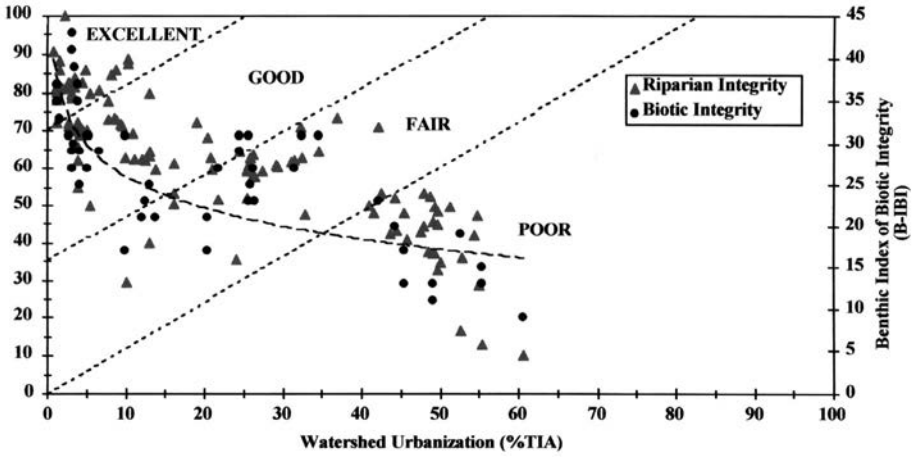

Figure 1.1 Relationship between basin development, riparian buffer width, and biological integrity in Puget Sound lowland streams. (From May, C.W. *Assessment of the Cumulative Effects of Urbanization on Small Streams in the Puget Sound Lowland Ecoregion: Implications for Salmonid Resource Management.* Ph.D. dissertation, University of Washington, Seattle. 1996. With permission.)

umented, but are even more difficult to assess, include accidental spills, unintended discharges, and atmospheric deposition.

The pollutants present in stormwater runoff vary with each watershed; however, certain pollutants are associated with specific activities (e.g., soybean farming, automobile service areas) and with area uses (e.g., parking lots, construction). By analyzing the land use patterns, watershed characteristics, and meteorological and hydrological conditions, an NPS assessment program can be focused and streamlined.

A number of studies have linked specific pollutants in stormwater runoff with their sources (Table 1.3). Pitt et al. (1995) reviewed the literature on stormwater pollutant sources and effects and also measured pollutants and sample toxicity from a variety of urban source categories of an impervious and pervious nature. The highest concentrations of synthetic organics were in roof runoff, urban creeks, and combined sewer overflows (CSOs). Zinc was highest from roof runoff (galvanized gutters). Nickel was highest in runoff from parking areas. Vehicle service areas produced the highest cadmium and lead concentrations, while copper was highest in urban creeks (Pitt et al. 1995). Most metals in stormwater runoff originate from streets (Table 1.4, FWHA 1987) and parking areas. Other metal sources include wood preservatives, algicides, metal corrosion, road salt, batteries, paint, and industrial electroplating waste. One large survey (EPA 1983) found only 13 organics occurring in at least 10% of the samples. The most common were 1,3-dichlorobenzene

Table 1.3 Potential Sources of Stormwater Toxicants

	Automobile Use	Pesticide Use	Industrial/Other
		Halogenated Aliphatics	
Methylene chloride		Fumigant[a]	Plastics, paint remover, solvent
Methyl chloride	Leaded gas[a]	Fumigant[a]	Refrigerant, solvent
		Phthalate Esters	
Di-N-butyl phthalate		Insecticide	Plasticizer[a], printing inks, paper, stain, adhesive
Bis (2-ethyhexyl) phthalate			Plasticizer[a]
Butylbenzyl phthalate			Plasticizer[a]
		Polycyclic Aromatic Hydrocarbons	
Chrysene	Gasoline[a], oil/grease		
Phenanthrene	Gasoline		Wood/coal combustion[a]
Pyrene	Gasoline, oil, asphalt	Wood preservatives	Wood/coal combustion[a]
		Volatiles	
Benzene	Gasoline[a]		Solvent formed from salt, gasoline and asphalt
Chloroform		Insecticide	Solvent, formed from chlorination[a]
Toluene	Gasoline[a], asphalt		Solvent
		Heavy Metals	
Chromium	Metal corrosion[a]		Paint, metal corrosion, electroplating waste[a]
Copper	Metal corrosion, brake linings	Algicide	Paint, metal corrosion, electroplating waste[a]
Lead	Gasoline, batteries		Paint
Zinc	Metal corrosion, road salt, rubber[a]	Wood preservative	Paint, metal corrosion[a]
		Organochlorides and Pesticides	
Lindane		Mosquito control[a]	
		Seed pretreatment	
Chlordane		Termite control[a]	
Pentachlorophenol		Wood preservative	Paint
			Wood processing
PCBs			Electrical, insulation, paper adhesives
Dieldrin			
Diazinon			
Chlorpyrifos			
Atrazine			

[a] Most significant sources.

Modified from Callahan, M.A., et al., *Water Related Environmental Fates of 129 Priority Pollutants.* U.S. Environmental Protection Agency, Monitoring and Data Support Division, EPA-4-79-029a and b. Washington D.C. 1979; Verschueren, K. *Handbook of Environmental Data on Organic Chemicals,* 2nd edition. Van Nostrand Reinhold Co., New York. 1983.

Table 1.4 Highway Runoff Constituents and Their Primary Sources

Constituents	Primary Sources
Particulates	Pavement wear, vehicles, atmosphere, maintenance
Nitrogen, phosphorus	Atmosphere, roadside fertilizer application
Lead	Leaded gasoline (auto exhaust), tire wear (lead oxide filler material, lubricating oil and grease, bearing wear)
Zinc	Tire wear (filler materials), motor oil (stabilizing additive), grease
Iron	Auto body rust, steel highway structures (guard rails, etc.), moving engine parts
Copper	Metal plating, bearing and bushing wear, moving engine parts, brake lining wear, fungicides and insecticides
Cadmium	Tire wear (filler material), insecticide application
Chromium	Metal plating, moving engine parts, break lining wear
Nickel	Diesel fuel and gasoline (exhaust), lubricating oil, metal plating, bushing wear, brake lining wear, asphalt paving
Manganese	Moving engine parts
Cyanide	Anticake compound (ferric ferrocyanide, sodium ferrocyanide, yellow prussiate of soda) used to keep deicing salt granular
Sodium, calcium, chloride	Deicing salts
Sulfate	Roadway beds, fuel, deicing salts
Petroleum	Spills, leaks, or blow-by of motor lubricants, antifreeze and hydraulic fluids, asphalt surface leachate
PCB	Spraying of highway rights-of-way, background atmospheric deposition, PCB catalyst in synthetic tires

From U.S. DOT, FHWA, Report No. FHWA/RD-84/056-060, June 1987.

and fluoranthene (23% of the samples). These 13 compounds were similar to those reported in most areas. The most common organic toxicants have been from automobile usage (polycyclic aromatic hydrocarbons, or PAHs), combustion of wood and coal (PAHs), industrial and home use solvents (halogenated aliphatics and other volatiles), wood preservatives (PAHs, creosote, pentachlorophenol), and a variety of agricultural, municipal, and highway compounds, and pesticides.

The major urban pollution sources are construction sites, on-site sewage disposal systems, households, roadways, golf courses, parks, service stations, and parking areas (Pitt et al. 1995). The primary pollutant from construction is eroded soils (suspended and bedload sediments, dissolved solids, turbidity), followed by hydrocarbons, metals, and fertilizers.

Silviculture is a major source of nonpoint pollution in many areas of the country. The primary pollutant is eroded soils, which result in elevated turbidity, silted substrates, altered habitat, higher dissolved solids, and altered ion ratios in the streams and lakes of the watershed. Water temperatures increase as tree canopies are removed and stream flow slows. Fertilizers and pesticides may also be used which are transported to the streams via surface runoff, groundwater, and drift.

Agricultural activities contribute a wide variety of stormwater pollutants, depending on the production focus and ecoregion. Major pollutants include eroded soils, fertilizers, pesticides, hydrocarbons (equipment-related), animal wastes, and soil salts.

The hydromodification category of NPS includes dredging, channelization, bank stabilization, and impoundments. Stormwaters obviously do not "run off" any of these sources, but stormwater (high flow) does degrade waters associated with these sources. Water quality parameters which may be affected by these sources during stormwater events include turbidity, sediment loading (habitat alteration), dissolved solids, temperature, nutrients, metals, synthetic organics, dissolved oxygen, pathogens, and toxicity.

Of a more site-specific nature, resource extraction, land waste disposal, and contaminated sediments are sources of pollutants during stormwater events. Activities such as sand and gravel, metal, coal, and oil and gas extraction from or near receiving waters may contribute to habitat alteration and increased turbidity, siltation, metals, hydrocarbons, and salt during storm events. Land waste disposal sources consist of sludge farm runoff, landfill and lagoon runoff and leachate, and on-site septic system (leachfield) overflows. These sources may contribute a variety of pollutants

to receiving waters such as nutrients, solids (dissolved and suspended), pathogens, metals, and synthetic organics. Contaminated sediments occur in numerous areas throughout the United States (EPA 1994). Many nutrients and toxic metals, metalloids, and synthetic organics readily sorb to particulates (organic or inorganic) which accumulate as bedded sediments. During storm events, these sediments may be resuspended and then become more biologically active by pollutant desorption, transformation, or particle uptake by organism ingestion.

The specific stormwater pollutants vary dramatically in their fate and effect characteristics. In most assessments of NPS pollution, there are many unknowns, such as:

- What are the pollutants of concern?
- What are the pollutant sources?
- What are the pollutant loadings?

These common unknowns provide the rationale for use of an integrated assessment strategy (see Unit 2) which incorporates several essential components of runoff-receiving water systems.

REGULATORY PROGRAM

In February 1987, amendments to the federal Clean Water Act (CWA) were passed by Congress and required states (Sections 101 and 319) to assess NPS pollution and develop management programs. These programs are to be tailored on a watershed-specific basis, although they are structured along political jurisdictions. There are also NPS requirements under Section 6217 of the Coastal Zone Act Reauthorization Amendments of 1990. The EPA published the Phase 1 stormwater discharge regulations for the CWA in the *Federal Register* on November 16, 1990. The regulations confirm stormwater as a point source that must be regulated through permits issued under the National Pollutant Discharge Elimination System (NPDES). Certain specified industrial facilities and large municipalities (>100,000 population) fell under the Phase 1 regulations. The Phase 2 regulations were enacted in October 1999, requiring municipalities of 10,000 and greater to comply with stormwater control guidelines.

Monitoring activities must be part of the Phase 1 NPDES stormwater permit requirements. One monitoring element is a field screening program to investigate inappropriate discharges to the storm drainage system (Pitt et al. 1993). The Phase 1 requirements also specified outfall monitoring during wet weather to characterize discharges from different land uses. Specified industries are also required to periodically monitor their stormwater discharges. Much of the local municipal effort associated with the Phase 1 permit requirements involved describing the drainage areas and outfalls. Large construction sites are also supposed to be controlled, but enforcement has been very spotty. Local governments have been encouraged by the EPA to develop local stormwater utilities to pay for the review and enforcement activities required by this regulation. The Phase 2 permit requirements are likely to have reduced required monitoring efforts for small communities and remaining industries.

The Stormwater Phase 2 Rule was published in early November 1999 in the *Federal Register.* The purpose of the rule is to designate additional sources of stormwater that need to be regulated to protect water quality. Two new classes of facilities are designated for automatic coverage on a nationwide basis:

1. Small municipal separate storm sewer systems located in urbanized areas (about 3500 municipalities) [Phase 1 included medium and large municipalities, having populations greater than 100,000]
2. Construction activities that disturb between 1 and 5 acres of land (about 110,000 sites a year) [Phase 1 included construction sites larger than 5 acres]

There is also a new "no exposure" incentive for Phase 1 sites having industrial activities. It is expected that this will exclude about 70,000 facilities nationwide from the stormwater regulations. The NPDES permitting authority would need to issue permits (most likely general permits) by May 31, 2002.

Proposed construction site regulations in the Phase 2 rule include:

1. Ensure control of other wastes at construction sites (discarded building materials, concrete truck washout, sanitary wastes, etc.)
2. Implement appropriate best management practices (such as silt fences, temporary detention ponds, etc.)
3. Require preconstruction reviews of site management plans
4. Receive and consider public information
5. Require regular inspections during construction
6. Have penalties to ensure compliance

If local regulations incorporate the following principles and elements into the stormwater program, they would be considered as "qualifying" programs that meet the federal requirements:

Five Principles
1. Good site planning
2. Minimize soil movement
3. Capture sediment
4. Good housekeeping practices
5. Mitigation of post-construction stormwater discharges

Eight Elements
1. Program description
2. Coordination mechanism
3. Requirements for nonstructural and structural BMPs
4. Priorities for site inspections
5. Education and training
6. Exemption of some activities due to limited impacts
7. Incentives, awards, and streamlining mechanisms
8. Description of staff and resources

Unfortunately, many common stormwater parameters which cause acute and chronic toxicity or habitat problems are not included in typical monitoring programs conducted under the NPDES stormwater permit program. Therefore, stormwater discharges that are degrading receiving waters may not be identified as significant outfalls from these monitoring efforts. Conversely, these data may suggest significant pollution is adversely affecting receiving waters, when in fact it is not. As discussed later in this book, the recent promotion and adoption of integrated assessment approaches which utilize stream biological community indices, toxicity, and habitat characterization of receiving waters provide much more reliable data on stormwater discharge effects and water quality.

Section 304 of the CWA directs EPA to develop and publish information on methods for measuring water quality and establishing water quality criteria for toxic pollutants. These other approaches include biological monitoring and assessment methods which assess the effects of pollutants on aquatic communities and factors necessary to restore and maintain the chemical, physical, and biological integrity of all waters. These "toolboxes" are intended to enable local users to make more efficient use of their limited monitoring resources. Of course, a primary purpose of this book is also to provide guidance to this user community. As such, it is hoped that this book can be considered a "super" toolbox, especially with its large number of references for additional information and its detailed case studies.

APPLICATIONS OF THE HANDBOOK

The first aspect of designing a monitoring program is describing how the data are to be used. This may include future uses of the data and must also include the necessary quality of the data (allowable errors). Many uses of the data may be envisioned, as shown in the following brief discussion. Data may be used in the evaluation of local stormwater problems (risk assessments) and identification of pollutant sources to support a comprehensive stormwater management program, compliance monitoring required by regulations, model calibration and verification for TMDL (total maximum daily load) evaluations, evaluation of the performance of control practices, screening analyses to identify sources of pollutants, etc. It is critical that an integrated assessment approach (designed on a site-specific basis) be used to improve the validity of the assessment and its resulting conclusions. Critical aspects of this are discussed below.

Stormwater Management Planning (Local Problem Evaluations and Source Identifications)

Stormwater management planning encompasses a wide range of site-specific issues. The local issues that affect stormwater management decisions include understanding local problems and the sources of pollutants or flows that affect these problems. Local monitoring therefore plays an important role in identifying local problems and sources.

The main purpose of treating stormwater is to reduce its adverse impacts on receiving water beneficial uses. Therefore, it is important in any stormwater runoff study to assess the detrimental effects that runoff is actually having on a receiving water. Receiving waters may have many beneficial use goals, including:

- Stormwater conveyance (flood prevention)
- Biological uses (warm water fishery, biological integrity, etc.)
- Noncontact recreation (linear parks, aesthetics, boating, etc.)
- Contact recreation (swimming)
- Water supply

As discussed in Chapter 2, it is unlikely that any of these uses can be fully obtained with full development in a watershed and with no stormwater controls. However, the magnitude of these effects varies greatly for different conditions. Obviously, local monitoring and evaluation of data are needed to describe specific local problems, especially through the use of an integrated monitoring approach that considers physical, chemical, and biological observations collectively. As described throughout this book, relying only on a single aspect of receiving water conditions, or applying general criteria to local data, can be very misleading, and ultimately expensive and ineffective.

After local receiving problems are identified, it is necessary to understand what is causing the problems. Again, this can be most effectively determined through local monitoring. Runoff is comprised of many separate source area flow components and phases that are discharged through the storm drainage system and includes warm weather stormwater, snowmelt, baseflows, and inappropriate discharges to the storm drainage (“dry-weather” flows). It may be important to consider all of these potential urban flow discharges when evaluating alternative stormwater management options.

It may be adequate to consider the combined outfall conditions alone when evaluating the long-term, area-wide effects of many separate outfall discharges to a receiving water. However, if better predictions of outfall characteristics (or the effects of source area controls) are needed, then the separate source area components must be characterized. The discharge at an outfall is made up of a mixture of contributions from different source areas. The “mix” depends on the characteristics

of the drainage area and the specific rain event. The effectiveness of source area controls is therefore highly site and storm specific.

Risk Assessments

Risk assessments contain four major components (NRC 1983):

- Hazard identification
- Effects characterization
- Exposure characterization
- Risk characterization

Hazard identification includes quantifying pollutant discharges, plus modeling the fate of the discharged contaminants. Obviously, substantial site-specific data are needed to prepare the selected model for this important aspect of a risk assessment. Knowledge about the mass and concentration discharges of a contaminant is needed so the transport and fate evaluations of the contaminant can be quantified. Knowledge of the variations of these discharges with time and flow conditions is needed to determine the critical dose–response characteristics for the contaminants of concern. A suitable model, supported by adequate data, is necessary to produce the likely dose–stressor response characteristics. Exposure assessment is related to knowledge of the users of receiving waters and contaminated components (such as contaminated fish that are eaten, contaminated drinking water being consumed, children exposed to contaminated swimming by playing in urban creeks, etc.). Finally, the risk is quantified based on this information, including the effects of all of the possible exposure pathways. Obviously, many types of receiving water and discharge data are needed to make an appropriate risk assessment associated with exposure to stormwater, especially related to discharge characteristics, fate of contaminants, and verification of contaminated components. The use of calibrated and validated discharge and fate models is therefore necessary when conducting risk assessments.

Total Maximum Daily Load (TMDL) Evaluations

The total maximum daily load (TMDL) for a stream is the estimated maximum discharge that can enter a water body without affecting its designated uses. TMDLs can be used to allocate discharges from multiple sources and to define the level of control that may be needed. Historically, assimilative capacities of many receiving waters were based on expected dissolved oxygen conditions using in-stream models. Point source discharges of BOD were then allocated based on the predicted assimilative capacity. Allowed discharges of toxic pollutants can be determined in a similar manner. Existing background toxicant concentrations are compared to water quality criteria under critical conditions. The margin in the pollutant concentration (difference between the existing and critical concentrations) is multiplied by the stream flow to estimate the maximum allowable increased discharge, before the critical criteria would likely be exceeded. There has always been concern about margins of safety and other pollutant sources in the simple application of assimilative capacity analyses. The TMDL process is a more comprehensive approach that attempts to examine and consider all likely pollutant sources in the watershed. The EPA periodically publishes guidance manuals describing resources available for conducting TMDL analyses (Shoemaker et al. 1997, for example).

Model Calibration and Validation

A typical use of stormwater monitoring data is to calibrate and validate models that can be used to examine many questions associated with urbanization, especially related to the design of

control programs to reduce problem discharges effectively. All models need to be calibrated for local conditions. Local rain patterns and development characteristics, for example, all affect runoff characteristics. Calibration usually involves the collection of an initial set of data that is used to modify the model for these local characteristics. Validation is an independent check to ensure that the calibrated model produces predictions within an acceptable error range. Unfortunately, many models are used to predict future conditions that are not well represented in available data sets, or the likely future conditions are not available in areas that could be monitored. These problems, plus many other aspects of modeling, require someone with good skill and support to ensure successful model use.

Model calibration and validation involves several steps that are similar for most stormwater modeling processes. The best scenario may be to collect all calibration information from one watershed and then validate the calibrated model using independent observations from another watershed. Another common approach is to collect calibration information for a series of events from one watershed, and then validate the calibrated model using additional data from other storms from the same watershed. Numerous individual rainfall-runoff events may need to be sampled to cover the range of conditions of interest. For most stormwater models, detailed watershed information is also needed. Jewell et al. (1978) presented one of the first papers describing the problems and approaches needed for calibrating and validating nonpoint source watershed scale models. Most models have descriptions of recommended calibration and validation procedures. Models that have been used for many years (such as SWMM and HSPF) also have many publications available describing the sensitivity of model components and the need for adequate calibration.

It is very important that adequate QA/QC procedures be used to ensure the accuracy and suitability of the data. Common problems during the most important rainfall-runoff monitoring activities are associated with unrepresentative rainfall data (using too few rain gauges and locating them incorrectly in the watershed), incorrect rain gauge calibrations, poor flow-monitoring conditions (surcharged flows, relying on Manning's equation for V and Q, poor conditions at the monitoring location), etc. The use of a calibrated flume is preferred, for example. Other common errors are associated with inaccurate descriptions of the watershed (incorrect area, amount of impervious area, understanding of drainage efficiency, soil characteristics, etc.). Few people appreciate the inherent errors associated with measuring rainfall and runoff. Most monitoring programs are probably no more than ±25% accurate for each event. It is very demanding to obtain rainfall and runoff data that is only 10% in error. This is most evident when highly paved areas (such as shopping centers or strip commercial areas) are monitored and the volumetric runoff coefficients are examined. For these areas, it is not uncommon for many of the events to have volumetric runoff coefficient (Rv) values greater than 1.0 (implying more runoff than rainfall). Similar errors occur with other sites but are not as obvious.

Data from several watersheds are available for the calibration and validation process. If so, start with data from the simplest area (mostly directly connected paved areas and roofs, with little unpaved areas). This area probably represents commercial roofs and parking/storage areas alone. These areas should be calibrated first, before moving on to more complex areas. The most complex areas, such as typical residential areas having large expanses of landscaped areas and with most of the roofs being disconnected from the drainage areas, should be examined last.

Effectiveness of Control Programs

Effective stormwater management programs include a wide variety of control options that can be utilized to reduce receiving water problems. With time and experience, some of these will be found to be more effective than others. In order to identify which controls are most cost-effective for a specific area, local performance evaluations should be conducted. In many cases, straightforward effectiveness monitoring (comparing influent with effluent concentrations for a stormwater filter, for example) can be utilized, while other program elements (such as public education or street

cleaning) can be much more difficult to evaluate. Therefore, this book presents monitoring approaches that can be utilized for a broad range of control programs. These monitoring activities may appear to be expensive. However, the true cost of not knowing how well currently utilized controls function under local conditions can be much more costly than obtaining accurate local data and making appropriate changes in design methods.

The first concern when investigating alternative treatment methods is determining the needed level of stormwater control. This determination has a great effect on the cost of the stormwater management program and needs to be made carefully. Problems that need to be addressed range from sewerage maintenance issues to protecting many receiving water uses. As an example, Laplace et al. (1992) recommends that all particles greater than about 1 to 2 mm in diameter be removed from stormwater in order to prevent deposition in sewerage. The specific value is dependent on the energy gradient of the flowing water in the drainage system and the hydraulic radius of the sewerage. This treatment objective can be easily achieved using a number of cost-effective source area and inlet treatment practices. In contrast, much greater levels of stormwater control are likely needed to prevent excessive receiving water degradation. Typical treatment goals usually specify about 80% reductions in suspended solids concentrations. For most stormwaters, this would require the removal of most particulates greater than about 10 μm in diameter, about 1% of the 1 mm size noted above to prevent sewerage deposition problems. Obviously, the selection of a treatment goal must be done with great care.

There are many stormwater control practices, but not all are suitable in every situation. It is important to understand which controls are suitable for the site conditions and can also achieve the required goals. This will assist in the realistic evaluation for each practice of the technical feasibility, implementation costs, and long-term maintenance requirements and costs. It is also important to appreciate that the reliability and performance of many of these controls have not been well established, with most still in the development stage. This is not to say that emerging controls cannot be effective; however, there is not a large amount of historical data on which to base designs or to provide confidence that performance criteria will be met under the local conditions. Local monitoring can be used to identify the most effective controls based on the sources of the identified problem pollutants, and monitoring can be utilized to measure how well in-place controls are functioning over the long term. These important data can be used to modify recommendations for the use of specific controls, design approaches, and sizing requirements.

Compliance with Standards and Regulations

The receiving water (and associated) monitoring tools described in this book can also be used to measure compliance with standards and regulations. Numerous state and local agencies have established regulatory programs for moderate and large-sized communities due to the EPA's NPDES (National Pollutant Discharge Elimination System) stormwater permit program. The recently enacted Phase 2 regulations will extend some stormwater regulations to small communities throughout the United States. In addition, the increasing interest in TMDL evaluations in critical watersheds also emphasizes the need for local receiving water and discharge information. These regulatory programs all require certain monitoring, modeling, and evaluation efforts that can be conducted using procedures and methods described in this book.

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CHAPTER 2

Receiving Water Uses, Impairments, and Sources of Stormwater Pollutants

"Bathing in sewage-polluted seawater carries only a negligible risk to health, even on beaches that are aesthetically very unsatisfactory."

Committee on Bathing Beach Contamination
Public Health Laboratory Service of the U.K.
1959

CONTENTS

INTRODUCTION

Wet-weather flow impacts on receiving waters have been historically misunderstood and de-emphasized, especially in times and areas of poorly treated municipal and industrial discharges. The above 1959 quote from the Committee on Bathing Beach Contamination of the Public Health Laboratory Service of the U.K. demonstrates the assumption that periodic combined sewer overflows (CSOs), or even raw sewage discharges, produced negligible human health risks. Is it any wonder then that the much less dramatically contaminated stormwater discharges have commonly been considered "clear" water by many regulators?

The EPA reported that only 57% of the rivers and streams in the United States fully support their beneficial uses (Figure 2.1). A wide variety of pollutants and sources are the cause of impaired

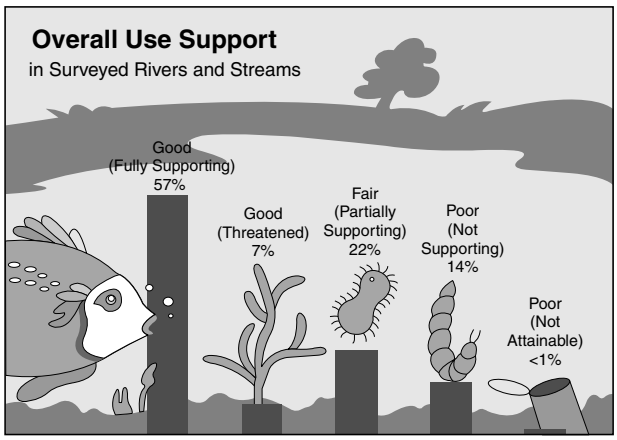

Figure 2.1 U.S. rivers and streams meeting designated beneficial uses. Note: Percentages do not add to 100% because more than one pollutant or source may impair a segment of ocean shoreline. (From U.S. Environmental Protection Agency. *National Water Quality Inventory. 1994 Report to Congress*. Office of Water. EPA 841-R-95-005. Washington, D.C. December 1995.)

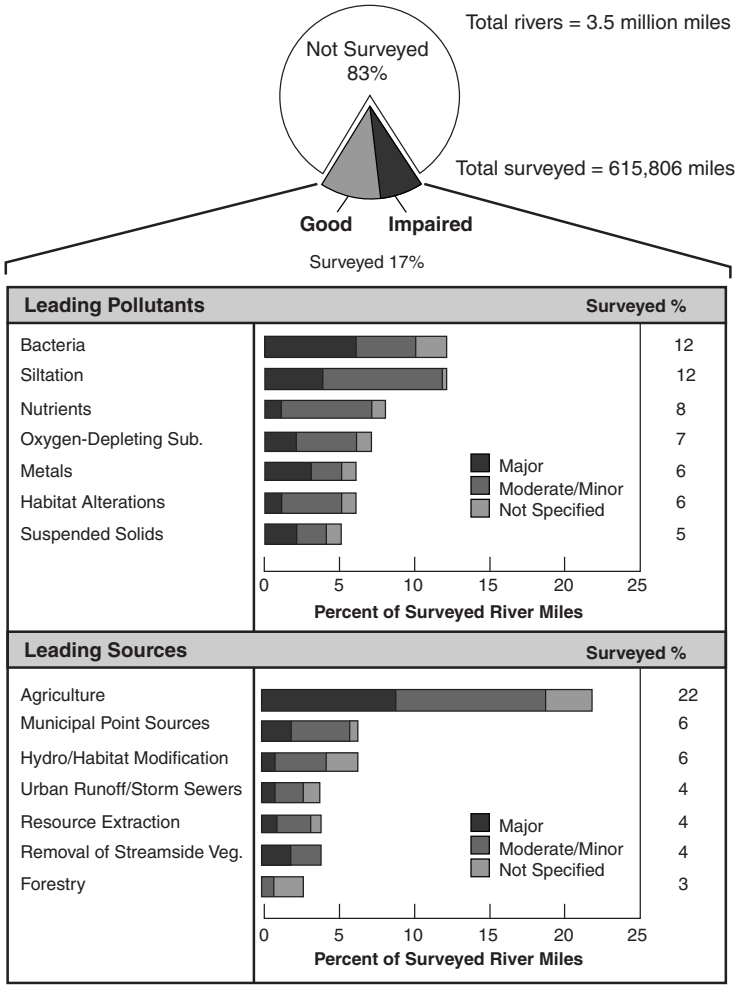

Figure 2.2 Pollutants and sources impairing U.S. rivers. Note: Percentages do not add to 100% because more than one pollutant or source may impair a segment of ocean shoreline. (From U.S. Environmental Protection Agency. *National Water Quality Inventory. 1994 Report to Congress*. Office of Water. EPA 841-R-95-005. Washington, D.C. December 1995.)

uses (Figures 2.2 through 2.6) but runoff from urban and agricultural sources dominate. This book contains discussions of instances of beneficial use impairments associated with stormwater runoff and the possible sources of the stressors of these effects. However, stormwater effects on receiving waters are not always clear and obvious. As will be evident to the reader, most stormwater runoff

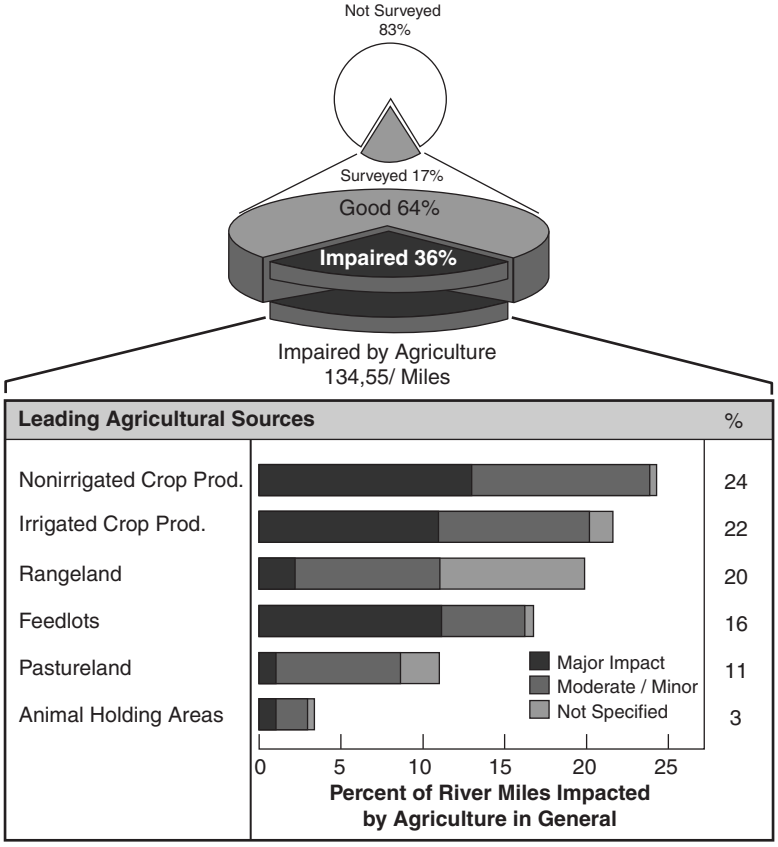

Figure 2.3 Agricultural activities affecting U.S. rivers and streams. Note: Percentages do not add to 100% because more than one pollutant or source may impair a segment of ocean shoreline. (From U.S. Environmental Protection Agency. *National Water Quality Inventory. 1994 Report to Congress*. Office of Water. EPA 841-R-95-005. Washington, D.C. December 1995.)

assessments have been conducted in urban waterways, with fewer examples for agricultural systems. However, many of the approaches, methods, and receiving water effects are similar in both urban and agriculturally dominated waterways. In completely urbanized watersheds, the small urban streams are commonly severely degraded, but they typically have no official beneficial uses or monitoring programs (and may be intermittent in flow), and are therefore unrecognized as being impacted or important. Unfortunately, these streams receive substantial recreational use by neighborhood children. Besides the obvious safety concerns and potential drowning fears, the water quality of urban streams can present significant risks. In older cities, stream sediments downstream from historical industrial areas can be heavily contaminated by heavy metals and organic compounds. Even in nonindustrialized areas, metallic and organic contamination can be high. Unfortunately, bacteria concentrations, especially near outfalls during and soon after rains, are always very high in these small streams, although the health risks are poorly understood. Sediment bacteria conditions are also always high, as the sediments appear to be an excellent sink for bacteria. Children, and others, playing in and near the streams therefore are exposed to potentially hazardous conditions. In addition, inner-city residents sometimes rely on nearby urban waterways for fishing opportunities, both for recreation and to supplement food supplies.

In contrast to the above obvious conditions associated with small streams in completely urbanized watersheds, wet-weather flows from relatively large cities discharging into large waterways may not be associated with obvious in-stream detrimental conditions. In one example, frequent CSO discharges from Nashville, TN, into the Cumberland River were not found to produce any significant dissolved oxygen (DO) or fecal coliform problems (Cardozo et al. 1994). However, Nashville is currently investigating sources of high bacteria levels in the small urban streams draining heavily urbanized city watersheds. A series of studies of airport deicing compound runoff

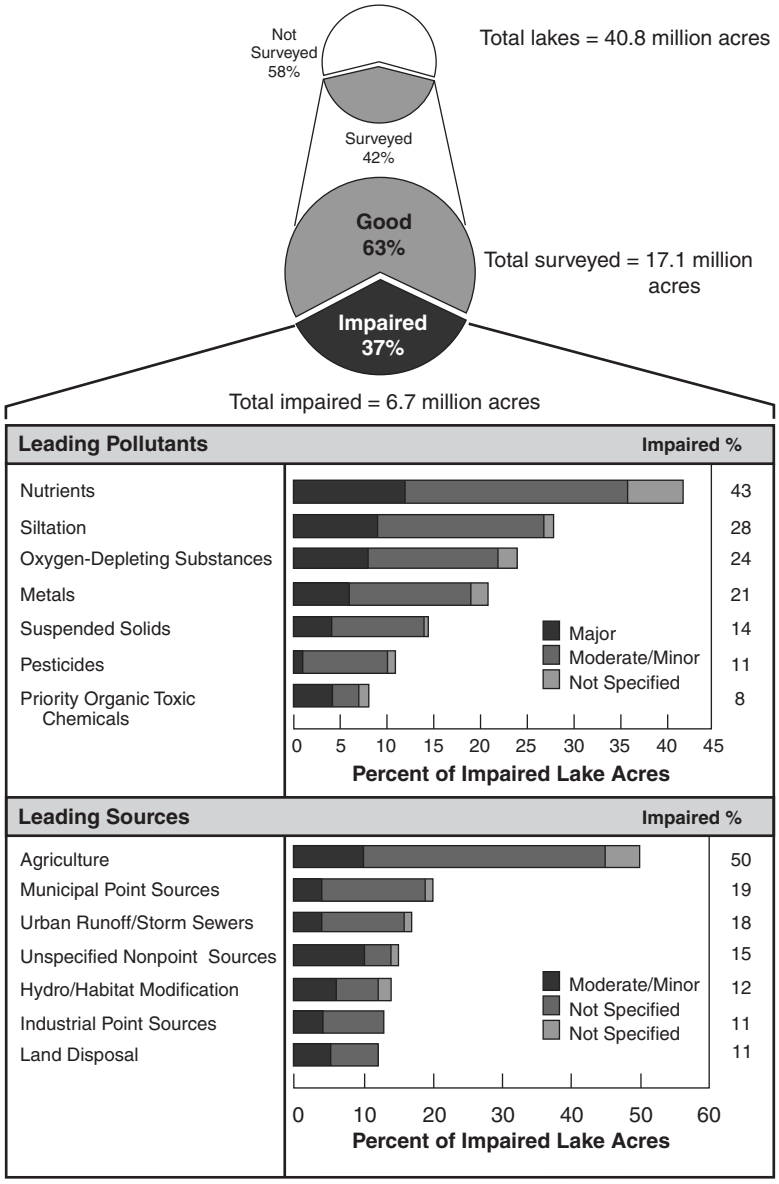

Figure 2.4 Pollutants and sources affecting U.S. lakes. Note: Percentages do not add to 100% because more than one pollutant or source may impair a segment of ocean shoreline. (From U.S. Environmental Protection Agency. *National Water Quality Inventory. 1994 Report to Congress*. Office of Water. EPA 841-R-95-005. Washington, D.C. December 1995.)

at Milwaukee's Mitchell Field is another example that demonstrates unique site-specific conditions affecting receiving water impacts. This study, conducted by the USGS and the Wisconsin Department of Natural Resources, found that the extremely high BOD concentrations (several thousand mg/L) associated with the deicing runoff had negligible effects on the DO levels in the small streams draining the airport area to Lake Michigan. They concluded that the cold temperatures occurring during the times of deicing runoff significantly reduced the BOD decomposition rate, and that the small streams had short travel times before discharging into Lake Michigan, where it was well mixed. Under laboratory conditions, the BOD rate would be much faster, and would be expected to produce dramatically low DO conditions for almost any condition in these small streams.

Other obvious receiving water problems, such as fish kills, are also rarely associated with stormwater discharges, as described in Chapter 3. Stormwater discharges occur frequently, and normally do not create acute toxicity problems (or extremely low DO conditions). Fish surviving

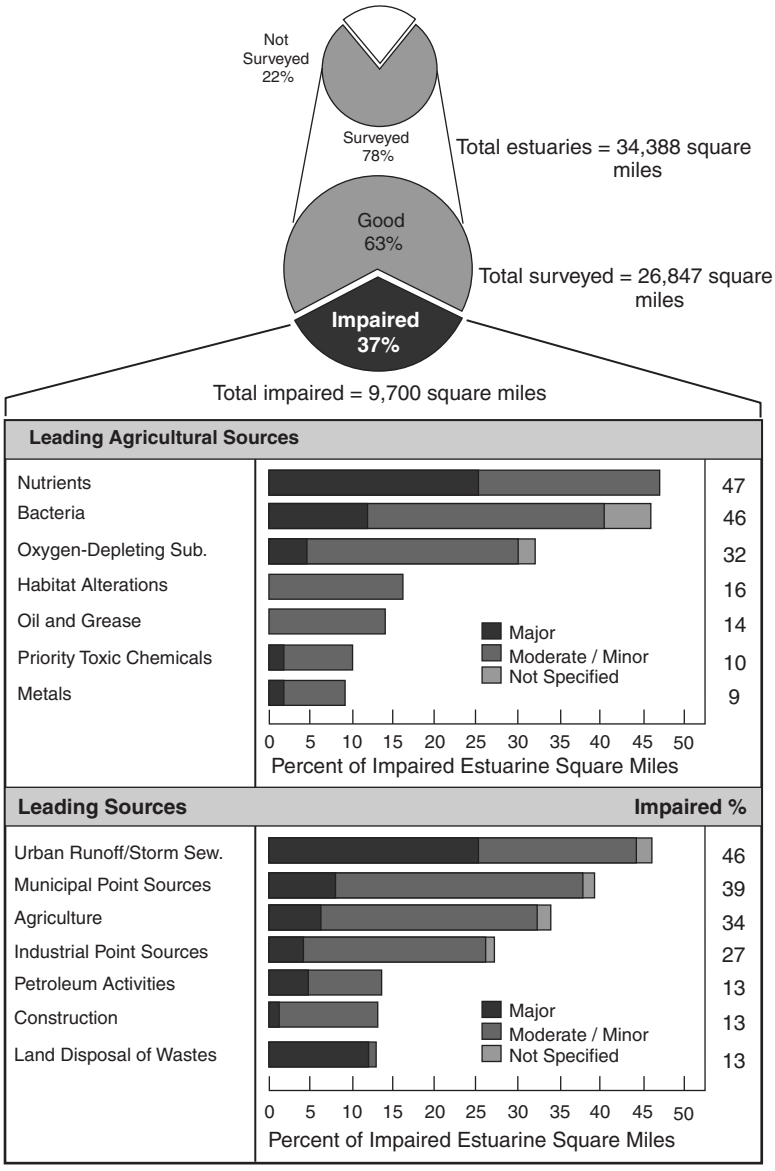

Figure 2.5 Pollutants and sources affecting U.S. estuaries. Note: Percentages do not add to 100% because more than one pollutant or source may impair a segment of ocean shoreline. (From U.S. Environmental Protection Agency. *National Water Quality Inventory. 1994 Report to Congress*. Office of Water. EPA 841-R-95-005. Washington, D.C. December 1995.)

in urban streams are tolerant species, with most of the intolerant organisms long since gone. It is therefore unusual for fish kills to occur, unless severe inappropriate discharges infrequently occur (such as those associated with industrial accidents, runoff from fire fighting, or improper waste disposal activities). However, chronic toxicity, mostly associated with contaminated sediments or suspended solids, is associated with stormwater. The effects of this chronic toxicity, plus habitat problems, are the likely causes of the commonly observed significant shifts in the in-stream biological community from naturally diverse (mostly intolerant) species to a much less diverse assemblage of introduced tolerant species. There is increasing evidence that stormwaters in urban and agriculturally dominated watersheds are often toxic (see Chapter 6). However, traditional toxicity approaches often do not detect problems associated with pulse exposures and or particulate-associated toxicity. More recently, both laboratory and in-stream (*in situ*) toxicity tests, especially associated with moderate to long-term exposures to contaminated sediments and particulates, have shown significant stormwater toxicity.

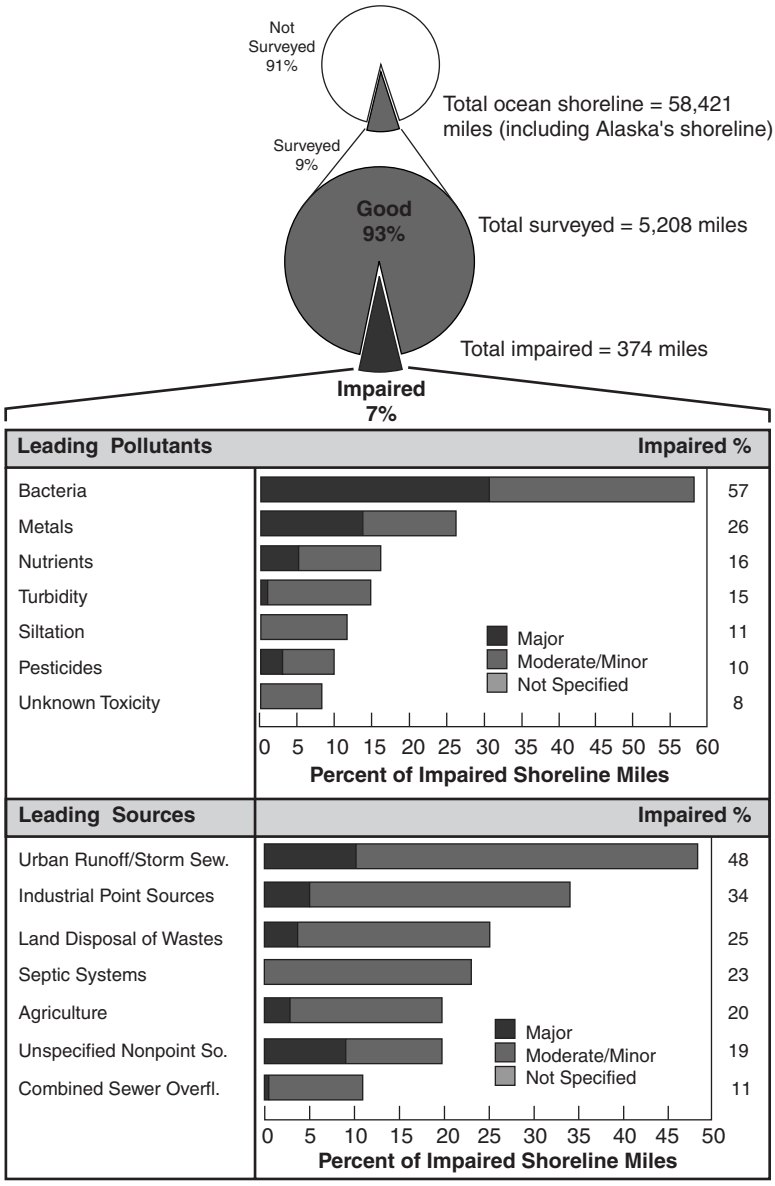

Figure 2.6 Pollutants and sources affecting U.S. ocean shorelines. Note: Percentages do not add to 100% because more than one pollutant or source may impair a segment of ocean shoreline. (From U.S. Environmental Protection Agency. *National Water Quality Inventory. 1994 Report to Congress*. Office of Water. EPA 841-R-95-005. Washington, D.C. December 1995.)

The discharges of stormwater are also periodic, causing different types of effects than the better-regulated continuous point source discharges. Stormwater causes episodic disturbances in aquatic ecosystems (Minshall 1988) whose patterns of occurrence are chaotic in nature (Pool 1989) and characteristics are unique to each event. The sciences of aquatic ecology and aquatic toxicology have progressed to the point where the effects of continuous levels of single stressors (e.g., dissolved oxygen, temperature, copper, DDT, diazinon, chlorpyrifos) on a wide variety of common aquatic species are known. The effects that the single stressors have, or may have, in stormwater are therefore known with reasonable certainty. However, as is shown in Table 2.1, nonpoint sources, including stormwater, contain multiple stressors that are applied intermittently, and science currently has a poor understanding of stressor interactions and effects.

The attributes of each stormwater event are a result of previous meteorological conditions (e.g., dry deposition, air patterns, humidity), land use patterns (e.g., traffic and parking patterns, construction and landscaping activities), storm intensity and duration, and other watershed character-

Table 2.1 Potential Effects of Some Sources of Alteration on Stream Parameters

Stream Parameter	Acid Mine Drainage or Acid Precipitation	Sewage Treatment Plant Discharge	Agriculture Runoff (pasture or cropland)	Urban Runoff	Channelization	Pulp and Paper	Textile	Metal Finishing and Electroplating	Petroleum	Iron and Steel	Paint and Ink	Dairy and Meat Products	Fertilizer Production and Lime Crushing	Plastics and Synthetics
pH	D					C	I	C		D	C		D,I	C
Alkalinity	D						I						D,I	
Hardness	I						I						I	
Chlorides		I	I	I							I	I		
Sulfates	I								I	I	I		I	I
TDS	I			I			I		I		I	I	I	
TKN		I	I	I					I	I	I	I	I	I
NH_3-N		I		I					I	I			I	I
Total-P		I	I	I				I	I			I	I	
Ortho-P		I		I				I			I		I	
BOD_5		I		I		I	I		I	I		I		I
COD	I	I		I		I	I			I	I	I		I
TOC		I	I	I		I			I	I	D	I		I
COD/BOD	I			I		I	I		D	I				
D.O.		D				D					I	D		
Volatile compounds			I	I		I			I					I
Fluoride						I		I			I		I	I
Cr				I		I	I	I	I					I
Cu	I			I				I			I			I
Pb				I				I	I	I	I			
Zn	I			I		I		I	I	I				I
Cd		I		I				I						I
Fe	I			I				I		I			I	I
Arsenic				I										
Mercury				I						I				I
Cyanide				I						I				I
Oil and grease				I		I	I	I	I			I		
Coliforms	D	I	I					D		D	I	I		
Chlorophyll	D	I	I		I	D		D	D	D	D	I	I	D
Diversity	D	D		D	D	D	D	D	D	D	D		D	D
Biomass	D	D	I		I		I		D		D	I	I	D
Riparian factors				C	C									
Temperature				I	I									
TSS			I	I	I	I	I	I					I	I
VSS				I	I	I	I							
Color						I						I		I
Conductivity	I	I					I						I	
Channel factors				C	C									

D = decrease, I = Increase, C = change.
From EPA (U.S. Environmental Protection Agency). *Results of the Nationwide Urban Runoff Program*. Water Planning Division, PB 84-185552, Washington, D.C. December 1983.

istics. Because of the potentials for extreme heterogeneity in stormwater and its associated quality, predicting effects to receiving waters is difficult and crude at best. Stormwaters often contain a large number of potential stressors to aquatic ecosystems. These stressors include oxygen demand, suspended solids, dissolved solids (including salts), altered ion ratios, nutrients, pathogens, metals, natural and synthetic organics, pH, and temperature. These stressors may interact to varying degrees in an antagonistic, additive, or synergistic fashion, affecting organisms in the receiving water.

There are numerous receiving water problems associated with stormwater that interfere with beneficial uses. The most obvious is the substantial increase in runoff causing increases in the frequency and magnitude of flooding along urban streams. Increases in stream flows also cause significant habitat problems in urban streams by attempting to enlarge the stream cross sections, causing significant channel erosion and unstable conditions. Stream-side residents also dramatically affect habitat by removing riparian vegetation and large organic debris from the streams. Another significant and obvious effect is the increase in sediment associated with poorly controlled construction site runoff. This sediment smothers coarse stream sediments that are needed by many spawning fish, and fills in stream pool areas. Another obvious receiving water problem associated with stormwater is the large amount of floating trash and litter (some hazardous) that is discharged by stormwater and that accumulates along urban waterways. This creates unsightly and potentially hazardous conditions interfering with noncontact recreational uses of the stream corridors.

The degree of impact on an exposed organism is dependent on numerous factors, such as the organism's sensitivity, life stage, feeding habits, frequency of exposure, and magnitude and duration of exposure. The organism or community affected by stormwater induces changes in other components of their ecosystem including habitat, food sources, predator–prey relationships, competition, and other behavior patterns. It is clear that there is no simple method by which to detect an effect of stormwaters on the receiving water ecosystem. Human health and safety concerns associated with stormwater discharges are also highly variable depending on many site conditions. Chapters 3 and 4 discuss ways in which effects can be assessed effectively, despite the complex, heterogeneous nature of the system, while Chapters 5 and 6 describe how specific monitoring activities can be carried out. Chapters 7 and 8 outline ways to evaluate the collected data to accomplish the study goals, outlined in Chapter 4.

The main purpose of treating stormwater is to reduce its adverse impacts on receiving water beneficial uses. Therefore, it is important in any stormwater runoff study to assess the detrimental effects that runoff is actually having on a receiving water. Below are discussions of the basic receiving water beneficial uses that need to be considered in all cases.

BENEFICIAL USE IMPAIRMENTS

Recognized Value of Human-Dominated Waterways

With full development in a watershed and with no stormwater controls, it is unlikely that any of the basic beneficial uses can be achieved. With less development, and with the application of stormwater controls, some uses may be possible. However, it is important that unreasonable expectations not be placed on urban or agricultural waters, as the cost to obtain these uses may be prohibitive. With full-scale development and lack of adequate stormwater controls, severely degraded streams will be common. In all cases, stormwater conveyance and aesthetics should be the basic beneficial use goals for all human-dominated waters. Biological uses should also be a goal, but with the realization that the natural stream ecosystem will be severely modified with urbanization and agricultural activities. Certain basic stormwater controls, installed at the time of development, plus protection of stream habitat, may enable partial to full use of some of these basic goals. Careful planning and optimal utilization of stormwater controls are necessary to obtain these basic goals in most watersheds. Water contact recreation, consumptive fisheries, and water

Figure 2.7 Original section of Riverwalk in San Antonio, TX.

Figure 2.8 New section of Riverwalk in San Antonio, TX.

supplies are not appropriate goals for most heavily developed watersheds. However, these higher uses may be possible in urban areas where the receiving waters are large and drain mostly undeveloped areas.

There are many examples throughout the world where local citizens have recognized the added value that aesthetically pleasing waters contribute to cities. With this recognition comes a local pride in these waters and a genuine desire to improve their condition. In many cases, water has played an important part in the economic renewal of an inner city area. Dreiseitl (1998) states that "stormwater is a valuable resource and opportunity to provide an aesthetic experience for the city dweller while furthering environmental awareness and citizen interest and involvement." He found that water flow patterns observed in nature can be duplicated in the urban environment to provide healthy water systems of potentially great beauty. Without reducing safety, urban drainage elements can utilize water's refractive characteristics and natural flow patterns to create very pleasing urban areas. Successful stormwater management in Germany has been best achieved by using several measures together. Small open drainage channels placed across streets have been constructed of cobbles. These collect and direct the runoff, plus slow automobile traffic and provide dividing lines for diverse urban landscaping elements. The use of rooftop retention and evaporation areas reduce peak flows. Dreiseitl has found that infiltration and retention ponds can also be used to great advantage by providing a visible and enjoyable design element in urban landscapes.

Probably the most famous U.S. example of the economic benefits that water has contributed in an older part of a city is Riverwalk in San Antonio, TX. Many cities would like to emulate Riverwalk, with the great economic benefit that it has provided to San Antonio (Figures 2.7 through 2.9). Riverwalk was conceived and constructed many decades ago, but only in recent years has its full value been realized. Bellingham, WA (Figure 2.10), Austin, TX (Figure 2.11), and Denver, CO (Figures 2.12 through 2.14) are some of the other U.S. cities that have long enjoyed central city urban creek corridors.

Dreiseitl (1998) described the use of stormwater as an important component of the Potsdamer Platz in the center of Berlin. Roof runoff will be stored in large underground cisterns, with some filtered and used for toilet flushing and irrigation. The rest of the roof runoff will flow into a 1.4-ha (3.8-acre) concrete-lined lake in the center of the project area. The small lake provides an important natural element in the center of this massive development and regulates the stormwater discharge rate to the receiving water (Landwehrkanal). The project is also characterized by numerous fountains, including some located in underground parking garages.

Figure 2.9 Litter control along Riverwalk, San Antonio, TX.

Figure 2.10 Bike and walking trail along Watcom Creek, Bellingham, WA.

Figure 2.11 Barton Springs swimming area, Austin, TX.

Figure 2.12 Cherry Creek walkway, downtown Denver, CO.

Figure 2.13 Cherry Creek walk in Denver, CO.

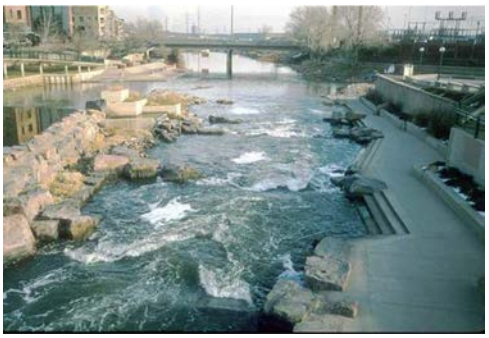

Figure 2.14 Cherry Creek and Platte River junction in Denver, CO.

Göransson (1998) also described the aesthetic use of stormwater in Swedish urban areas. The main emphasis was to retain the stormwater in surface drainages instead of rapidly diverting it to underground conveyances. Small, sculpted rainwater channels are used to convey roof runoff downspouts to the drainage system. Some of these channels are spiral in form and provide much visual interest in areas dominated by the typically harsh urban environment. Some of these spirals are also formed in infiltration areas and are barely noticeable during dry weather. During rains, increasing water depths extenuate the patterns. Glazed tile, small channels with perforated covers, and geometrically placed bricks with large gaps to provide water passage slightly below the surface help urban dwellers better appreciate the beauty of flowing water.

Tokyo has instituted major efforts to restore historical urban rivers that have been badly polluted, buried, or have had all of their flows diverted. Fujita (1998) describes how Tokyo residents place great value on surface waterways: "Waterfront areas provide urban citizens with comfort and joy as a place to observe nature and to enjoy the landscape." Unfortunately, the extensive urbanization that has taken place in Tokyo over the past several decades has resulted in severe stream degradation, including the disappearance of streams altogether. However, there has recently been a growing demand for the restoration of polluted urban watercourses in Tokyo. This has been accomplished in many areas by improved treatment of sanitary sewage, reductions in combined sewer overflows, and by infiltration of stormwater.

Fujita (1998) repeatedly states the great importance the Japanese place on nature, especially flowing water and the associated landscaping and attracted animals. They are therefore willing to perform what seems to be extraordinary efforts in urban stream recovery programs in one of the world's largest cities. The stream recovery program is but one element of the local efforts to provide a reasonably balanced urban water program. Water reuse and conservation are also important elements in their efforts. Stormwater infiltration to recharge groundwaters and the use of treated wastewaters for beneficial uses (including stream restoration, plus landscaping irrigation, train washing, sewer flushing, fire fighting, etc.) are all important elements of these efforts, although this reuse currently only amounts to about 7% of the total annual water use in Tokyo.

At many U.S. wet detention pond project sites, the stormwater treatment pond is used to increase the value of the property. Figures 2.15 and 2.16 show two examples (in Austin, TX, and in Lake Oswego, OR, respectively). Many people live near wet detention ponds because of the close presence of the wetlands, and their property values are typically greater than lots farther from the ponds (Marsalek et al. 1982). They also reported that small (well-maintained) wet detention ponds are less subject to controversy than larger ponds (that are more commonly neglected). Debo and Ruby (1982) summarized a survey conducted in Atlanta, GA, of residents living near and downstream of 15 small detention ponds and found that almost half the people surveyed who lived in the immediate areas of the ponds did not even know that they existed. Wiegand et al. (1986) found that wet detention ponds, when properly maintained, are preferred by residents over any other urban runoff control practice.

Figure 2.15 Advertising the benefits of a stormwater pond (Austin, TX).

Figure 2.16 Stormwater pond adding value to apartment complex (Lake Oswego, OR).

Emmerling-DiNovo (1995) reported on a survey of homeowners in the Champaign-Urbana, IL, area living in seven subdivisions having either dry or wet detention ponds. She reported that past studies have recognized that developers are well aware that proximity to water increases the appeal of a development. Detention ponds can create a sense of identity, distinguishing one development from another, and can be prominent design elements. Increased value is important because the added cost of the detention facility, including loss of developable land, must be recovered by increasing the housing costs. Others have also found that the higher costs of developments having stormwater detention facilities can also be offset by being able to sell the housing faster. In a survey in Columbia, MD, 73% of the respondents were found to be willing to pay more for property located in an area having a wet detention pond if designed to enhance fish and wildlife use. Although the residents were concerned about nuisances and hazards, they felt that the benefits outweighed these concerns. In her survey, Emmerling-DiNovo (1995) received 143 completed surveys. Respondents reported that the overall attractiveness of the neighborhood was the most important factor in their decision to purchase their home. Resale value was the second most important factor, while proximity to water was slightly important. More than 74% of the respondents believed that wet detention ponds contributed positively to the image of the neighborhood and that they were a positive factor in choosing that subdivision. In contrast, the respondents living in the subdivisions with dry ponds felt that the dry ponds were not a positive factor for locating in their subdivision. Respondents living adjacent to wet ponds felt that the presence of the pond was very positive in the selection of their specific lot. The lots adjacent to the wet ponds were reported to be worth about 22% more than lots that were not adjacent to the wet ponds. Lots adjacent to the dry ponds were actually worth less (by about 10%) than other lots in two of the three dry basin subdivisions studied. The respondents favored living adjacent to wet ponds even more than next to golf courses. Living adjacent to dry ponds was the least preferred location.

Stormwater Conveyance (Flood Prevention)

This is a basic beneficial use of streams and storm drainage systems that must be considered. Problems are caused by increases in peak runoff flow rates that are associated with large increases in runoff volume and decreases in the drainage time of concentration. Because of high flows during wet weather, it is common for urban streams to have much lower flows during dry weather due to lack of recharge from shallow groundwaters (Color Figure 2.1).* Debris and obstructions in the receiving waters, which assist aquatic life uses, typically degrade flooding and drainage uses and are often cleared to provide better drainage. Other common conflicts are associated with the desire to have homogeneous channels (smooth bottoms and straight alignments) for drainage (Figure 2.17), while aquatic life requires diversity in the channel characteristics. These conflicts must be resolved through comprehensive planning, including source controls and drainage controls that have minimal effects on aquatic life. The best solutions would provide for the necessary flooding and drainage benefits while also providing suitable biological habitat (including improved channel stability, decreased bank erosion, artificial pools and riffle areas, overstory shading, gravel linings, low flow meandering channel alignments, and other refuge areas).

Figure 2.17 Channelized urban stream, Nor-X-Way, Menomonee Falls, WI.

Recreation (Non-water Contact) Uses

This basic beneficial use is concerned with odors, trash, beauty, access, and rapidly fluctuating flows. Safety is an important issue in urban

* Color figures follow p. 370.

Figure 2.18 Degraded stream banks along New York City shoreline.

Figure 2.19 Debris in riparian area, New York City.

Figure 2.20 Algal mats and other floating debris, Orlando, FL.

Figure 2.21 Litter controlled behind floating booms, New York City.

areas where children frequently play near small streams. Bank stability and rapidly fluctuating flows are, therefore, of prime importance (Figures 2.18 and 2.19). Many communities have also established linear parks along urban streams as part of their flood control and parks programs. In these cases, aesthetics (trash, odor, and beauty), access (paths and bridges), and the above safety issues are also important. Excessive algal growths, with attendant odors and unsightly conditions, may also occur along stressed urban waterways (Figures 2.20 and Color Figure 2.2). Some simple controls have been instituted in some areas to reduce aesthetic impacts (Figure 2.21). Human health may be an issue if water contact (especially by wading children) or if consumptive fishing occurs. These human health uses will be very difficult to maintain in urban areas.

Biological Uses (Warm-Water Fishery, Aquatic Life Use, Biological Integrity, etc.)

This basic beneficial use is also important, but it is defined differently by different people. It is unreasonable to expect natural receiving water conditions in agricultural or urbanized streams. Some degradation is inevitable. The goal is to have an acceptable diversity of aquatic life and an absence of episodic fish kills, at a minimum. It is unfortunate if sensitive and important species exist in an agricultural or urbanized stream and need special protection, as it is probably unrealistic to believe that it is possible to maintain these species in the absence of dramatic and extensive stormwater controls (which are not likely to occur). The most significant impairments to aquatic life beneficial uses are likely: habitat destruction (including channel and bank instability, sedimen-

tation, and loss of refuge areas and vegetative overstory/canopy), highly fluctuating flow rates, inappropriate dry-weather contaminated discharges (toxicants and pathogens), polluted sediment (toxicants and oxygen-demanding materials), and possibly wet weather water quality degradation. Decreases in groundwater recharge and increased peak flows during periods of storm events are obviously associated with decreased flows during dry periods. Aquatic life undergoes additional stress during periods of low flow due to associated increased water temperatures, decreased pollutant mixing and transport, and simple decreased mobility and forage opportunities.

It may be possible to obtain significant short-term biological beneficial use improvements in a degraded stream with improvements in habitat conditions alone. Longer-term benefits would likely require sediment removal and control, plus the control of inappropriate dry-weather toxic discharges. It is unlikely that large improvements in wet weather water quality would be possible in heavily developed watersheds, nor may it be needed to obtain acceptable (but degraded) biological uses. The retrofitting of stormwater controls to improve wet-weather runoff quality in an urban area is very costly and is limited in effectiveness. However, the basic use of construction site erosion controls and biofiltration/infiltration and sedimentation stormwater controls in newly developing areas should be mandatory to decrease the further degradation of biological conditions in receiving waters.

Human Health-Related Uses (Swimming, Fishing, and Water Supply)

In many areas of the country, urban and agricultural runoff drains into public water supplies, swimming areas, or fisheries. In these cases, additional concerns need to be considered, especially relating to toxicants and pathogens. Public water supplies are frequently affected by upstream wastewater discharges (both point and nonpoint sources) and are designed to reduce and monitor constituents of concern. As upstream discharges increase, water treatment becomes more difficult and costly, with increased probabilities of waterborne disease outbreaks and increased (but "legal") taste and odor problems. Swimming areas in urban receiving waters (large rivers and lakes) have also been more frequently closed to the public because of high bacteria counts for extended periods after rains, and because of other unsafe conditions (Figures 2.22 through 2.25 and Color Figure 2.3). In addition, although fishing in urban and agricultural areas is relatively common (Figures 2.26 and 2.27), many communities are posting fishing advisories to discourage this practice (Figure 2.28).

Figure 2.22 Swimming restriction in urban lake, San Francisco, CA.

Figure 2.23 Swimming near stormwater outfall, Navesink River, NJ.

Figure 2.24 Children playing in Lincoln Creek, Milwaukee, WI. (Courtesy of Wisconsin Department of Natural Resources.)

Figure 2.25 Floatable trash from CSO and stormwater discharges, New York City.

Figure 2.26 Fishing in urban stream, Birmingham, AL.

Figure 2.27 Urban fishing in Neva River, St. Petersburg, Russia.

Unfortunately, pathogen levels in stormwater may be high. Fecal coliform levels can be very high, but fecal coliform levels are not thought to be a good indicator of pathogens in stormwater (see also Chapter 4). Direct pathogen monitoring in stormwater has shown very large numbers of some specific pathogens, however, requiring careful consideration for human health issues. In addition, sediments may contain elevated levels of pathogens which live for extended periods following high flow events (Burton et al. 1987). It is very difficult to reduce the high levels using typical stormwater controls. Common disinfection controls are also very costly and may create additional problems associated with trihalomethane production. The consumption of fish or shellfish in waters receiving agricultural and urban runoff is also a cause of concern because of pathogens and toxicants. This has been shown with the recent outbreaks of *Pfiesteria* in nutrient-laden waters of the East Coast. Many of the toxic compounds found in stormwater may readily bioaccumulate in aquatic organisms, and pathogens can also contaminate the aquatic organisms. All of these human health issues require careful study by epidemiologists and public health professionals.

Figure 2.28 Fish advisory for Village Creek, Jefferson Co., AL.

LIKELY CAUSES OF RECEIVING WATER USE IMPAIRMENTS

In general, monitoring of urban and agricultural stormwater runoff has indicated that the biological beneficial uses of receiving waters are most likely affected by habitat destruction and long-term pollutant exposures (especially to macroinvertebrates via contaminated sediment). Pulse exposures to suspended solids and toxicants and contaminated sediments have also been shown to be common in urban and agricultural waterways (see Chapter 6; also review by Burton et al. 2000). Mancini and Plummer (1986) have long been advocates of numeric water quality standards for stormwater that reflect the partitioning of the toxicants and the short periods of exposure during rains. Unfortunately, this approach attempts to isolate individual runoff events and does not consider the accumulative adverse effects caused by the frequent exposures of receiving water organisms to stormwater (Davies 1995; Herricks et al. 1996a,b). Recent investigations have identified acute toxicity problems associated with intermediate-term (about 10 to 20 days) exposures to adverse toxicant concentrations in urban receiving streams (Crunkilton et al. 1996). The most severe receiving water problems may be associated with chronic exposures to contaminated sediment and to habitat destruction.

Heaney et al. (1980) conducted a comprehensive evaluation of the early literature pertaining to urban runoff effects on receiving waters. They found that well-documented cases of receiving water detrimental effects were scarce. Through their review of many reports, they found several reasons to question the implied cause-and-effect relationships between urban runoff and receiving water conditions. Impacts that were attributed to urban runoff were probably caused, in many cases, by other water pollution sources (such as combined sewer overflows, agricultural nonpoint sources, etc.). One of the major difficulties encountered in their study was the definition of "problem" that had been used in the reviewed projects. They found that very little substantive data had been collected to document beneficial use impairments. In addition, urban runoff impacts are most likely to be associated with small receiving waters, while most of the existing urban water quality monitoring information exists for larger bodies of water. It was also very difficult for many researchers to isolate urban runoff effects from other water pollutant sources, such as municipal and industrial wastes. This was especially important in areas that had combined sewers that overflowed during wet weather, contributing to the receiving water impacts during wet-weather conditions.

Claytor (1996a) summarized the approach developed by the Center for Watershed Protection as part of their EPA-sponsored research on stormwater indicators (Claytor and Brown 1996). The 26 stormwater indicators used for assessing receiving water conditions were divided into six broad categories: water quality, physical/hydrological, biological, social, programmatic, and site. These were presented as tools to measure stress (impacting receiving waters), to assess the resource itself, and to indicate stormwater control program implementation effectiveness. The biological communities in Delaware's Piedmont streams have been severely impacted by stormwater, after the extent of imperviousness in the watersheds exceeded about 8 to 15%, according to a review article by Claytor (1996b). If just conventional water quality measures are used, almost all (87%) of the state's nontidal streams supported their designated biological uses. However, when biological assessments are included, only 13% of the streams were satisfactory.

MAJOR URBAN RUNOFF SOURCES

Soil erosion from construction sites and increased stormwater runoff generated from newly established urban areas cause significant economic, social, and environmental problems. These problems may result from all land development activities such as subdivision development, individual homesite construction, large-scale construction projects such as shopping centers and industrial sites, highway construction, and public utility construction projects. Problems caused by construction site erosion and stormwater runoff include sediment that destroys fish habitat and fills in lakes; urban runoff volumes and flow rates that increase flooding; nutrient discharges that produce nuisance algae growths; toxic heavy metal and organic discharges that result in inedible fish, undrinkable water, and shifts in aquatic life to more pollution-tolerant species; and pathogenic bacteria discharges that necessitate swimming beach closures.

Erosion losses and downstream sedimentation peak during construction, when soil exposure is greatest, and decline after construction is completed. Thus, while the impacts of erosion and sedimentation may be severe, they are relatively short term in nature for any specific construction site.

Stormwater runoff and pollutant discharges, on the other hand, increase steadily as development progresses and remain at an elevated level for the lifetime of the development. This happens because impervious surfaces such as roads, sidewalks, driveways, rooftops, etc., permanently reduce infiltration of rainfall and runoff into the ground.

Accelerated stormwater runoff rates also occur with development and can significantly increase the water's ability to detach sediment and associated pollutants, to carry them off site, and to deposit them downstream. Increased runoff rates may also cause stream bank and channel erosion. Increased stormwater runoff volumes and flow rates also increase urban flooding and the resultant loss of human life and property.

Urbanization may also affect groundwater adversely. In some cases, polluted stormwater contaminates groundwater. More frequently, impervious surfaces block infiltration of rainfall and runoff that otherwise would recharge groundwater supplies. Reduced infiltration affects not only groundwater levels but also the amount of groundwater-derived stream flow available during low flow periods. From a water quality standpoint, low flow periods are critical because the amount of water available to dilute stream pollutants is at a minimum at those times. Reduced flows during extended dry periods also adversely affect aquatic life.

Urban runoff, which includes stormwater, construction site runoff, snowmelt, and contaminated baseflows, has been found to cause significant receiving water impacts on aquatic life. The effects are obviously most severe for small receiving waters draining heavily urbanized and rapidly developing watersheds. However, some studies have shown important aquatic life impacts for streams in watersheds that are less than 10% urbanized.

In order to best identify and understand these impacts, it is necessary to include biological monitoring (using a variety of techniques) and sediment quality analyses in a monitoring program. Water column testing alone has been shown to be very misleading. Most aquatic life impacts associated with urbanization are probably related to chronic long-term problems caused by habitat destruction, polluted sediments, and food web disruption. Transient water column quality conditions associated with urban runoff probably rarely cause significant direct aquatic life acute impacts.

The underlying theme of many researchers is that an adequate analysis of receiving water biological impacts must include investigations of a number of biological organism groups (fish, benthic macroinvertebrates, algae, rooted macrophytes, etc.) in addition to studies of water and sediment quality. Simple studies of water quality alone, even with possible comparisons with water quality criteria for the protection of aquatic life, are usually inadequate to predict biological impacts associated with urban runoff.

Duda et al. (1982) presented a discussion on why traditional approaches for assessing water quality, and selecting control options, in urban areas have failed. The main difficulties of traditional

approaches when applied to urban runoff are the complexity of pollutant sources, wet weather monitoring problems, and limitations when using water quality standards to evaluate the severity of wet weather receiving water problems. They also discuss the difficulty of meeting water quality goals (that were promulgated in the Water Pollution Control Act of 1972) in urban areas.

Relationships between observed receiving water biological effects and possible causes have been especially difficult to identify, let alone quantify. The studies reported in this chapter have identified a wide variety of possible causative agents, including sediment contamination, poor water quality (low dissolved oxygen, high toxicants, etc.), and factors affecting the physical habitat of the stream (high flows, unstable stream beds, absence of refuge areas, etc.). It is expected that all of these factors are problems, but their relative importance varies greatly depending on the watershed and receiving water conditions. Horner (1991), as an example, notes that many watershed, site, and organism-specific factors must be determined before the best combination of runoff control practices to protect aquatic life can be determined.

Construction Site Erosion Characterization

Sediment is, by weight, the greatest pollutant of water resources. Willett (1980) estimated that approximately 5 billion tons of sediment reach U.S. surface waters annually, of which 30% is generated by natural processes and 70% by human activities. Half of this 70% is attributed to eroding croplands. Although urban construction accounts for only 10%, this amount equals the combined contributions of forestry, mining, industrial, and commercial activities (Willett 1980; Virginia 1980).

Construction accounts for a much greater proportion of the sediment load in urban areas — sometimes more than 50% — than it does in the nation as a whole. Urban areas experience large sediment loads from construction site erosion because construction sites usually have extremely high erosion rates and because urban construction sites are efficiently drained by stormwater drainage systems. Construction sites at most U.S. locations have an erosion rate of approximately 20 to 200 tons per acre per year, a rate that is about 3 to 100 times that of croplands. Construction site erosion losses vary greatly depending on local rain, soil, topographic, and management conditions. As an example, the Birmingham, AL, area may have some of the highest erosion rates in the nation because of its combination of very high-energy rains, moderately erosive soils, and steep topography. The typically high erosion rates mean that even a small construction project may have a significant detrimental effect on local water bodies. While construction occurs on only about 0.007% of U.S. land, it accounts for about 10% of the sediment load to U.S. surface waters (Willett 1980).

Data from the highly urbanized Menomonee River watershed in southeastern Wisconsin illustrate the impact of construction on water quality. These data indicate that construction sites have much greater potential for generating sediment and phosphorus than do areas in other land uses (Chesters et al. 1979). For example, construction sites can generate approximately 8 times more sediment and 18 times more phosphorus than industrial sites, the land use that contributes the second highest amount of these pollutants, and 25 times more sediment and phosphorus than row crops. In fact, construction contributes more sediment and phosphorus to the river than any other land use. In 1979, construction comprised only 3.3% of the watershed's total land area, but it contributed about 50% of the suspended sediment and total phosphorus loading at the river mouth (Novotny et al. 1979).

Similar conclusions were reported by the Southeastern Wisconsin Regional Planning Commission in a 1978 modeling study of the relative pollutant contributions of 17 categories of point and nonpoint pollution sources to 14 watersheds in the southeast Wisconsin regional planning area (SEWRPC 1978). This study revealed construction as the first or second largest contributor of sediment and phosphorus in 12 of the 14 watersheds. Although construction occupied only 2% of the region's total land area in 1978, it contributed approximately 36% of the sediment and 28% of the total phosphorus load to inland waters, making construction the region's second largest

source of sediment and phosphorus. The largest source of sediment was estimated to be cropland; livestock operations were estimated to be the largest source of phosphorus. By comparison, cropland comprised 72% of the region's land area and contributed about 45% of the sediment and only 11% of the phosphorus to regional watersheds. This study again points out the high pollution-generating ability of construction sites and the significant water quality impact a small amount of construction may have on a watershed.

A monitoring study of construction site runoff water quality in the Village of Germantown (Washington County, WI) yielded similar results (Madison et al. 1979). Several large subdivisions being developed with single and multifamily residences were selected for runoff monitoring. All utility construction, including the storm drainage system and streets, was completed before monitoring began.

Analysis of the monitoring data showed that sediment leaving the developing subdivisions averaged about 25 to 30 tons per acre per year (Madison et al. 1979). Construction practices identified as contributing to these high yields were removing surface vegetation; stripping and stockpiling topsoil; placing large, highly erodible mounds of excavated soil on and near the streets; pumping water from flooded basement excavations; and tracking of mud into the streets by construction vehicles. If the amount of sediment leaving the sites during utility development had been added in, the total amount of eroded sediment leaving the site would have been substantially greater.

Analysis of the Germantown data also showed that the amount of sediment leaving areas undergoing development is a function of the extent and duration of development and is independent of the type of development. In other words, there is no difference in the per acre sediment loads produced by single-family or multifamily construction. This finding is significant because local and state regulatory programs sometimes exempt single-family home construction from erosion control requirements.

Almost all eroded sediment from the Germantown construction areas entered the receiving waters. The delivery of sediment to the receiving waters was found to be nearly 100% when 10% or more of the watershed was experiencing development. The smallest delivery value obtained during the Germantown monitoring was 50%, observed when only 5% of the watershed was undergoing development. These high delivery values occurred (even during periods with small amounts of development) because storm drainage systems, which efficiently transport water and its sediment load, had been installed during an early stage of development.

Local Birmingham, AL, erosion rates from construction sites can be 10 times the erosion rates from row crops and 100 times the erosion rates from forests or pastures (Nelson 1996). The site-specific factors affecting construction site erosion include:

- Rainfall energy (Alabama has the highest in the nation)
- Soil erodibility (northern part of the state has fine-grained, highly erosive soils)
- Site topography (northeastern part of the state has steep hills under development)
- Surface cover (usually totally removed during initial site grading)

The rain energy is directly related to rainfall intensity, and the rainfall erosion index varies from 250 to 550+ for Alabama (most of the state is about 350), which is the highest in the United States. The months having the greatest erosion potential are February and March, while September through November have the lowest erosion potential. Nelson (1996) monitored sediment quantity and particle size from 70 construction site runoff samples from the Birmingham area. He measured suspended solids concentrations ranging from 100 to more than 25,000 mg/L (overall median about 4000 mg/L), while the turbidity values ranged from about 300 to >50,000 NTU (average of about 4000 NTU). About 90% of the particles (by mass) were smaller than about 20 μm (0.02 mm) in diameter, and the median size was about 5 μm (0.005 mm). The local construction site erosion discharges were estimated to be about 100 tons/acre/year. Table 2.2 summarizes the measured suspended solids and median particle sizes as a function of rain intensity. High-intensity rains were found to have the most severe erosion problems, as expected, with much greater suspended solids

Table 2.2 Birmingham (AL) Construction Site Erosion Runoff Characteristics

	Low-Intensity Rains (<0.25 in/hr)	Moderate-Intensity Rains (about 0.25 in/hr)	High-Intensity Rains (>1 in/hr)
Suspended solids, mg/L	400	2000	25,000
Particle size (median), μm	3.5	5	8.5

Data from Nelson, J. *Characterizing Erosion Processes and Sediment Yields on Construction Sites.* M.S.C.E. thesis. Department of Civil and Environmental Engineering, University of Alabama at Birmingham. 94 pp. 1996.

concentrations. Typical small particle sizes of erosion particulates make it very difficult to remove these particulates after they have been eroded from the site. The extreme turbidity values also cause very high in-stream turbidity conditions in local receiving waters for great distances downstream of eroding sites.

Urban Runoff Contaminants

Urban runoff is comprised of many different flow phases. These may include dry-weather base flows, stormwater runoff, combined sewer overflows (CSOs), and snowmelt. The relative magnitudes of these discharges vary considerably, based on a number of factors. Season (such as cold vs. warm weather, or dry vs. wet weather) and land use have been identified as important factors affecting baseflow and stormwater runoff quality.

Land development increases stormwater runoff volumes and pollutant concentrations. Impervious surfaces, such as rooftops, driveways, and roads, reduce infiltration of rainfall and runoff into the ground and degrade runoff quality. The most important hydraulic factors affecting urban runoff volume (and therefore the amount of water available for groundwater infiltration) are the quantity of rain and the extent of impervious surfaces directly connected to a stream or drainage system. Directly connected impervious areas include paved streets, driveways, and parking areas draining to curb and gutter drainage systems, and roofs draining directly to a storm or combined sewer pipe. Table 2.3 presents older stormwater quality data (APWA 1969), while Table 2.4 is a summary of the Nationwide Urban Runoff Program (NURP) stormwater data collected from about 1979 through 1982 (EPA 1983). The NURP data are the most comprehensive stormwater data available from throughout the nation. The recently collected data for the stormwater NPDES permits is a potentially large and important database of information, but it has not been made conveniently available. Land use and source areas (parking areas, rooftops, streets, landscaped areas, etc.) all have important effects on stormwater runoff quality. BOD_5 bacteria and nutrient concentrations in stormwater are lower than in raw sanitary wastewater. However, urban stormwater still has relatively high concentrations of bacteria, along with high concentrations of many metallic and some organic toxicants.

NURP found that stormwater pollutant concentrations, runoff volumes, and therefore annual pollutant yields often vary with land use. Although inconsistencies in local development practices within a single land use category make land use a less than perfect indicator of urban runoff characteristics, land use must serve as a surrogate for more appropriate indicators because development data are typically reported in land use categories. The amount of directly connected impervious area is a very good indicator of an area's runoff volume. The extent of "effective" impervious surfaces, however, is a function of local development customs (lot sizes, use of swale drainages, single or multilevel buildings, type of landscaping, etc.), which can vary significantly within a single land use category (such as medium-density residential). Development characteristics are not uniform throughout a region, and they may also vary by age of development or location within a single city.

Bannerman et al. (1979) found a high correlation between pollutant loading values and percent connected-imperviousness during monitoring of seven subwatersheds of the Menomonee River basin: pollutant loading to the river increased as the extent of impervious areas directly connected to the storm drainage system increased. Although larger amounts of runoff and pollutants were

Table 2.3 Characteristics of Stormwater Runoff from Early Studies

City	BOD_5 (mg/L)	Total Solids (mg/L)	Suspended Solids (mg/L)	Chlorides (mg/L)	COD (mg/L)
East Bay Sanitary District:					
Oakland, California					
Minimum	3	726	16	300	
Maximum	7700		4400	10,260	
Average	87	1401	613	5100	
Cincinnati, Ohio					
Maximum Seasonal Means	12	260			110
Average	17		227		111
Los Angeles County					
Average 1962–63	161	2909		199	
Washington, D.C.					
Catch-basin samples during storm	6		26	11	
Minimum	625		36,250	160	
Maximum	126		2100	42	
Average					
Seattle, Washington	10				
Oxney, England	100[a]	2045			
Moscow, Russia	186–285	1000–3500[a]			
Leningrad, Russia	36	14,541			
Stockholm, Sweden	17–80	30–8000			18–3100
Pretoria, South Africa					
Residential	30				29
Business	34				28
Detroit, Michigan	96–234	310–914	102–213[b]		

[a] Maximum.
[b] Mean.

From APWA (American Public Works Association). *Water Pollution Aspects of Urban Runoff*. Water Pollution Control Research Series WP-20-15, Federal Water Pollution Control Administration. January 1969.

Table 2.4 Median Stormwater Pollutant Concentrations for All Sites by Land Use

Constituent	Residential		Mixed Land Use		Commercial		Open/Non-urban	
	Median	COV[a]	Median	COV	Median	COV	Median	COV
BOD_5, mg/L	10	0.41	7.8	0.52	9.3	0.31	—	—
COD, mg/L	73	0.55	65	0.58	57	0.39	40	0.78
TSS, mg/L	101	0.96	67	1.14	69	0.85	70	2.92
Total Kjeldahl nitrogen, µg/L	1900	0.73	1288.8	0.50	1179	0.43	965	1.00
$NO_2 + NO_3$ (as N) µg/L	736	0.83	558	0.67	572	0.48	543	0.91
Total P, µg/L	383	0.69	263	0.75	201	0.67	121	1.66
Soluble P, µg/L	143	0.46	56	0.75	80	0.71	26	2.11
Total lead, µg/L	144	0.75	114	1.35	104	0.68	30	1.52
Total copper, µg/L	33	0.99	27	1.32	29	0.81	—	—
Total zinc, µg/L	135	0.84	154	0.78	226	1.07	195	0.66

[a] COV: coefficient of variation = standard deviation/mean.

From EPA (U.S. Environmental Protection Agency). *Results of the Nationwide Urban Runoff Program*. Water Planning Division, PB 84-185552, Washington, D.C. December 1983.

generated in low-density residential areas, compared to undisturbed areas, runoff and pollutant delivery from the source areas to streams was still low due to the use of grass-lined roadside drainage channels. Soil and vegetation have a greater chance to reduce runoff water and pollutants in areas drained by grass-lined drainage channels than in similar areas drained by conventional curb-and-gutter drainage systems.

Table 2.5 presents estimates of typical urban area pollutant yields from several separate studies. Local conditions and development characteristics significantly affect these estimates. The most significant factor is the drainage efficiency of the areas, specifically if the areas are drained by grass swales. The low-density residential area values shown on this table reflect grass swale drained areas. If conventional curbs and gutters were used instead of grass swales, the yields would be about 10 times greater. Other important development characteristics affecting runoff yields include roof drainage connections and the presence of alleyways. Increased drainage efficiency invariably leads to increased pollutant discharges.

A number of urban runoff monitoring projects (such as EPA 1983; Pitt and McLean 1986) have found inorganic and organic hazardous and toxic substances in urban runoff. The NURP data, collected from mostly residential areas throughout the United States, did not indicate any regional differences in the substances detected, or in their concentrations. However, residential and industrial data obtained by Pitt and McLean (1986) in Toronto found significant concentration and yield differences for these two land uses and for dry weather and wet weather urban runoff flows.

Tables 2.6 and 2.7 list the toxic and hazardous organic substances that have been found in greater than 10% of industrial and residential urban runoff samples. NURP data do not reveal toxic urban runoff conditions significantly different for different geographical areas throughout North America (EPA 1983). The pesticides shown were mostly found in urban runoff from residential areas, while other hazardous materials were much more prevalent in industrial areas. Urban runoff dry weather baseflows may also be important contributors of hazardous and toxic pollutants.

Urban Runoff Pollutant Sources

Sources of the toxic and hazardous substances found in urban runoff vary widely. Table 1.3 listed the major expected sources of these substances. Automobile use contributes significantly to many of these materials. Polycyclic aromatic hydrocarbons (PAHs), the most commonly detected toxic organic compounds found in urban runoff, are mostly from fossil fuel combustion. Phthalate esters, another group of relatively common toxic organic compounds, are derived from plastics. Pentachlorophenol, also frequently found, comes from preserved wood. Such compounds are very hard to control at their sources, and, unfortunately, their control by typical stormwater management practices is little understood.

Urban runoff includes warm and cold weather baseflows, stormwater runoff, and snowmelt. Table 2.8 shows median concentrations of some of the pollutants monitored in a mixed residential and commercial catchment and from an industrial area in Toronto, Ontario, for these different flow phases (Pitt and McLean 1986). Samples were obtained from baseflow discharges, stormwater runoff, and snowmelt. The baseflows had surprisingly high concentrations of several pollutants, especially dissolved solids (filtrate residue) and fecal coliforms from the residential catchment. The concentrations of some constituents in the stormwater from the industrial watershed were typically much greater than the concentrations of the same constituents in the residential stormwater. The industrial warm weather baseflows were also much closer in quality to the industrial stormwater quality than the residential baseflows were to the residential stormwater quality. The data collected for pesticides and PCBs indicate that the industrial stormwater and baseflows typically contained much greater concentrations of these pollutants than the residential waters. Similarly, the more commonly analyzed heavy metals were also more prevalent in the industrial stormwater. However, herbicides were only detected in residential urban runoff, especially the baseflows.

During cold weather, the increases in filtrate residue were quite apparent for both study catchments and for both baseflows and snowmelt. These increases were probably caused by high chlorides from road salt applications. In contrast, bacteria populations were noticeably lower in all outfall discharges during cold weather. Few changes were noted in concentrations of nutrients and heavy metals at the outfall, between cold- and warm-weather periods.

Table 2.5 Typical Urban Area Pollutant Yields (lb/acre/year or kg/ha/yr)[a]

Land Use	Total Solids	Suspended Solids	Chloride	Total Phosphorus	TKN	NH_3	NO_3 plus NO_2	BOD_5
Commercial	2100	1000	420	1.5	6.7	1.9	3.1	62
Parking lot	1300	400	300	0.7	5.1	2.0	2.9	47
High-density residential	670	420	54	1.0	4.2	0.8	2.0	27
Medium-density residential	450	250	30	0.3	2.5	0.5	1.4	13
Low-density residential[b]	65	10	9	0.04	0.3	0.02	0.1	1
Freeways	1700	880	470	0.9	7.9	1.5	4.2	NA[b]
Industrial	670	500	25	1.3	3.4	0.2	1.3	NA
Parks	NA[c]	3	NA	0.03	NA	NA	NA	NA
Shopping center	720	440	36	0.5	3.1	0.5	1.7	NA

Land Use	COD	Lead[d]	Zinc	Chromium	Copper	Cadmium	Arsenic
Commercial	420	2.7	2.1	0.15	0.4	0.03	0.02
Parking lot	270	0.8	0.8	NA	0.06	0.01	NA
High-density residential	170	0.8	0.7	NA	0.03	0.01	NA
Medium-density residential	50	0.05	0.1	0.02	0.03	0.01	0.01
Low-density residential[e]	7	0.01	0.04	0.002	0.01	0.001	0.001
Freeways	NA	4.5	2.1	0.09	0.37	0.02	0.02
Industrial	200	0.2	0.4	0.6	0.10	0.05	0.04
Parks	NA	0.005	NA	NA	NA	NA	NA
Shopping center	NA	1.1	0.6	0.04	0.09	0.01	0.02

[a] The difference between lb/acre/year and kg/ha/yr is less than 15%, and the accuracy of the values shown in this table cannot differentiate between such close values.
[b] The monitored low-density residential areas were drained by grass swales.
[c] NA = Not available.
[d] The lead unit area loadings shown on this table are currently expected to be significantly less than shown on this table, as these values are from periods when leaded gasoline adversely affected stormwater lead quality.
[e] The monitored low-density residential areas were drained by grass swales.

Data from Bannerman et al. (1979, 1983); Madison et al. (1979); EPA (1983); Pitt and McLean (1986).

Table 2.6 Hazardous Substances Observed in Urban Runoff

Hazardous Substances	Residential Areas	Industrial Areas
Benzene	5 µg/L	5 µg/L
Chlordane	17 ng/L	—
Chloroform	—	5 µg/L
Dieldrin	2 to 6 ng/L	—
Endrin	44 ng/L	—
Methoxychlor	20 ng/L	—
Pentachlorophenol	70 to 280 ng/L	50 to 710 ng/L
Phenol	1 µg/L	4 µg/L
Phosphorus	0.1 mg/L	0.5 µg/L
Toluene	—	5 µg/L

Data from EPA 1983; Pitt and McLean 1986 (Toronto); and Pitt et al. 1996 (Birmingham).

Table 2.7 Other Toxic Substances Observed in Urban Runoff

GC/MS Volatiles	Residential Areas	Industrial Areas
1,2-Dichloroethane	—	6 µg/L
Methylene chloride	—	5 µg/L
Tetrachloroethylene	—	High in some source areas
GC/MS Base/Neutrals		
Bis (2-ethylene) phthalate	8 µg/L	18 µg/L
Butyl benzyl phthalate	5 µg/L	58 µg/L
Diethyl phthalate	—	20 µg/L
Di-N-butyl phthalate	3 µg/L	4 µg/L
Isophorone	2 µg/L	—
N-Nitrosodimethylamine	—	3 µg/L
Phenanthrene	—	High in some source areas
Pyrene	—	High in some source areas
GC/MS Pesticides		
BHC	up to 20 ng/L	—
Chlordane	up to 15 ng/L	—
Dieldrin	up to 6 ng/L	—
Endosulfan sulfate	up to 10 ng/L	—
Endrin	up to 45 ng/L	—
PCB-1254	—	up to 630 ng/L
PCB-1260	—	up to 440 ng/L

Data from EPA 1983; Pitt and McLean 1986 (Toronto); and Pitt et al. 1996 (Birmingham).

Table 2.9 compares the estimated annual discharges from the residential and industrial catchments during the different runoff periods. The unit area annual yields for many of the heavy metals and nutrients are greater from the industrial catchment. Industrial catchments contribute most of the chromium to the local receiving waters, and approximately equal amounts with the residential and commercial catchments for phosphorus, chemical oxygen demand, copper, and zinc. This table also shows the great importance of warm weather baseflow discharges to the annual urban runoff pollutant yields, especially for industrial areas. Cold weather bacteria discharges are insignificant when compared to the warm weather bacteria discharges, but chloride (and filtrate residue) loadings are much more important during cold weather.

Table 2.10 shows the fraction of the annual estimated yields for different warm and cold periods (warm weather baseflow, stormwater flows, cold weather baseflow, and snowmelt). Typical storm-

Table 2.8 Median Urban Runoff Pollutant Concentrations

Constituent	Warm-Weather Baseflow		Warm-Weather Stormwater	
	Residential	Industrial	Residential	Industrial
Total residue	979	554	256	371
Filterable residue	973	454	230	208
Particulate residue	<5	43	22	117
Total phosphorus	0.09	0.73	0.28	0.75
Total Kjeldahl N	0.9	2.4	2.5	2.0
Phenolics (μg/L)	<1.5	2.0	1.2	5.1
COD	22	108	55	106
Fecal coliforms (no./100 mL)	33,000	7000	40,000	49,000
Fecal streptococci (no./100 mL)	2300	8800	20,000	39,000
Chromium	<0.06	0.42	<0.06	0.32
Copper	0.02	0.045	0.03	0.06
Lead	<0.04	<0.04	<0.06	0.08
Zinc	0.04	0.18	0.06	0.19
Constituent	**Cold-Weather Baseflow**		**Cold-Weather Melting Periods**	
	Residential	**Industrial**	**Residential**	**Industrial**
Total residue	2230	1080	1580	1340
Filterable residue	2210	1020	1530	1240
Particulate residue	21	50	30	95
Total phosphorus	0.18	0.34	0.23	0.50
Total Kjeldahl N	1.4	2.0	1.7	2.5
Phenolics (μg/L)	2.0	7.3	2.5	15.0
COD	48	68	40	94
Fecal coliforms (no./100 mL)	9800	400	2320	300
Fecal streptococci (no./100 mL)	1400	2400	1900	2500
Chromium	<0.01	0.24	<0.01	0.35
Copper	0.015	0.04	0.04	0.07
Lead	<0.06	<0.04	0.09	0.08
Zinc	0.065	0.15	0.12	0.31

From Pitt, R. and J. McLean. *Humber River Pilot Watershed Project*, Ontario Ministry of the Environment, Toronto, Canada. 483 pp. June 1986.

water flow contributions from these separate stormwater outfalls were only about 20 to 30% of the total annual discharges (by volume). Baseflows contributed the majority of flows. Many constituents were also contributed mostly by snowmelt and baseflows, with the stormwater contributions being less than 50% of the total annual yields. The ratios of expected annual pollutant yields from the industrial catchment divided by the yields from the residential/commercial catchment can be high, as summarized below.

Ratios of Industrial to Mixed Residential/Commercial Unit Area Yields	
Particulate residue (suspended solids)	4.4
Phosphorus	3.0
Phosphates	5.1
Chemical oxygen demand	2.0
Fecal streptococci bacteria	2.6
Chromium	53.0
Zinc	2.5

The only constituents with annual unit area yields that were lower in the industrial catchment than in the mixed residential/commercial catchment were chloride and filtrate residue (dissolved

Table 2.9 Monitored Annual Pollutant Discharges for Toronto's Humber River Watershed Test Sites

		Thistledowns (Residential/Commercial)					Emery (Industrial)						
		Warm		Cold			Warm		Cold				
Constituent	Units	Base-flow	Storm-water	Base-flow	Melt-water	Approx. Total	Base-flow	Storm-water	Base-flow	Melt-water	Approx. Total	Approx. Indus. to Resid. Total Yield Ratios	Weighted Indus. to Resid. Total Yield Ratios[a]
Runoff	m^3/ha	1700	950	1100	1800	5600	2100	1500	660	830	5100	0.9	0.3
Total residue	kg/ha	1700	240	2400	1700	6100	1100	670	710	1500	4000	0.7	0.2
Chlorides	kg/ha	480	33	1200	720	2400	160	26	310	700	1200	0.5	0.2
Total P	g/ha	150	290	200	570	1200	1500	1300	220	540	3600	3.0	1.0
Total Kjeldahl N	g/ha	1500	2800	1500	3500	9300	4900	3400	1300	2800	12,000	1.3	0.4
Phenolics	g/ha	<2.6	1.2	2.3	23	26	4.1	8.1	4.8	14	31	1.2	0.4
COD	kg/ha	38	51	52	130	270	220	170	45	91	530	2.0	0.7
Chromium	g/ha	<100	21	<10	15	36	860	600	160	290	1900	50	18
Copper	g/ha	35	30	16	77	160	92	120	26	76	310	1.9	0.7
Lead	g/ha	<70	41	<70	170	210	<75	170	<25	150	320	1.5	0.5
Zinc	g/ha	70	74	70	270	480	370	430	100	350	1200	2.5	0.8
Fecal coliform	10^9 org/ha	560	480	110	62	1200	144	760	3	6	910	0.8	0.3

"Warm weather" is for the period from about March 15 through December 15, while "cold weather" is for the period from about December 15 through March 15.

[a] The Humber River basin is about 25% industrial and 75% residential and commercial.

From Pitt, R. and J. McLean. *Humber River Pilot Watershed Project*, Ontario Ministry of the Environment, Toronto, Canada. 483 pp. June 1986.

Table 2.10 Major Concentration Periods by Parameter

	Runoff Volume		Total Residue		Filtrate Residue		Particulate Residue		Chlorides	
	Residential	Industrial	Residential	Industrial	Residential	Industrial	Residential	Industrial	Residential	Industrial
Warm baseflow	31%	41%	28%	28%	28%	30%	4%	16%	20%	13%
Stormwater	17	29	4	17	4	10	18	53	1	2
Cold baseflow	20	13	40	18	40	18	14	5	49	26
Meltwater	33	16	29	38	27	41	63	26	29	58

	Phosphorus		Phosphate		Total Kjeldahl Nitrogen		Ammonia Nitrogen	
	Residential	Industrial	Residential	Industrial	Residential	Industrial	Residential	Industrial
Warm baseflow	12	42	—	35	16	39	—	—
Stormwater	24	36	24	51	30	27	21	24
Cold baseflow	16	6	—	—	16	10	—	—
Meltwater	47	15	76	14	38	23	78	76

	Phenolics		COD		Fecal Coliform		Fecal Streptococci		*Pseudomonas aeruginosa*	
	Residential	Industrial	Residential	Industrial	Residential	Industrial	Residential	Industrial	Residential	Industrial
Warm baseflow	—	13	14	42	46	16	12	20	53	41
Stormwater	5	27	19	32	40	84	61	73	46	58
Cold baseflow	9	16	19	9	9	—	4	2	1	—
Meltwater	87	45	48	17	5	—	22	4	—	1

	Chromium		Copper		Lead		Zinc	
	Residential	Industrial	Residential	Industrial	Residential	Industrial	Residential	Industrial
Warm baseflow	—	45	22	29	—	—	14	30
Stormwater	59	31	19	38	19	54	15	35
Cold baseflow	—	8	10	8	—	—	14	8
Meltwater	41	16	49	24	81	46	56	27

Warm period included samples from Thistledowns from July 28 through Nov. 15, 1983, and from Emery from May 14 through Nov.15, 1983. Cold period samples from Thistledowns were from Feb. 2 through March 25, 1984, and from Emery from Jan. through March 22, 1984.

From Pitt, R. and J. McLean. *Humber River Pilot Watershed Project*, Ontario Ministry of the Environment, Toronto, Canada. 483 pp. June 1986.

solids). The annual unit area yields from the residential/commercial catchment were approximately twice the annual unit area yields from the industrial catchment for these constituents.

If only warm weather stormwater runoff is considered (and not baseflows and snowmelts), then significant yield and control measure selection errors are probable. Residential/commercial unit area annual yields for total residue (total solids) for stormwater alone are approximately 240 kg/ha, compared with approximately 670 kg/ha for the industrial catchment. These yields are similar to yields reported elsewhere for total annual total residue unit area yields. However, these warm weather stormwater runoff yields only contributed approximately 5 to 20% of the total annual total residue yields for these study catchments. Annual yields of several constituents were dominated by cold weather processes irrespective of the land use monitored. These constituents include total residue, filtrate residue, chlorides, ammonia nitrogen, and phenolics. The only constituents for which the annual yields were dominated by warm weather processes, irrespective of land use, were bacteria (fecal coliforms, fecal streptococci, and *Pseudomonas aeruginosa*), and chromium. Lead and zinc were both dominated by either stormwater or snowmelt runoff, with lower yields of these heavy metals originating from baseflows.

Warm weather stormwater runoff alone was the most significant contributor to the annual yields for a number of constituents from the industrial catchment. These constituents included particulate residue, phosphorus, phosphates, the three bacteria types, copper, lead, and zinc. In the residential/commercial catchment, only fecal streptococcus bacteria and chromium were contributed by warm weather stormwater runoff more than by the other three sources of water shown. Either warm or cold weather baseflows were most responsible for the yields of many constituents from the industrial catchment. These constituents included runoff volume, phosphorus, total Kjeldahl nitrogen, chemical oxygen demand, and chromium. Important constituents that have high yields in the baseflow from the residential/commercial catchment included total residue, filtrate residue, chlorides, and fecal coliform and *P. aeruginosa* bacteria. More recently, agricultural pesticides have been detected in urban rainfall and urban pesticides in agricultural rainfall and have also been detected in receiving waters.

SUMMARY

This chapter reviewed some of the major receiving water use impairments that have been associated with urban stormwater discharges. The problems associated with urban stormwater discharges can be many, but varied, depending on the specific site conditions. It is therefore important that local objectives and conditions be considered when evaluating local receiving water problems. There has been a great deal of experience in receiving water assessments over the past decade, especially focusing on urban nonpoint source problems. The main purpose of this book is to provide techniques and direction that can be applied to local waters to assess problems based on actual successful field activities. Of course, monitoring and evaluation techniques are constantly changing and improving, and this book also periodically presents short summaries of emerging techniques that hold promise, but may require additional development to be easily used by most people.

Generally, receiving water problems are not readily recognized or understood if one relies on only a limited set of tools. It is critical that conventional water quality measurements be supplemented with habitat evaluations and biological studies, for example. In many cases, receiving water problems caused by urbanization may be mostly associated with habitat destruction, contaminated sediment, and inappropriate discharges, all of which would be poorly indicated by relying only on conventional water quality measurements. In contrast, eliminating water quality measurements from an assessment and relying only on less expensive indicators, such as the currently popular citizen monitoring of benthic conditions, is also problematic, especially from a human health perspective.

A well-balanced assessment approach is therefore needed to understand the local problems of most concern and is the focus of this book.

This chapter also summarized stormwater characteristics. Runoff from established urban areas may not be the major source of some of the problem pollutants in urban areas. Obviously, construction site runoff is typically the major source of sediment in many areas, but snowmelt contributions of sediment (and many other constituents) is also very important in northern areas. Dry weather flows in separate storm drainage systems can be contaminated with inappropriate discharges from commercial and industrial establishments and sewage. Obviously, these inappropriate discharges need to be identified and corrected.

The rest of this book establishes an approach for investigating receiving water use impairments and in identifying the likely causes for these problems. When this information is known, it is possible to begin to develop an effective stormwater management program.

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CHAPTER 3

Stressor Categories and Their Effects on Humans and Ecosystems

"As for Paris, within the last few years, it has been necessary to move most of the mouths of the sewers down stream below the last bridge."

Victor Hugo, 1862

CONTENTS

EFFECTS OF RUNOFF ON RECEIVING WATERS

Many studies have shown the severe detrimental effects of urban and agricultural runoff on receiving waters. These studies have generally examined receiving water conditions above and below a city, by comparing two parallel streams, or by comparing to an ecoregion reference. However, only a few studies have examined direct cause-and-effect relationships of runoff for receiving water aquatic organisms (Heaney and Huber 1984; Burton and Moore 1999; Werner et

al. 2000; Vlaming et al. 2000; Bailey et al. 2000; Wenholz and Crunkilton 1995). Chapter 4 presents several case studies representing the major approaches to assessing receiving water problems, while this chapter presents a review of the major stressor categories and summarizes their observed effects.

Indicators of Receiving Water Biological Effects and Analysis Methodologies

There are a number of useful, well-proven tools that can detect adverse biological effects in receiving waters (see also Chapter 6). When these tools are used correctly and combined in the proper framework, they can be used to identify runoff-related problems. Kuehne (1975) studied the usefulness of aquatic organisms as indicators of pollution. He found that invertebrate responses are indicative of pollution for some time after an event, but they may not give an accurate indication of the nature of the pollutants. In-stream fish studies were not employed as biological indicators much before 1975, but they are comparable in many ways to invertebrates as quality indicators and can be more easily identified. However, because of better information pertaining to invertebrates and due to their limited mobility, certain invertebrate species may be sensitive to minor changes in water quality. Fish can be highly mobile and cover large sections of a stream, as long as their passage is not totally blocked by adverse conditions. Fish disease surveys were also used during the Bellevue, WA, urban runoff studies as an indicator of water quality problems (Scott et al. 1982; Pitt and Bissonnette 1984). McHardy et al. (1985) examined heavy metal uptake in green algae (*Cladophora glomerata*) from urban runoff for use as a biological monitor of specific metals.

It is necessary to use a range of measurement endpoints to characterize ecosystem quality in systems that receive multiple stressors (Marcy and Gerritsen 1996; Baird and Burton 2001). Dyer and White (1996) examined the problem of multiple stressors affecting toxicity assessments. They felt that field surveys can rarely be used to verify simple single parameter laboratory experiments. They developed a watershed approach integrating numerous databases in conjunction with *in situ* biological observations to help examine the effects of many possible causative factors (see also Chapter 6).

The interactions of stressors such as suspended solids and chemicals can be confounding and easily overlooked. Ireland et al. (1996) found that exposure to UV radiation (natural sunlight) increased the toxicity of PAH-contaminated sediments to *C. dubia*. The toxicity was removed when the UV wavelengths did not penetrate the water column to the exposed organisms. Toxicity was also reduced significantly in the presence of UV when the organic fraction of the stormwater was removed. Photo-induced toxicity occurred frequently during low flow conditions and wet-weather runoff and was reduced during turbid conditions.

Johnson et al. (1996) and Herricks et al. (1996a,b) describe a structured tier testing protocol to assess both short-term and long-term wet-weather discharge toxicity that they developed and tested. The protocol recognizes that the test systems must be appropriate to the time-scale of exposure during the discharge. Therefore, three time-scale protocols were developed, for intra-event, event, and long-term exposures. The use of standard whole effluent toxicity (WET) tests were found to overestimate the potential toxicity of stormwater discharges.

The effects of stormwater on Lincoln Creek, near Milwaukee, WI, were described by Crunkilton et al. (1996). Lincoln Creek drains a heavily urbanized watershed of 19 mi^2 that is about 9 miles long. On-site toxicity testing was conducted with side-stream flow-through aquaria using fathead minnows, plus in-stream biological assessments, along with water and sediment chemical measurements. In the basic tests, Lincoln Creek water was continuously pumped through the test tanks, reflecting the natural changes in water quality during both dry and wet-weather conditions. The continuous flow-through mortality tests indicated no toxicity until after about the 14th day of exposure, with more than 80% mortality after about 25 days, indicating that short-term toxicity tests likely underestimate stormwater toxicity. The biological and physical habitat assessments supported a definitive relationship between degraded stream ecology and urban runoff.

Rainbow (1996) presented a detailed overview of heavy metals in aquatic invertebrates. He concluded that the presence of a metal in an organism cannot tell us directly whether that metal is poisoning the organism. However, if compared to concentrations in a suite of well-researched biomonitors, it may be possible to determine if the accumulated concentrations are atypically high, with a possibility that toxic effects may be present. The user should be cautious, however, when attempting to relate tissue concentrations to effects or with bioconcentration factors. Many metals are essential and/or regulated by organisms and their internal concentrations might bear no relationship to the concentrations in surrounding waters or sediments.

A battery of laboratory and *in situ* bioassay tests are most useful when determining aquatic biota problems (Burton and Stemmer 1988; Burton et al. 1996; Chapter 6). The test series may include microbial activity tests, along with exposures of zooplankton, amphipods, aquatic insects, bivalves, and fish. Indigenous microbial activity responses correlated well with *in situ* biological and chemical profiles. Bascombe et al. (1990) also reported on the use of *in situ* biological tests, using an amphipod exposed for 5 to 6 weeks in urban streams, to examine urban runoff receiving water effects. Ellis et al. (1992) examined bioassay procedures for evaluating urban runoff effects on receiving water biota. They concluded that an acceptable criteria for protecting receiving water organisms should not only provide information on concentration and exposure relationships for *in situ* bioassays, but also consider body burdens, recovery rates, and sediment-related effects.

During the Coyote Creek, San Jose, CA, receiving water study, 41 stations were studied in both urban and non-urban perennial flow stretches of the creek. Short- and long-term sampling techniques were used to evaluate the effects of urban runoff on water quality, sediment properties, fish, macroinvertebrates, attached algae, and rooted aquatic vegetation (Pitt and Bozeman 1982).

Fish Kills and Advisories

Runoff impacts are sometimes difficult for many people to appreciate in urban and agricultural areas. Fish kills are the most obvious indication of water quality problems for many people. However, because receiving water quality is often so poor, the aquatic life in typical urban and agricultural receiving waters is usually limited in abundance and diversity, and quite resistant to poor water quality. Sensitive native organisms have typically been displaced, or killed, long ago, and it usually requires an unusual event to cause a fish fill (Figure 3.1). Ray and White (1979) stated that one of the complicating factors in determining fish kills related to heavy metals is that the fish mortality may lag behind the first toxic exposure by several days and is usually detected many miles downstream from the discharge location. The actual concentrations of the water quality constituents that may have caused the kill could then be diluted beyond detection limits, making probable sources of the toxic materials impossible to determine in many cases.

Heaney et al. (1980) reviewed fish kill information reported to government agencies from 1970 to 1979. They found that less than 3% of the reported 10,000 fish kills was identified as having been caused by urban runoff. This is fewer than 30 fish kills per year nationwide. However, the cause of most of these 10,000 fish kills could not be identified. It is expected that many of these fish kills could have been caused by runoff, or a combination of problems that could have been worsened by runoff. For example, elevated nutrient loading causes eutrophication that may lead to dissolved oxygen deficits and subsequent fish kills. These events are exacerbated by natural stressors such as low flow conditions. More recent surveys have found nearly 30% of fish kills is attributable to runoff (Figure 3.2; EPA 1995).

During the Bellevue, WA, receiving water studies, some fish kills were noted in the unusually clean urban streams (Pitt and Bissonnette 1984). The fish kills were usually associated with inappropriate discharges to the storm drainage system (such as cleaning materials and industrial chemical spills) and not from "typical" urban runoff. However, as noted later, the composition of the fish in the Bellevue urban stream was quite different, as compared to the control stream (Scott et al. 1986).

Figure 3.1 Fish kill in Village Creek, Birmingham, AL, due to Dursban entering storm drainage during warehouse fire.

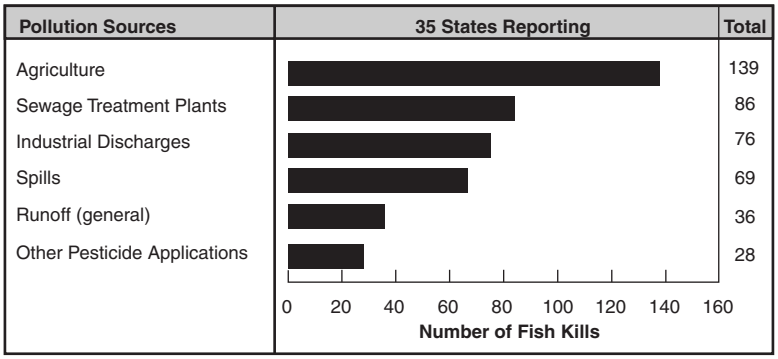

Figure 3.2 Sources associated with fish kills. (From U.S. Environmental Protection Agency. *National Water Quality Inventory. 1994 Report to Congress*. Office of Water. EPA 841-R-95-005. Washington, D.C. December 1995.)

Fish kill data have, therefore, not been a good indicator for identifying stressor categories or types. However, the composition of the fisheries and other aquatic life taxonomic information are sensitive indicators of receiving water problems in streams.

In addition to fish kills, a significant concern is the increasing number of fish advisories being issued by states across the nation (Figure 3.3; EPA 1995). The causes of fish contamination and fish kills vary, but runoff is a primary contributor.

Adverse Aquatic Life Effects Caused by Runoff

Aquatic organisms are sensitive indicators of water quality. There have been many studies that describe aquatic life impairments that may result from exposure to contaminated runoff and/or habitat degradation. The following section summarizes some of these studies, which are typical of urban and agricultural watersheds.

Klein (1979) studied 27 small watersheds having similar characteristics, but having varying land uses, in the Piedmont region of Maryland. During an initial phase of the study, definite relationships were found between water quality and land use. Subsequent study phases examined aquatic life relationships in the watersheds. The principal finding was that stream aquatic life problems were first identified with watersheds having imperviousness areas comprising at least 12% of the watershed. Severe problems were noted after the imperviousness quantities reached 30%.

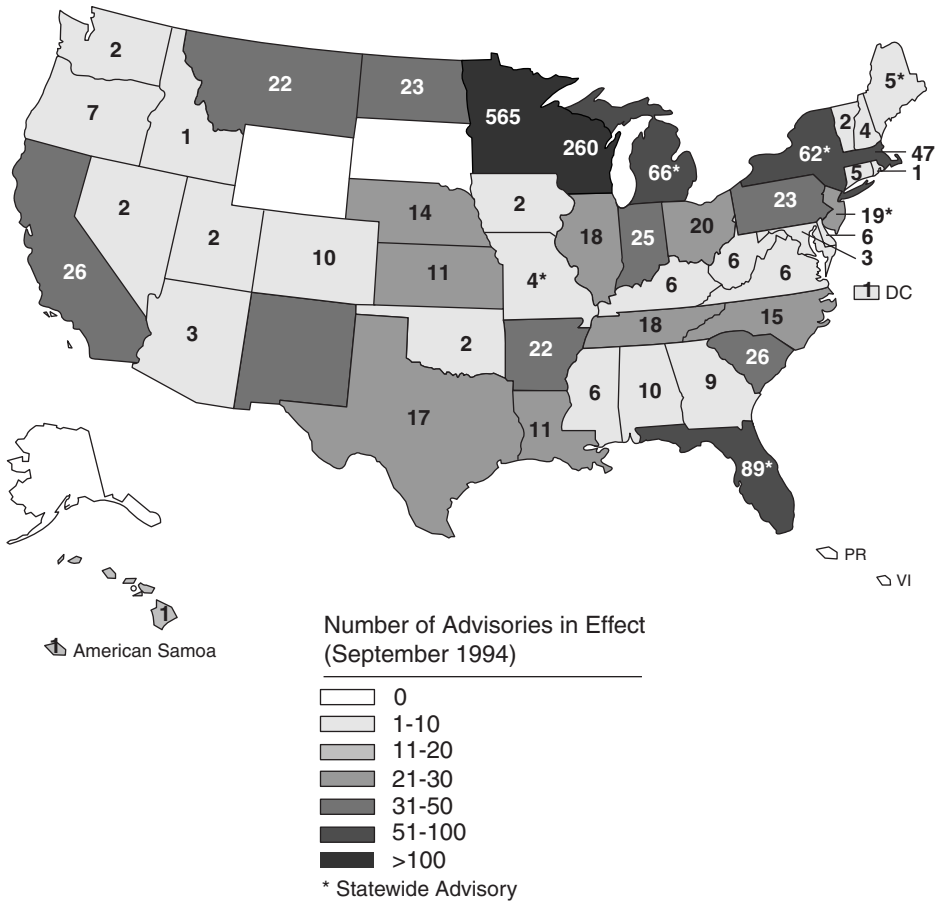

Figure 3.3 U.S. fish consumption advisories. **Note:** States that perform routine fish tissue analysis (such as Great Lake States) will detect more cases of fish contamination and issue more advisories than states with less rigorous fish sampling programs. In many cases, the states with the most fish advisories support the best monitoring programs for measuring toxic contamination in fish, and their water quality is no worse than the water quality in other states. (From U.S. Environmental Protection Agency. *National Water Quality Inventory. 1994 Report to Congress*. Office of Water. EPA 841-R-95-005. Washington, D.C. December 1995.)

Receiving water impact studies were also conducted in North Carolina by Lenat et al. (1979), Lenat and Eagleson (1981), and Lenat et al. (1981). The benthic fauna occurred mainly on rocks. As sedimentation increased, the amount of exposed rocks decreased, with a decreasing density of benthic macroinvertebrates. Data from 1978 and 1979 in five cities showed that urban streams were grossly polluted by a combination of toxicants and sediment. Chemical analyses, without biological analyses, would have underestimated the severity of the problems because the water column quality varied rapidly, while the major problems were associated with sediment quality and effects on macroinvertebrates. Macroinvertebrate diversities were severely reduced in the urban streams, compared to the control streams. The biotic indices indicated "very poor" conditions for all urban streams. Occasionally, high populations of pollutant-tolerant organisms were found in the urban streams, but would abruptly disappear before subsequent sampling efforts. This was probably caused by intermittent discharges of spills or illegal dumping of toxicants. Although the cities studied were located in different geographic areas of North Carolina, the results were remarkably uniform.

A major nonpoint runoff receiving water impact research program was conducted in Georgia (Cook et al. 1983). Several groups of researchers examined streams in major areas of the state.

Benke et al. (1981) studied 21 stream ecosystems near Atlanta having watersheds of 1 to 3 square miles each and land uses ranging from 0 to 98% urbanization. They measured stream water quality but found little relationship between water quality and degree of urbanization. The water quality parameters also did not identify a major degree of pollution. In contrast, there were major correlations between urbanization and the number of species. They had problems applying diversity indices to their study because the individual organisms varied greatly in size (biomass). CTA (1983) also examined receiving water aquatic biota impacts associated with nonpoint sources in Georgia. They studied habitat composition, water quality, macroinvertebrates, periphyton, fish, and toxicant concentrations in the water, sediment, and fish. They found that the impacts of land use were the greatest in the urban basins. Beneficial uses were impaired or denied in all three urban basins studied. Fish were absent in two of the basins and severely restricted in the third. The native macroinvertebrates were replaced with pollution-tolerant organisms. The periphyton in the urban streams were very different from those found in the control streams and were dominated by species known to create taste and odor problems.

Pratt et al. (1981) used basket artificial substrates to compare benthic population trends along urban and nonurban areas of the Green River in Massachusetts. The benthic community became increasingly disrupted as urbanization increased. The problems were not only associated with times of heavy rain, but seemed to be affected at all times. The stress was greatest during summer low flow periods and was probably localized near the stream bed. They concluded that the high degree of correspondence between the known sources of urban runoff and the observed effects on the benthic community was a forceful argument that urban runoff was the causal agent of the disruption observed.

Cedar swamps in the New Jersey Pine Barrens were studied by Ehrenfeld and Schneider (1983). They examined 19 swamps subjected to varying amounts of urbanization. Typical plant species were lost and replaced by weeds and exotic plants in urban runoff-affected swamps. Increased uptakes of phosphorus and lead in the plants were found. It was concluded that the presence of stormwater runoff to the cedar swamps caused marked changes in community structure, vegetation dynamics, and plant tissue element concentrations.

Medeiros and Coler (1982) and Medeiros et al. (1984) used a combination of laboratory and field studies to investigate the effects of urban runoff on fathead minnows. Hatchability, survival, and growth were assessed in the laboratory in flow-through and static bioassay tests. Growth was reduced to one half of the control growth rates at 60% dilutions of urban runoff. The observed effects were believed to be associated with a combination of toxicants.

The benthos in the upper reaches of Coyote Creek (San Jose, CA) consisted primarily of amphipods and a diverse assemblage of aquatic insects (Pitt and Bozeman 1982). Together those groups comprised two thirds of the benthos collected from the non-urban portion of the creek. Clean water forms were abundant and included amphipods (*Hyaella azteca*) and various genera of mayflies, caddisflies, black flies, crane flies, alderflies, and riffle beetles. In contrast, the benthos of the urban reaches of the creek consisted almost exclusively of pollution-tolerant oligochaete worms (tubificids). Tubificids accounted for 97% of the benthos collected from the lower portion of Coyote Creek.

There were significant differences in the numbers and types of benthic organisms found during the Bellevue Urban Runoff Program (Pederson 1981; Perkins 1982; Richey et al. 1981; Richey 1982; Scott et al. 1982). Mayflies, stoneflies, caddisflies, and beetles were rarely observed in urbanized Kelsey Creek, but were quite abundant in rural Bear Creek. These organisms are commonly regarded as sensitive indicators to environmental degradation. As an example of a degraded aquatic habitat, a species of clams (*Unionidae*) was not found in Kelsey Creek, but was found in Bear Creek. These clams are very sensitive to heavy siltation and unstable sediments. Empty clam shells, however, were found buried in the Kelsey Creek sediments indicating their previous presence in the creek and their inability to adjust to the changing conditions. The benthic organism composition in Kelsey Creek varied radically with time and place, while the organisms were much more stable in Bear Creek.

Introduced fishes often cause radical changes in the nature of the fish fauna present in a given water body. In many cases, they become the dominant fishes because they are able to outcompete the native fishes for food or space, or they may possess greater tolerance to environmental stress. In general, introduced species are most abundant in aquatic habitats modified by man, while native fishes tend to persist mostly in undisturbed areas. Such is apparently the case within Coyote Creek, San Jose, CA (Pitt and Bozeman 1982).

Samples from the non-urban portion of the study area were dominated by an assemblage of native fish species such as hitch, three spine stickleback, Sacramento sucker, and prickly sculpin. Rainbow trout, riffle sculpin, and Sacramento squawfish were captured only in the headwater reaches and tributary streams of Coyote Creek. Collectively, native species comprised 89% of the number and 79% of the biomass of the 2379 fishes collected from the upper reaches of the study area. In contrast, native species accounted for only 7% of the number and 31% of the biomass of the 2899 fishes collected from the urban reach of the study area.

Hitch was the most numerous native fish species present. Hitch generally exhibit a preference for quiet water habitat and are characteristic of warm, low elevation lakes, sloughs, sluggish rivers, and ponds. Mosquitofish dominated the collections from the urbanized section of the creek and accounted for over two thirds of the total number of fish collected from the area. This fish is particularly well adapted to withstand extreme environmental conditions, including those imposed by stagnant waters with low dissolved oxygen concentrations and elevated temperatures. The second most abundant fish species in the urbanized reach of Coyote Creek, the fathead minnow, is equally well suited to tolerate extreme environmental conditions. The species can withstand low dissolved oxygen, high temperature, high organic pollution, and high alkalinity. Often thriving in unstable environments such as intermittent streams, the fathead minnow can survive in a wide variety of habitats.

The University of Washington (Pederson 1981; Perkins 1982; Richey et al. 1981; Richey 1982; Scott et al. 1982) conducted a series of studies to contrast the biological and chemical conditions in urban Kelsey Creek with rural Bear Creek. The urban creek was significantly degraded when compared to the rural creek, but still supported a productive but limited and unhealthy salmonid fishery. Many of the fish in the urban creek, however, had respiratory anomalies. The urban creek was not grossly polluted, but flooding from urban developments has increased dramatically in recent years. These increased flows have dramatically changed the urban stream's channel, by causing unstable conditions with increased stream bed movement, and by altering the availability of food for the aquatic organisms. The aquatic organisms are very dependent on the few relatively undisturbed reaches. Dissolved oxygen concentrations in the sediments depressed embryo salmon survival in the urban creek. Various organic and metallic priority pollutants were discharged to the urban creek, but most of them were apparently carried through the creek system by the high storm flows to Lake Washington. The urbanized Kelsey Creek also had higher water temperatures (probably due to reduced shading) than Bear Creek. This probably caused the faster fish growth in Kelsey Creek.

The fish population in Kelsey Creek had adapted to its degrading environment by shifting the species composition from coho salmon to less sensitive cutthroat trout and by making extensive use of less-disturbed refuge areas (Figure 4.22). Studies of damaged gills found that up to three fourths of the fish in Kelsey Creek were affected with respiratory anomalies, while no cutthroat trout and only two of the coho salmon sampled in Bear Creek had damaged gills. Massive fish kills in Kelsey Creek and its tributaries were observed on several occasions during the project due to the dumping of toxic materials down the storm drains.

Urban runoff impact studies were conducted in the Hillsborough River near Tampa Bay, FL, as part of the NURP program (Mote Marine Laboratory 1984). Plants, animals, sediment, and water quality were all studied in the field and supplemented by laboratory bioassay tests. Effects of saltwater intrusion and urban runoff were both measured because of the estuarine environment. During wet weather, freshwater species were found closer to the bay than during dry weather. In coastal areas, these additional natural factors make it even more difficult to identify the

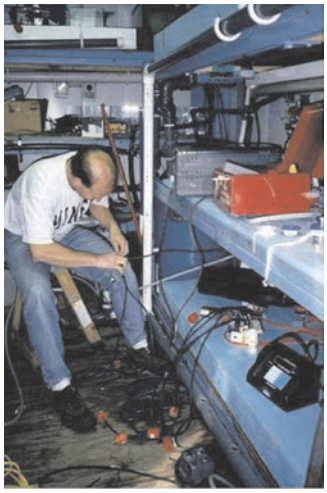

Figure 3.4 Installation of side-stream fish bioassay test facilities at Lincoln Creek, Milwaukee, WI.

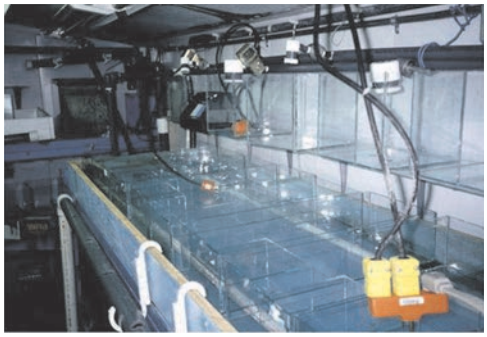

Figure 3.5 Lincoln Creek side-stream fish bioassay test facilities nearing completion.

cause-and-effect relationships for aquatic life problems. During another NURP project, Striegl (1985) found that the effects of accumulated pollutants in Lake Ellyn (Glen Ellyn, IL) inhibited desirable benthic invertebrates and fish and increased undesirable phytoplankton blooms. LaRoe (1985) summarized the off-site effects of construction sediment on fish and wildlife. He noted that physical, chemical, and biological processes all affect receiving water aquatic life.

The number of benthic organism taxa in Shabakunk Creek in Mercer County, NJ, declined from 13 in relatively undeveloped areas to 4 below heavily urbanized areas (Garie and McIntosh 1986, 1990). Periphyton samples were also analyzed for heavy metals, with significantly higher metal concentrations found below the heavily urbanized area than above.

The Wisconsin Department of Natural Resources, in conjunction with the USGS and the University of Wisconsin, conducted side-stream fish bioassay tests in Lincoln Creek in Milwaukee (Figures 3.4 and 3.5) (Crunkilton et al. 1996). They identified significant acute toxicity problems associated with intermediate-term (about 10 to 20 day) exposures to adverse toxicant concentrations in urban receiving streams, with no indication of toxicity for shorter exposures. These toxicity effects were substantially (but not completely) reduced through the removal of stormwater particulates using a typical wet detention pond designed to remove most of the particles larger than 5 µm.

Observed Habitat Problems Caused by Runoff

Some of the most serious effects of urban and agricultural runoff are on the aquatic habitat of the receiving waters. These habitat effects are in addition to the pollutant concentration effects. The major effects of sediment on the aquatic habitat include silting of spawning and food production areas and unstable bed conditions (Cordone and Kelley 1961). Other major habitat destruction problems include rapidly changing flows and the absence of refuge areas to protect the biota during these flow changes. Removal of riparian vegetation can increase water temperatures and eliminate a major source of debris, which provides important refuge areas. The major source of these habitat problems is the increased discharge volumes and flow rates associated with stormwater in developing areas that cause significant enlargements and unstable banks of small and moderate sized streams (Figures 3.6 and 3.7). Other habitat problems are caused by attempts to "correct" these problems by construction of lined channels (Figures 3.8 and 3.9) or small drop structures which hinder migration of aquatic life and create areas for the accumulation of contaminated silt (Figure 3.10).

Figure 3.6 Creek blowout after initial significant spring rains in newly developed area. (Courtesy of Wisconsin Department of Natural Resources.)

Figure 3.7 Unstable banks and trash along Five-Mile Creek, Birmingham, AL.

Figure 3.8 Lined embankment along Waller Creek, Austin, TX.

Figure 3.9 Lined channel in Milwaukee, WI.

Schueler (1996) stated that channel geometry stability can be a good indicator of the effectiveness of stormwater control practices. He also found that once a watershed area has more than about 10 to 15% effective impervious cover, noticeable changes in channel morphology occur, along with quantifiable impacts on water quality and biological conditions. Stephenson (1996) studied changes in streamflow volumes in South Africa during urbanization. He found increased stormwater runoff, decreases in the groundwater table, and dramatically decreased times of concentration. The peak flow rates increased by about twofold, about half caused by increased pavement (in an area having only about 5% effective impervious cover), with the remainder caused by decreased times of concentration.

Figure 3.10 Small drop structure obstruction in Lincoln Creek, Milwaukee, WI.

Brookes (1988) has documented many cases in the United States and Great Britain of stream morphological changes associated with urbanization. These changes are mostly responsible for habitat destruction which is usually the most significant detriment to aquatic life. In many cases, water quality improvement would result in very little aquatic life benefit if the physical habitat is grossly modified. The most obvious habitat problems are associated with stream "improvement" projects, ranging from removal of debris, to straightening streams, to channelization projects. Brookes (1988, 1991) presents a number of ways to minimize habitat problems associated with stream channel projects, including stream restoration.

Wolman and Schick (1967) observed deposition of channel bars, erosion of channel banks, obstruction of flows, increased flooding, shifting of channel bottoms, along with concurrent changes in the aquatic life, in Maryland streams affected by urban construction activities. Robinson (1976) studied eight streams in watersheds undergoing urbanization and found that the increased magnitudes and frequencies of flooding, along with the increased sediment yields, had considerable impact on stream morphology (and therefore aquatic life habitat).

The aquatic organism differences found during the Bellevue Urban Runoff Program were probably most associated with the increased peak flows in Kelsey Creek caused by urbanization and the resultant increase in sediment-carrying capacity and channel instability of the creek (Pederson 1981; Perkins 1982; Richey et al. 1981; Richey 1982; Scott et al. 1982). Developed Kelsey Creek had much lower flows than rural Bear Creek during periods between storms. About 30% less water was available in Kelsey Creek during the summers. These low flows may also have significantly affected the aquatic habitat and the ability of the urban creek to flush toxic spills or other dry-weather pollutants from the creek system (Ebbert et al. 1983; Prych and Ebbert undated). Kelsey Creek had extreme hydrologic responses to storm. Flooding substantially increased in Kelsey Creek during the period of urban development; the peak annual discharges have almost doubled in the last 30 years, and the flooding frequency has also increased due to urbanization (Ebbert et al. 1983; Prych and Ebbert undated). These increased flows in urbanized Kelsey Creek resulted in greatly increased sediment transport and channel instability. The Bellevue studies (summarized by Pitt and Bissonnette 1984) indicated very significant interrelationships between the physical, biological, and chemical characteristics of the urbanized Kelsey Creek system. The aquatic life beneficial uses were found to be impaired, and stormwater conveyance was most likely associated with increased flows from the impervious areas in the urban area. Changes in the flow characteristics could radically alter the ability of the stream to carry the polluted sediments into the other receiving waters. If the stream power (directly related to sediment-carrying capacity) of Kelsey Creek were reduced, these toxic materials could be expected to be settled into its sediment, with increased effects on the stream's aquatic life. Reducing peak flows would also reduce the flushing of smaller fish and other aquatic organisms from the system.

Many recent studies on urban stream habitats and restoration efforts have been conducted, especially in the Pacific Northwest. In one example, May et al. (1999) found that maintaining natural land cover offers the best protection for maintaining stream ecological integrity and that best management practices have generally been limited in their ability to preserve appropriate conditions for lowland salmon spawning and rearing streams. They found that Puget Sound watersheds having a 10% impervious cover (likely resulting in marginal in-stream conditions) maintained at least 50% forested cover.

Groundwater Impacts from Stormwater Infiltration

There have been some nationwide studies that have shown virtually every agricultural and urban watershed contains elevated levels of nutrients, pesticides, and other organic chemicals in surface and groundwaters, sediments, and fish tissues (e.g., USGS 1999). Since groundwaters are widely used as a drinking water and irrigation source and recharge many surface water bodies, the implications of chemical contamination are quite serious.

Prior to urbanization, groundwater recharge resulted from infiltration of precipitation through pervious surfaces, including grasslands and woods. This infiltrating water was relatively uncontam-

Figure 3.11 Groundwater recharge basin in Long Island, NY, using stormwater. (Courtesy of New York Department of USGS).

Figure 3.12 Karst geology at an Austin, TX, roadcut showing major channeling opportunities for surface water to enter the Edwards Aquifer.

Figure 3.13 Public education roadside sign in Austin, TX, warning about sensitive recharge zone.

Figure 3.14 Paver blocks for on-site infiltration in Essen, Germany.

inated. Urbanization reduced the permeable soil surface area through which recharge by infiltration could occur. This resulted in much less groundwater recharge and greatly increased surface runoff. In addition, the waters available for recharge generally carried increased quantities of pollutants. With urbanization, new sources of groundwater recharge also occurred, including recharge from domestic septic tanks, percolation basins (Figure 3.11), and industrial waste injection wells, and from agricultural and residential irrigation. Special groundwater contamination problems may occur in areas having Karst geology where surface waters can be easily and quickly directed to the subsurface (Figures 3.12 and 3.13). Of course, there are many less dramatic opportunities for stormwater to enter the groundwater, including areas of porous paver blocks (Figures 3.14 through 3.16), grass swales (Figures 3.17 and 3.18), infiltration trenches (Figure 3.19), biofiltration areas (Figure 3.20), and simply from runoff flowing across grass (Figure 3.21). Many of these infiltration practices are done to reduce surface water impacts associated with stormwater discharges. If the infiltration is conducted through surface soils (such as for grass swales and grass landscaped areas), groundwater contamination problems are significantly reduced. However, if subsurface infiltration is used (especially through the use of injection wells), the risk of groundwater contamination for many stormwater pollutants substantially increases (Pitt et al. 1994, 1996).

Figure 3.15 Paver blocks for emergency and utility vehicle access, Madison, WI (under construction).

Figure 3.16 Paver blocks for occasional access road, Seattle Science Center, WA.

Figure 3.17 Grass swale in residential area, Milwaukee, WI.

Figure 3.18 Grass swale in office park area, Milwaukee, WI.

The Technical University of Denmark (Mikkelsen et al. 1996a,b) has been involved in a series of tests to examine the effects of stormwater infiltration on soil and groundwater quality. It found that heavy metals and PAHs present little groundwater contamination threat if surface infiltration systems are used. However, it expresses concern about pesticides, which are much more mobile. Squillace et al. (1996) along with Zogorski et al. (1996) presented information concerning stormwater and its potential as a source of groundwater MTBE contamination. Mull (1996) stated that traffic areas are the third most important source of groundwater contamination in Germany (after abandoned industrial sites and leaky sewers). The most important contaminants are chlorinated hydrocarbons, sulfate, organic compounds, and nitrates. Heavy metals are generally not an important groundwater contaminant because of their affinity for soils. Trauth and Xanthopoulus (1996) examined the long-term trends in groundwater quality at Karlsruhe, Germany. They found that urban land use is having a long-term influence on the groundwater quality. The concentration of many pollutants has increased by about 30 to 40% over 20 years. Hütter and Remmler (1996)

Figure 3.19 Stormwater infiltration through infiltration trench, office park, Lake Oswego, OR.

Figure 3.20 Biofiltration in parking area (Photo used with permission of Center for Watershed Protection.)

describe a groundwater monitoring plan, including monitoring wells that were established during the construction of an infiltration trench for stormwater disposal, in Dortmund, Germany. The worst problem expected is with zinc if the infiltration water has a pH value of 4.

The following paragraphs (summarized from Pitt et al. 1994, 1996) describe the stormwater pollutants that have the greatest potential of adversely affecting groundwater quality during inadvertent or intentional stormwater infiltration, along with suggestions on how to minimize these potential problems.

Figure 3.21 Infiltration through grassed areas.

Nutrients

Groundwater contamination with phosphorus has not been as widespread, or as severe, as with nitrogen compounds. Nitrates are one of the most frequently encountered contaminants in groundwater. Whenever nitrogen-containing compounds come into contact with soil, a potential for nitrate leaching into groundwater exists, especially in rapid-infiltration wastewater basins, stormwater infiltration devices, and in agricultural areas. Nitrate has leached from fertilizers and affected groundwaters under various turf grasses in urban areas, including golf courses, parks, and home lawns. Significant leaching of nitrates occurs during the cool, wet seasons. Cool temperatures reduce denitrification and ammonia volatilization, and limit microbial nitrogen immobilization and plant uptake. The use of slow-release fertilizers is recommended in areas having potential groundwater nitrate problems. The slow-release fertilizers include urea formaldehyde (UF), methylene urea, isobutyldiene diurea (IBDU), and sulfur-coated urea. Residual nitrate concentrations are highly variable in soil due to soil texture, mineralization, rainfall and irrigation patterns, organic matter content, crop yield, nitrogen fertilizer/sludge rate, denitrification, and soil compaction. Nitrate is highly soluble (>1 kg/L) and will stay in solution in the percolation water, after leaving the root zone, until it reaches the groundwater.

Nitrate has a low to moderate groundwater contamination potential for both surface percolation and subsurface infiltration/injection practices because of its relatively low concentrations found in most stormwaters. However, if the stormwater nitrate concentration were high, then the groundwater contamination potential would also likely be high.

Pesticides

Pesticide contamination of groundwater can result from agricultural, municipal, and homeowner use of pesticides for pest control and their subsequent collection in stormwater runoff. A wide range of pesticides and their metabolites have been found in watersheds, which include typical urban pesticides in agricultural areas, and vice versa. This cross-contamination of pesticides into areas in which they are not being used is attributed to atmospheric transport. Heavy repetitive use of mobile pesticides on irrigated and sandy soils likely contaminates groundwater. Some insecticides, fungicides, and nematocides must be mobile in order to reach the target pest and, hence, they generally have the highest contamination potential. Pesticide leaching depends on patterns of use, soil texture, total organic carbon content of the soil, pesticide persistence, and depth to the water table.

The greatest pesticide mobility occurs in areas with coarse-grained or sandy soils without a hardpan layer, having low clay and organic matter content and high permeability. Structural voids, which are generally found in the surface layer of finer-textured soils rich in clay, can transmit pesticides rapidly when the voids are filled with water and the adsorbing surfaces of the soil matrix are bypassed. In general, pesticides with low water solubilities, high octanol-water partitioning coefficients, and high carbon partitioning coefficients are less mobile. The slower-moving pesticides have been recommended in areas of groundwater contamination concern. These include the fungicides iprodione and triadimefon, the insecticides isofenphos and chlorpyrifos, and the herbicide glyphosate. The most mobile pesticides include 2,4-D, acenaphthylene, alachlor, atrazine, cyanazine, dacthal, diazinon, dicamba, malathion, and metolachlor.

Pesticides decompose in soil and water, but the total decomposition time can range from days to years. Literature half-lives for pesticides generally apply to surface soils and do not account for the reduced microbial activity found deep in the vadose zone. Pesticides with a 30-day half-life can show considerable leaching. An order-of-magnitude difference in half-life results in a five- to tenfold difference in percolation loss. Organophosphate pesticides are less persistent than organochlorine pesticides, but they also are not strongly adsorbed by the sediment and are likely to leach into the vadose zone and the groundwater. Perhaps a greater concern that has recently emerged is the widespread prevalence of toxic pesticide metabolites (breakdown products) that are not routinely analyzed. The ecological and human health significance of this is not known at present, but will be a future topic of investigation.

Lindane and chlordane have moderate groundwater contamination potentials for surface percolation practices (with no pretreatment) and for subsurface injection (with minimal pretreatment). The groundwater contamination potentials for both of these compounds would likely be substantially reduced with adequate sedimentation pretreatment. Pesticides have mostly been found in urban runoff from residential areas, especially in dry-weather flows associated with landscaping irrigation runoff.

Other Organics

The most commonly occurring organic compounds that have been found in urban groundwaters include phthalate esters (especially bis(2-ethylhexyl)phthalate) and phenolic compounds. Other organics more rarely found, possibly due to losses during sample collection, have included the volatiles: benzene, chloroform, methylene chloride, trichloroethylene, tetrachloroethylene, toluene,

and xylene. PAHs (especially benzo(a)anthracene, chrysene, anthracene, and benzo(b)fluoroanthenene) have also been found in groundwaters near industrial sites.

Groundwater contamination from organics, as from other pollutants, occurs more readily in areas with sandy soils and where the water table is near the land surface. Removal of organics from the soil and recharge water can occur by one of three methods: volatilization, sorption, and degradation. Volatilization can significantly reduce the concentrations of the most volatile compounds in groundwater, but the rate of gas transfer from the soil to the air is usually limited by the presence of soil water. Hydrophobic sorption onto soil organic matter limits the mobility of less soluble base/neutral and acid extractable compounds through organic soils and the vadose zone. Sorption is not always a permanent removal mechanism, however. Organic resolubilization can occur during wet periods following dry periods. Many organics can be at least partially degraded by microorganisms, but others cannot. Temperature, pH, moisture content, ion-exchange capacity of soil, and air availability may limit the microbial degradation potential for even the most degradable organic.

1,3-Dichlorobenzene may have a high groundwater contamination potential for subsurface infiltration/injection (with minimal pretreatment). However, it would likely have a lower groundwater contamination potential for most surface percolation practices because of its relatively strong sorption to vadose zone soils. Both pyrene and fluoranthene would also likely have high groundwater contamination potentials for subsurface infiltration/injection practices, but lower contamination potentials for surface percolation practices because of their more limited mobility through the unsaturated zone (vadose zone). Others (including benzo(a)anthracene, bis(2-ethylhexyl) phthalate, pentachlorophenol, and phenanthrene) may also have moderate groundwater contamination potentials if surface percolation with no pretreatment or subsurface injection/infiltration is used. These compounds would have low groundwater contamination potentials if surface infiltration was used with sedimentation pretreatment. Volatile organic compounds (VOCs) may also have high groundwater contamination potentials if present in the stormwater (likely for some industrial and commercial facilities and vehicle service establishments). The other organics, especially the volatiles, are mostly found in industrial areas. The phthalates are found in all areas. The PAHs are also found in runoff from all areas, but they are in higher concentrations and occur more frequently in industrial areas.

Pathogenic Microorganisms

Viruses have been detected in groundwater where stormwater recharge basins are located short distances above the aquifer. Enteric viruses are more resistant to environmental factors than enteric bacteria and they exhibit longer survival times in natural waters. They can occur in potable and marine waters in the absence of fecal coliforms. Enteroviruses are also more resistant to commonly used disinfectants than are indicator bacteria, and can occur in groundwater in the absence of indicator bacteria.

The factors that affect the survival of enteric bacteria and viruses in the soil include pH, antagonism from soil microflora, moisture content, temperature, sunlight, and organic matter. The two most important attributes of viruses that permit their long-term survival in the environment are their structure and very small size. These characteristics permit virus occlusion and protection within colloid-size particles. Viral adsorption is promoted by increasing cation concentration, decreasing pH, and decreasing soluble organics. Since the movement of viruses through soil to groundwater occurs in the liquid phase and involves water movement and associated suspended virus particles, the distribution of viruses between the adsorbed and liquid phases determines the viral mass available for movement. Once the virus reaches the groundwater, it can travel laterally through the aquifer until it is either adsorbed or inactivated.

The major bacterial removal mechanisms in soil are straining at the soil surface and at intergrain contacts, sedimentation, sorption by soil particles, and inactivation. Because they are larger than viruses, most bacteria are retained near the soil surface due to this straining effect. In general, enteric bacteria survive in soil for 2 to 3 months, although survival times up to 5 years have been documented.

Enteroviruses likely have a high groundwater contamination potential for all percolation practices and subsurface infiltration/injection practices, depending on their presence in stormwater (likely, if contaminated with sanitary sewage). Other pathogens, including *Shigella*, *Pseudomonas aeruginosa*, and various protozoa, would also have high groundwater contamination potentials if subsurface infiltration/injection practices are used without disinfection. If disinfection (especially by chlorine or ozone) is used, then disinfection by-products (such as trihalomethanes or ozonated bromides) would have high groundwater contamination potentials. Pathogens are most likely associated with sanitary sewage contamination of storm drainage systems, but several bacterial pathogens are commonly found in surface runoff in residential areas.

Heavy Metals and Other Inorganic Compounds

The heavy metals and other inorganic compounds in stormwater of most environmental concern, from a groundwater pollution standpoint, are chromium, copper, lead, nickel, and zinc. However, the majority of metals, with the consistent exception of zinc, are mostly found associated with the particulate solids in stormwaters and are thus relatively easily removed through sedimentation practices. Filterable forms of the metals may also be removed by either sediment adsorption or organically complexing with other particulates.

In general, studies of recharge basins receiving large metal loads found that most of the heavy metals are removed either in the basin sediment or in the vadose zone. Dissolved metal ions are removed from stormwater during infiltration mostly by adsorption onto the near-surface particles in the vadose zone, while the particulate metals are filtered out near the soil surface. Studies at recharge basins found that lead, zinc, cadmium, and copper accumulated at the soil surface with little downward movement over many years. However, nickel, chromium, and zinc concentrations have exceeded regulatory limits in the soils below a recharge area at a commercial site. Elevated groundwater heavy metal concentrations of aluminum, cadmium, copper, chromium, lead, and zinc have been found below stormwater infiltration devices where the groundwater pH has been acidic. Allowing percolation ponds to go dry between storms can be counterproductive to the removal of lead from the water during recharge. Apparently, the adsorption bonds between the sediment and the metals can be weakened during the drying period.

Similarities in water quality between runoff water and groundwater have shown that there is significant downward movement of copper and iron in sandy and loamy soils. However, arsenic, nickel, and lead did not significantly move downward through the soil to the groundwater. The exception to this was some downward movement of lead with the percolation water in sandy soils beneath stormwater recharge basins. Zinc, which is more soluble than iron, has been found in higher concentrations in groundwater than has iron. The order of attenuation in the vadose zone from infiltrating stormwater is zinc (most mobile) > lead > cadmium > manganese > copper > iron > chromium > nickel > aluminum (least mobile).

Nickel and zinc would likely have high groundwater contamination potentials if subsurface infiltration/injection were used. Chromium and lead would have moderate groundwater contamination potentials for subsurface infiltration/injection practices. All metals would likely have low groundwater contamination potentials if surface infiltration were used with sedimentation pretreatment.

Salts

Salt applications for winter traffic safety is a common practice in many northern areas, and the sodium and chloride, which are collected in the snowmelt, travel down through the vadose zone

to the groundwater with little attenuation. Soil is not very effective at removing salts. Salts that are still in the percolation water after it travels through the vadose zone will contaminate the groundwater. Infiltration of stormwater has led to increases in sodium and chloride groundwater concentrations above background concentrations. Fertilizer and pesticide salts also accumulate in urban areas and can leach through the soil to the groundwater.

Studies of depth of pollutant penetration in soil have shown that sulfate and potassium concentrations decrease with depth, while sodium, calcium, bicarbonate, and chloride concentrations increase with depth. Once contamination with salts begins, the movement of salts into the groundwater can be rapid. The salt concentration may not decrease until the source of the salts is removed.

Chloride would likely have a high groundwater contamination potential in northern areas where road salts are used for traffic safety, irrespective of the pretreatment, infiltration, or percolation practice used. Salts are at their greatest concentrations in snowmelt and in early spring runoff in northern areas.

STRESSOR CATEGORIES AND THEIR EFFECTS

There are several ways in which stormwater stressors may be grouped. Overlap between these categories will occur since the ecosystem is comprised of interrelated, interactive components. Attempts at studying single stressors or single categories represents a "reductionist" approach as opposed to a more realistic "holistic" ecosystem approach (Chapman et al. 1992). However, for one to understand the whole system and its response to stormwater stressors, there must first be a basic understanding of single component effects and patterns (see also Chapters 3 through 6). The adverse effect of stormwater runoff has been mainly documented indirectly in NPS effect studies in urban and agricultural watersheds. The aquatic ecosystems in these environments typically show a loss of sensitive species, loss of species numbers (diversity and richness), and increases in numbers of pollution-tolerant organisms (e.g., Schueler 1987; EPA 1987a; Pitt and Bozeman 1982; Pitt 1995). These trends are observed at all levels of biological organization including fish, insects, zooplankton, phytoplankton, benthic invertebrates, protozoa, bacteria, and macrophytes. These alterations tend to change an aquatic ecosystem from a stable system to an unstable one, and from a complex system to an overly simplistic one. As disturbances (e.g., toxic stormwater discharges) increase in frequency and severity, the recovery phase will increase and the ability to cope with a disturbance will decrease. The following categories are but a generalized summary of commonly observed characteristics and effects in previous stormwater and ecotoxicological studies.

Stream Flow Effects and Associated Habitat Modifications

Some of the most serious effects of urban and agricultural runoff are on the aquatic habitat of the receiving waters. A major threat to habitat comes from the rapidly changing flows and the absence of refuge areas to protect the biota during these flow changes. The natural changes in stream hydrology will change naturally at a slow, relatively nondetectable rate in most areas of the United States where stream banks are stabilized by riparian vegetation. In other areas, however, natural erosion and bank slumping will occur in response to high flow events. This "natural" contribution to stream solids is accelerated by hydromodifications, such as increases in stream power due to upstream channelization, installation of impervious drainage networks, increased impervious areas in the watershed (roof tops, roadways, parking areas), and removal of trees and vegetation. All of these increase the runoff volume and stream power, and decrease the time period for stream peak discharge.

In moderately developed watersheds, peak discharges are two to five times those of predevelopment levels (Leopold 1968; Anderson 1970). These storm events may have 50% greater volume, which may result in flooding. The quicker runoff periods reduce infiltration; thus, interflows and

baseflows into the stream from groundwater during drought periods are reduced, as are groundwater levels. As stream power increases, channel morphology will change with an initial widening of the channel to as much as two to four times its original size (Robinson 1976; Hammer 1972). Floodplains increase in size, stream banks are undercut, and riparian vegetation lost. The increased sediment loading from erosion moves through the watershed as bedload, covering sand, gravel, and cobble substrates.

The aquatic organism differences found during the Bellevue Urban Runoff Program were probably most associated with the increased peak flows in Kelsey Creek caused by urbanization and the resultant increase in sediment-carrying capacity and channel instability of the creek (Pederson 1981; Perkins 1982; Richey et al. 1981; Richey 1982; Scott et al. 1982). Kelsey Creek had much lower flows than Bear Creek during periods between storms. About 30% less water was available in Kelsey Creek during the summers. These low flows may also have significantly affected the aquatic habitat and the ability of the urban creek to flush toxic spills or other dry-weather pollutants from the creek system (Ebbert et al. 1983; Prych and Ebbert undated). Kelsey Creek had extreme hydrologic responses to storms. Flooding substantially increased in Kelsey Creek during the period of urban development; the peak annual discharges have almost doubled in the last 30 years, and the flooding frequency has also increased due to urbanization (Ebbert et al. 1983; Prych and Ebbert undated). These increased flows in urbanized Kelsey Creek resulted in greatly increased sediment transport and channel instability.

The Bellevue studies (Pitt and Bissonnette 1984) indicated very significant interrelationships among the physical, biological, and chemical characteristics of the urbanized Kelsey Creek system. The aquatic life beneficial uses were found to be impaired, and stormwater conveyance was most likely associated with increased flows from the impervious areas in the urban area. Changes in the flow characteristics could radically alter the ability of the stream to carry the polluted sediments into the other receiving waters.

Stephenson (1996) studied changes in stream flow volumes in South Africa during urbanization. He found increased stormwater runoff, decreases in the groundwater table, and dramatically decreased times of concentration. The peak flow rates increased by about twofold, about half caused by increased pavement (in an area having only about 5% effective impervious cover), with the remainder caused by decreased times of concentration.

Bhaduri et al. (1997) quantified the changes in stream flow and decreases in groundwater recharge associated with urbanization. They point out that the most widely addressed hydrologic effect of urbanization is the peak discharge increases that cause local flooding. However, the increase in surface runoff volume also represents a net loss in groundwater recharge. They point out that urbanization is linked to increased variability in volume of water available for wetlands and small streams, causing "flashy" or "flood-and-drought" conditions. In northern Ohio, urbanization at a study area was found to have caused a 195% increase in the annual volume of runoff, while the expected increase in the peak flow for the local 100-year event was 26% for the same site. Although any increase in severe flooding is problematic and cause for concern, the much larger increase in annual runoff volume, and associated decrease in groundwater recharge, likely has a much greater effect on in-stream biological conditions.

A number of presentations concerning aquatic habitat effects from urbanization were made at the *Effects of Watershed Development and Management on Aquatic Ecosystems* conference held in Snowbird, UT, in August of 1996, and sponsored by the Engineering Foundation and the ASCE. MacRae (1997) presented a review of the development of the common zero runoff increase (ZRI) discharge criterion, referring to peak discharges before and after development. This criterion is commonly met using detention ponds for the 2-year storm. MacRae shows how this criterion has not effectively protected the receiving water habitat. He found that stream bed and bank erosion is controlled by the frequency and duration of the mid-depth flows (generally occurring more often than once a year), not the bank-full condition (approximated by the 2-year event). During monitoring

Table 3.1 Hours of Exceedance of Developed Conditions with Zero Runoff Increase (ZRI) Controls Compared to Predevelopment Conditions

Recurrence Interval (yrs)	Existing Flow Rate (m^3/s)	Exceedance for Predevelopment Conditions (hrs per 5 yrs)	Exceedance for Existing Development Conditions, with ZRI Controls (hrs per 5 yrs)	Exceedance for Ultimate Development Conditions, with ZRI Controls (hrs per 5 yrs)
1.01 (critical mid-bank-full conditions)	1.24	90	380	900
1.5 (bank-full conditions)	2.1	30	34	120

near Toronto, he found that the duration of the geomorphically significant predevelopment mid-bank-full flows increased by a factor of 4.2 times, after 34% of the basin had been urbanized, compared to flow conditions before development. The channel had responded by increasing in cross-sectional area by as much as three times in some areas, and was still expanding. Table 3.1 shows the modeled durations of critical discharges for predevelopment conditions, compared to current and ultimate levels of development with "zero runoff increase" controls in place. At full development and even with full ZRI compliance in this watershed, the hours exceeding the critical mid-bank-full conditions will increase by a factor of 10, with significant effects on channel stability and the physical habitat.

MacRae (1997) also reported other studies that found channel cross-sectional areas began to enlarge after about 20 to 25% of the watershed was developed, corresponding to about a 5% impervious cover in the watershed. When the watersheds are completely developed, the channel enlargements were about five to seven times the original cross-sectional areas. Changes from stable stream bed conditions to unstable conditions appear to occur with basin imperviousness of about 10%, similar to the value reported for serious biological degradation. He also summarized a study conducted in British Columbia that examined 30 stream reaches in natural areas, in urbanized areas having peak flow attenuation ponds, and in urbanized areas not having any stormwater controls. The channel widths in the uncontrolled urban streams were about 1.7 times the widths of the natural streams. The streams having the ponds also showed widening, but at a reduced amount compared to the uncontrolled urban streams. He concluded that an effective criterion to protect stream stability (a major component of habitat protection) must address mid-bank-full events, especially by requiring similar durations and frequencies of stream power (the product of shear stress and flow velocity, not just flow velocity alone) at these depths, compared to satisfactory reference conditions.

Urbanization radically affects many natural stream characteristics. Pitt and Bissonnette (1984) reported that the coho and cutthroat were affected by the increased nutrients and elevated temperatures of the urbanized streams in Bellevue, as studied by the University of Washington as part of the EPA NURP project (EPA 1983). These conditions were probably responsible for accelerated growth of the fry, which were observed to migrate to Puget Sound and the Pacific Ocean sooner than their counterparts in the control forested watershed that was also studied. However, the degradation of sediments, mainly the decreased particle sizes, adversely affected their spawning areas in streams that had become urbanized. Sovern and Washington (1997) reported that, in Western Washington, frequent high flow rates can be 10 to 100 times the predevelopment flows in urbanized areas, but that the low flows in the urban streams are commonly lower than the predevelopment low flows. They have concluded that the effects of urbanization on western Washington streams are dramatic, in most cases permanently changing the stream hydrologic balance, by increasing the annual water volume in the stream, increasing the volume and rate of storm flows, decreasing the low flows during dry periods, and increasing the sediment and pollutant discharges from the

watershed. With urbanization, the streams increase in cross-sectional area to accommodate these increased flows, and headwater downcutting occurs to decrease the channel gradient. The gradients of stable urban streams are often only about 1 to 2%, compared to 2 to 10% gradients in natural areas. These changes in width and the downcutting result in very different and changing stream conditions. For example, the common pool/drop habitats are generally replaced by pool/riffle habitats, and the stream bed material is comprised of much finer material. Along urban streams, fewer than 50 aquatic plant and animal species are usually found. Researchers have concluded that once urbanization begins, the effects on stream shape are not completely reversible. Developing and maintaining quality aquatic life habitat, however, is possible under urban conditions, but it requires human intervention and it will not be the same as for forested watersheds.

Increased flows due to urban and agricultural modification obviously cause aquatic life impacts due to destroyed habitat (unstable channel linings, scour of sediments, enlarging stream cross sections, changes in stream gradient, collapsing of riparian stands of mature vegetation, siltation, embeddedness, etc.) plus physical flushing of aquatic life from refuge areas downstream. The increases in peak flows, annual runoff amounts, and associated decreases in groundwater recharge obviously cause decreased dry-weather flows in receiving streams. Many small and moderate-sized streams become intermittent after urbanization, causing extreme aquatic life impacts. Even with less severe decreased flows, aquatic life impacts can be significant. Lower flows are associated with increased temperatures, increased pollutant concentrations (due to decreased mixing and transport), and decreased mobility and forage opportunities.

Safety Concerns with Stormwater

There are many aspects of safety associated with urban and agricultural waters, including:

- Exposure to pathogens and toxicants
- Flows (rapidly changing and common high flows)
- Steep banks/cut banks/muddy/slippery banks
- Mucky sediments
- Debris (sharps and strainers)
- Habitat for nuisance organisms (e.g., mosquitoes, rats, snakes)

Most urban receiving waters having direct storm drainage outfalls are quite small and have no formally designated beneficial uses. Larger receiving waters typically have basic uses established, but few urban receiving waters have water contact recreation as a designated beneficial use. Unfortunately, these small waters typically attract local children who may be exposed to some of the hazards associated with stormwater, as noted above. Conditions associated with pathogens and toxicants are likely a serious problem, but the other hazards listed are also very serious. Obviously, drowning should be a concern to all and is often a topic of heated discussion at public meetings where wet detention ponds for stormwater treatment are proposed. However, drowning hazards may be more common in typical urban streams than in well-designed wet detention ponds. These hazards are related to rapidly changing water flows, high flow rates, steep and muddy stream banks, and mucky stream deposits. These hazards are all increased with stormwater discharges and are typically much worse than in predevelopment times when the streams were much more stable. This can be especially critical in newly developing areas where the local streams are thought to be relatively safe from prior experience, but rapidly degrade with increased development and associated stormwater discharges. Other potentially serious hazards are related to debris thrown into streams or trash dumped along stream banks. In unstable urban streams, banks are often continuously cut away, with debris (bankside trees, small buildings, trash piles, and even automobiles) falling into the waterway.

Many people also see untidy urban stream corridors as habitat for snakes and other undesirable creatures and like to clearcut the riparian vegetation and plant grass to the water's edge. Others see creeks as convenient dumping grounds and throw all manner of junk (yard wastes, old appliances, etc.) over their back fences or off bridges into stream corridors. Both of these approaches greatly hinder the use of streams. In contrast, residents of Bellevue, WA, have long accepted the value of small urban streams as habitat for fish. As an example, they have placed large amounts of gravel into streams to provide suitable spawning habitat. In other Northwest area streams, large woody debris is carefully placed into urban streams (using large street-side cranes, and sometimes even helicopters) to improve the aquatic habitat. In these areas, local residents are paying a great deal of money to improve the habitat along the streams and are obviously much more careful about creating hazards associated with trash and other inappropriate debris or discharges.

Drowning Hazards

Marcy and Flack (1981) state that drownings in general most often occur because of slips and falls into water, unexpected depths, cold water temperatures, and fast currents. Four methods to minimize these problems include eliminating or minimizing the hazard, keeping people away, making the onset of the hazard gradual, and providing escape routes.

Jones and Jones (1982) consider safety and landscaping together because landscaping should be used as an effective safety element. They feel that appropriate slope grading and landscaping near the water's edge can provide a more desirable approach than widespread fencing around wet detention ponds. Fences are expensive to install and maintain and usually produce unsightly pond edges. They collect trash and litter, challenge some individuals who like to defy barriers, and impede emergency access if needed. Marcy and Flack (1981) state that limited fencing may be appropriate in special areas. When the side slopes of a wet detention pond cannot be made gradual (such as when against a railroad right-of-way or close to a roadway), steep sides with submerged retaining walls may be needed. A chain-link fence located directly on the top of the retaining wall very close to the water's edge may be needed (to prevent human occupancy of the narrow ledge on the water side of the fence). Another area where fencing may be needed is at the inlet or outlet structures of wet detention ponds. However, fencing usually gives a false sense of security, because most can be easily crossed (Eccher 1991).

Common recommendations to maximize safety near wet detention ponds include suggestions that the pond side slopes be gradual near the water's edge, with a submerged ledge close to shore. Aquatic plants on the ledge would decrease the chance of continued movement to deeper water, and thick vegetation on shore near the water's edge would discourage access to the water and decrease the possibility of falling accidentally. Pathways should not be located close to the water's edge, or turn abruptly near the water. Marcy and Flack (1981) also encourage the placement of escape routes in the water whenever possible. These could be floats on cables, ladders, hand-holds, safety nets, or ramps. They should not be placed to encourage entering the water.

The use of inlet and outlet trash racks and antivortex baffles is also needed to prevent access to locations with dangerous water velocities. Several types are recommended by the NRCS (SCS 1982). Racks need to have openings smaller than about 6 in, to prevent people from passing through them, and they need to be placed where water velocities are less than 3 ft/s, to allow people to escape (Marcy and Flack 1981). Besides maintaining safe conditions, racks also help keep trash from interfering with the operation of the outlet structure.

Eccher (1991) lists the following pond attributes to ensure maximum safety, while having good ecological control:

1. There should be no major abrupt changes in water depth in areas of uncontrolled access.
2. Slopes should be controlled to ensure good footing.

3. All slope areas should be designed and constructed to prevent or restrict weed and insect growth (generally requiring some form of hardened surface on the slopes).
4. Shoreline erosion needs to be controlled.

Obviously, many of these suggestions to improve safety near wet detention ponds may also be applicable to urban stream corridors. Of course, streams can periodically have high water velocities, and steep banks may be natural. However, landscaping and trail placement along urban stream corridors can be carefully done to minimize exposure to the hazardous areas.

Aesthetics, Litter/Floatables, and Other Debris Associated with Stormwater

One of the major problems with the aesthetic degradation of receiving waters in urban areas is a general lack of respect for the local water bodies. In areas where stormwater is considered a beneficial component of the urban water system, these problems are not as severe, and inhabitants and visitors enjoy the local waterscape. The following list indicates the types of aesthetic problems that are common for neglected waters:

- Low flows
- Mucky sediments
- Trash from illegal dumping
- Floatables from discharges of litter
- Unnatural riparian areas
- Unnatural channel modifications
- Odiferous water and sediment
- Rotting vegetation and dead fish
- Objectionable sanitary wastes from CSOs and SSOs

The above list indicates the most obvious aesthetic problems in receiving waters. Many of these problems are directly associated with poor water quality (such as degraded sediments, eutrophication, and fish kills). Other direct problems associated with runoff include massive modifications of the hydrologic cycle with more severe and longer durations of low flow periods due to reduced infiltration of rainwater. Many of the other problems on the above list are related to indirect activities of the inhabitants of the watershed, namely, illegal dumping of trash into streams, littering in the drainage area, and improper modifications. In many areas, separate sewer overflows (SSOs) and combined sewer overflows (CSOs) also contribute unsightly and hazardous debris to urban receiving waters.

Floatable Litter Associated with Wet-Weather Flows

As previously indicated, aesthetics is one of the most important beneficial uses recognized for urban waterways. Floatable litter significantly degrades the aesthetic enjoyment of receiving waters. The control of floatables has therefore long been a goal of most communities.

In coastal areas, land-based sources of beach debris and floatable material have generally been found to originate from wet-weather discharges from the land, and not from marine sources (such as shipping). Of course, in areas where solid wastes (garbage or sewage sludge, for example) have been (or are still being) dumped in the sea, these sources may also be significant beach litter sources. In CSO areas, items of sanitary origin are found in the receiving waters and along the beaches, but stormwater discharges are responsible for most of the bulk litter material, including much of the hazardous materials. In inland areas, marine contributions are obviously not an issue. Therefore, with such direct linkages to the drainage areas, much of the floatable material control efforts have focused on watershed sources and controls (including being part of the “nine minimum” controls

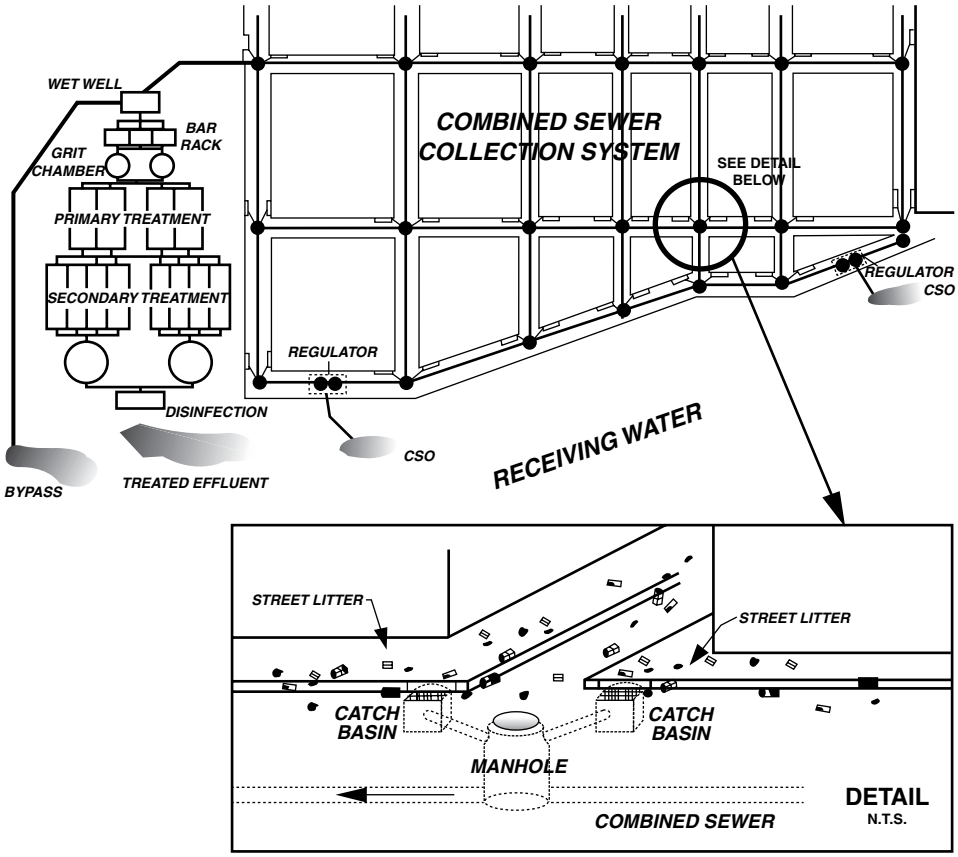

Figure 3.22 Schematic of transport of street and sidewalk litter into receiving waters. (From HydroQual, Inc. *Floatables Pilot Program Final Report: Evaluation of Non-Structural Methods to Control Combined and Storm Sewer Floatable Materials.* City-Wide Floatables Study, Contract II. Prepared for New York City, Department of Environmental Protection, Bureau of Environmental Engineering, Division of Water Quality Improvement. NYDP2000. December 1995.)

for CSOs required by the EPA). Figure 3.22 shows a schematic of how street and sidewalk litter enter the receiving waters (HydroQual 1995).

An example of an investigation of beach litter sources was conducted by Williams and Simmons (1997) along the Bristol Channel in the U.K. They concluded that most of the litter accumulating on the beaches originated from river discharges, and not from litter being deposited directly on the beaches by visitors or from shipping or other oceanic sources. The sources of the litter into the major rivers were the many combined sewer overflows in the area. About 3000 CSOs exist in Wales, and 86 of the 126 CSOs discharging into the study area receive no treatment. They summarized previous studies that have concluded that about half of Britain's coastline is contaminated, with an average of 22 plastic bottles, 17 cans, and 20 sanitary items occurring per km of coast. In some areas, the beach litter can exceed 100 items per category per kilometer. Their survey found that low energy (relatively flat) sandy beaches collected the most debris. Winter litter loadings were generally higher than during the summer, further indicating that storm-related sources were more important than visitor-related sources. They concluded that the linear strip development in South Wales' valleys had led to rivers being used as open sewers and as general dumping grounds.

One of the largest and most comprehensive beach litter and floatable control investigations and control efforts in the United States has been conducted by New York City. At the beginning of their description of this floatable control program, Grey and Olivieri (1998) stated that "one of the major

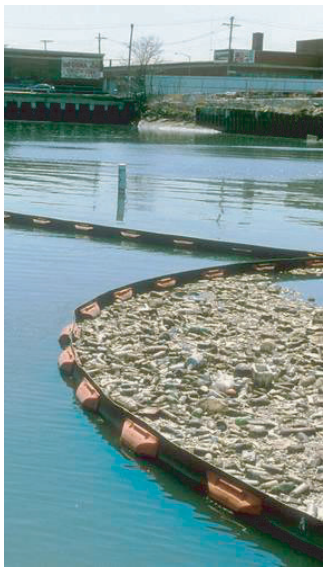

Figure 3.23 Trash boom, New York City.

Figure 3.24 New York booms and skimmers for the control of floatable discharges.

issues of urban wet-weather pollution is the control of floatable pollution." The comprehensive New York City program included investigations of the sources of the litter contributing to the floatable discharges (mostly street and sidewalk litter) and the effectiveness of many floatable control practices (including public education, enhanced street cleaning, catchbasin hoods, floatable capture nets, and booming and skimmer boats) (Figures 3.23 through 3.26).

New York City used in-line net boxes installed below catchbasin inlets to capture the discharge of floatables for identification and quantification. Much of the work was directed at the capture efficiency of the floatable material in catchbasins. It was found that it was critical that hoods (covers over the catchbasin outlets that extended below the standing water) be used in the catchbasins to help retain the captured material. The hoods increased the capture of the floatables by 70 to 85%. Unhooded catchbasins were found to discharge about 11 g/100 ft of curb length per day, while

Figure 3.25 TrashTrap™ at Fresh Creek, Brooklyn, NY.

Figure 3.26 New York City's use of end-of-pipe TrashTrap systems.

Table 3.2 Floatable Litter Characteristics Found on New York City Streets

	No. of Items (%)	Weight of Items (%)	Density of Items (lb/ft^3)
Plastic	57.2	44.3	2.8
Metal	18.9	12.0	3.8
Paper (coated/waxed)	5.9	4.0	2.0
Wood	5.9	5.3	7.7
Polystyrene	5.4	1.3	0.7
Cloth/fabric	2.5	12.5	8.3
Sensitive items	1.7	0.4	na
Rubber	1.1	1.1	10.5
Misc.	1.0	3.6	9.8
Glass	0.4	15.6	13.8

From HydroQual, Inc. *Floatables Pilot Program Final Report: Evaluation of Non-Structural Methods to Control Combined and Storm Sewer Floatable Materials.* City-Wide Floatables Study, Contract II. Prepared for New York City, Department of Environmental Protection, Bureau of Environmental Engineering, Division of Water Quality Improvement. NYDP2000. December 1995.

hooded catchbasins reduced this discharge to about 3.3 g/100 ft of curb length per day. It was also found that the hoods greatly extended the period of time between cleanings and the depth of accumulated litter that could be captured in the catchbasins without degraded capture performance.

There are about 130,000 stormwater inlet structures in New York City's 190,000 acres served by combined and separate sewers, or about 1.5 acres served by each inlet. They are surveying all of these inlet structures, replacing damaged or missing hoods, and accurately measuring their dimensions and indicating their exact locations for a citywide GIS system. Catchbasin cleaning costs are about $170 per inlet, while the inspection and mapping costs are about $45 per inlet. Replacement hoods cost about $45 per inlet.

Litter surveys conducted by the New York City Department of Sanitation (DOS) in 1984 and 1986 found that 70% of the street litter items consisted of food and beverage wrappers and containers (60%) and the paper and plastic bags (10%) used to carry these items. The early studies also found that litter levels on the streets and sidewalks were about 20 to 25% higher in the afternoon than in the morning. The DOS conducted similar surveys in 1993 at 90 blockfaces throughout the city (HydroQual 1995). Each litter monitoring site was monitored several times simultaneously when the surveys were conducted with the floatable litter separated into 13 basic categories. They found that twice as much floatable litter was located on the sidewalks compared to the streets (especially glass) and that land use had little effect on the litter loadings (except in the special business districts where enhanced street cleaning/litter control was utilized, resulting in cleaner conditions). Their baseline monitoring program determined that an average of 2.3 floatable litter items were discharged through the catchbasin inlets per day per 100 ft of curb. This amount was equivalent to about 6.2 in^2 and 0.013 lb (8.5 g) of material. The total litter load discharged was about twice this floatable amount. Table 3.2 summarizes the characteristics of the floatable litter found on the streets.

Solids (Suspended, Bedded, and Dissolved)

The detrimental effects of elevated suspended and dissolved solids and increases in siltation and fine-grained bedded sediments have been well documented (EPA 1987b). The sources of these solids are primarily from dry deposition, roadways, construction, and channel alteration and have significant effects on receiving-system habitats. Solids concentrations are directly related to watershed use characteristics and watershed hydrology.

In the United States, 64% of the land is dominated by agriculture and silviculture from which the major pollutant is sediment (approximately 1.8 billion metric tons per year) (EPA 1977). The suspended sediments transport toxicants, nutrients, and lower the aesthetic value of the waterways

Table 3.3 Classification of Suspended and Dissolved Solids and Their Probable Major Impacts on Freshwater Ecosystems

	Chemical and Physical Effects	Biochemical and Biological Effects
	Suspended Solids	
Clays, silts, sand	Sedimentation, erosion, and abrasion turbidity (light reduction), habitat change	Respiratory interference habitat restriction, light limitation
Natural organic matter	Sedimentation, DO utilization	Food sources, DO effects
Wastewater organic particles	Sedimentation, DO utilization	DO effects, eutrophication, nutrient source
Toxicants sorbed to particles	All of the above	Toxicity
	Dissolved Solids	
Major inorganic salts	Salinity, buffering, precipitation, element ratios	Nutrient availability, succession, salt effects
Important nutrients		Eutrophication, DO production
Natural organic matter		DO effects and utilization
Wastewater organic matter		DO effects and utilization
Toxicants		Toxicity and effects on DO

From EPA (U.S. Environmental Protection Agency). *Suspended and Dissolved Solids Effects on Freshwater Biota: A Review,* Environmental Research Laboratory, U.S. Environmental Protection Agency, Corvallis, OR, EPA 600/3-77/042. 1977.

(EPA 1977). Suspended sediments decrease light penetration and photosynthesis, clog gills and filtering systems of aquatic organisms, reduce prey capture, reduce spawning, reduce survival of sensitive species, and carry adsorbed pollutants (Tables 3.3 through 3.5). Acute effects of suspended solids are commonly observed at 80,000 mg/L with death at 200,000 mg/L. Recovery is quick at lower exposures (EPA 1977). As the suspended sediments settle, they cover silt-free spawning substrates, suffocating embryos, and alter the sediment environment. Suspended solids reduce primary productivity and alter temperatures, thus affecting summer stratification. Solids should not reduce photosynthesis by more than 10% of the seasonal average, using the "light–dark" bottle method (APHA 1992). Reduced productivity may then reduce zooplankton populations. Desirable benthic species may be smothered, and tolerant species, such as oligochaetes, will increase in numbers. The sediment environment plays a major role in aquatic ecosystem functioning and overlying water quality (Wetzel 1975). These new bedded sediments may possess different chemical, physical, and biological characteristics from pre-impact sediments. So any alteration to the micro-, meio-, and macrobenthic communities, sorption and desorption dynamics of essential and toxic chemical species, and organic matter and nutrient cycling processes may profoundly influence the aquatic ecosystem (Power and Chapman 1992). As the rate of bedload sediment movement increases and the frequency of occurrence of bedload movement increases, the stress to the system increases.

Dissolved solids concentrations can often be very high in stormwaters and baseflows. The associated dissolved constituents consist primarily of road salts and salts from exposed soils. Though the major cations and anions are nontoxic to most species in relatively high concentrations, stormwaters may exceed threshold levels (EPA 1977) and alter ion ratios, which may cause chronic toxicity effects. In addition, toxic trace metal-metalloids such as selenium may be dissolved from natural soil matrices (as dramatically demonstrated in the San Joaquin Valley's Kesterson Reservoir of California), or dissolved zinc may be discharged from roof runoff components of urban runoff. Long-term and repeated exposures result as the dissolved species accumulate in interstitial water, bacteria, macrophytes, phytoplankton, and other food chain components (Burton et al. 1987; EPA 1977) and result in increased mortality, teratogenicity and other adverse effects (EPA 1977).

Table 3.4 Summary of Suspended Solids Effects on Aquatic Macroinvertebrates

Organisms	Effect	Suspended Solid Concentration	Source of Suspended Solids	Comment
Mixed populations	Lower summer populations		Mining area	
	Reduced populations to 25%	261–390 NTU (turbidity)	Log dragging	
	Densities 11% of normal	1000–6000 mg/L		Normal populations at 60 mg/L
	No organisms in the zone of setting	>5000 mg/L	Glass manufacturing	Effect noted 13 miles downstream
Chironomus and Tubificidae	Normal fauna replaced by species selection		Colliery	Reduction in light-reduced submerged plants
Chematopsyche (net spinners)	Number reduced	(High concentrations)	Limestone quarry	Suspended solids as high as 250 mg/L
Tricorythoides	Number increased		Limestone quarry	Due to preference for mud or silt
Mixed populations	90% increase in drift	80 mg/L	Limestone quarry	
	Reduction in numbers	40–200 NTU	Manganese strip mine	Also caused changes in density and diversity
Chironomidae	Increased drift with suspended sediment		Experimental sediment addition	
Ephemoptera, Simuliidae, Hydracarina	Inconsistent drift response to added sediment		Experimental sediment addition	

From EPA (U.S. Environmental Protection Agency). *Suspended and Dissolved Solids Effects on Freshwater Biota: A Review,* Environmental Research Laboratory, U.S. Environmental Protection Agency, Corvallis, OR, EPA 600/3-77/042. 1977.

Dissolved Oxygen

Historically, dissolved oxygen has received much attention when researchers investigate biological receiving water effects of pollutant discharges. Therefore, the earliest efforts to evaluate the potential problems caused by urban runoff included investigations of dissolved oxygen conditions in urban receiving waters.

Bacteria respond rapidly (within minutes) in temperate streams and lakes to their surrounding environment. Due to the low level of nutrients normally present, most of the indigenous bacteria are dominant. During a storm event, however, micro- to submicrogram levels of organic nutrients (e.g., carbon, nitrogen, phosphorus, and sulfur-containing compounds) suddenly increase by orders of magnitude. Consequently, bacterial reproduction and respiration rates increase dramatically; thus exerting biochemical oxygen demand (BOD). Oxygen depletion problems may occur during the high flow event, but it is likely more serious days later when associated with organic material affecting the sediment oxygen demand (Pitt 1979). BOD_5 levels may exceed 20 mg/L during storm events, which may result in anoxia in downstream receiving waters (Schueler 1987). Predicting this problem is complicated by toxicants that may be present and interfere with the BOD test (OWML 1982). Sediment resuspension contributes to both BOD and chemical oxygen demand (COD). BOD_5 values were elevated tenfold (10 to 20 days after a storm event) related to sediment oxygen demand (SOD). Stormwater dissolved oxygen (DO) levels less than 5 mg/L are common (Keefer et al. 1979).

Aquatic macrofauna are cold-blooded and sensitive to temperature changes. In cold water systems, sustained temperatures in excess of 21°C are stressful to resident biota. Many agricultural and urban watersheds contribute to thermal pollution by removing shade canopies over streams, and runoff temperatures increase rapidly as water flows over impervious surfaces (Schueler 1987).

Table 3.5 Summary of Suspended Solids Effects on Fish[a]

Fish (Special)	Effect	Concentration of Suspended Solids (mg/L)	Source of Suspended Materials
Rainbow trout (*Salmo gairdneri*)	Survived 1 day	80,000	Gravel washing
	Killed in 1 day	160,000	Gravel washing
	50% Mortality in 3½ weeks	4250	Gypsum
	Killed in 20 days	1000–2500	Natural sediment
	50% mortality in 16 weeks	200	
	1/5 mortality in 37 days	1000	Spruce fiber
	No deaths in 4 weeks	553	Cellulose fiber
	No deaths in 9–10 weeks	200	Gypsum
	20% mortality in 2–6 months	90	Coal washery waste
	No deaths in 8 months	100	Kaolin and diatomaceous earth
	No deaths in 8 months	50	Spruce fiber
	No increased mortality	30	Coal washery waste
	Reduced growth	50	Kaolin or diatomaceous earth
	Reduced growth	50	Wood fiber
	Fair growth	200	Coal washery waste
	"Fin-rot" disease	270	Coal washery waste
	"Fin-rot" disease	100	Diatomaceous earth
	No "fin-rot"	50	Wood fiber
	Reduced egg survival	(Siltation)	Wood fiber
	Total egg mortality in 6 days	1000–2500	
	Reduced survival of eggs	(Silting)	Wood fiber
	Supports populations	(Heavy loads)	Mining operations
	Avoid during migration	(Muddy waters)	Glacial silt
Brown trout (*Salmo trutta*)	Do not dig redds	(Sediment in gravel)	
	Reduced populations to 1/7 of clean streams	1000–6000	China-clay waste
Cutthroat trout (*Salmo clarkii*)	Abandon redds	(If silt is encountered)	
	Sought cover and stopped feeding	35	
Brook trout (*Salvelinus fontinalis*)	No effect on movement	(Turbidity)	
Golden shiner (*Notemigonus crysoleucas*)	Reaction	20,000–50,000	
	Death	50,000–100,000	
Carp (*Cyrinus carpio*)	Reaction	20,000	
	Death	175,000–250,000	
Largemouth black bass (*Micropterus salmoides*)	Reaction	20,000	
	Death	101,000 (average)	
Smallmouth bass (*Micropterus dolomieu*)	Successful nesting, spawning, hatching	(Sporadic periods of high turbidity)	

[a] See EPA 1977 for additional species-specific effect information.

From EPA (U.S. Environmental Protection Agency). *Suspended and Dissolved Solids Effects on Freshwater Biota: A Review,* Environmental Research Laboratory, U.S. Environmental Protection Agency, Corvallis, OR, EPA 600/3-77/042. 1977.

Acid precipitation and acid mine drainage cause NPS pollution problems in some parts of the United States which are, at times, aggravated by storm events. During the spring in areas where snows have accumulated, rain events intensify the snowmelt process. This results in pulses of low pH runoff and snowmelts which may be stressful or lethal to aquatic macrofauna, particularly the sensitive life stages of fish occurring during the spring spawning period.

Keefer et al. (1979) examined the data from 104 water quality monitoring sites near urban areas throughout the country for DO conditions. These stations were selected from more than 1000 nationwide monitoring stations operated by various federal and state agencies. They conducted

analyses of daily DO data for 83 of these sites. About one half of the monitoring stations examined showed a 60% or greater probability of a higher than average dissolved oxygen deficit occurring at times of higher than average stream flow, or on days with rainfall. This result was based on daily data for entire water years; not all years at any given location exhibited this 60% probability condition. They found that the DO levels fell to less than 75% saturation at most of the stations that had this 60% or greater probability condition. They also found that DO concentrations of less than 5 mg/L were common. Keefer et al. (1979) examined hourly DO data at 22 nationwide sites to find correlations between flows and DO deficit. They found that for periods of steady low flows, the DO fluctuated widely on a daily cycle, ranging from 1 to 7 mg/L. During rain periods, however, the flow increased, of course, but the diurnal cycle of this DO fluctuation disappeared. The minimum DO dropped from 1 to 1.5 mg/L below the minimum values observed during steady flows, and remained constant for periods ranging from 1 to 5 days. They also reported that as the high flow conditions ended, the DO levels resumed diurnal cyclic behavior. About 50% of the stations examined in detail on an hour-by-hour basis would not meet a 5 mg/L DO standard, and about 25% of these stations would not even meet a 2.0 mg/L standard for 4-hour averages. The frequency of these violations was estimated to be up to five times a year per station.

Ketchum (1978) conducted another study in Indiana that examined DO depletion on a regional basis. Sampling was conducted at nine cities, and the project was designed to detect significant DO deficits in streams during periods of rainfall and runoff. The results of this study indicated that wet-weather DO levels generally appeared to be similar or higher than those observed during dry-weather conditions in the same streams. They found that significant wet-weather DO depletions were not observed, and due to the screening nature of the sampling program, more subtle impacts could not be measured.

Heaney et al. (1980), during their review of studies that examined continuous DO stations downstream from urbanized areas, indicated that the worst DO levels occurred after the storms in about one third of the cases studied. This lowered DO could be due to urban runoff moving downstream, combined sewer overflows, and/or resuspension of benthic deposits. Resuspended benthic deposits could have been previously settled urban runoff solids.

Pitt (1979) found that the BOD of urban runoff, after a 10- to 20-day incubation period, can be more than five to ten times the BOD of a 1- to 5-day incubation period (Figure 3.27). Therefore, urban runoff effects on DO may occur at times substantially different from the actual storm period and be associated with interaction between sediment and the overlying water column. It is especially important to use acclimated microorganisms for the BOD test seed for stormwater BOD analyses. The standard activated sludge seed may require substantial acclimation periods. Even in natural waters, several-day acclimation periods may be needed (see Lalor and Pitt 1998; P/R *in situ* test descriptions in Chapter 6).

Temperature

In-stream temperature increases have been noted in many studies as being adversely affected by urbanization. Rainwater flowing across heated pavement can significantly elevate stormwater temperatures. This temperature increase can be very detrimental in steams having sensitive cold-water fisheries. Removal of riparian vegetation can also increase in-stream water temperatures. Higher water temperatures increase the toxicity of ammonia and also affect the survival of pathogens. The temperature increases in urban streams are most important during the hot summer months when the natural stream temperatures may already be nearing critical conditions and when the stream flows are lowest. Pavement is also the hottest at this time and stormwater temperature increases are therefore the highest. Much of the habitat recovery efforts in urban streams focus on restoring an overstory for the streams to provide shading, refuge areas, and bank stability. Wet detention ponds in urban areas have also been shown to cause significant temperature increases. Grass-lined channels, however, provide some relief, compared to rock-lined or asphalt-lined drain-

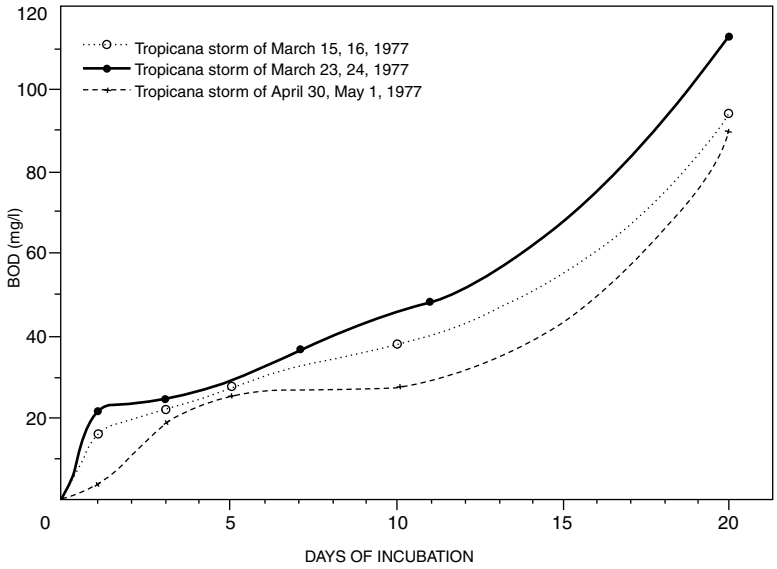

Figure 3.27 BOD rate curve for stormwater, showing dramatic increase after 10 days of incubation. (From Pitt, R. *Demonstration of Nonpoint Pollution Abatement through Improved Street Cleaning Practices,* EPA-600/2-79-161, U.S. Environmental Protection Agency, Cincinnati, OH. 270 pp. 1979.)

age channels. Since temperature is simple to monitor and is a critical stressor for many aquatic organics, it should be included in most monitoring efforts.

Nutrients

In general, urban stormwater is relatively low in organic matter and nutrients and high in toxicants. However, the nutrient levels in stormwaters can periodically be high and produce large mass discharges of nitrogen and phosphorus compounds (e.g., EPA 1977, 1983; Schueler 1987). Single spring storm events have been shown to contribute 90% of the annual phosphorus input into receiving impoundments. However, urban and agricultural runoff may contain nutrient concentrations which exceed the normal (predevelopment) ranges, and result in adverse responses such as cyanobacterial (blue-green algae) and green algal blooms. Many of the nutrients present in urban runoff are soluble and thus readily assimilated by planktonic organisms (Schueler 1987). Sources include rain, dry deposition, soils, fertilizers, and animal wastes. Impoundments receiving contaminated runoff, with retention times of 2 weeks or longer, may develop symptoms of eutrophication. Blue-green algal blooms can produce hepato- and neurotoxins implicated in cattle deaths, human liver cancer, and allergic responses (Zhang et al. 1991). As algal blooms eventually decompose, bacterial respiration may result in DO sags and anoxia, with associated fish kills.

A large amount of the nutrients enter receiving waters adsorbed to suspended solids (Lin 1972; Middlebrooks 1974; Carlile et al. 1974). These fractions will largely end up as bedded sediments which may or may not be subsequently released to overlying waters. The sediment nutrients may stimulate bacterial activity, ammonia production, and rooted macrophyte growth.

Toxicants

Heavy Metals

Stormwater runoff commonly contains elevated levels of metals and metalloids, particularly in urban areas (EPA 1983; Pitt et al. 1995; Schueler 1987). Some of these constituents are very toxic at relatively low concentrations (Table 3.6). The metals of principal concern that often occur in

Table 3.6 U.S. EPA Trace Metal Criteria for Human Health and Aquatic Life Beneficial Uses

Trace Metal Contaminant	Water Hardness (mg/L as $CaCO_3$)	Human[a] Ingestion (food/drink) (μg/L)	Ambient Life Criteria for Intermittent Exposure (μg/L)[b]	
			Threshold[c] Effect	Significant[d] Mortality
Copper	50	—	20	50–90
	100	—	35	90–150
	200	—	80	120–350
Cadmium	50	10	3	7–160
	100	10	6.6	15–350
	300	10	20	45–1070
Lead	50	50	150	350–3200
	100	50	360	820–7500
	200	50	850	1950–17850
Zinc	50	—	380	870–3200
	100	—	680	1550–4500
	200	—	1200	2750–8000
Nickel	—	13.4	—	—

[a] Derived from EPA drinking water criteria.
[b] EPA estimate of toxicity under intermittent, short-duration exposure (several hours once every several days).
[c] Concentration causing mortality to the most sensitive individual of the most sensitive species.
[d] Significant mortality shown as a range: 50% mortality in the most sensitive species, and mortality of the most sensitive individual in the species in the 25th percentile of sensitivity.

From EPA (U.S. Environmental Protection Agency). *Quality Criteria for Water*. EPA 440/5-86-001. U.S. Environmental Protection Agency, Washington, D.C. May 1986.

urban runoff are arsenic, cadmium, copper, lead, mercury, and zinc (EPA 1983). Metal bioavailability is reduced in waters of higher hardness (Table 3.6) by sorption to solids and by stormwater dilution. However, acute and chronic effects have been attributed to stormwater metals (Ray and White 1979; Ellis 1992). The highest metal concentrations are not always associated with the "first flush," but are better correlated with the peak flow period (Heaney 1978). Most metals are bound to street and parking area particulates and subsequently deposited in stream and lake sediments (Pitt et al. 1995). Sediment metal concentrations are dependent on particle size (Wilber and Hunter 1980). Wilber and Hunter (1980) suggest that larger particle sizes are better indicators of urban inputs since they are less affected by scouring. Zinc and copper are often present in runoff as soluble forms (Schueler 1987; Pitt et al. 1995).

Predicting detrimental effects from water or sediment metal concentration or loading data is difficult due to the myriad of processes which control bioavailability and fate. Speciation, availability, and toxicity are affected by pH, redox potential, temperature, hardness, alkalinity, solids, iron and manganese oxyhydroxides, sulfide fractions, and other organic-inorganic chelators. These constituents and conditions are often rapidly changing during a storm event and processes which increase and decrease bioavailability (e.g., loss of sulfide complexes and formation of oxyhydroxide complexes) may occur simultaneously. This makes accurate modeling of toxicity difficult, if not impossible.

Episodic exposures of organisms to stormwaters laden with metals can produce stress and lethality (see also Chapter 6). Ray and White (1976) observed fish death days after exposure and miles downstream after metals were diluted to nondetectable levels. Ellis et al. (1992) showed amphipods bioaccumulated zinc from episodic, *in situ* exposures. Repeated exposures increased their sensitivity, and mortality was observed 3 weeks after the storm event.

Toxic Organic Compounds

The types and concentrations of toxic organic compounds that are in stormwaters are driven primarily by land use patterns and automobile activity in the watershed. Most nonpesticide organic

compounds originate as washoff from impervious areas in commercial areas having large numbers of automobile startups and/or other high levels of vehicle activities, including vehicle maintenance operations and heavily traveled roads. The compounds of most interest are the polycyclic aromatic hydrocarbons (PAHs). Other organics include phthalate esters (plasticizers) and aliphatic hydrocarbons. Other compounds frequently detected in residential and agricultural areas are cresol constituents (and other wood preservatives), herbicides, and insecticides. Many of these organic compounds are strongly associated with the particulate fraction of stormwater. Volatile organic compounds (VOCs) are rarely found in urban runoff. While most organics are not detected or are detected at low µg/L concentrations, some are acutely toxic, including freshly applied pesticides and photoactivated PAHs (Skalski 1991; Oris and Giesy 1986). The extent of detrimental impact from these constituents has not been well documented, but likely is significant in some areas.

Environmental Fates of Runoff Toxicants

The fate of runoff toxicants after discharge significantly determines their associated biological effects. If the pollutants are discharged in a soluble form and remain in solution, they may have significant acute toxicity effects on fish, for example. However, if discharged soluble pollutants form insoluble complexes or sorb onto particulates, chronic toxicity effects associated with contaminated sediments are more likely. For many of the metallic and organic toxicants discharged in urban runoff, the particulate fractions are much greater than the soluble fractions (Pitt et al. 1995). Particulate forms of pollutants may remain in suspension, if their settling rates are low and the receiving water is sufficiently turbulent. However, polluted sediments are common in many urban and agricultural streams, indicating significant accumulations of runoff particulate pollutants (Pitt 1995).

Tables 3.7 through 3.9 summarize the importance of various environmental processes for the aquatic fates of some runoff heavy metals and organic priority pollutants, as described by Callahan et al. (1979). Photolysis (the breakdown of the compounds in the presence of sunlight) and volatilization (the transfer of the materials from the water into the air as a gas or vapor) are not nearly as important as the other mechanisms for heavy metals. Chemical speciation (the formation of chemical compounds) is very important in determining the solubilities of the specific metals. Sorption (adsorption is the attachment of the material onto the outside of a solid, and absorption is the attachment of the material within a solid) is very important for all of the heavy metals shown. Sorption can typically be the controlling mechanism affecting the mobility and the precipitation of most heavy metals. Bioaccumulation (the uptake of the material into organic tissue) can occur for all of the heavy metals shown. Biotransformation (the change of chemical form of the metal by organic processes) is very important for some of the metals, especially mercury, arsenic, and lead. In many cases, mercury, arsenic, or lead compounds discharged in forms that are unavailable can be accumulated in aquatic sediments. They are then exposed to various benthic organisms that can biotransform the material through metabolization to methylated forms, which can be highly toxic and soluble.

Tables 3.8 and 3.9 also summarize various environmental fates for some of the toxic organic pollutants found in typical runoff from human-modified watersheds, mainly various phenols, polycyclic aromatic hydrocarbons (PAHs), and phthalate esters. Photolysis may be an important fate process for phenols and PAHs but is probably not important for the phthalate esters. Oxidation or hydrolysis may be important for some phenols. Volatilization may be important for some phenols and PAHs. Sorption is an important fate process for most of the materials, except for phenols. Bioaccumulation, biotransformation, and biodegradation are important for many of these organic materials.

Pathogens

Water Environment & Technology (1996) reported that the latest National Water Quality Inventory released by the EPA only showed a slight improvement in the attainment of beneficial uses in

Table 3.7 Importance of Environmental Processes on the Aquatic Fates of Selected Urban Runoff Heavy Metals

Environmental Process	Arsenic	Cadmium	Copper	Mercury	Lead	Zinc
Photolysis	Not important	Not important	Not important	May be important in some aquatic environments	Determines the form of lead entering the aquatic system	Not important
Chemical speciation	Important in determining distribution and mobility[a]	Complexation with organics; most important in polluted waters	Complexation with organics; most important in polluted waters	Conversion to complex species; HgS will precipitate in reducing sediments	Determines which solid phase controls solubility	Complexation predominates in polluted waters
Volatilization	Important when biological activity or highly reducing conditions produce AsH_3 or methyl-arsenic	Not important	Not important	Important	Not important	Not important
Sorption	Sorption onto clays, oxides, and organic material important	Sorption onto organic materials, clays, hydrous iron and manganese oxides most important	Can reduce Cu mobility and enrich suspended and bed sediments; sorption onto organics in polluted waters, clay minerals or hydrous iron and manganese oxides	Strongest onto organic material, results in partitioning of mercury into suspended and bed sediments	Adsorption to inorganic solids, organic materials and hydrous iron and manganese oxides control mobility of lead	Strong affinity for hydrous metal oxides, clays, and organic matter; adsorption increases with pH
Bioaccumulation	Most important at lower trophic levels; toxicity limits bioaccumulation	Biota strongly bioaccumulate cadmium	Biota strongly bioaccumulate copper	Occurs by many mechanisms, most connected to methylated forms of mercury	Biota strongly bioaccumulates lead	Zinc is strongly bioaccumulated
Biotransformation	Arsenic can be metabolized to organic arsenicals	Not methylized biologically, organic ligands may affect solubility and adsorption	Source Cu complexes may be metabolized; organic ligands are important in sorption and complexation processes	Can be metabolized by bacteria to methyl and dimethyl forms which are quite mobile	Biomethylation of lead in sediments can remobilize lead	Not evident; organic ligands of biological origin may affect solubility and adsorption

[a] Conversion of As^{3+} and As^{5+} and organic complexation most important.

From Callahan, M.A. et al. *Water Related Environmental Fates of 129 Priority Pollutants.* U.S. Environmental Protection Agency, Monitoring and Data Support Division, EPA-4-79-029a and b. Washington, D.C. 1979.

Table 3.8 Importance of Environmental Processes on the Aquatic Fates of Various Polycyclic Aromatic Hydrocarbons and Phthalate Esters

Environmental Process[a]	Anthracene	Fluoranthene	Phenanthrene	Diethyl Phthalate (DEP)	Di-n-Butyl Phthalate (DBP)	Bis (2-Ethyl-hexyl) Phthalate (DEHP)	Butyl Benzyl Phthalate (BBP)
Photolysis	Dissolved portion may undergo rapid photolysis	Dissolved portion may undergo rapid photolysis	Dissolved portion may undergo rapid photolysis	Not important	Not important	Not important	Not important
Volatilization	May be competitive with adsorption	May be competitive with adsorption	May be competitive with adsorption	Not important	Not important	Not important	Not important
Sorption	Adsorbs onto suspended solids; movement by suspended solids is important transport process	Adsorbs onto suspended solids; movement by suspended solids is important transport process	Adsorbs onto suspended solids; movement by suspended solids is important transport process	Sorbed onto suspended solids and biota; complexation with humic substances most important transport process	Sorbed onto suspended solids and biota; complexation with humic substances most important transport process	Sorbed onto suspended solids and biota; complexation with humic substances most important transport process	Sorbed onto suspended solids and biota; complexation with humic substances most important transport process
Bioaccumulation	Short-term process; is readily metabolized	Short-term process; is readily metabolized	Short-term process; is readily metabolized	Variety of organisms accumulate phthalates (lipophilic)	Variety of organisms accumulate phthalates (lipophilic)	Variety of organisms accumulate phthalates (lipophilic)	Variety of organisms accumulate phthalates (lipophilic)
Biotransformation	Readily metabolized by organisms and biodegradation, probably ultimate fate mechanisms	Readily metabolized by organisms and biodegradation, probably ultimate fate mechanism	Readily metabolized by organisms and biodegradation, probably ultimate fate mechanisms	Can be metabolized	Can be metabolized	Can be metabolized	Can be metabolized

[a] Oxidation and hydrolysis are not important fate mechanisms for any of these compounds.

From Callahan, M.A. et al. *Water Related Environmental Fates of 129 Priority Pollutants.* U.S. Environmental Protection Agency, Monitoring and Data Support Division, EPA-4-79-029a and b. Washington, D.C. 1979.

Table 3.9 Importance of Environmental Processes on the Aquatic Fates of Various Phenols and Pyrene

Environmental Process	Phenol	Pentachlorophenol (PCP)	2,4,6-Trichlorophenol	2,4-Dimethyl Phenol (2,4-Xylenol)	Pyrene
Photolysis	Photooxidation may be important degradation process in aerated, clear, surface waters	Reported to occur in natural waters; important near water surface	Reported, but importance is uncertain	May be important degradation process in clear aerated surface waters	Dissolved portion may undergo rapid photolysis
Oxidation	Metal-catalyzed oxidation may be important in aerated surface waters	Not important	Not important	Metal-catalyzed oxidation may be important in aerated surface waters	Not important
Volatilization	Possibility of some phenol passing into the atmosphere	Not important	Not important	Not important	Not as important as adsorption
Sorption	Not important	Sorbed by organic litter in soil and sediments	Potentially important for organic material; not important for clays	Not important	Adsorption onto suspended solids important; movement by suspended solids important
Bioaccumulation	Not important	Bioaccumulates in numerous aquatic organisms	Not important	Not important	Short-term process not significant; metabolized over long term
Biotransformation	Very significant	Can be metabolized to other phenol forms	Reported in soil and sewage sludge; uncertain for natural surface waters	Inconclusive information	Readily metabolized; biodegradation probably ultimate fate process

From Callahan, M.A. et al. *Water Related Environmental Fates of 129 Priority Pollutants*. U.S. Environmental Protection Agency, Monitoring and Data Support Division, EPA-4-79-029a and b. Washington, D.C. 1979.

the nation's waters. Urban runoff was cited as the leading source of problems in estuaries, with nutrients and bacteria as the primary problems. Problems in rivers and lakes were mostly caused by agricultural runoff, with urban runoff the third ranked source for lakes and the fourth ranked source for rivers. Bacteria, siltation, and nutrients were the leading problems in the nation's rivers and lakes.

Pathogens in stormwater are a significant concern potentially affecting human health. The use of indicator bacteria is controversial for stormwater, as is the assumed time of typical exposure of swimmers to contaminated receiving waters. However, recent epidemiological studies have shown significant health effects associated with stormwater-contaminated marine swimming areas. Protozoan pathogens, especially associated with likely sewage-contaminated stormwater, are also a public health concern.

Fecal indicators (i.e., fecal coliforms, fecal streptococci, *Escherichia coli*, and enterococci) are usually found in elevated concentrations in stormwater runoff, greatly exceeding water quality criteria and standards for primary and secondary contact (MWCOG 1984). This suggests that fecal pathogen levels are also elevated, though significant correlations with fecal coliforms are tenuous (EPA 1986). Die-off of fecal organisms in receiving waters during summer months is relatively rapid, with 99% dying within 24 to 48 hours (Burton 1985). However, fecal microorganisms also accumulate in sediments where survival is extended for weeks to months (Burton et al. 1987). Recent sediment bacteriological analyses conducted by UAB in local Birmingham (AL) area urban lakes have found elevated pore water concentrations (several hundred to several thousand organisms/100 mL) of *E. coli* and enterococci extending to at least 0.1 m into the sediments. Also, when gently disturbed, the water layer over the sediments is also found to significantly increase in microorganism concentrations. *In situ* die-off studies also indicated that bacteria sedimentation may be a more important fate mechanism of stormwater bacteria than die-off (Easton 2000).

Good correlations between the incidence of gastroenteritis in swimmers and *E. coli* and enterococci concentrations in water have resulted in new recreational water criteria (EPA 1986). High fecal microorganism concentrations in stormwaters originate from wastes of wildlife, pets, livestock, septic systems, and combined sewer overflows (CSOs). The ecological effects of these inputs of fecal organisms are unknown; however, public health is at risk in swimming areas that receive stormwaters.

Urban Bacteria Sources

The Regional Municipality of Ottawa–Carleton (1972) recognized the importance of rooftop, street surface, and field runoff in contributing bacteria contaminants to surface waters in the Ottawa area. Gore & Storrie/Proctor and Redfern (1981) also investigated various urban bacteria sources affecting the Rideau River. They examined dry-weather continuous coliform sources, the resuspension of contaminated river bottom sediments, exfiltration from sanitary sewers, and bird feces. These sources were all considered in an attempt to explain the relatively high dry-weather coliform bacteria concentrations found in the river. They concluded, however, that stormwater runoff is the most probable source for the wet-weather and continuing dry-weather bacteria concentrations in the Rideau River. The slow travel time of the river water usually does not allow the river to recover completely from one rainstorm before another begins.

The Regional Municipality of Ottawa–Carleton (1972) noted the early Ottawa activities in correcting stormwater and sanitary sewage cross-connections. Since that time, many combined sewer overflows have also been eliminated from the Rideau River. Loijens (1981) stated that, as a result of sewer separation activities, only one overflow remained active by 1981 (Clegg Street). During river surveys in 1978 and 1979 in the vicinity of this outfall, increased bacteria levels were not found. Gore & Storrie/Proctor and Redfern (1981) stated that there was no evidence that combined sewer overflows are causing the elevated fecal coliform bacteria levels in the river. Environment Canada (1980), however, stated that high dry-weather bacteria density levels, espe-

cially when considering the fecal coliform to fecal streptococci ratio, constitutes presumptive evidence of low-volume sporadic inputs of sanitary sewage from diverse sources into the downstream Rideau River sectors.

Street surfaces have been identified as potential major sources of urban runoff bacteria. Pitt and Bozeman (1982) found that parking lots, street surfaces, and sidewalks were the major contributors of indicator bacteria in the Coyote Creek watershed in California. Gupta et al. (1981) found high concentrations of fecal coliforms at a highway runoff site in Milwaukee. This site was entirely impervious and located on an elevated bridge deck. The only likely sources of fecal coliforms at this site were atmospheric deposition, bird droppings, and possibly feces debris falling from livestock trucks or other vehicles.

Several studies have found that the bacteria in stormwater in residential and light commercial areas were from predominantly nonhuman origins. Geldreich and Kenner (1969) stated that the fecal coliforms in stormwater are from dogs, cats, and rodents in city areas, and from farm animals and wildlife in rural areas. Qureshi and Dutka (1979) found that there may be an initial flush of animal feces when runoff first develops. The most important source, however, may be feces bacteria that are distributed in the soil and not the fresh feces washing off the impervious surfaces.

Some studies have investigated vegetation sources of coliform bacteria. For example, Geldreich (1965) found that the washoff of bacteria from vegetation does not contribute significant bacteria to the runoff. They also found that most of the bacteria on vegetation is of insect origin. Geldreich et al. (1980) found that recreation activities in water bodies also increase the fecal coliform and fecal streptococci concentrations. These organisms of intestinal origin will concentrate in areas near the shore or in areas of stratification. Fennell et al. (1974) found that open dumps containing domestic refuse can be a reservoir of *Salmonella* bacteria that can be spread to nearby water bodies by foraging animals and birds.

When a drainage basin has much of its surface paved, the urban runoff bacteria concentrations can be expected to peak near the beginning of the rainfall event and then decrease as the event continues. Initial high levels of bacteria may be associated with direct flushing of feces from paved surfaces. These feces are from dogs defecating on parking lots and street areas and from birds roosting on rooftops. When a drainage area has a lot of landscaped areas or open land, relatively high bacteria concentrations in the urban runoff may occur throughout the rain event associated with continuous erosion of contaminated soils.

Fecal Coliform to Fecal Streptococci Bacteria Ratios

Geldreich (1965) found that the ratio of fecal coliform to fecal streptococci bacteria concentrations may be indicative of the probable fecal source. In fresh human fecal material and domestic wastes, he found that the fecal coliform densities were more than four times the fecal streptococcal densities. However, this ratio for livestock, poultry, dogs, cats, and rodents was found to be less than 0.6. These ratios must be applied carefully because of the effects of travel time and various chemical changes (especially pH) on the die-off rates of the component bacteria. This can result in the ratio changing, as the fecal coliform organisms tend to die faster than the fecal streptococcal bacteria. As a generality, he stated that fecal coliform to fecal streptococci ratios greater than 4 indicate that the bacteria pollution is from domestic wastes, which are composed mostly of human fecal material, laundry wastes, and food refuse. If the ratio is less than 0.6, the bacteria are probably from livestock or poultry in agricultural areas or from stormwater runoff in urban areas. He found that agricultural and stormwater runoff can be differentiated by studying the types of fecal streptococci bacteria found in the water samples. Geldreich and Kenner (1969) further stressed the importance of using this ratio carefully. They stressed that samples must be taken at the wastewater outfalls. At these locations, domestic waste, meat packing wastes, stormwater discharges, and feedlot drainage contain large numbers of fecal organisms recently discharged from warm-blooded animals. Once these organisms are diffused into the receiving stream, however, water temperature,

Table 3.10 Fecal Coliform to Fecal Streptococci Bacteria Population Ratios in Study Area

Source Areas	FC/FS Ratio
Rooftop runoff	0.5
Vacant land sheetflow	0.3
Parking lot sheetflow	0.2
Gutter flows	0.2
Average of source area values	0.3
Rideau River segment	
A	1.2
B	0.6
C	0.5
D	0.5
E	1.0
Average of river segment values	0.7
River swimming beaches	
Strathcona	2.8
Brantwood	2.3
Brighton	2.1
Mooney's Bay	1.7
Average of swimming beach values	2.2

From Pitt, R. *Urban Bacteria Sources and Control by Street Cleaning in the Lower Rideau River Watershed.* Rideau River Stormwater Management Study Technical Report. Prepared for the Ontario Ministry of the Environment, Environment Canada, Regional Municipality of Ottawa-Carleton, City of Ottawa, and Nepean. 1983.

organic nutrients, toxic metals, and adverse pH values may alter the relationship between the indicator organisms. This ratio should only be applied within 24 hours following the discharge of the bacteria.

Feachem (1975) examined how these ratios could be used with bacteria observations taken over a period of time. Because the fecal coliform and fecal streptococci bacteria die-off rates are not the same, the ratio gradually changes with time. He found that bacteria are predominantly from human sources if the FC/FS ratios are initially high (greater than 4) and then decrease with time. Nonhuman bacteria sources would result in initially low FC/FS ratios (less than 0.7), which then rise with time.

Pitt (1983) examined the FC/FS bacteria population ratios observed in the Rideau River study area in Ottawa, as shown in Table 3.10. These ratios were divided into groups corresponding to source area samples, Rideau River water samples, and water samples collected at the swimming beaches farther downstream. The source area sheet-flow samples contained the most recent pollution, while the river segment and beach samples contained "older" bacteria. The initial source area samples all had ratios of less than 0.7. However, the river averages ranged from 0.5 to 1.2, and the beach samples (which may be "older" than the river samples) ranged from 1.7 to 2.8. These ratios are seen to start with values less than 0.7 and increase with time. Based on Feachem's (1975) work, this would indicate that the major bacteria sources in the Rideau River are from nonhuman sources. Periodic high bacteria ratios in the river and at the beaches could be caused by the greater die-off ratio of fecal streptococci as compared to fecal coliform. The observed periodic high Rideau River FC/FS ratios (which can be greater than 4) may therefore be from old, nonhuman fecal discharges and not from fresh human fecal discharges.

Human Health Effects of Stormwater

There are several mechanisms whereby stormwater exposure can cause potential human health problems. These include exposure to stormwater contaminants at swimming areas affected by stormwater discharges, drinking water supplies contaminated by stormwater discharges, and the consumption of fish and shellfish that have been contaminated by stormwater pollutants. Understanding the risks associated with these exposure mechanisms is difficult and not very clear. Receiving waters where human uses are evident are usually very large, and the receiving waters are affected by many sanitary sewage and industrial point discharges, along with upstream agricultural nonpoint discharges, in addition to the local stormwater discharges. In receiving waters having only stormwater discharges, it is well known that inappropriate sanitary and other wastewaters are also discharging through the storm drainage system. These "interferences" make it especially difficult to identify specific cause-and-effect relationships associated with stormwater discharges alone, in contrast to the many receiving water studies that have investigated ecological problems that can more easily study streams affected by stormwater alone. Therefore, much of the human risk assessment associated with stormwater exposure must use theoretical evaluations relying on stormwater characteristics and laboratory studies in lieu of actual population studies. However, some site investigations, especially related to swimming beach problems associated with nearby stormwater discharges, have been conducted and are summarized (from Lalor and Pitt 1998) in the following discussion.

Contact recreation in pathogen-contaminated waters has been studied at many locations. The sources of the pathogens are typically assumed to be sanitary sewage effluent, or periodic industrial discharges from certain food preparation industries (especially meat packing and fish and shellfish processing). However, several studies have investigated pathogen problems associated with stormwater discharges. It has generally been assumed that the source of pathogens in stormwater are from inappropriate sanitary connections. However, stormwater unaffected by these inappropriate sources still contains high counts of pathogens that are also found in surface runoff samples from many urban surfaces. Needless to say, sewage contamination of urban streams is an important issue that needs attention during a receiving water investigation.

Inappropriate Sanitary Sewage Discharges into Urban Streams

Urban stormwater runoff includes waters from many other sources that find their way into storm drainage systems, besides from precipitation. There are cases where pollutant levels in storm drainage are much higher than they would otherwise be because of excessive amounts of contaminants that are introduced into the storm drainage system by various non-stormwater discharges. Additionally, baseflows (during dry weather) are also common in storm drainage systems. Dry-weather flows and wet-weather flows have been monitored during numerous urban runoff studies. These studies have found that discharges observed at outfalls during dry weather were significantly different from wet-weather discharges and may account for the majority of the annual discharges for some pollutants of concern from the storm drainage system.

In many cases, sanitary sewage was an important component (although not necessarily the only component) of the dry-weather discharges from the storm drainage systems. From a human health perspective (associated with pathogens), it may not require much raw or poorly treated sewage to cause a receiving water problem. However, at low discharge rates, the DO receiving water levels may be minimally affected. The effects these discharges have on receiving waters is therefore highly dependent on many site-specific factors, including frequency and quantity of sewage discharges and the creek flows. In many urban areas, the receiving waters are small creeks in completely developed watersheds. These creeks are the most at risk from these discharges as dry baseflows may be predominantly dry-weather flows from the drainage systems. In Tokyo (Fujita 1998), for example, numerous instances were found where correcting inappropriate sanitary sewage discharges

resulted in the urban streams losing all of their flow. In cities adjacent to large receiving waters, these discharges likely have little impact (such as DO impacts from Nashville, TN, CSO discharges on the Cumberland River, as studied by Cardozo et al. 1994). The presence of pathogens from raw or poorly treated sewage in urban streams, however, obviously presents a potentially serious public health threat. Even if the receiving waters are not designated as water contact recreation, children are often seen playing in small city streams.

There have been a few epidemiology studies describing the increased health risks associated with contaminated dry-weather flows affecting public swimming beaches. The following discussion presents an overview of the development of water quality criteria for water contact recreation, plus the results of a recent epidemiological study that specifically examined human health problems associated with swimming in water affected by stormwater. In most cases, the levels of indicator organisms and pathogens causing increased illness were well within the range found in urban streams.

Runoff Pathogens and Their Sanitary Significance

The occurrence of *Salmonella* biotypes is typically low, and their reported density is less than one organism/100 mL in stormwater. *Pseudomonas aeruginosa* are frequently encountered at densities greater than 10 organisms/100 mL, but only after rains. The observed ranges of concentrations and percent isolations of bacterial biotypes vary significantly from site to site and at the same location for different times. Many potentially pathogenic bacteria biotypes may be present in urban runoff. Because of the low probability of ingestion of urban runoff, many of the potential human diseases associated with these biotypes are not likely to occur. The pathogenic organisms of most concern in urban runoff are usually associated with skin infections and body contact. The most important biotype causing skin infections would be *P. aeruginosa*. This biotype has been detected frequently in most urban runoff studies in concentrations that may cause infections. However, there is little information associating the cause and effect of increased *P.* concentrations with increased infections. *Shigella* may be present in urban runoff and receiving waters. This pathogen, when ingested in low numbers, can cause dysentery.

Salmonella

Salmonella has been reported in some, but not all, urban stormwaters. Qureshi and Dutka (1979) frequently detected *Salmonella* in southern Ontario stormwaters. They did not find any predictable patterns of *Salmonella* isolations; they were found throughout the various sampling periods. Olivieri et al. (1977a) found *Salmonella* frequently in Baltimore runoff, but at relatively low concentrations. Typical concentrations were from 5 to 300 *Salmonella* organisms/10 L. The concentrations of *Salmonella* were about ten times higher in the stormwater samples than in the urban stream receiving the runoff. The researchers also did not find any marked seasonal variations in *Salmonella* concentrations. Almost all of the stormwater samples that had fecal coliform concentrations greater that 2000 organisms/100 mL had detectable *Salmonella* concentrations, while about 275 of the samples having fecal coliform concentrations less than 200 organisms/100 mL had detectable *Salmonella*.

Quite a few urban runoff studies have not detected *Salmonella*. Schillinger and Stuart (1978) found that *Salmonella* isolations were not common in a Montana subdivision runoff study and that the isolations did not correlate well with fecal coliform concentrations. Environment Canada (1980) stated that *Salmonella* were virtually absent from Ottawa storm drainage samples in 1979. It concluded that *Salmonella* are seldom present in significant numbers in Ottawa urban runoff. The types of *Salmonella* found in southern Ontario were *S. thompson* and *S. typhimurium* var. *copenhagen* (Qureshi and Dutka 1979).

Olivieri et al. (1977b) stated that the primary human enteric disease producing *Salmonella* biotypes associated with the ingestion of water include *S. typhi* (typhoid fever), *S. paratyphi* (paratyphoid fever), and *Salmonella* species (salmonellosis). These biotypes are all rare except for

Salmonella sp. The dose of *Salmonella* sp. required to produce an infection is quite large (approximately 10^5 organisms). The salmonellosis health hazard associated with water contact in urban streams is believed to be small because of this relatively large infective dose. If 2 L of stormwater having typical *Salmonella* concentrations (10 *Salmonella* organisms/10 L) is ingested, less than 0.001 of the required infective dose would be ingested. If a worst-case *Salmonella* stormwater concentration of 10,000 organisms/10 L occurred, the ingestion of 20 L of stormwater would be necessary for an infective dose. They stated that the low concentrations of *Salmonella*, coupled with the unlikely event of consuming enough stormwater, make the *Salmonella* health hazard associated with urban runoff small.

Staphylococcus

Staphylococcus aureus is an important human pathogen it can cause boils, carbuncles, abscesses, and impetigo on skin on contact. Olivieri et al. (1977b) stated that the typical concentrations of *Staphylococci* are not very high in urban streams. They also noted that there was little information available relating the degree of risk of staph infections with water concentrations. They concluded that *Staphylococcus aureus* appears to be the most potentially hazardous pathogen associated with urban runoff, but there is no evidence available that skin, eye, or ear infections can be caused by the presence of this organism in recreational waters. They concluded that there is little reason for extensive public health concern over recreational waters receiving urban storm runoff containing staph organisms.

Shigella

Olivieri et al. (1977b) stated that there is circumstantial evidence that *Shigella* is present in urban runoff and receiving waters and could present a significant health hazard. *Shigella* species causing bacillary dysentery are one of the primary human enteric disease-producing bacteria agents present in water. The infective dose of *Shigella* necessary to cause dysentery is quite low (10 to 100 organisms). Because of this low required infective dose and the assumed presence of *Shigella* in urban waters, it may be a significant health hazard associated with urban runoff.

Streptococcus

Streptococcus faecalis and atypical *S. faecalis* are of limited sanitary significance (Geldreich 1976). *Streptococcus* determinations on urban runoff are most useful for identifying the presence of *S. bovis* and *S. equinus,* which are specific indicators of nonhuman, warm-blooded animal pollution. However, it is difficult to interpret fecal streptococcal data when their concentrations are lower than 100 organisms/100 mL because of the ubiquitous occurrence of *S. faecalis* var. *liquifaciens.* This biotype is generally the predominant streptococcal biotype occurring at low fecal streptococcal concentrations.

Pseudomonas aeruginosa

Pseudomonas is reported to be the most abundant pathogenic bacteria in urban runoff and streams (Olivieri et al. 1977b). This pathogen is associated with eye and ear infections and is resistant to antibiotics. Oliveri et al. also stated that past studies have failed to show any relationships between *P. aeruginosa* concentrations in bathing waters and ear infections. However, *Pseudomonas* concentrations in urban runoff are significantly higher (about 100 times) than the values associated with past bathing beach studies. Cabelli et al. (1976) stated that *P. aeruginosa* is indigenous in about 15% of the human population. Swimmer's ear or other *Pseudomonas* infections may, therefore, be caused by trauma to the ear canals associated with swimming and diving, and not exposure to *Pseudomonas* in the bathing water.

Environment Canada (1980) stated that there is preliminary evidence of the direct relationship between very low levels of *P. aeruginosa* and an increase in incidents of ear infections in swimmers. It stated that a control level for this *Pseudomonas* biotype of between 23 and 30 organisms/100 mL was considered. Cabelli et al. (1976) stated that *P. aeruginosa* densities greater than 10 organisms/100 mL were frequently associated with fecal coliform levels considerably less than 200 organisms/100 mL. *Pseudomonas aeruginosa* densities were sometimes very low when the fecal coliform levels were greater than 200 organisms/100 mL. An average estimated *P. aeruginosa* density associated with a fecal coliform concentration of 200 organisms/100 mL is about 12/100 mL. It further stated that *P. aeruginosa* by itself cannot be used as a basis for water standards for the prevention of enteric diseases during recreational uses of surface waters. The determinations of this biotype should be used in conjunction with fecal coliform or other indicator organism concentrations for a specific location. It recommended that bathing beaches that are subject to urban runoff be temporarily closed until the *P. aeruginosa* concentrations return to a baseline concentration.

Campylobacter

Koenraad et al. (1997) investigated the contamination of surface waters by *Campylobacter* and its associated human health risks. They reported that campylobacteriosis is one of the most frequently occurring acute gastroenteritis diseases in humans. Typical investigations have focused on the consumption of poultry, raw milk, and untreated water as the major sources of this bacterial illness. Koenraad et al. (1997) found that human exposures to *Campylobacter*-contaminated surface waters is likely a more important risk factor than previously considered. In fact, they felt that *Campylobacter* infections may be more common than *Salmonella* infections. The incidence of campylobacteriosis due to exposure to contaminated recreational waters has been estimated to be between 1.2 to 170 per 100,000 individuals. The natural habitat of *Campylobacter* is the intestinal tract of warm-blooded animals (including poultry, pigs, cattle, gulls, geese, pigeons, magpies, rodents, shellfish, and even flies). It does not seem to multiply outside of its host, but it can survive fairly well in aquatic environments. It can remain culturable and infective for more than 2 months under ideal environmental conditions. Besides runoff, treated wastewater effluent is also a major source of *Campylobacter* in surface waters. Sanitary wastewater may contain up to 50,000 MPN of *Campylobacter* per 100 mL, with 90 to 99% reductions occurring during typical wastewater treatment.

Cryptosporidium, Giardia, *and* Pfiesteria

Protozoa became an important public issue with the 1993 *Cryptosporidium*-caused disease outbreak in Milwaukee when about 400,000 people become ill from drinking contaminated water. Mac Kenzie et al. (1994) prepared an overview of the outbreak, describing the investigation of the causes of the illness and the number of people affected. They point out that *Cryptosporidium*-caused disease in humans was first documented in 1976, but had received little attention and no routine monitoring. *Cryptosporidium* is now being monitored routinely in many areas and is the subject of much research concerning its sources and pathways. At the time of the Milwaukee outbreak, both of the city's water treatment plants (using water from Lake Michigan) were operating within acceptable limits, based on required monitoring. However, at one of the plants (which delivered water to most of the infected people), at the time of the outbreak the treated water underwent a large increase in turbidity (from about 0.3 NTU to about 1.5 NTU) that was not being well monitored (the continuous monitoring equipment was not functioning, and values were obtained only every 8 hours). More than half of the residents receiving water from this plant became ill. The plant had recently changed its coagulant from polyaluminum chloride to alum, and equipment to assist in determining the correct chemical dosages was not being used. The finished water had apparently relatively high levels of *Cryptosporidium* because some individuals became ill after drinking less than 1 L of water.

Cryptosporidium oocysts have often been found in untreated surface waters, and it was thought that *Cryptosporidium* oocysts entered the water treatment supply before the increase in turbidity was apparent. MacKenzie et al. (1994) point out that monitoring in the United Kingdom has uncovered sudden, irregular, community-wide increases in cryptosporidiosis that were likely caused by waterborne transmission. They also stated that the source of the *Cryptosporidium* oocysts was speculative, but could have included cattle feces contamination in the Milwaukee and Menomonee Rivers, slaughterhouse wastes, and human sewage. The rivers were also swelled by high spring rains and snowmelt runoff that may have aided the transport of upstream *Cryptosporidium* oocysts into the lake near the water intakes.

The *Journal of the American Water Works Association* has published numerous articles on protozoa contamination of drinking water supplies. Crockett and Haas (1997) describe a watershed investigation to identify sources of *Giardia* and *Cryptosporidium* in the Philadelphia watershed. They describe the difficulties associated with monitoring *Cryptosporidium* and *Giardia* in surface waters because of low analytical recoveries and the cost of analyses. Large variations in observed protozoa concentrations made it difficult to identify major sources during the preliminary stages of their investigations. They do expect that wastewater treatment plant discharges are a major local source, although animals (especially calves and lambs) are likely significant contributors. Combined sewer overflows had *Giardia* levels similar to raw sewage, but the CSOs had much less *Cryptosporidium* than the raw sewage. LeChevallier et al. (1997) investigated *Giardia* and *Cryptosporidium* in open reservoirs storing finished drinking water. This gave them an opportunity to observe small increases in oocyst concentrations associated from nonpoint sources of contamination from the highly controlled surrounding area. They observed significantly larger oocyst concentrations at the effluent (median values of 6.0 *Giardia*/100 L and 14 *Cryptosporidium*/100 L) in the reservoirs than in the influents (median values of 1.6 *Giardia*/100 L and 1.0 *Cryptosporidium*/100 L). No human wastes could influence any of the tested reservoirs, and the increases were therefore likely caused by wastes from indigenous animals or birds, either directly contaminating the water or through runoff from the adjacent wooded areas.

A Management Training Audioconference Seminar on *Cryptosporidium* and Water (MTA 1997) was broadcast in May of 1997 to familiarize state and local agencies about possible *Cryptosporidium* problems that may be evident as a result of the EPA's Information Collection Rule which began in July of 1997. This regulation requires all communities serving more than 100,000 people to monitor their source water for *Cryptosporidium* oocysts. If the source water has more than 10 *Cryptosporidium* oocysts/L, the finished water must also be monitored. It is likely that many source waters will be found to be affected by *Cryptosporidium*. The researchers reviewed one study that found the percentage of positive samples of *Cryptosporidium* in lakes, rivers, and springs was about 50 to 60% and about 5% in wells. In contrast, the percentage of samples testing positive for *Giardia* was about 10 to 20% in lakes and rivers, and very low in springs and wells.

Special human health concerns have also been recently expressed about *Pfiesteria piscicida*, a marine dinoflagellate that is apparently associated with coastal eutrophication caused by runoff nutrients (Maguire and Walker 1997). Dramatic blooms and resulting fish kills have been associated with increased nutrient loading from manure-laden runoff from large livestock feedlot operations. This organism has garnered much attention in the popular press, usually called the "cell from hell" (Zimmerman 1998). It has been implicated as causing symptoms of nausea, fatigue, memory loss, and skin infections in south Atlantic coastal bay watermen. *Pfiesteria* and *Pfiesteria*-like organisms have also been implicated as the primary cause of many major fish kills and fish disease events in Virginia, Maryland, North Carolina, and Delaware. In August 1997, hundreds of dead and dying fish were found in the Pocomoke River, near Shelltown, MD, in the Chesapeake Bay, prompting the closure of a portion of the river. Subsequent fish kills and confirmed occurrences of *Pfiesteria* led to further closures of the Manokin and Chicamacomico Rivers. The Maryland Department of Health and Mental Hygiene also presented preliminary evidence that adverse public health effects could result from exposure to the toxins released by *Pfiesteria* and *Pfiesteria*-like organisms. The

increasing numbers of fish kills of Atlantic menhaden (an oily, non-game fish) motivated Maryland's governor to appoint a Citizens *Pfiesteria* Action Commission. The commission convened a forum of noted scientists to examine the existing information on *Pfiesteria.* The results of the State of Maryland's *Pfiesteria* monitoring program are available on the Maryland Department of Natural Resources' Web site: http://www.dnr.state.md.us/pfiesteria/.

Pfiesteria has a complex life cycle, including at lease 24 flagellated, amoeboid, and encysted stages. Only a few of these stages appear to be toxic, but their complex nature makes them difficult to identify by non-experts (Maguire and Walker 1997). *Pfiesteria* spends much of its life span in a nontoxic predatory form, feeding on bacteria and algae, or as encysted dormant cells in muddy sediment. Large schools of oily fish (such as the Atlantic menhaden) trigger the encysted cells to emerge and excrete toxins. These toxins make the fish lethargic, so the fish remain in the area where the toxins attack the fish skin, causing open sores to develop. The *Pfiesteria* then feed on the sloughing fish tissue. Unfortunately, people working in the water during these toxin releases may also be affected (Zimmerman 1998).

Researchers suggest that excessive nutrients (causing eutrophication) increase the algae and other organic matter that the *Pfiesteria* and Atlantic menhaden use for food. The increased concentrations of *Pfiesteria* above natural background levels increase the likelihood of toxic problems. Maguire and Walker (1997) state that other factors are also apparently involved, including stream hydraulics, water temperature, and salinity. They feel that *Pfiesteria* is only one example of the increasing threats affecting coastal ecosystems that are experiencing increased nutrient levels. Most of the resulting algal blooms only present nuisance conditions, but a small number can result in human health problems (mostly as shellfish poisonings). The increased nutrient discharges are mostly associated with agricultural operations, especially animal wastes from large poultry and swine operations. In the Pocomoke River watershed, the Maryland Department of Natural Resources estimates that about 80% of the phosphorus and 75% of the nitrogen load is from agricultural sources. Urban runoff may also be a causative factor of eutrophication in coastal communities, especially those having small enclosed coastal lagoons or embayments, or in rapidly growing urban areas. Zimmerman (1998) points out that the Chesapeake Bay area is one of the country's most rapidly growing areas, with the population expected to increase by 12% by the year 2010.

Viruses

It is believed that approximately half of all waterborne diseases are of viral origin. Unfortunately, it is very difficult and time-consuming to identify viruses from either environmental samples or sick individuals. When the EPA conducted its extensive epidemiological investigations of freshwater and marine swimming beaches in the 1980s, two viruses common to human gastrointestinal tracts (coliphage and enterovirus) were evaluated as potential pathogen indicators. These two indicators did not show good correlations between their presence and the incidence of gastroenteritis. Viruses tend to survive for slightly longer periods in natural waters than do Gram-negative bacteria. It is believed that the high correlation observed between gastroenteritis and the presence of enterococci may be because the Gram-positive enterococci's longer survival more closely mimics viral survival. Therefore, enterococci may serve as a good recreational water indicator for the presence of viral pathogens.

RECEIVING WATER EFFECT SUMMARY

Recent studies have combined chemical-physical characterizations of water and sediment with biosurveys and laboratory/*in situ* toxicity surveys (low and high flow) to effectively characterized major water column and sediment stressors (Burton and Rowland 1999; Burton et al. 1998; Dyer and White 1996; Burton and Moore 1999). Suspended solids, ammonia, sediments, temperature,

PAHs, and/or stormwater runoff were observed to be primary stressors in these test systems. These primary stressors could not have been identified without low and high flow and sediment quality assessments both in the laboratory and field. It is apparent that to determine the role of chemicals as stressors in the receiving waters, the role of other stressors (both natural and anthropogenic) must be assessed (see also Chapters 6 and 8).

Johnson et al. (1996) and Herricks et al. (1996a,b) describe a structured tier testing protocol to assess both short-term and long-term wet-weather discharge toxicity. The protocol recognizes that the test systems must be appropriate to the time-scale of exposure during the discharge. Therefore, three time-scale protocols were developed, for intra-event, event, and long-term exposures.

There is a natural tendency in the popular "weight-of-evidence" or "sediment quality triad" approaches to look for "validation" of one assessment tool with another (see also Chapters 6 and 8). For example, matching a toxic response in a WET test with that of an impaired community gives a greater weight of evidence. This does not, however, necessarily "validate" the results (or invalidate, if there are differences) (Chapman 1995). Natural temporal changes in aquatic populations at different sites within a study system need not be the same (Power et al. 1988; Resh 1988; Underwood 1993); therefore, predictions of effect or no-effect from WET testing of reference sites may be in error. Each monitoring tool (i.e., chemical, physical, and indigenous biota characterizations, laboratory and field toxicity, and bioaccumulation) provides unique and often essential information (Burton 1995; Chapman et al. 1992; Burton et al. 1996; Baird and Burton 2001). If the responses of each of the biological tools disagree, it is likely due to species differences or a differing stressor exposure dynamic/interaction. These critical exposures issues can be characterized through a systematic process of separating stressors and their respective dynamics into low and high flow and sediment compartments using both laboratory and field exposures. Then, a more efficient and focused assessment can identify critical stressors and determine their ecological significance with less uncertainty than the more commonly used approaches. The chronic degradation potential of complex ecosystems receiving multiple stressors cannot be adequately evaluated without a comprehensive assessment that characterizes water, sediment, and biological dynamics and their interactions.

Because most sites have multiple stressors (physical, chemical, and biological), it is essential that the relative contributions of these stressors be defined to design effective corrective measures. The integrated laboratory and field approach rigorously defines the exposures of organisms (media of exposure and contaminant concentration), separating it into overlying water, surficial sediment, historical sediment, and interstitial water. The degree of contaminant-associated toxicity can best be assessed using a combination of laboratory and field screening methods which separate stressors (i.e., a Stressor Identification Evaluation (SIE) approach) (Burton et al. 1996), into different, major stressor categories, including metals, nonpolar organics, photoinduced toxicity from PAHs, ammonia, suspended solids, predators, dissolved oxygen, and flow. There is much research to be done to refine these approaches, but the tools are there to make ecologically relevant assessments of aquatic ecosystem contamination with reasonable certainty.

The effects of urban runoff on receiving water aquatic organisms or other beneficial uses is also very site specific. Different land development practices may create substantially different runoff flows. Different rain patterns cause different particulate washoff, transport, and dilution conditions. Local attitudes also define specific beneficial uses and desired controls. There are also a wide variety of water types receiving urban and agricultural runoff, and these waters all have watersheds that are urbanized to various degrees. Therefore, it is not surprising that runoff effects, though generally dramatic, are also quite variable and site specific.

Previous attempts to identify runoff problems using existing data have not generally been conclusive because of differences in sampling procedures and the common practice of pooling data from various sites or conditions. It is therefore necessary to carefully design comprehensive, long-term studies to investigate runoff problems on a site-specific basis. Sediment transport, deposition, and chemistry play key roles in receiving waters and need additional research. Receiving water

aquatic biological conditions, especially compared to unaffected receiving waters, should be studied in preference to laboratory bioassays.

These specific studies need to examine beneficial uses directly, and not rely on published water quality criteria and water column measurements alone. Published criteria are usually not applicable to urban runoff because of the sluggish nature of runoff and the unique chemical speciation of its components.

The long-term aquatic life effects of runoff are probably more important than short-term effects associated with specific events. The long-term effects are probably related to the deposition and accumulation of toxic sediments, or the inability of the aquatic organisms to adjust to repeated exposures to high concentrations of toxic materials or high flow rates.

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UNIT 2

Components of the Assessment

CHAPTER 4

Overview of Assessment Problem Formulation

"If the Lord Almighty had consulted me before embarking on the Creation, I would have recommended something simpler."

Alfonso X of Castile (Alfonso the Wise), 1221–1284

CONTENTS

INTRODUCTION

This chapter summarizes various approaches that have been used and recommended for evaluating receiving water effects. It outlines a reasonable method that allows the study designer to consider many factors that may affect the outcome of the project. Major study approaches are presented with extensive case study examples. The chapters and appendices in this book complement this material by providing guidance for developing an experimental design, methods for the collection of samples and their analysis, various other field evaluation efforts, and the statistical analysis of the data.

Rationale for an Integrated Approach to Assessing Receiving Water Problems

During the past decade, it has become apparent from numerous water and sediment quality assessment studies that no one single approach (e.g., chemical-specific criteria) can be routinely used to accurately determine or predict ecosystem health and beneficial use impairment. In Ohio, evaluation of indigenous biota showed that many of the impaired stream segments could not be detected using chemical criteria alone (EPA 1990b). In an intensive survey, 431 sites in Ohio were assessed using in-stream chemical and biological surveys. In 36% of the cases, chemical evaluations implied no impairment, but the biological survey evaluations did show impairment. In 58% of the cases the chemical and biological assessments agreed. Of these, 17% identified waters with no impairment, while 41% identified waters which were considered impaired. Realization of the inadequacy of nationwide criteria prompted the EPA to look for other site-specific criteria modifications. Numerous studies of bulk sediment contaminant concentrations failed to show significant correlations with toxic effects to test species (Burton 1991).

Each assessment approach or component has associated strengths and weaknesses (Table 4.1). The ultimate objective of the CWA (Sec. 101(a)) is "to restore and maintain the chemical, physical,

Table 4.1 Components of an Integrated Approach to Assess Receiving Water Quality

Control Approach	What It Provides	What It Doesn't Provide:
Chemical specific	Human health protection	All toxics present
	Complete toxicology	Bioavailability
	Straightforward treatability	Interactions of mixtures (e.g., additivity)
	Familiarity with control	Poor trend analysis
	Persistency coverage	Accurate toxicology (false assumptions)
	Regulatory ease	Actual and direct evaluations of receiving water beneficial use impairments
Toxicity	Aggregate toxicity	Human health protection
	All toxicants present	Complete toxicology (few species may be tested)
	Bioavailability	Simple treatability
	Accurate toxicology	Persistency coverage
	Good trend analysis	
	Lab or *in situ* testing	
Bioassessments	Actual receiving water effects	Critical flow effects
	Trend analysis	Straightforward interpretation of results
	Severity of impact	Cause of impact
	Total effect of all sources	Differentiation of sources
		Habitat and site variation influence

Modified from EPA. Wisconsin legislature establishes a nonpoint pollution committee. *Nonpoint Source EPA News-Notes.* #8. October 1990a.

and biological integrity of the Nation's waters." These three components define the overall ecological integrity of an aquatic ecosystem (EPA 1990a). Pollutant loadings into receiving waters from point and nonpoint sources vary in magnitude, frequency, duration, and type. They are also strongly influenced by meteorological and hydrologic conditions, terrestrial processes, and land use activities.

A myriad of potential stressor combinations are possible in waters that are in human-dominated watersheds. In the laboratory, it would be impossible to evaluate even a small number of the possible stressor combinations, varying the magnitude, frequency, and duration of each stressor. Traditional bioassay methods simply look at one simple exposure scenario. Chemical criteria provide a benchmark from which to evaluate the significance of contaminant concentrations and direct further monitoring resources. Biological assessments indicate if the aquatic community is of a pollution- and/or habitat-tolerant or sensitive nature by showing the effect of long-term exposures. By considering habitat influence and comparing to reference sites, evaluations of ecological integrity (health) can be made. Habitat (physical) evaluations are essential to separate point source and nonpoint source toxicity effects from physical effects. As an example, some NPS pollution effects from stormwater may be of a physical nature, such as habitat alteration and destruction from increased stream flow, increased suspended and bedload sediments, or elevated water temperatures. In addition, a fourth major assessment component (toxicity) is needed beyond the three components of chemical, physical, and biological integrity (EPA 1990a). Biosurvey data may not detect subtle, short-term, or recent toxic effects due to the natural variation (spatial and temporal) that occurs in aquatic communities. Toxicity testing also removes the effects of habitat problems relatively well, focusing on the availability of chemical contaminants alone. The EPA (1990a) states that when any assessment approach (i.e., chemical-specific, toxicity, or biosurvey) shows water quality standards not being achieved, regulatory action should be taken.

The complexity of ecosystems dictates that these assessment tools be used in an integrated fashion. Scientists in any of the traditional disciplines (such as chemistry, microbiology, ecology, limnology, oceanography, hydrology, agronomy) are quick to point out the multitude of ecosystem complexities associated with their science. Many of these complexities influence chemical fate and effects and, more importantly, affect natural and anthropogenic stressor fate and effects. For example, it is well documented that many natural factors may act as significant stressors to organisms in aquatic systems, including light, temperature, flow, dissolved oxygen, sediment particle size, suspended solids, habitat quality, ammonia, salinity, food quality and quantity, predators, parasites, and pathogens. In addition, ecotoxicologists have long been aware of the differences between species and their life stages in regard to toxicant sensitivity. Unfortunately, toxicity information exists only for a fraction of the 1.5 to 100 million species (Wilson 1992; May 1994) and 7 million chemicals (U.S. General Accounting Office 1994) in the world. This reality makes extrapolations between species and chemicals tenuous at best. Despite these many and often interacting complexities, some excellent and proven tools exist for conducting ecologically relevant assessments of contamination.

The necessity of using each of the above assessment components and the degree to which each is utilized is a site-specific issue. At sites of extensive chemical pollution, extreme habitat destruction, or absence of desirable aquatic organisms, the impact can be clearly established with only one or two components, or simply qualitative measures. However, at most study sites, there will be "gray" areas where the ecosystem's integrity (quality) is less clear and should be measured via multiple components, using a weight-of-evidence approach to evaluate adverse effects.

WATERSHED INDICATORS OF BIOLOGICAL RECEIVING WATER PROBLEMS

The EPA (1996) published a list of 18 indicators to track the health of the nation's aquatic ecosystems. These indicators are intended to supplement conventional water quality analyses in compliance-monitoring activities. The use of broader indicators of environmental health is increasing. As an example, by 1996, 12 states were using biological indicators and 27 states were

developing local biological indicators, according to Pelley (1996). Because of the broad nature of the nation's potential receiving water problems, this list is more general than typically used for any one specific discharge type (such as stormwater, municipal wastewaters, or industrial wastewaters). These 18 indicators are (EPA 1996):

1. Population served by drinking water systems violating health-based requirements
2. Population served by unfiltered surface water systems at risk from microbiological contamination
3. Population served by community drinking water systems exceeding lead action levels
4. Drinking water systems with source water protection programs
5. Fish consumption advisories
6. Shellfish-growing waters approved for harvest for human consumption
7. Biological integrity of rivers and estuaries
8. Species at risk of extinction
9. Rate of wetland acreage loss
10. Designated uses: drinking water supply, fish, and shellfish consumption, recreation, aquatic life
11. Groundwater pollutants (nitrates)
12. Surface water pollutants
13. Selected coastal surface water pollutants in shellfish
14. Estuarine eutrophication conditions
15. Contaminated sediments
16. Selected point source loadings to surface water and groundwater
17. Nonpoint source sediment loadings from cropland
18. Marine debris

In one example of the use of watershed indicators, Claytor (1996, 1997) summarized the approach developed by the Center for Watershed Protection as part of its EPA-sponsored research for assessing the effectiveness of stormwater management programs (Claytor and Brown 1996). The indicators selected are direct or indirect measurements of conditions or elements that indicate trends or responses of watershed conditions to stormwater management activities. Categories of these environmental indicators are shown in Table 4.2, ranging from conventional water quality measurements to citizen surveys. Biological and habitat categories are also represented. Table 4.3 lists 26 indicators, by category. It was recommended that appropriate indicators be selected from each category for a specific area under study. This will enable a better understanding of the linkage of what is done on the land, how the sources are regulated or managed, and the associated receiving water problems. The indicators were selected to (1) measure stress or the activities that lead to

Table 4.2 Stormwater Indicator Categories

Category	Description	Principal Element Being Assessed
Water quality	Specific water quality characteristics	Receiving water quality
Physical/hydrologic	Measure changes to, or impacts on, the physical environment	Receiving water quality
Biological	Use of biological communities to measure changes to, or impacts on, biological parameters	Receiving water quality
Social	Responses to surveys or questionnaires to assess social concerns	Human activity on the land surface
Programmatic	Quantify various nonaquatic parameters for measuring program activities	Regulatory compliance or program initiatives
Site	Indicators adapted for assessing specific conditions at the site level	Human activity on the land surface

From Claytor, R.A. An introduction to stormwater indicators: urban runoff assessment tools. Presented at the *Assessing the Cumulative Impacts of Watershed Development on Aquatic Ecosystems and Water Quality* conference. March 20–21, 1996. Northeastern Illinois Planning Commission. pp. 217–224. Chicago, IL. April 1997.

Table 4.3 Environmental Indicators

Indicator Category	Indicator Name
Water quality indicators	Water quality pollutant constituent monitoring
	Toxicity testing
	Nonpoint source loadings
	Exceedance frequencies of water quality standards
	Sediment contamination
	Human health criteria
Physical and hydrologic indicators	Stream widening/downcutting
	Physical habitat monitoring
	Impacted dry-weather flows
	Increased flooding frequency
	Stream temperature monitoring
Biological indicators	Fish assemblage
	Macroinvertebrate assemblage
	Single species indicator
	Composite indicators
	Other biological indicators
Social indicators	Public attitude surveys
	Industrial/commercial pollution prevention
	Public involvement and monitoring
	User perception
Programmatic indicators	Illicit connections identified/corrected
	BMPs installed, inspected, and maintained
	Permitting and compliance
	Growth and development
Site indicators	BMP performance monitoring
	Industrial site compliance monitoring

From Claytor, R.A. An introduction to stormwater indicators: urban runoff assessment tools. Presented at the *Assessing the Cumulative Impacts of Watershed Development on Aquatic Ecosystems and Water Quality* conference. March 20–21, 1996. Northeastern Illinois Planning Commission. pp. 217–224. Chicago, IL. April 1997.

impacts on receiving waters, (2) assess the resource itself, and (3) measure the regulatory compliance or program initiatives. Claytor (1997) presented a framework for using stormwater indicators that is similar to many others recommended in hazard and risk assessment, as shown below:

Level 1 (Problem Identification):

1. Establish management sphere (who is responsible, other regulatory agencies involved, etc.).
2. Gather and review historical data.
3. Identify local uses that may be impacted by stormwater (flooding/drainage, biological integrity, noncontact recreation, drinking water supply, contact recreation, and aquaculture).
4. Inventory resources and identify constraints (time frame, expertise, funding and labor limitations).
5. Assess baseline conditions (use rapid assessment methods).

Obviously, the selection of the indicators to assess the baseline conditions should be based on the local uses of concern. Most of the anticipated important uses are shown to require indicators selected for each of the categories. However, the indicator selection process requires more than just a beneficial use consideration. Additional issues, such as the questions being asked, regulatory and societal concerns, the characteristics of the ecoregion, sensitive and threatened indigenous species, resource availability, and time constraints, are also important considerations.

Claytor (1997) also recommends a Level 2 assessment strategy for examining the local management program as outlined below:

Level 2:

1. State goals for program (based on baseline conditions, resources, and constraints)
2. Inventory prior and ongoing efforts (including evaluating the success of ongoing efforts)
3. Develop and implement management program
4. Develop and implement monitoring program (more quantitative indicators than typically used for the Level 1 evaluations above)
5. Assess indicator results (does the stormwater indicator monitoring program measure the overall watershed health?)
6. Reevaluate management program (update and revise management program based on measured successes and failures)

While the approach and recommendations of Claytor (1997) have merit and provide a good overall framework, they may not adequately consider all the important study design issues for every specific area. Most important, their indicator guidance for determining receiving water effects from stormwater runoff may not provide a characterization of all the important stressors. For example, short-term pulses of polycyclic aromatic hydrocarbons from roadways and parking lots may be creating photoinduced toxicity problems not detected by traditional bioassessment approaches.

Another example of the effective use of environmental indicators is in the Detroit, MI, area. Cave (1998) described how they are being used to summarize the massive amounts of data being generated by the Rouge River National Wet Weather Demonstration Project in Wayne County. This large project is examining existing receiving water problems, the performance of stormwater and CSO management practices, and receiving water responses in a 438 mi^2 watershed having more than 1.5 million people in 48 separate communities. The baseline monitoring program has now more than 4 years of continuous monitoring of flow, pH, temperature, conductivity, and DO, supplemented by automatic sampling for other water quality constituents, at 18 river stations. More than 60 projects are examining the effectiveness of stormwater management practices, and 20 projects are examining the effectiveness of CSO controls, each also generating large amounts of data. Toxicants are also being monitored in sediment, water, fish tissue, and with semipermeable membranes to help evaluate human health and aquatic life effects. Habitat surveys were conducted at 83 locations along more than 200 miles of waterway. Algal diversity and benthic macroinvertebrate assessments were also conducted at these survey locations. Electrofishing surveys were conducted at 36 locations along the main river and in tributaries. Several computer models were also used to predict sources, loadings, and wet-weather flow management options for the receiving waters and for the drainage systems. A geographic information system was used to manage and provide spatial analyses of the massive amounts of data collected. However, there was still a great need to simplify the presentation of the data and findings, especially for public presentations. Cave described how they developed a short list of 35 indicators, based on the list of 18 from EPA and on discussions with state and national regulatory personnel. They then developed seven indices that could be color-coded and placed on maps to indicate areas of existing problems and projected conditions based on alternative management scenarios. These indices are described as follows:

Condition Quality Indicators:

1. Dissolved oxygen. Concentration and % saturation values (ecologically important)
2. Fish consumption index. Based on advisories from the Michigan Department of Public Health
3. River flow. Significant for aquatic habitat and fish communities
4. Bacteria count. *E. coli* counts based on Michigan Water Quality Standards, distinguished for wet and dry conditions

Multifactor Indices:

1. Aquatic biology index. Composite index based on fish and macroinvertebrate community assessments (populations and individuals)

2. Aquatic habitat index. Habitat suitability index, based on substrate, cover, channel morphology, riparian/bank condition, and water quality
3. Aesthetic index. Based on water clarity, color, odor, and visible debris

These seven indicators represent 30 physical, chemical, and biological conditions that directly impact the local receiving water uses (water contact recreation, warm water fishery, and general aesthetics). Cave presented specific descriptions for each of the indices and gave examples of how they are color-coded for map presentation. These data presentations have clearly demonstrated how the Rouge River is degraded in specific areas and show the relationships of these critical river areas with adjacent watershed activities.

SUMMARY OF ASSESSMENT TOOLS

Almost all states using bioassessment tools have relied on the EPA reference documents as the basis for their programs. Common components of these bioassessment programs (in general order of popularity) include:

- Macroinvertebrate surveys (almost all programs, but with varying identification and sampling efforts)
- Habitat surveys (almost all programs)
- Some simple water quality analyses
- Some watershed characterizations
- Few fish surveys
- Limited sediment quality analyses
- Limited stream flow analyses
- Hardly any toxicity testing
- Hardly any comprehensive water quality analyses

Normally, numerous metrics are used, typically only based on macroinvertebrate survey results, which are then assembled into a composite index. Many researchers have identified correlations between these composite index values and habitat conditions. Water quality analyses in many of these assessments are seldom comprehensive, a possible overreaction to conventional, very costly programs that have typically resulted in minimally worthwhile information. This book recommends a more balanced assessment approach, using toxicity testing and carefully selected water and sediment analyses to supplement the needed biological and habitat monitoring activities. A multi-component assessment enables a more complete evaluation of causative factors and potential mitigation approaches.

STUDY DESIGN OVERVIEW

The study design must be developed based on the study objectives, preliminary site-problem assessments, regulatory mandates, and available resources. This chapter includes detailed information for developing the experimental design aspects of the study design. Many of the typical monitoring subcomponents of each approach are listed in Table 4.4. All of these parameters cannot realistically be evaluated in routine water quality assessments. The amount and type of monitoring hinges not only on the above issues but the degree of confidence and accuracy expected from the results. This issue falls under the Data Quality Objectives process and is also discussed in later chapters.

The most commonly used test hypotheses in assessing receiving water impacts is that the designated use or integrity of the water body is not impaired (null hypothesis), or the alternative hypotheses that it or some component is impaired or some specific factor (e.g., stormwater) is

Table 4.4 Summary of Recommended Aquatic Ecosystem Assessment Parameters

Physical Evaluations	Chemical Evaluations	Indigenous Biota Evaluations	Toxicity Evaluations
In-stream characteristics	Dissolved oxygen (W)	Biological inventory	Acute/Short-term
Size (mean width/depth)	Toxicants (WS)	(Existing Use Analysis):	Chronic
Flow/velocity	Nutrients (W)	Fish	Responses(WS):
Total volume	Nitrogen	Macroinvertebrates	Fish (*Pimephales promelas*)
Reaeration rates	Phosphorus	Microinvertebrates	Zooplankton (*Ceriodaphnia dubia*)
Gradient/pools/riffles	Biochemical oxygen demand (W)	Phytoplankton	Benthic macroinvertebrates (*Selenastrum capricornutum*)
Temperature	Sediment oxygen demand (S)	Macrophytes	Other (microbial, protozoan, macrophytes, amphibian, or indigenous species)
Suspended solids	Conductivity/salinity(W)	Biological Condition/Health	
Sedimentation	Hardness (W)	Analysis:	
Channel modifications	Alkalinity (W)	Diversity indices	
Channel stability	pH (WS)	HIS models	
Substrate composition and characteristics	Temperature (W)	Tissue analysis	
Particle size distribution	Dissolved solids (W)	Recovery index	
Sediment dry weight	Total organic carbon (S)	Intolerant species	
Channel debris	Acid volatile sulfides (S)	Omnivore-carnivore analysis	
Sludge deposits	Ammonia (WS)	Biological potential analysis	
Riparian characteristics		Reference reach comparison	
Downstream characteristics			

W = Water

S = Sediment

causing impairment. To detect differences between ambient and/or reference (nonimpacted) conditions in an aquatic system and the test system, it is important to establish the appropriate level of sensitivity. A 5% difference in condition or integrity is more difficult to detect than a 50% difference. The level of detection needs to be predetermined to establish the sample size (see Chapter 5).

A thorough assessment of ecosystem impact, hazard, or risk may follow the general approach proposed by EPA for ecological risk assessments. The toxicity assessment process consists of identifying the stressors (hazards), using various measurement endpoints to determine concentration (exposure)–response gradients, and then characterizing the stressor–effect level (threshold) and degree of impact, hazard, or risk that exists so that management decisions regarding remediation (corrective action) can be made. The impact characterization step is the most difficult given the many natural and anthropogenic unknowns, such as spatial and temporal variation; chemical fate, effects, and interactions through time and food webs; and biotic and abiotic patch interactions. For these reasons, the weight-of-evidence approach is the most reliable, as discussed in Chapter 8. The most effective use of resources in routine stormwater assessments is via a tiered monitoring approach (see also Chapter 8).

BEGINNING THE ASSESSMENT

Designing and implementing an assessment study requires careful and methodical planning to ensure that the study objectives will be accomplished. The preceding section described the watershed indicator approach recommended by Claytor (1996, 1997) and the EPA. The following sections in this chapter will provide additional critical considerations, approach details, and method options for conducting receiving water impact assessments.

The main objectives of most environmental monitoring studies may be divided into two general categories: characterization and/or comparisons. Characterization pertains to quantifying a few simple attributes of the parameter of interest. As an example, the concentration of copper in the

sediment near an outfall may be of concern. The important question would be, "What is the most likely concentration of the copper?" Other questions of interest include changes in the copper concentrations between surface deposits and buried deposits, or in upstream vs. downstream locations. These additional questions are considered in the second category, namely, comparisons. Other comparison questions may relate to comparing the observed copper concentrations with criteria or standards. Finally, many researchers would also be interested in quantifying trends in the copper concentrations. This extends beyond the above comparison category, as trends usually consider more than just two locations or conditions. Examples of trend analyses would examine copper gradients along the receiving stream, or trends of copper concentrations with time. Another type of analysis related to comparisons is the identification of hot spots, where the gradient of concentrations in an area is used to identify areas having unusually high concentrations.

An adequate experimental design enables a researcher to efficiently investigate a study hypothesis. The results of the experiments will theoretically either prove or disprove the hypothesis. In reality, the experiments will tend to shed some light on the real problem and will probably result in many more questions that need addressing. In many cases, the real question may not have even been recognized initially. Therefore, even though it is very important to have a study hypothesis and appropriate experimental design, it may be important to reserve enough study resources to enable additional unanticipated experiments. In this discussion, sampling plans and specific statistical tools will be briefly examined.

Experimental design covers several aspects of a monitoring program. The most important aspect of an experimental design is being able to write down the study objectives and why the data are needed. The quality of the data (accuracy of the measurements) must also be known. Allowable errors need to be identified based on how the information will change a conclusion. Specifically, how sensitive are the data that are to be collected in defining the needed answer? A logical experimental process that can be used to set up an assessment of receiving waters consists of several steps:

1. Establish clear study objectives and goals (hypothesis to be tested, calibration of equation or model to be used, etc.).
2. Assess initial site assessment and identify preliminary problem.
3. Review historical site data. Collect information on the physical conditions of the system to be studied (watershed characteristics, etc.), estimate the time and space variabilities of the parameters of interest (assumed, based on prior knowledge, or other methods).
4. Formulate a conceptual framework (e.g., the EPA ecological risk framework) and model.
5. Determine optimal assessment parameters. Determine the sampling plan (strata and relationships that need to be defined), including the number of samples needed (when and where, within budget restraints).
6. Establish data quality objectives (DQO) and procedures needed for QA/QC during sample collection, processing, analysis, data management, and data analyses.
7. Locate sampling sites.
8. Establish field procedures, including the sampling specifics (volumes, bottle types, preservatives, samplers to be used, etc.).
9. Review QA/QC issues.
10. Construct data analysis plan by determining the statistical procedures that will be used to analyze the data (including field data sheets and laboratory QA/QC plan).
11. Implement the study.

Preliminary project data obtained at the beginning of the project should be analyzed to verify assumptions used in the experimental design process. However, one needs to be cautious and not make major changes until sufficient data have been collected to verify new assumptions. After the data have been analyzed and evaluated, it is likely that follow-up monitoring should be conducted to address new concerns uncovered during the project.

Table 4.5 Principles for Designing Successful Environmental Studies

1. State concisely to someone what question you are asking. Your results will be as coherent and as comprehensible as your initial conception of the problem.
2. Take replicate samples within each combination of time, location, and any other controlled variable. Differences between groups can only be demonstrated by comparison to differences within groups.
3. To test whether a condition has an effect, collect samples both where the condition is present and where the condition is absent (reference site) but all else is the same. An effect can only be demonstrated by comparison with a control.
4. Carry out some preliminary sampling to provide a basis for evaluation of sampling design and statistical analysis options. Deleting this step to save time usually results in losing time.
5. Verify that the sampling device or method is sampling the population it should be sampling, and with equal and adequate efficiency over the entire range of sampling conditions to be encountered. Variation in efficiency of sampling from area to area biases among-area comparisons.
6. If the area to be sampled has a large-scale environmental pattern, break the area up into relatively homogeneous subareas and allocate samples to each in proportion to the size of the subarea. If it is an estimate of total abundance over the entire area that is desired, make the allocation proportional to the number of organisms in the subarea.
7. Verify that the sample unit size is appropriate to the size, densities, and spatial distributions of the organisms being sampled. Then estimate the number of replicate samples required to obtain the needed precision.
8. Test the data to determine whether the error variation is homogeneous, normally distributed, and independent of the mean. If it is not, as will be the case for most field data, then (a) appropriately transform the data, (b) use a distribution-free (nonparametric procedure, (c) use an appropriate sequential sampling design, or (d) test against simulated H_0 data.
9. Having chosen the best statistical method to test the hypothesis, stick with the result. An unexpected or undesired result is *not* a valid reason for rejecting the method and searching for a "better" one.

Green, R.H. *Sampling Design and Statistical Methods for Environmental Biologists*. John Wiley & Sons, New York. 1979.

Most of the first six of these elements are described in this chapter, while the remaining ones are included in the later chapters. If any of these process components are inadequately addressed, the study outputs may not achieve the necessary study goals and/or may lead to erroneous conclusions. An early paper by Green (1979) lists principles (Table 4.5) that are still valid for preparing environmental study designs.

Specific Study Objectives and Goals

The study objectives and goals should be clearly defined, addressing ecosystem characterization and protection concerns and also the role of the assessment in the decision-making process for managing the particular problem. There are four primary reasons for an assessment program: planning, research or design, control and process optimization, and corrective action/regulation. The overall scope of planning studies is often general, while the other program types are more specific in nature. Study goals may range from establishing trends or background levels to optimizing control design or even enforcement actions. Once the objectives are defined, the needed sensitivity of the evaluation can be determined in the DQO process.

Initial Site Assessment and Problem Identification

It is essential that a reconnaissance survey be conducted or an individual who has previously studied the site be included in the design process. A substantial degree of qualitative site characterization information is gained through this process and cannot be acquired through reading report descriptions. These preliminary studies should be conducted by personnel with expertise in evaluating pollution effects on aquatic ecosystems. The preliminary survey should focus on several watershed characteristics (Table 4.6) that will need to be addressed in the study design and final assessment. Most of these factors are interwoven in a cause–effect relationship, but will often affect the study design and field methods as separate, influencing components. As an example, the most

Table 4.6 Stream Assessment Factors for Nonpoint Source-Affected Streams

Watershed development factor	Imperviousness of contributing watershed and drainage efficiency of land use. Watershed area. Age of development. Nature of upstream land use. Percent forest cover. Pollutant (NPS and PS) input locations and dynamics.
Best management practice	Proportion of contributing watershed effectively controlled by a proposed BMP or retrofit. Type and performance of BMP.
Hydrologic change factor	Drainage efficiency (such as pre- vs. post-development runoff coefficients and times of concentrations). Dry-weather flow rate in modified vs. reference watershed. Frequent return period flows and associated channel dimensions.
Channel form/stability factor	Natural, eroded, open, lined, protected or enclosed channel form. Dry-weather wetted perimeter vs. reference watershed. Evidence of widening or downcutting. Bedrock controlled channel. Consolidated or unconsolidated banks. Channel gradient.
Substrate quality factor	Median diameter or bed sediment. Degree of embeddedness. Reference substrate in undeveloped stream. Existing and future disturbed areas. Evidence of shifting sand bars, discolored cobbles.
Water quality factor	Summer maximum temperature. Benthic algal growth. Organic slime on rocks. Silt and sand deposits in stream. Presence/absence of point source discharge or pipes along stream. Type and height of debris jams. Discolored or black rocks upon turning. Dry-weather water velocity.
Stream community factor	Reference macroinvertebrate and fish species expected. Evidence of benthic algae or leaf processing. Rock turning or kick sampling. Cold, cool, or warm water community.
Refugia factor	Presence of refuge habitats allowing species escape and reintroduction.
Riparian cover factor	Presence or absence of riparian canopy cover over stream. Width of buffer 2½ H max. Is vegetation stabilizing banks?
Stream reach factor	Presence or absence of pool and riffle structure. Minimum dry-weather flow. Sinuosity of channel. Open or closed to fish migration. Creation of linear barrier across stream.
Contiguous wetland factor	Presence or absence of nontidal wetlands in riparian, floodplain, or BMP zone. Quality, area, and function of wetlands present. Downstream wetlands to be affected?
Floodplain change factor	Constrained or unconstrained floodplain. Extent of ultimate flood plain. Property in floodplain.
Receiving water target factor	Are there any unique watershed water quality targets in a downstream river, lake, or estuary?

Modified from Schueler, T.R. *Controlling Urban Runoff: A Practical Manual for Planning and Designing Urban BMPs*. Department of Environmental Programs. Metropolitan Washington Council of Governments. Water Resources Planning Board. 1987.

important factors at the root of most nonpoint source pollution-related problems include watershed development characteristics whether of an urban, agricultural, or silviculture nature. Therefore, the preliminary problem identification process should begin with observations on the type, number, size, and location of point source discharges, stormwater inputs, upstream land use drainage patterns, and combined sewer overflows (CSOs).

A reference watershed should be located in the same type of ecoregion, but which has an undeveloped (unimpacted) watershed of a similar size with a stream (or lake) of a similar size. It is not practical to expect to find a completely natural and totally unimpacted watershed that can be used as a reference. The amount of allowable impact in the reference watershed will depend on the frequency and degree of exposure, persistence of the stressors, substrate composition, habitat and riparian quality, ecoregion and species sensitivity, and the range in water quality conditions.

The use of reference sites is common to most bioassessment approaches. Reference sites are typically selected to represent natural conditions as nearly as possible. However, it is not possible to identify such pristine locations representing varied habitat conditions in most areas of the country. Schueler (1997) points out that in many cases, a completely natural forested area is not a suitable

benchmark for current conditions before urbanization. In many areas of the country, land that has long been in agricultural use is being converted to urban land, and the in-stream changes expected should therefore be more reasonably compared to agricultural conditions.

The Ohio EPA has been recognized for having one of the more advanced biological assessments in place, especially in its efforts to incorporate biological criteria as part of the regulatory program. It relies heavily on a large network of reference sites representing the various ecological conditions throughout the state. Many of the states waterways were channelized decades ago. This severe habitat disruption prevents them from ever attaining as high a quality as a similar unchannelized waterway. Therefore, Ohio EPA established "modified" warm water habitat designations with appropriate modified reference sites. Few of these reference sites are completely unimpacted by modifications or human activity in the watersheds. Yoder and Rankin (1997) reported that biological monitoring of small streams in Ohio has indicated a general lowering of biological index scores with increasing urbanization, especially in areas having CSOs and industrial discharges. Of 110 sampling sites, only 23% had good to exceptional biological resources. Poor or very poor scores were evident in 85% of the urbanized areas. They also found that more than 40% of the suburban, urbanizing sites were impaired, due to increasing residential and commercial developments. An earlier Ohio study found that biological impairments were evident in about half the locations where no impairments were indicated, based on chemical ambient monitoring data alone. They have, therefore, come to rely on biological monitoring, such as expressed in the Index of Biotic Integrity (IBI) and the Invertebrate Community Index (ICI), as a less expensive and more accurate overall indication of receiving water problems than conventional chemical water pollutant monitoring.

Crawford and Lenat (1989) examined the differences between streams located in forested, agricultural, and urban watersheds in North Carolina. The USGS study found that the stream impacted by agricultural operations was intermediate in quality, with higher nutrient and worse substrate conditions than the urban stream, but better macroinvertebrate and fish conditions. The forested watershed had the best conditions (good conditions for all categories), except for somewhat higher heavy metal concentrations in sediment than expected. Even though the agricultural watershed had little impervious area, it had high sediment and nutrient discharges, plus some impacted stream corridors. The urban stream had poor macroinvertebrate and fish conditions, poor sediment and temperature conditions, and fair substrate and nutrient conditions.

Review of Historical Site Data

As in any environmental assessment process, historical site data should be reviewed initially. Municipal, county, regional, state, and federal information sources of public information may be available concerning:

1. Predevelopment water quality, fisheries, and flow conditions (e.g., state and EPA STORET database)
2. Annual hydrological conditions vs. development area (e.g., USGS)
3. Business and industrial categories (e.g., municipality)
4. Historical hazardous spills, large quantity toxicant releases and storage (e.g., fire department, state EPA, and EPA's Toxics Release Inventory), and hazardous waste and sanitary landfill locations (e.g., state and EPA)

The initial information search should review land use patterns from a chronological approach and attempt to correlate development with hydrological data and previous water quality surveys. Unfortunately, these data are often nonexistent for the small and more heavily impacted urban streams (headwaters). If the contaminants (stressors) of concern are known, site or area stream quality survey data can be used to determine the likely background levels in water, sediment, soil,

and fish. Also, one should determine what the effects and threshold levels are likely to be, and whether any rare, threatened, or endangered species are indigenous to the area. Sources of the above information may include state environmental and natural resource agencies; state game and fish agencies; conservation agencies; societies; citizens' and sportsman's groups; state agricultural agencies; relevant university departments; museums; park officials; local water and wastewater utilities; and regional offices of federal agencies (i.e., U.S. Fish and Wildlife Service, U.S. Environmental Protection Agency, U.S. Department of Agriculture, and Natural Resources Conservation Service). From this information, it is possible to determine which species are most likely to be present and what problems may exist in an area.

Formulation of a Conceptual Framework

A conceptual framework is similar to logistical critical-path control schedules, where the major components of the study (i.e., investigation of pollutant sources, hydrologic analyses, and stream and ecosystem monitoring) are blended to describe source movement, distribution, and interaction with the receiving water ecosystem. Once the previous steps are completed, it should be possible to formulate a suitable assessment problem formulation. This process is improved if there are adequate knowledge and expertise to address the key issues of pollutant types expected, predicted pollutant fate and effects, beneficial use designations, stream hydrological characteristics, meteorological characteristics, reference and test stream water quality, and key indicator aquatic organisms present at the reference and test locations. This design stage leads directly to the next step of defining measurement endpoints.

This process should be tailored toward addressing the study objectives. If the study is to be an "endangerment," "hazard," or "risk" assessment to meet EPA regulatory requirements (e.g., RCRA, CERCLA), it would be best to follow their assessment paradigm:

1. Hazard identification: qualitative stress (e.g., lead) and receptor (e.g., trout) identification
2. Exposure assessment: contaminant (stress) dynamics vs. receptor patterns and characteristics
3. Toxicity assessment: stress–response relationship quantified
4. Hazard or risk characterization: combine above information to predict or assign adverse effects vs. source exposure

The specifics of these approaches are currently still under development by the EPA. This book could possibly be used to support any program directive which includes assessing the effects of stormwater runoff on receiving water ecosystems.

Selecting Optimal Assessment Parameters (Endpoints)

Characterization of the ecosystem should allow for differentiation of its present "natural" status from its present condition caused by polluted discharges and/or other anthropogenic stressors. This requires that a number of chemical, biological, and physical parameters be monitored, including flow and habitat. There are a wide variety of potentially useful study parameters which vary in importance with the study objectives and program needs, as shown in Table 4.7. Many of the chemical endpoints would be specifically selected based on the likely pollutant sources in the watershed. Those shown in Table 4.7 are a general list.

The selection of the specific endpoints for monitoring should be based on expected/known receiving water problems. The parameters being monitored should confirm if these uses are being impaired. If they are, then more detailed investigations can be conducted to understand the discharges of the problem pollutants, or the other factors, causing the documented problems. Finally, control programs can be designed, implemented, and monitored for success. Therefore, any receiving water investigation should proceed in stages if at all possible. It is much more cost-effective

Table 4.7 Useful Receiving Water Assessment Parameters

Chemical	Physical	Biological
Oxygen	Habitat quantification[a]	*Escherichia coli*
Dissolved	Flow, velocity	Enterococci
Biochemical demand	Temperature	Fecal coliforms
Carbonaceous	Conductivity, salinity	Benthic macroinvertebrate indices[a]
Nitrogenous	Suspended solids	Fish community indices[a]
Ultimate	Dissolved solids	Blue-green algal (cyanobacteria) blooms
Chemical demand	Reach lengths	Toxicity tests[b]
Sediment demand	Channel morphology	*Pimephales promelas* early-life stage
Nutrients	Tributary loadings	*Ceriodaphnia* or *Daphnia* sp.
Nitrogen: Total, Organic, Nitrate,	Point source loadings	*Selenastrum capricornutum*
Nitrite, Ammonia (total, un-ionized)	Nonpoint source loadings	Microtox
Phosphorus	Particle size distributions	*Hyalella azteca*
Total, Organic	Bedload	*Chironomus tentans*
Carbon	Precipitation	Tissue contaminants[b]
Total, Dissolved		Fish or bivalve tissue residues
pH		Bioaccumulation testing with *Lumbriculus variegatus*, bivalves, or fish
Alkalinity		Uptake in semipermeable membrane devices (SPMD)
Hardness		
Metals: Cd, Cu, Zn, Pb		
Organics: Polycyclic aromatic hydrocarbons (PAHs)		
Aliphatic hydrocarbons		
Pesticides (chlorinated and new age)		
Oil and grease		

[a] Comprised of multiple endpoints (see EPA 1989 and OEPA 1989 and Chapter 5).
[b] Water, whole sediment, and effluent exposures (see Chapter 5 for specific effect endpoints).

to begin with a relatively simple and inexpensive monitoring program to document the problems that may exist in a receiving water than it is to conduct a large and comprehensive monitoring program with little prior knowledge. Without having information on the potential existing problems, the initial list of parameters to be monitored has to be based on best judgment. Chapter 3 contains a review of the potential problems caused by stormwater in urban streams. The parameters to be monitored can be taken from Table 4.7 and grouped into general categories depending on expected beneficial use impairments, as follows:

- Flooding and drainage: debris and obstructions affecting flow conveyance are parameters of concern.
- Biological integrity: habitat destruction, high/low flows, inappropriate discharges, polluted sediment (SOD and toxicants), benthic macroinvertebrate and fish species impairment (toxicity and bioaccumulation of contaminants), and wet-weather quality (toxicants, nutrients, DO) are key parameters.
- Noncontact recreation: odors, trash, high/low flows, aesthetics, and public access are the key parameters.
- Swimming and other contact recreation: pathogens and above-listed noncontact parameters are key parameters.
- Water supply: water quality standards (especially pathogens and toxicants) are key parameters.
- Shellfish harvesting and other consumptive fishing: pathogens, toxicants, and those listed under biological integrity are key parameters.

Point source discharges, stormwater runoff, snowmelt, baseflows in receiving waters, sediments, and biological specimens may all need to be sampled and analyzed to obtain a complete understanding of receiving water effects from pollutant discharges.

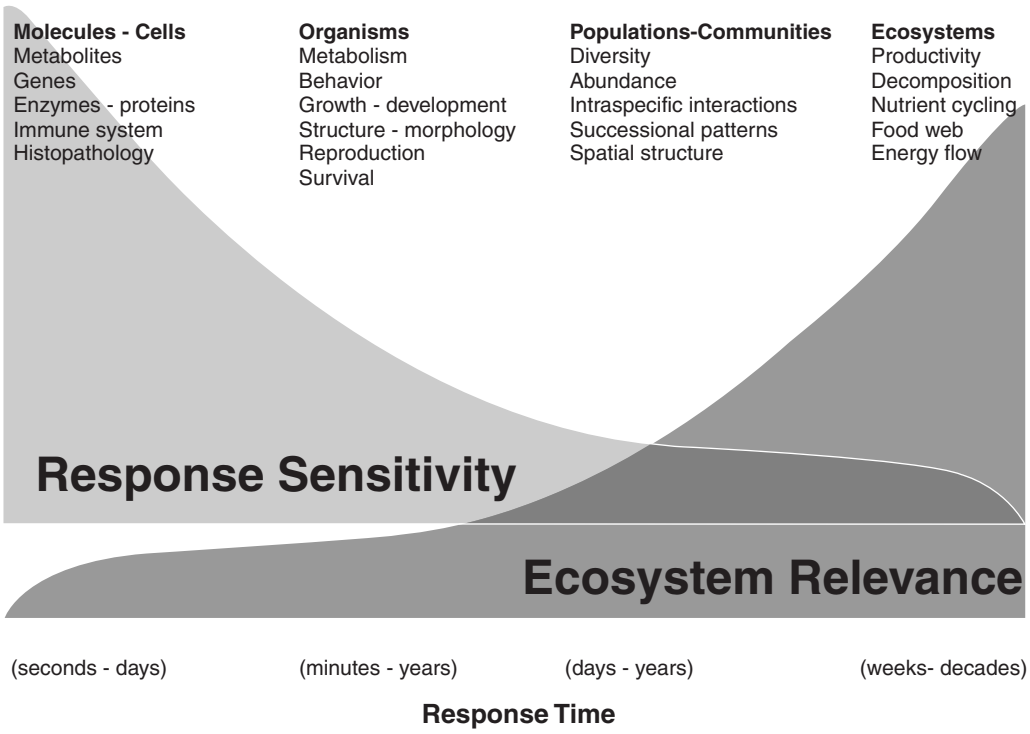

Figure 4.1- Ecotoxicological endpoints: sensitivity and relevance. (Reprinted with permission from Burton, G.A., Jr. Assessing freshwater sediment toxicity. *Environ. Toxicol. Chem.*, 10: 1585–1627, 1991. © SETAC, Pensacola, FL, U.S.A.)

Selection of Biological Endpoints for Monitoring

The optimal assessment parameters which should be included depend on the project objectives. These parameters can be defined as measured characteristics, responses, or endpoints. For example, if the affected stream is classified as a high quality water and cold water fishery, then possible assessment or measured responses (endpoints) could include trout survival and hatchability, population and community indices (e.g., species richness), spawning area quantity and quality, dissolved oxygen, suspended solids, and water temperature. Endpoints vary dramatically in their sensitivity to pollutants and ecological relevance (Figure 4.1). The endpoints that are more sensitive are often more variable or respond to natural "nonpollutant" factors, so that adverse effects (stressors) are more difficult to classify with certainty. The most commonly and successfully used biotic indicators and endpoints are discussed in subsequent sections.

Aquatic ecosystems are quite complex, consisting of a wide variety of organisms. These organisms have their own unique function in the ecosystem and are directly or indirectly linked with other organisms. For example, bacteria, fungi, insects, and other invertebrates that inhabit the bottom of the waterways each need the others to assist in the decomposition of organic matter (such as leaves) so that they may consume it as food. If any one of these groups of organisms is lost or reduced, then the others will also be adversely affected. If the invertebrates are lost, their fish predators will be impacted. These groups are made up of a number of species with varying tolerance levels to stressors, and each possesses unique or overlapping functional characteristics (e.g., organic matter processing, nitrogen cycling). By carefully selecting the biological monitoring parameters, a broad range of relevant and sensitive indicator organisms can be used to efficiently assess ecosystem quality.

The most commonly used biological groups in aquatic assessments are fish, benthic macroinvertebrates, zooplankton, and algae. In lotic (flowing water) systems, fish and benthic macroinvertebrates are often chosen as monitoring tools. Benthic refers to sediment or bottom surfaces (organic and inorganic). Macroinvertebrates are typically classified as those organisms which are retained in sieves larger than 0.3 to 0.5 mm. They include a wide range of invertebrates, such as worms, insect larvae, snails, and bivalves. They are excellent indicators of water quality because they are relatively sedentary and do not move between different parts of a stream or lake. In addition, a great deal is known about their life histories and pollution sensitivity. Algae, zooplankton, and fish are used more in lentic (lake) environments. Of these, fish are most often used (both in lotic and lentic habitats). Fish are transient, moving between sites, so it is more difficult to determine their source of exposure to stressors; however, they are excellent indicators of water quality and provide a direct link to human health and wildlife consumption advisories. Rooted macrophytes and terrestrial plant species are good wetland health indicators, but are used less frequently.

In order to effectively and accurately evaluate ecosystem integrity, biosurveys should use two to three types of organisms which have different roles in the ecosystem, such as decomposers (bacteria), producers, primary to tertiary consumers (EPA 1990b). This same approach should be used in toxicity testing (Burton et al. 1989, 1996; Burton 1991). This increases the power of the assessment, providing greater certainty that if there is a type of organism(s) (species, population, or community) in the ecosystem being adversely affected, either directly or indirectly, it will be detected. This also allows for better predictions of effects, such as in food chain bioaccumulation with subsequent risk to fish-eating organisms (e.g., birds, wildlife, humans). A large database exists for many useful indicator species concerning their life history, distribution, abundance in specific habitats or ecoregions, ecological function, and pollutant (stressor) sensitivity.

In the monitoring of fish and benthic macroinvertebrate communities, a wide variety of approaches have been used. A particularly popular approach recommended by the U.S. EPA, Ohio EPA, state volunteer monitoring programs, and other agencies is a multimetric approach, as summarized previously. The multimetric approach uses the basic data of which organisms are present at the site and analyzes the data using a number of different metrics, such as richness (number of species present), abundance (number of individuals present), and groups types of pollution-sensitive and resistant species. The various metrics provide unique and sometimes overlapping information on the quality of the aquatic community. Structural metrics describe the composition of a community, that is, the number and abundance of different species, with associated tolerance rankings. Functional metrics may measure photosynthesis, respiration, enzymatic activity, nutrient cycling, or proportions of feeding groups, such as omnivores, herbivores, insectivores, shredders, collectors, and grazers. The U.S. EPA and Ohio EPA approaches are described in more detail in Chapter 6 and Appendices A, B, and C.

The Microtox™ (from Azur) toxicity screening test has been successfully used in numerous studies to indicate the sources and variability of toxicant discharges. However, these tests have not been standardized by the U.S. EPA or state environmental agencies but have been in Europe. More typically, whole effluent toxicity test methods are employed (see Chapter 6, and also review by Burton et al. 2000). These tests may miss toxicant pulses and do not reflect real-world exposure dynamics. Many of the *in situ* toxicity tests, especially in conjunction with biological surveys (at least habitat and benthic macroinvertebrate evaluations) and sediment chemical analyses, can provide more useful information to document actual receiving water toxicity problems than relying on water analyses alone. If a water body is shown to have toxicant problems, it is best to conduct a toxicity identification evaluation (TIE) to attempt to isolate the specific problematic compounds (or groups of compounds) before long lists of toxicants are routinely analyzed.

Selection of Chemical Endpoints for Monitoring

An initial monitoring program must include parameters associated with the above beneficial uses. However, as the receiving water study progresses, it is likely that many locations and some

beneficial uses may not be found to be problematic. This would enable a reduction in the list of parameters to be routinely monitored. Similarly, additional problems may also become evident with time, possibly requiring an expansion of the monitoring program. The following paragraphs briefly describe the main chemical monitoring parameters that could be included for the beneficial use impact categories listed previously for a receiving water only affected by stormwater. However, it might be a good idea to periodically conduct a more-detailed analysis as a screening tool to observe less obvious, but persistent problems. If industrial or municipal point discharges or other nonpoint discharges (such as from agriculture, forestry, or mining activities) also affect the receiving water under study, additional constituents might need to be added to this list.

Obviously, chemical analyses can be very expensive. Therefore, care should be taken to select an appropriate list of parameters for monitoring. However, the appropriate number of samples must be collected (see Chapter 5) to ensure reliable conclusions. Chemical analyses of sediments may be more informative of many receiving water problems (especially related to toxicants) than chemical analyses of water samples. This is fortunate because sediment chemical characteristics do not change much with time, so generally fewer sediment samples need to be analyzed during a study period, compared to water samples. In addition, the concentrations of many of the constituents are much higher in sediment samples than in water samples, requiring less expensive methods for analyses. Unfortunately, sediment sample preparation (especially extractions for organic toxicant analyses and digestions for heavy metal analyses) can be much more difficult for sediments than for water.

Sediment Chemical Analyses

The basic list for chemical analyses for sediment samples, depending on beneficial use impairments, includes toxicants and sediment oxygen demand. The toxicants should include heavy metals (likely routine analyses for copper, zinc, lead, and cadmium, in addition to periodic ICP analyses for a broad list of metals). Acid volatile sulfides (AVS) are also sometimes analyzed to better understand the availability of the sediment heavy metals. Other sediment toxicant analyses may include PAHs and pesticides. Particle size analyses should also be routinely conducted on the sediment samples. Sediment oxygen demand analyses, in addition to an indication of sediment organic content (preferably particulate organic carbon, or at least COD and volatile solids), and nutrient analyses are important in areas having nutrient enrichment or oxygen depletion problems. Microorganisms (*Escherichia coli*, enterococci, and fecal coliforms) should also be evaluated in sediments in areas having likely pathogen problems (all urban areas). Interstitial water may also need to be periodically sampled and analyzed at important locations for the above constituents.

Water Chemical Analyses

The basic list for chemical analyses for water samples, depending on beneficial use impairments, includes toxicants, nutrients, solids, dissolved oxygen, and pathogens.

The list of specific toxicants is similar to that for the sediments (copper, zinc, lead, and cadmium, plus PAHs and pesticides). However, because of the generally lower concentrations of the constituents in the sample extracts for these analyses, more difficult analytical methods are generally needed, but the extraction and digestion processes are usually less complex than for sediments. In addition, because of the high variability of the constituent concentrations with time, many water samples are usually required to be analyzed for acceptable error levels. Therefore, less costly screening methods should be stressed for indicating toxicants in water. Because of the their strong associations with particulates, the toxicants should also be periodically analyzed in both their total and filterable forms. This increases the laboratory costs, but is necessary to understand the fates and controllability of the toxicant discharges. Typical chemical analyses for stormwater toxicants may include:

- Metals (lead, copper, cadmium, and zinc using graphite furnace atomic adsorption spectrophotometry, or other methods having comparable detection limits), periodic total and filtered sample analyses
- Organics (PAHs, phenols, and phthalate esters using GC/MSD with SIM, or HPLC), pesticides (using GC/ECD, or immunoassays), periodic total and filtered sample analyses

Pesticides in urban stormwater have recently started to receive more attention (USGS 1999). The USGS's National Water Quality Assessment (NAWQA) program has extensively sampled urban and rural waters throughout the nation. Herbicides commonly detected in urban water samples include simazine, prometon, 2,4-D, diuron, and tebuthiuron. These herbicides are extensively used in urban areas. However, other herbicides frequently found in urban waters are used in agricultural areas almost exclusively (and likely drift in to urban lands from adjacent farm lands) and include atrazine, metolachlor, deethylatrazine, alachlor, cyanezine, and EPTC. Insecticides commonly detected in urban waters include diazinon, carbaryl, chlorpyrifos, and malathion.

Nutrient analyses are also important when evaluating several beneficial uses. These analyses are not as complex as the toxicants listed above and are therefore much less expensive. However, relatively large numbers of analyses are still required. Water analyses may include the following typical nutrients: total phosphorus, inorganic phosphates (and, by difference, organic phosphates), ammonia, Kjeldahl nitrogen (or the new HACH total nitrogen), nitrate plus nitrite, and TOC. Periodic analyses for total and filtered forms of the phosphorus and TOC should also be conducted.

Dissolved oxygen is a basic water quality parameter and is important for several beneficial uses. Historical discharge limits have typically been set based on expected DO conditions in the receiving water. The typical approach is to use a portable DO meter for grab analyses of DO. Continuous *in situ* monitors, described in Chapter 6, are much more useful, especially the new units that have much more stable DO monitoring capabilities and can also frequently record temperature, specific conductance, turbidity, pH, and ORP. These long-term analyses are especially useful when evaluating diurnal variations or storm-induced discharges.

Pathogens should be monitored frequently in most receiving waters. Both urban and rural streams are apparently much more contaminated by problematic pathogenic conditions than has previously been assumed. Historically monitored organisms (such as fecal coliforms), in addition to *E. coli* and enterococci which are now more commonly monitored, can be present at very high levels and be persistent in urban streams. Specific pathogens (such as *Pseudomonas aeruginosa* and *Shigella*) can also be more easily monitored now than in the past. Most monitoring efforts should probably focus on fecal coliforms, *E. coli*, and enterococci.

Additional conventional parameters affecting fates and effects of pollutants in receiving waters should also be routinely monitored, including hardness, alkalinity, pH, specific conductivity, COD, turbidity, suspended solids (SS), volatile suspended solids (VSS), and total dissolved solids (TDS).

Selection of Additional Endpoints Needed for Monitoring

Several other stream parameters also need to be evaluated when investigating beneficial uses. These may include debris and flow obstructions, high/low flow variations, inappropriate discharges, aesthetics (odors and trash), and public access.

Data Quality Objectives and Quality Assurance Issues

For each study parameter, the precision and accuracy needed to meet the project objectives should be defined. After this is accomplished, the procedures for monitoring and controlling data quality must be specific and incorporated within all aspects of the assessment, including sample collection, processing, analysis, data management, and statistical procedures (see also Chapter 7).

When designing a plan one should look at the study objectives and ask:

- How will the data be used to arrive at conclusions?
- What will the resulting actions be?
- What are the allowable errors?

This process establishes the Data Quality Objectives (DQOs), which determine the level of uncertainty that the manager is willing to accept in the results. DQOs, in theory, require the study designers (decision makers and technical staff) to decide what are allowable probabilities for Type I and II errors (false-positive and false-negative errors) and issues such as what difference in replicate means is significant. The DQO process is a pragmatic approach to environmental studies, where limited resources prevent the collection of data not essential to the decision-making process. Uncertainty in ecological impact assessments is natural due to variability and unknowns, sampling measurement errors, and data interpretation errors. Determining the degree of uncertainty in any of these areas can be difficult or impractical. Yet an understanding of these uncertainties and their relative magnitudes is critical to the QA objectives of producing meaningful, reliable, and representative data. The more traditional practices of QA/QC should be expanded to encompass these objectives and thus help achieve valid conclusions on the test ecosystem's health (Burton 1992).

The first stage in developing DQOs requires the decision makers to determine what information is needed, reasons for the need, how it will be used, and to specify time and resource limits. During the second stage, the problem is clarified and constraints on data collection identified. The third stage develops alternative approaches to data selection, selecting the optimal approach, and establishing the DQOs (EPA 1984, 1986). Chapter 5 includes detailed information concerning the required sampling efforts to achieve the necessary DQOs, based on measured or estimated parameter variabilities and the uncertainty goals.

EXAMPLE OUTLINE OF A COMPREHENSIVE RUNOFF EFFECT STUDY

The following is an outline of the specific steps that generally need to be followed when designing and conducting a receiving water investigation. This outline includes the topics that are described in detail in later chapters of this book.

Step 1. What's the Question?

For example: Does site runoff degrade the quality of the receiving-stream ecosystem? Chapter 3 is a summary of documented receiving water problems associated with urban stormwater, for example. That chapter will enable the investigator to identify the likely problems that may be occurring in local receiving waters, and to identify the likely causes.

Step 2. Decide on Problem Formulation

Candidate experimental designs can be organized in one of the following basic patterns:

1. Parallel watersheds (developed and undeveloped)
2. Upstream and downstream of a city
3. Long-term trend
4. Preferably, most elements of all of the above approaches combined in a staged approach

Examples of these problem formulations are included at the end of this chapter, while Chapter 5 describes basic study designs, such as stratified random sampling, cluster sampling, and search sampling.

Another important issue is determining the appropriate study duration. In most cases, at least 1 year should be planned in order to examine seasonal variations, but a longer duration may be

needed if unusual or dynamic conditions are present. As shown in Chapter 7, trend analyses can require many years. In addition, variations in the parameters being investigated will require specific numbers of observations in order to obtain the necessary levels of errors in the program (as described in Chapter 5). If the numbers of observations relate to events (such as runoff events), the study will need to last for the duration necessary to observe and monitor the required number of events.

Step 3. Project Design

1. Qualitative watershed characterization
   A. Establish degree of residential, commercial, and industrial area to predict potential stressors. Typically, elevated solids, flows, and temperatures are stressors common to all urban land uses. The following lists typical problem pollutants that may be associated with each of these land uses:
      1. Residential: nutrients, pesticides, fecal pathogens, PAHs, and metals
      2. Commercial: petroleum compounds, metals
      3. Industrial: petroleum compounds, other organics, metals
      4. Construction: suspended solids

   Topographical maps are used to determine watershed areas and drainage patterns.
2. Stream characterization
   A. Identify potential upstream stressor sources and potential stressors
      1. Photograph and describe sites.
   B. Survey upstream and downstream (from outfall to 1 km minimum) quality. Record observations on physical characteristics, including channel morphology (pools, riffles, runs, modification), flow levels, habitat (for fish and benthos), riparian zone, sediment type, organic matter, oil sheens, and odors. Record observations on biological communities, such as waterfowl, fish-eating birds or mammals, fish, benthic invertebrates, algal blooms, benthic algae, and filamentous bacteria.
   C. Identify appropriate reference site upstream and/or in a similar sized watershed with same ecoregion.
   D. Collect historical data on water quality and flows.
3. Select monitoring parameters
   A. Habitat evaluation. Should be conducted at project initiation and termination. Includes Quantitative Habitat Evaluation Index (QHEI), bed instability survey (bed lining materials and channel cross-sectional area changes), aesthetic/litter survey, inappropriate discharges (field screening), etc.
   B. Stressors and their indicators:
      1. Physical: flow, temperature, turbidity. Determine at intervals throughout base to high flow conditions.
      2. Chemical: conductivity, dissolved oxygen, hardness, alkalinity, pH, nutrients (nitrates, ammonia, orthophosphates), metals (cadmium, copper, lead, and zinc), and immunoassays (pesticides and polycyclic aromatic hydrocarbons) and/or toxicity screening (Microtox). The necessity of testing nutrients, metals, and organics will depend on the watershed characteristics. Determine at intervals throughout base to high flow conditions.
      3. Biological: benthic community structure (e.g., RBP), fish community structure, and tissue residues (confirmatory studies only). Benthic structure should be determined at the end of the project. Sediment bioaccumulation potential can be determined using the benthic invertebrate *Lumbriculus variegatus*.
      4. Toxicity: short-term chronic toxicity assays of stream water, outfalls, and sediment. Sediment should be sampled during baseflow conditions and tested before and after a high flow event. Water samples should be collected during baseflow and during pre-crest levels. Test species selection is discussed in Chapter 6 and in Appendix D. Expose test chambers with and without sunlight-simulating light (containing ultraviolet light wavelengths) to detect PAH toxicity. *In situ* toxicity assays should be deployed in the stream for confirmatory studies during base and high flow periods.

4. Data quality objectives. Determine the kinds of data needed and the levels of accuracy and precision necessary to meet the project objectives. These decisions must consider that there is typically a large amount of spatial and temporal variation associated with runoff study parameters. Chapter 5 relates sampling efforts associated with actual variability and accuracy and precision goals. This requires additional resources for adequate quantification.
5. Triggers and tiered testing. Establish the trigger levels or criteria that will be used to determine when there is a significant effect, when the objective has been answered, and/or when additional testing is required. Appropriate trigger levels may include:
   A. An arbitrary 20% difference in the test site sample, as compared to the reference site, might constitute a significant effect. (However, as noted in Chapter 5, a difference this small for many parameters may be difficult and therefore expensive to detect because of the natural variability.)
   B. An exceedance of the 95% statistical confidence intervals as compared to the reference sample.
   C. High toxicity in the test site sample, measured as Toxic Units (TUs) (e.g., 1/LC50).
   D. Exceedance of biotic integrity, sediment, or water quality criteria/guidelines/standards at the test site
   E. Exceedance of a hazard quotient of 1 (e.g., site concentration/environmental effect or background concentration).

   A tiered or a phased testing approach is most cost effective, if time permits. A qualitative or semiquantitative study may include a greater number of indicator or screening parameters, such as turbidity, temperature, DO, specific conductivity, and pH using a continuous recording water quality sonde, plus artificial substrate macroinvertebrate colonization tests, and "quick" sediment toxicity tests. If possible, Microtox screening toxicity tests, immunoassay tests for pesticides and PAHs, and sediment metal analyses should also be added to this initial effort. These simple tests can be conducted with more widespread sampling to better focus later tiers on quantifying appropriate stressors in critical sampling areas and times. Final project tiers can identify specific stressors, their contribution to the problem, their sources, or simply confirm the ecological significance of the observed effects.
6. Sampling station selection. Select the study sites, such as upstream reference sites, outfall(s), and downstream impacted sites. In the selection of the upstream/reference and downstream sites, consider flow dynamics, stressor sources, and reference habitat similarities.
7. Quality assurance project plans (QAPP). It is essential that the quality of the project be ensured with adequate quality assurance and quality control measures. This will include routine laboratory and field documentation of operator and instrumentation performance, chain-of-custody procedures, adequate sample replication, QA/QC samples (blanks and spikes, etc.), performance criteria, and ensuring data validity. Appropriate experimental design (study design and sampling efforts) is also a critical component of a QAPP.

Step 4. Project Implementation (Routine Initial Semiquantitative Survey)

1. Baseflow conditions
   A. Habitat survey (e.g., Qualitative Habitat Evaluation Index)
   B. Benthic RBP
   C. Test water and sediment from all test sites for short-term chronic toxicity with two species.
   D. Establish spatial and diurnal variation (YSI 6000 for several weeks, plus grab samples or time composites).
   E. Set up automatic stream samplers/monitors, stream depth gauges, and rain gauges.
   F. Establish local contacts to oversee field equipment and provide rain event notification.
   G. Conduct field screening survey at outfalls to identify sources of dry-weather flows.
2. High flow conditions
   A. Confirm that the samplers and monitors are operational. Collect grab samples if necessary (for microbiological and VOC analyses, for example).
   B. Deploy *in situ* toxicity test assays.
   C. Measure flow and note staff gauge depth, using manual or automatic samplers and flow recorders. Repeat flow measurements at intervals of 0.5- to 1.0-ft stream depth intervals as the stream rises, noting time and depth. Focus on first flush to crest period.

   D. Measure DO, temperature, turbidity, conductivity, and stage at each station following each flow measurement. Establish spatial variance. May use continuous recording water quality sondes.
   E. Collect flow-weighted composited (or combine many discrete) samples for other analyses.
3. Sample analyses
   A. Filter, preserve, and chill samples, as required.
   B. Deliver samples to analytical laboratories with chain-of-custody forms.
   C. Initiate toxicity testing and other chemical and microbiological analyses within required time period since sample collection.
   D. Document QA/QC.
4. Follow-up (post-event) monitoring
   A. Check *in situ* assay chambers at 24 and 48 hours and at 7 and 14 days if deployed.
   B. Conduct benthic RBP.
   C. Conduct QHEI, noting bedload movement.
   D. Collect fish for tissue residue analyses.

Step 5. Data Evaluation

1. Plot flow vs. physical and chemical analysis results.
2. Statistically compare responses/loadings during base, first flush, and post-crest conditions. This will provide a characterization of flow dynamics and its effect on stressor profiles.
3. Statistically compare stations (instantaneous, mean periods) for significant differences and correlations.
4. Calculate and compare physical, chemical, and toxicity (using Toxicity Units) loadings. This will show the relative load contribution of stressors from reference (upstream) vs. impacted (downstream) reach.
5. Identify magnitude and duration of trigger exceedances.
6. Identify sources of uncertainty.
7. Identify potential sources of pollutants and stressors.
8. Determine literature value thresholds for key stressors on key indigenous species.

Step 6. Confirmatory Assessment (Optional Tier 2 Testing)

1. Repeat Steps 2 and 3 using Tier 1 information to select fewer test parameters with increased sampling frequency and/or select more descriptive methods. Increased sampling will better quantify the magnitude and duration of stressor dynamics. Expanded sampling will better document the quality of the receiving water. More definitive testing could include:
   A. Short-term chronic toxicity testing with additional species (lab and *in situ*)
   B. Increased testing of toxicants
   C. Characterizing fish, plankton, periphyton, or mussel populations
   D. Measuring assimilative capacity via long-term BOD and SOD testing
   E. Measuring productivity with light/dark bottle BOD *in situ* tests
2. Conduct toxicity identification evaluation (TIE) study of water, outfalls, and/or sediment to determine contribution of each stressor to total toxicity. This information can better determine which stressors are important to control and can also identify sources of toxicity.
3. Conduct bioaccumulation testing of site sediments. Some pollutants, such as highly chlorinated organic compounds (e.g., chlordane, DDT, PCBs, dioxins) are readily bioaccumulated, yet may not be detected using the above study design. The EPA has a benthic invertebrate 28-day assay to measure sediment bioaccumulation potential. Also SPMDs may be used.
4. Indigenous biological community characterization and tissue analysis. More in-depth quantification of benthic and/or fish community structure on a seasonal basis will better identify significant ecological effects. Tissue sampling of fish for contaminants will provide information on bioaccumulative pollutants and potential food web or human health effects from consumption.

Table 4.8 Watershed Study Complexity Matrix

Situation: Complexity Scale (Simple to Complex)	Primary Considerations
Single outfall	Focus on loading of site stressors from site and from upstream. Reference upstream.
Small stream (small watershed)	
Large stream (larger watershed)	Determine if upstream inputs are degrading water quality. Upstream and separate ideal reference sites.
Pristine estuary	Focus on outfall quality and mixing zone. Deploy *in situ* monitors. Use far-field reference.
Multiple outfalls	
River (multi-watersheds)	Multistation network with habitat, benthos, and select toxicity evaluations of water and sediments. Tiered study with TIE, outfall, and *in situ* studies to find major problem sources. Use upstream and adjacent watershed references. Focus on tributary mouths for initial sampling and use SPMDs.
Coastal harbor	Focus on outfall quality and near-field mixing zones. Deploy *in situ* monitors. Use far-field, adjacent watershed references.

Step 7. Project Conclusions

1. List probable stressors.
2. Document trigger exceedances.
3. Discuss relative contribution of stressors(s) to ecosystem degradation. Support documentation may include:
   A. Literature threshold values
   B. Criteria exceedances
   C. Toxicity observed (from TIE, photoactivation, or *in situ* assays)
   D. Bioaccumulation factors and potential for food web contamination
4. Provide recommendations for stressor reduction and ecosystem enhancement.
5. Include suggestions on habitat improvement, flow reduction, turbidity removal, and reduced siltation.

Table 4.8 summarizes the primary considerations that should be examined for different levels of receiving water complexity. Obviously, increasingly complex situations require more complex study designs and elements. However, this table briefly outlines the major issues that should be considered.

CASE STUDIES OF PREVIOUS RECEIVING WATER EVALUATIONS

This section presents several case studies that have been conducted to investigate receiving water problems associated with runoff. These case studies illustrate the major approaches used to identify a potentially affected area through comparisons with a control area. The basic experimental designs are:

- Above/below longitudinal study where a stream is studied as it flows from above a city through a city. Obviously, the upstream control reach must be in a relatively undisturbed portion of the watershed and only wet-weather flows of interest affect any of the test reaches.
- Parallel stream study where two (or more) streams are studied. One of the streams is a control stream in a relatively undisturbed area, while the other stream is in an urbanized area.
- Trend analyses with time in a single stream to investigate changes that may occur with time as a watershed becomes urbanized, or with the application of stormwater controls.

The selection of suitable test areas is critical. As noted, the control water body should be minimally affected by urbanization, while the urban test water body should be affected only by urban runoff (and not municipal or industrial discharges, for example) if possible. In addition, the test and control water bodies must be otherwise very similar (especially as watershed area, topography, habitat potential, etc., are concerned). In a longitudinal study, the watershed area obviously increases in a downstream (urbanized) direction. In addition, the urban water body has a substantially different flow regime than an undisturbed water body. These differences should be the result of urbanization and not other factors. A successful receiving water study usually requires several years of study at many locations in each stream segment. As noted throughout this book, the selection of monitoring parameters is also critical. In most cases, varied and complementary analyses should be conducted, covering a range of biological, physical, and chemical parameters. However, carefully designed investigations can be more successfully focused on limited project objectives.

The first three case studies are examples of these three basic experimental designs for conducting a receiving water investigation and include both test and control conditions. Most of the receiving water studies reported in the literature only focus on potentially impacted water bodies, without any adequate control sites. This may be suitable in an area where the receiving water potential is well understood through extensive prior studies (such as in Ohio). However, it is very problematic to rely solely on various criteria to identify the magnitude of receiving water problems, without extensive local expertise on relatively natural conditions.

The identification of a "problem" is also highly dependent on desired beneficial uses. The local perception of use is critical. Obviously, human health considerations associated with potentially contaminated water supplies, consumptive fisheries, or contact recreation areas must be stringently addressed. Biological uses may be more open to local interpretation, however. It is unreasonable to expect completely natural receiving water conditions in an urban area. There are unavoidable impacts that will prevent the best natural conditions from occurring in an urbanized watershed. Obviously, general biological uses can still be met by providing suitable habitat and somewhat degraded conditions that would allow a reasonable assemblage of aquatic organisms to exist in an area. Noncontact recreational uses (especially the aesthetic factors of odors and trash) should also be provided in urban receiving waters. Test and control receiving water investigations are very useful in that they enable contrasting of existing degraded conditions with less impacted conditions. Perhaps the control reference sites should include not natural conditions, but acceptable degraded conditions associated with partial urbanization. This is possible with a longitudinal study where a receiving water is studied as it flows through an urban area, becoming more degraded in the downstream direction. Parallel stream studies can also include partially degraded, but acceptable, sites. In addition, trend analyses with time will indicate when unacceptable degradation occurs.

Example of a Longitudinal Experimental Design — Coyote Creek, San Jose, CA, Receiving Water Study

The Coyote Creek study is an example of an investigation of the effects of stormwater on the biological conditions in an urban creek as it passed through the City of San Jose, CA. This was an early comprehensive receiving water study that examined many attributes of the creek above and within the city.

This research project included many different biological, chemical, and physical parameters to quantify biological effects. The project was conducted by Pitt and Bozeman (1982) from 1977 through 1982, with funding from the Storm and Combined Sewer Section of the U.S. Environmental Protection Agency. The objective of this 3-year field monitoring study was to evaluate the sources and impacts of urban runoff on water quality and biological conditions in Coyote Creek. In many cases, very pronounced gradients of water and biological quality indicators were observed. Cause-

and-effect relationships cannot be conclusively proven in a study such as this; the degradation of conditions in Coyote Creek may be due to several factors, including urban runoff, stream flows (both associated and not associated with urban runoff), and natural conditions (e.g., drought, stream gradient, groundwater infiltration, etc.). Information collected during this study implied that the effects of various urban runoff constituents, especially organics and heavy metals in the water and in the polluted sediment, may be responsible for many of the adverse biological conditions observed.

The beginning of the project followed 2 years of severe drought. The first major rains occurred the previous November (1977), and seasonal rains that occurred during the study period were considered normal. Typical rainfall averaged 33 cm (13 in) per year in the area below Lake Anderson, and 50 to 71 cm (20 to 28 in) per year in the watershed above Lake Anderson. During the drought, which preceded this study, rainfall was only about one half of these amounts.

Step 1. What's the Question?

The major questions that were to be addressed during the Coyote Creek study were:

1. Identify and describe important sources of urban runoff pollutants.
2. Describe the effects of those pollutants on water quality, sediment quality, aquatic organisms, and the creek's associated beneficial uses.
3. Assess potential measures for controlling the problem pollutants in urban runoff.

Step 2. Decide on Problem Formulation

This project was designed to examine the changes in conditions in Coyote Creek as it passed through San Jose, CA. It was therefore a longitudinal study. The several-year duration of the study also enabled year-to-year variations to be compared to the differences in locations.

Step 3. Project Design

Qualitative Watershed Characterization

Figure 4.2 is a map of the San Francisco Bay area showing the location of the Coyote Creek watershed, while Figure 4.3 is a detailed map of the Coyote Creek watershed. The watershed itself is about 70 km (45 miles) long, 15 km (10 miles) wide, and contains about 80,000 ha (200,000 acres). Nearly 15% of the watershed consisted of developed urban areas during the study period. Most of the urban development is located in the northwest portion of the watershed.

Stream Characterization

For much of its length, Coyote Creek flows northwesterly along the western edge of the watershed. Elevations in the watershed range from sea level to nearly 920 m (3000 ft). Figure 4.4 shows the elevations of the various major sampling locations. Near the San Jose urban area, the watershed can be characterized as a broad plain with rolling foothills to the east. A portion of the watershed (i.e., the narrow strip between Lake Anderson and the urban area) is used for light but productive agriculture. The upper reaches and the headwaters of Coyote Creek are in extremely rugged terrain, with slopes commonly exceeding 30%. These upper areas can be characterized as chaparral-covered hills and gullies in a fairly natural state; they receive little use by man. Much of this land is within the Henry Coe State Park; non-park land is used primarily for low-density cattle grazing. Even though the watershed is very large and has upstream dams, the flow variations are extreme. Figure 4.5 shows the creek during a wet-weather period where the flows are overtopping

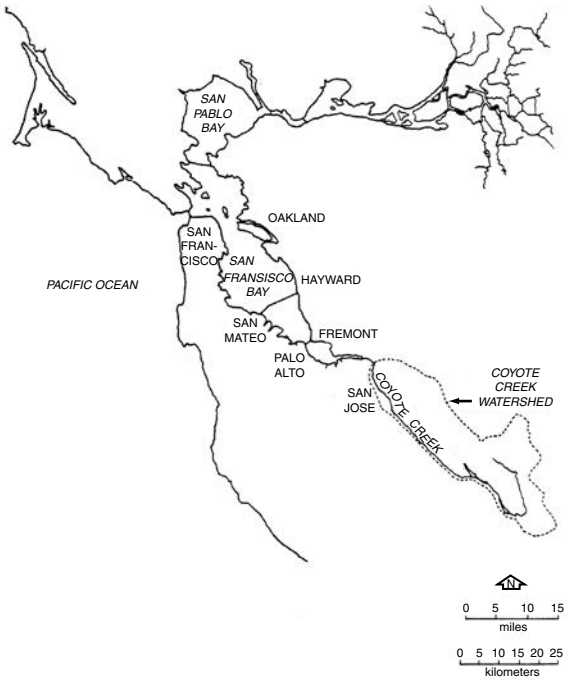

Figure 4.2 San Francisco Bay area and the location of the Coyote Creek watershed. (From Pitt, R. and M. Bozeman. *Sources of Urban Runoff Pollution and Its Effects on an Urban Creek*, EPA-600/S2-82-090, U.S. Environmental Protection Agency, Cincinnati, OH. 1982.)

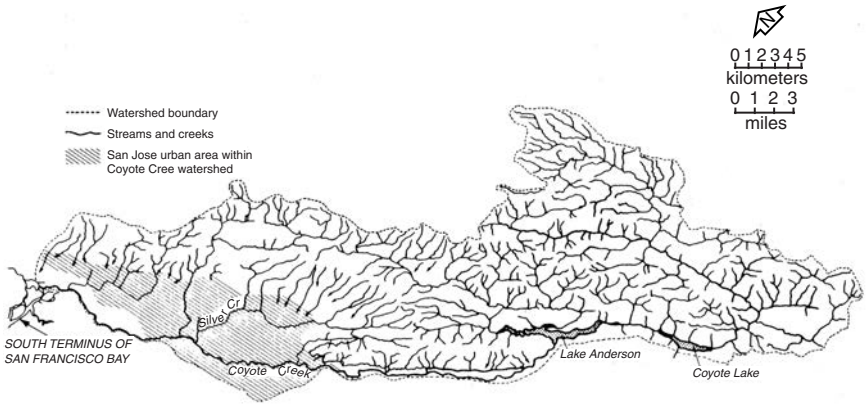

Figure 4.3 Detailed map of the Coyote Creek watershed. (From Pitt, R. and M. Bozeman. *Sources of Urban Runoff Pollution and Its Effects on an Urban Creek*, EPA-600/S2-82-090, U.S. Environmental Protection Agency, Cincinnati, OH. 1982.)

a road culvert, while Figure 4.6 shows the creek during a typical dry period (commonly lasting for 100 days without rain during summer months).

Several major facilities have been built on Coyote Creek to provide flood control and groundwater recharge. The largest are the dams, which contain man-made reservoirs: Lake Anderson and Coyote Lake. Discharges from these lakes are controlled by the Santa Clara Valley Water District. The major study area was located between the farthest downstream dam (Lake Anderson) and the first major confluence (where Coyote Creek meets Silver Creek, within the City of San Jose). Within this 39-km (24-mile) study area, approximately 16 km (10 miles) are urban and 23 km (14 miles) are non-urban. Sampling stations were located in both the urban and non-urban reaches of the stream for comparison.

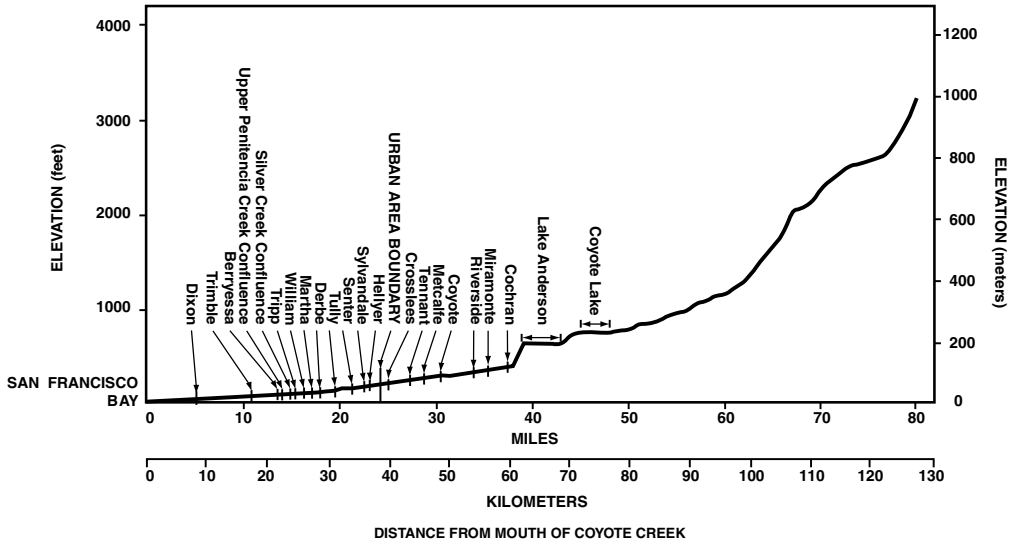

Figure 4.4 Elevations of the major sampling locations. (From Pitt, R. and M. Bozeman. *Sources of Urban Runoff Pollution and Its Effects on an Urban Creek*, EPA-600/S2-82-090, U.S. Environmental Protection Agency, Cincinnati, OH. 1982.)

Figure 4.5 High flows in Coyote Creek overtopping road culvert.

Figure 4.6 Low flows in Coyote Creek during typically extended summer dry period.

Average daily flows in the northern part of the creek during dry weather were typically less than 1.5 m^3/s (50 cfs). Major storm flows, however, approach 30 m^3/s (1000 cfs). The flows in the northern part of the creek were controlled largely by the discharges from Lake Anderson and Coyote Lake.

Coyote Creek is an important element of the Santa Clara Valley Water District's groundwater recharge program. Several recharge basins have been established adjacent to the stream channel within the study area. Diversion channels withdraw water from Coyote Creek, route it into these large basins, and return it back to the creek, depending upon such factors as season, stream flow, and groundwater level.

There is an average of 0.6 to 3 storm drain outfalls per kilometer (1 to 5 per mile) along the urban reach of Coyote Creek that was studied. The outfalls ranged from 20 to 180 cm (8 to 70 in) in diameter, but most are about 75 cm (30 in) in diameter. The drainage area per outfall

Table 4.9 Coyote Creek Drainage Areas above Each Monitoring Station

Sampling Station	Total Area (hectares)	Urban Area (hectares)	Non-urban Area (hectares)	Percent Urban
Cochran	49,510	<5	49,510	<0.01
Miramonte	50,260	<5	50,260	<0.01
Metcalfe	52,360	<50	54,360	<0.1
Crosslees	54,030	50	53,980	0.1
Hellyer	54,400	350	54,050	0.6
Sylvandale	54,720	450	54,320	0.7
Senter	55,300	800	50,500	1.5
Derbe	56,300	1740	54,560	3.2
William	56,920	2150	54,770	3.9
Tripp	57,260	2460	54,800	4.5

From Pitt, R. and M. Bozeman. *Sources of Urban Runoff Pollution and Its Effects on an Urban Creek*, EPA-600/S2-82-090, U.S. Environmental Protection Agency, Cincinnati, OH. 1982.

ranged from 2 to 320 ha (5 to 800 acres), but most of the outfalls drained areas smaller than 40 ha (100 acres).

Table 4.9 describes the drainage areas which cumulatively contribute runoff flows to selected monitoring stations. The urban area stations had about 3 to 5% (1700 to 2500 ha or 4000 to 6000 acres) of their total drainage areas urbanized, whereas the non-urban area stations had less than 0.1% of their drainage areas urbanized. The three stations designated as Hellyer, Sylvandale, and Senter were transition stations (about 0.6 to 1.5% of their drainage areas were urbanized).

Select Monitoring Parameters

The project involved conducting field measurements, observations, sampling, and other studies of Coyote Creek from March 1977 through August 1980. The study focused on the urban reaches of Coyote Creek, extending from Lake Anderson to the confluence with Silver Creek. In this reach of Coyote Creek, there are no known flow or pollutant contributions other than urban runoff. The sampling areas were selected such that each included a stretch of stream several hundred meters long, which met prescribed criteria for physical, biological, and chemical homogeneity.

The following parameters were typically examined at each sampling location:

- Basic hydrologic conditions
- Water quality
- Sediment properties
- General habitat characteristics
- Fish
- Benthic organisms (e.g., aquatic insects, crustaceans, mollusks)
- Attached algae
- Rooted aquatic vegetation (e.g., cattails)

Step 4. Project Implementation (Routine Initial Semiquantitative Survey)

Sampling took place during all months during the complete project period. As an example, the biological sampling stressed the spring and summer seasons of all project years, while the water column and sediment samples were conducted approximately monthly.

All water and sediment sampling was conducted manually using either plastic (HDPE) or glass wide-mouth bottles. Sediment core samples were obtained using a liquid carbon dioxide freezing core sampler. All water and sediment samples were comprised of at least six subsamples from the sampling location reach that were composited before analysis. The samples were then appropriately preserved and delivered to a commercial analytical laboratory for EPA-approved analyses.

Biological samples for lead and zinc bioaccumulation measurements (e.g., mosquito fish, filamentous algae, crayfish, cattail plant segments) were obtained at selected sampling stations during the routine fish sampling activities.

Fish were collected by seining and electroshocking representative pool and riffle habitats at 40 locations within the Coyote Creek system. Most of the collection efforts (conducted during the spring and summer of the project years) were focused on the portion of Coyote Creek between Lake Anderson and the confluence of Silver Creek. However, to further define the species composition and distribution of fishes, additional samples were obtained from both the upper and lower reaches of Coyote Creek, as well as from several locations within major tributaries. Captured fishes were identified and counted. The total length and weight were recorded for each specimen. Where numerous individuals of a particular species were encountered, only length range and aggregate weight were recorded, along with any abnormalities.

Quantitative collections of benthic macroinvertebrates were made at nine locations in Coyote Creek. Benthic macroinvertebrate samples were collected from natural substrates (e.g., cobbles, gravel, sand) in both pool and riffle habitats by means of an Ekman dredge (sample area of 0.023 m^2) or a Surber sampler (sample area of 0.093 m^2). Additionally, artificial substrates were used at six sampling locations. These consisted of pairs of Hester-Dendy multiplate samplers constructed of multiple, parallel plates of tempered hardboard (sample area of 0.120 m^2). The Hester-Dendy samplers were left in riffle sections of the stream for 8 weeks and then removed and examined in the laboratory.

Qualitative benthic collections were also made with the use of a D-frame sweep net at all biological monitoring stations. The benthic samples were washed through a sieve having a mesh size of 500 mm. Organisms retained on the screen were removed and preserved in 10% formalin, transferred to 70% ethanol, identified to the lowest practicable taxon, and enumerated.

Attached algae samples were obtained from both natural and artificial substrates throughout the various reaches of Coyote Creek. Qualitative samples of attached algae were collected by scraping uniform areas of natural substrates such as logs and rocks. Quantitative collections of attached algae were made with the use of artificial substrates consisting of diatometers equipped with glass slides. These were suspended in the water column at six locations within the study area for 8 weeks, then removed and examined in the laboratory.

Rooted aquatic plants were sampled qualitatively whenever they were encountered in the study area. Plant specimens were collected, pressed or preserved, and identified.

Step 5. Data Evaluation and Step 6. Confirmatory Assessment

Observed Conditions in Coyote Creek

Water Quality — The purpose of the water quality monitoring program in Coyote Creek was to define receiving water conditions in the urban and non-urban areas during dry-weather conditions. Data on wet-weather Coyote Creek water quality conditions were also obtained from other sources for comparison (Pitt 1979; Metcalf and Eddy 1978; Pitt and Shawley 1982; SCVWD 1978; USDA 1978). Table 4.10 summarizes Coyote Creek water quality data for the wet- and dry-weather conditions and for both the urban and non-urban creek reaches. Dry-weather concentrations of many constituents exceeded corresponding wet-weather concentrations by factors of two to five times. For example, during dry weather, many of the major constituents (e.g., major ions, hardness, alkalinity, total solids, total dissolved solids, specific conductance, ammonia nitrogen, and orthophosphate) were significantly greater in both the urban and non-urban reaches. These constituents were all found at substantially lower concentrations in the urban runoff affecting Coyote Creek (Pitt 1979). Temperature, pH, dissolved oxygen, nitrate nitrogen, and arsenic were found to be about the same for wet and dry weather, for both the urban and non-urban areas. Within the urban area, several constituents were found in greater concentrations during wet weather than during dry

Table 4.10 Typical Coyote Water Quality Condition by Location and Season (mg/L unless otherwise noted)

	Urban Area		Non-Urban Area	
	Wet Weather	Dry Weather	Wet Weather	Dry Weather
Common Parameters and Major Ions				
pH	7	8		8
Temperature	16	17	—	16
Calcium — dissolved	20	100	40	100
Magnesium — dissolved	6	70	20	60
Sodium — dissolved	0.01	—	—	20
Potassium — dissolved	2	4	2	2
Bicarbonate	50	150	—	200
Sulfate	20	60	—	40
Chloride	10	60	—	20
Total hardness	70	500	200	600
Total alkalinity	50	300	150	300
Residuals				
Total solids	350	1000	600	1000
Total dissolved solids	150	1000	300	1000
Suspended solids	300	4	600	20
Volatile suspended solids	60	2	90	10
Turbidity (NTU)	50	15	—	20
Specific conductance (μmhos/cm)	200	500	—	400
Organics and Oxygen Demand Material				
Dissolved oxygen (DO)	8	7	—	9
Biochemical oxygen demand (5-day) (BOD_5)	25	—	5	—
Chemical oxygen demand (COD)	100	40	90	30
Total organic carbon (TOC)	110	—	—	0.6
Nutrients				
Total Kjeldahl nitrogen (TKN)	7	0.5	2	<0.3
Nitrate (as N)	0.7	0.8	—	1.2
Nitrite (as N)	—	0.02	—	<0.002
Ammonia (as N)	0.1	0.8	0.1	0.3
Orthophosphate	0.2	0.5	0.1	0.4
Heavy Metals				
Lead (μg/L)	2000	40	200	2
Zinc (μg/L)	400	30	200	20
Copper (μg/L)	20	10	50	5
Chromium (μg/L)	20	10	5	5
Cadmium (μg/L)	5	<1	5	<1
Mercury (μg/L)	1	0.2	1	0.2
Arsenic (μg/L)	4	3	5	2
Iron (μg/L)	10,000	1000	20,000	2000
Nickel (μg/L)	40	<1	80	<1

From Pitt, R. and M. Bozeman. *Sources of Urban Runoff Pollution and Its Effects on an Urban Creek*, EPA-600/S2-82-090, U.S. Environmental Protection Agency, Cincinnati, OH. 1982.

weather (e.g., suspended solids, volatile suspended solids, and turbidity). COD and organic nitrogen were also present in the urban area in greater abundance during wet weather than dry, as were heavy metals (e.g., lead, zinc, copper, cadmium, mercury, iron, and nickel).

Water quality upstream of the urbanized area was fairly consistent from site to site, but the quality changed markedly as the creek passed through the urbanized area. The water quality within the urbanized reach was generally poorer than at the stations upstream. Similar differences between wet and dry weather were also noted for the non-urban area. However, the wet-weather concentrations were typically much higher in the urban area than in the non-urban area. Several other constituents were also found in higher concentrations in the urban area than in the non-urban area during wet weather. Lead concentrations were more than seven times greater in the urban reach than in the non-urban reach during dry weather. Nitrite concentrations were almost seven times greater in the urban area. Ammonia nitrogen values in the urban area were 2.8 times greater than in the non-urban area. Other significant increases in urban area concentrations included chloride, nitrate, orthophosphate, COD, specific conductance, sulfate, and zinc. Conversely, the dissolved oxygen measurements were about 20% less in the urban reach than in the non-urban reach of the creek.

Selected water and sediment samples from the urban area reaches of Coyote Creek were analyzed as part of a nationwide screening effort to assess priority pollutant concentrations in urban runoff and urban receiving waters. Three samples were collected in January 1979, during a major storm. These included a runoff sample and samples of sediment and water from Coyote Creek. The sampling was conducted in and near the Martha Street outfall, which is located in a heavily urbanized area. Only 18 of the approximately 120 priority pollutants analyzed were detected (base-neutrals: fluoranthene, diethyl phthalate, di-*n*-butyl phthalate, bis(2-ethyl hexyl)phthalate, anthracene, phenanthrene, and pyrene; the phenols: 2,4,6-tricholorphenol, 2,4-dimethylphenol, pentachlorophenol, and phenol; and heavy metals: arsenic, cadmium, copper, lead, mercury, and zinc). These priority pollutants are generally the same as those found in most other urban runoff and receiving water samples collected nationwide (EPA 1983, Pitt et al. 1995).

Sediment Quality — Sediment samples were collected at the major sampling locations three times during the study. Table 4.11 summarizes all of the Coyote Creek sediment quality measurements obtained during the entire project. Orthophosphates, TOC, BOD_5, sulfates, sulfur, and lead were all found in higher concentrations in the sediments from the urban area stations, as compared with those from the upstream, non-urban area stations. The median sediment particle sizes were also found to be significantly smaller at the urban area stations, reflecting a higher silt content. Sulfur, lead, and arsenic were found in substantially greater concentrations (4 to 60 times greater) for the urban area sediments compared to the non-urban area sediments.

When all of the sediment data from the three monitoring periods were combined, very few differences were found between the urban and non-urban area values for COD, total phosphate, arsenic, and median particle size. However, seasonal variations were found to be important. When the data from just one sampling period were considered alone, greater and more significant variations in constituent concentrations between the two reaches were observed.

Lead concentrations in the urban area sediments were markedly greater than those from the non-urban area, by a factor of about six times (which is the widest margin for any constituent monitored). Large differences were also found between the urban and non-urban area data for both sulfate and phosphate. Average zinc concentrations in the sediments were found to increase by only about 1.5 times, but with a high degree of confidence.

The largest difference between urban and non-urban area sediment (mg/kg) to water (mg/L) concentration ratios (S/W) was for lead, where the S/W ratio was over 3000 for the urban area and only about 400 for the non-urban area. The total Kjeldahl nitrogen S/W ratio was about 5500 for the urban area but exceeded 22,000 for the non-urban area. For the other constituents studied, the differences between the urban and non-urban area S/W ratios were much less. Lead, zinc, arsenic,

Table 4.11 Coyote Creek Sediment Quality

All Units Are mg/kg Total Solids, Except for Particle Size	Non-Urban Area Stations below Anderson Dam						Urban Area Stations above Silver Creek						Urban and Non-Urban Differences	
	No. of Obs.	Mean	Min	Max	St. Dev.	COV	No. of Obs.	Mean	Min	Max	St. Dev.	COV	Ratio of Means	Confidence that Urban/ Non-Urban Values
Chemical oxygen demand	7	35,500	7400	98,000	34,800	0.98	13	39,300	4600	131,000	41,000	1.0	1.1	<60%
Total phosphate	4	148	7.5	344	168	1.1	10	168	14	406	161	0.96	1.1	<60%
Orthophosphate	3	1.2	0.46	1.7	0.65	0.54	3	3.6	1.2	6.6	2.8	0.78	3.0	85%
Total Kjeldahl nitrogen	7	6500	138	29,000	10,500	1.6	13	2490	146	14,000	4100	1.7	0.4	85%
Sulfate	7	136	<200	478	229	1.7	13	430	<200	3670	1010	2.4	3.2	80%
Arsenic	7	11.1	<1.0	28	11.1	1.0	13	13.0	1.5	45	10.3	0.79	1.2	65%
Lead	7	18.8	6.7	37	10.2	0.54	13	114	20	400	132	1.2	6.1	96%
Zinc	7	64	14	90	25	0.39	13	96	30	170	37	0.39	1.5	97%
Median particle size (μm)	7	4350	210	8760	4085	0.94	13	4480	70	8600	3650	0.81	1.0	<60%

From Pitt, R. and M. Bozeman. *Sources of Urban Runoff Pollution and Its Effects on an Urban Creek*, EPA-600/S2-82-090, U.S. Environmental Protection Agency, Cincinnati, OH. 1982.

and total Kjeldahl nitrogen all had S/W ratios of between 2000 and 5000 in the urban area. COD and total phosphate had S/W ratios of 1300 and 670, respectively, while orthophosphate and sulfate had S/W ratios of only about 20 and 6, respectively.

Because of these high observed sediment pollutant concentrations, it is likely that urban runoff-affected sediment is an important factor in the general decline in biological quality as Coyote Creek passes through the San Jose urban area. Other natural factors (e.g., stream gradient, temperature, and velocity changes) also probably contribute to this decline. For example, relatively flat creek gradients in the urban reach lead to low velocities which, in turn, encourage sedimentation of polluted particulates and allow temperatures to rise. Decreased flows in the urban area (due to diversions and infiltration) are an additional cause for changes in flow regime, water quality, and biological conditions.

Bioaccumulation of Lead and Zinc — Biological samples were collected from six stations in Coyote Creek and were analyzed to determine the lead and zinc they had accumulated while living in the creek. This sampling program was restricted to a single collection of organisms, with representative samples obtained from throughout the urban and non-urban stretches of the creek. Fish (*Gambusia affinis*), filamentous algae (*Cladophora* sp.), crayfish (*Procambarus clarkii*), and cattail plant segments (*Typha* sp.) were collected for analysis. An effort was made to collect similar specimens of the same species from each sampling location. All samples were rinsed to remove adhering sediment and were then chemically digested and analyzed for total lead and zinc content.

Some evidence of bioaccumulation of lead and zinc was found in many of the samples of algae, crayfish, and cattails. The measured concentrations of these metals in organisms (mg/kg) exceeded concentrations in the sediments (mg/kg) by up to a maximum factor of about 6. Concentrations of lead and zinc in the organisms exceeded water column concentrations by factors of 100 to 500 times, depending on the organism. Lead concentrations in urban area samples of algae, crayfish, and cattails were found to be two to three times as high as in non-urban area samples (Table 4.12), whereas zinc concentrations in urban area algae and cattail samples were about three times as high as the concentrations in the samples from the non-urban areas (Table 4.13). Lead and zinc concentrations in fish tissue were not significantly different between the urban and non-urban area samples.

Several early studies examined metal bioaccumulations in urban aquatic environments (Wilber and Hunter 1980; Neff et al. 1978; Phillips and Russo 1978; Ray and While 1976; Rolfe et al. 1977; Spehan et al. 1978). The lead concentrations in Coyote Creek waters are probably lower than the critical levels necessary to cause significant bioaccumulation in most aquatic organisms. The whole-body concentrations of zinc for the fish and crayfish were greater than many of the whole-body concentrations reported in the literature. The zinc concentrations in the Coyote Creek plants, however, were smaller than concentrations reported elsewhere for polluted waters.

Table 4.12 Lead Concentrations (mg lead/kg dry tissue) in Biological Samples[a]

	Non-Urbanized Area Stations			Urbanized Area Stations		
	Cochran	Miramonte	Metcalfe	Derbe	William	Tripp
Fish	<40	NS	NS	<30	<40	<50
Attached algae	<20	<30	<30	200	170	70
Crayfish	14	NS	<30	29	<36	40
Higher aquatics	<20	<30	<30	<30	<50	60
Sediment	28	37	16	37	370	400

[a] During storm events, lead concentrations in the urban reaches of Coyote Creek averaged about 2 mg/L. Dry weather, lead concentrations averaged about 0.04 mg/L in the urban reach. Non-urbanized reaches had lead water concentrations about 1/10 these values.

NS = No sample collected.

From Pitt, R. and M. Bozeman. *Sources of Urban Runoff Pollution and Its Effects on an Urban Creek*, EPA-600/S2-82-090, U.S. Environmental Protection Agency, Cincinnati, OH. 1982.

Table 4.13 Zinc Concentrations (mg zinc/kg dry tissue) in Biological Samples[a]

	Non-Urbanized Area Stations			Urbanized Area Stations		
	Cochran	Miramonte	Metcalfe	Derbe	William	Tripp
Fish	135	NS	NS	100	120	130
Attached algae	6.5	24	17	160	135	69
Crayfish	80	NS	90	89	140	62
Higher aquatics	9	78	26	40	150	210
Sediment	70	70	14	30	120	70

[a] During storm events, zinc concentration in the urban reaches of Coyote Creek averaged about 0.4 mg/L. Dry-weather zinc concentration in the urban reaches averaged about 0.03 mg/L. Non-urban reach water sample zinc concentrations were about half of these values.

NS = No sample collected.

From Pitt, R. and M. Bozeman. *Sources of Urban Runoff Pollution and Its Effects on an Urban Creek*, EPA-600/S2-82-090, U.S. Environmental Protection Agency, Cincinnati, OH. 1982.

Aquatic Biota Conditions

Fish — The fish fauna known to exist in the Coyote Creek drainage system at the time of the study was comprised of 27 species, 11 of which are native California fishes. The remainder were introduced through stocking by the California Department of Fish and Game and by the activities of bait dealers, fisherman, farm pond owners, and others. Although a relatively large variety of fish species was present in the Coyote Creek drainage, the existing distribution of some species was not widespread. Both Lake Anderson and Coyote Lake reservoirs sustained warm-water sport fisheries, and several of the fish species reported from the drainage were apparently confined to the specific habitat provided by those reservoirs. This included brown bullhead, channel catfish, Mississippi silverside, pumpkinseed, and redear sunfish. Of the remaining 22 species of fish known in Coyote Creek, 21 were encountered during this study, in which a total of 7198 fish were collected from 40 locations throughout the drainage. Rainbow trout and riffle sculpin were captured only in the headwater reaches and tributary streams of Coyote Creek. Likewise, Sacramento squawfish were found only in the upper reaches of the creek and reportedly have not been encountered downstream of Lake Anderson since 1960 (Scoppettone and Smith 1978). Seventeen fish species were collected from the major study area between Lake Anderson and the confluence of Silver Creek. Speckled dace, a native species previously reported to occur in the study area, was not encountered. Pacific lamprey, an anadromous species which moves into fresh water to spawn, was found only in and around the mouth of Upper Penitencia Creek, a tributary that enters the lower reaches of Coyote Creek.

Introduced fishes often cause radical changes in the nature of the fish fauna present in a given water body or drainage system. In many cases, they become the dominant fishes because they are able to outcompete the native fish for food or space, or they may possess greater tolerance to environmental stress. In general, introduced species are most abundant in aquatic habitats modified by man, while native fish tend to persist mostly in undisturbed areas (Moyle and Nichols 1973). Such was apparently the case within Coyote Creek. As seen in Table 4.14, samples from the non-urban portion of the study area were dominated by an assemblage of native fish species such as hitch, threespine stickleback, Sacramento sucker, and prickly sculpin. Collectively, native species comprised 89% of the number and 79% of the biomass of the 2379 fish collected from the upper reaches of the study area. In contrast, native species accounted for only 7% of the number and 31% of the biomass of the 2899 fish collected from the urban reach of the study area.

Hitch was the most numerous native fish species present. Hitch generally exhibit a preference for quiet water habitat and are characteristic of warm, low elevation lakes, sloughs, sluggish rivers, and ponds (Calhoun 1966; Moyle and Nichols 1976). In streams of the San Joaquin River system in the Sierra Nevada foothills of central California, Moyle and Nichols (1973) found hitch to be

Table 4.14 Relative Abundance of Fish in Coyote Creek

	Urban Reach	Rural Reach
Native Fish		
Hitch	4.9%	34.8%
Threespine stickleback	0.8	27.3
Sacramento sucker	0.1	12.6
Prickly sculpin	<0.1	8.2
Introduced Fish		
Mosquitofish	66.9	5.6
Fathead minnow	20.6	0.6
Threadfin shad	2.4	nd
Green sunfish	1.2	<0.1
Bluegill	1.0	0.2

From Pitt, R. and M. Bozeman. *Sources of Urban Runoff Pollution and Its Effects on an Urban Creek,* EPA-600/S2-82-090, U.S. Environmental Protection Agency, Cincinnati, OH. 1982.

most abundant in warm, sandy-bottomed streams with large pools, where introduced species such as green sunfish, largemouth bass, and mosquitofish were common. Likewise, during this Coyote Creek study, hitch were found to be associated with green sunfish, fathead minnows, and mosquitofish in the lower portions of Coyote Creek. However, mosquitofish dominated the collections from the urbanized section of the creek and accounted for over two thirds of the total number of fish collected from that area. In foothill streams of the Sierra Nevada, Moyle and Nichols (1973) found mosquitofish to be most abundant in disturbed portions of the intermittent streams, especially in warm, turbid pools. The fish is particularly well adapted to withstand extreme environmental conditions, including those imposed by stagnant waters with low dissolved oxygen concentrations and elevated temperature. The second most abundant fish species in the urbanized reach of Coyote Creek, the fathead minnow, is equally well suited to tolerate extreme environmental conditions. The species can withstand low dissolved oxygen, high temperature, high organic pollution, and high alkalinities. Often thriving in unstable environments such as intermittent streams, the fathead minnow can survive in a wide variety of habitats. However, the species seems to do best in pools of small, muddy streams and in ponds (Moyle and Nichols 1976).

Benthic Macroinvertebrates — The taxonomic composition and relative abundance of benthic macroinvertebrates were collected from both natural and artificial substrates in Coyote Creek (Figures 4.7 through 4.9). The abundance and diversity of benthic taxa were greatest in the non-urbanized sections of the stream. Figure 4.10 shows the trend of the overall decrease in the total number of benthic taxa encountered in the urbanized sections of the study area during 1978 and 1979. An overall increase in number and diversity of benthic organisms was encountered in 1979, compared to 1978 collections. This may be attributed to further recovery from the drought conditions that preceded this study. The benthos in the upper reaches of Coyote Creek consisted primarily of amphipods and a diverse assemblage of aquatic insects. Together those groups comprised two thirds of the benthos collected from the non-urban portion of the creek. Clean-water forms were abundant and included amphipods (*Hyalella azteca*) and various genera of mayflies, caddisflies, black flies, crane flies, alderflies, and riffle beetles. In contrast, the benthos of the urban reaches of the creek consisted almost exclusively of pollution-tolerant oligochaete worms (tubificids). Tubificids accounted for 97% of the benthos collected from the lower portion of Coyote Creek.

Crayfish were present throughout the study area and were collected in conjunction with the fish sampling effort. Two species of crayfish were encountered in Coyote Creek waters — *Pacifastacus leniusculus* and *Procambarus clarkii*. Neither species is native to California waters. *Pacifastacus*

Figure 4.7 Natural substrate sampling using a Surber sampler in Coyote Creek.

Figure 4.8 Removing benthic macroinvertebrate samples from Surber sampler.

Figure 4.9 Artificial substrate sampling using a Hester-Dendy multiplate sampler in Coyote Creek.

leniusculus was collected in the non-urbanized section of the study area. It is typically found in a wide variety of habitats including large rivers, swift or sluggish streams, lakes, and, occasionally, muddy sloughs. *Procambarus clarkii* was collected in both the urbanized and non-urbanized sections of the stream. The species prefers sloughs where the water is relatively warm and vegetation plentiful; however, it is also found in large streams. Because of its burrowing activities *P. clarkii* often becomes a nuisance by damaging irrigation ditches and earthen dams.

Attached Algae — Qualitative samples from natural substrates indicated that the filamentous alga *Cladophora* sp. was found throughout the study area. However, its growth reached greatest proportions in the upper sections of the stream. Table 4.15 presents the taxonomic composition and relative abundance of diatoms collected from artificial substrates (Figure 4.11) placed at selected sample locations. The periphyton of the non-urban reaches of the stream was dominated by the genera *Cocconeis* and *Achnanthes*. The genera *Nitzschia* and *Navicula*, generally accepted to be more pollution-tolerant forms, dominated the periphyton of the urbanized reaches of Coyote Creek.

Rooted Aquatic Vegetation — Rooted aquatic plants were not greatly abundant in the Coyote Creek study area. Submerged macrophytes were restricted entirely to the upper reaches of the study area and consisted of occasional stands of sago pondweed (*Potamogeton pectinatus*) and curly-leaf pondweed (*P. crispus*). Emergent forms consisted of water primrose (*Jussiaea* sp.), confined to several areas in the non-urban reach of the stream, and numerous small stands of cattails (*Typha* sp.) sparsely distributed throughout the length of the study area.

Step 7. Project Conclusions

The biological investigations in Coyote Creek indicated distinct differences in the taxonomic composition and relative abundance of the aquatic biota present in Coyote Creek. The non-urban

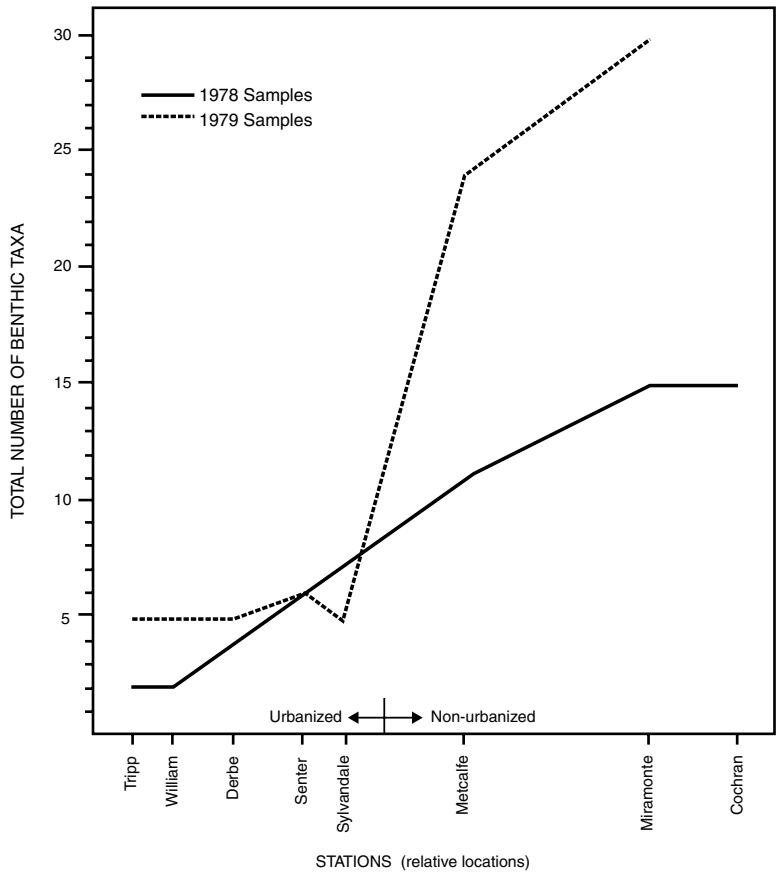

Figure 4.10 Trend of total number of benthic taxa observed during 1978 and 1979 (From Pitt, R. and M. Bozeman. *Sources of Urban Runoff Pollution and Its Effects on an Urban Creek*, EPA-600/S2-82-090, U.S. Environmental Protection Agency, Cincinnati, OH. 1982.)

sections of the creek supported a comparatively diverse assemblage of aquatic organisms, including an abundance of native fishes and numerous benthic macroinvertebrate taxa. In contrast, however, the urban portions of the creek comprised an aquatic community generally lacking in diversity and was dominated by pollution-tolerant organisms such as mosquitofish and tubificid worms.

Although certain differences in physical habitat occurred in the downstream reaches of the study area (e.g., a decrease in stream gradient, shorter riffles, wider, deeper pools, etc.), such differences were not thought to be responsible for the magnitude of change noted in the aquatic biota of the urban reach of Coyote Creek.

Urban runoff monitoring during this project showed that stormwater was the significant contributor to the high levels of many toxic materials in the receiving water and sediments of the stream. In addition, changes in the nature of the stream substrate occurred as a result of the deposition of silt and debris, which largely originate from urban runoff. Such changes were likely the primary reason for the decline in species abundance and diversity observed in the urban reaches of Coyote Creek.

Critique of the Longitudinal Analyses in Coyote Creek

The Coyote Creek study was very comprehensive, and therefore costly. This was probably the earliest large-scale receiving water study conducted to investigate urban runoff effects on in-stream

Table 4.15 Taxonomic Composition and Relative Abundance of Diatoms Collected on Glass Slides in Coyote Creek during the Spring of 1978

Taxon	Relative Abundance (%) of each Taxon within the Sample					
	Non-Urban Area Stations			Urban Area Stations		
	Cochran	Miramonte	Metcalfe	Derbe	Williams	Tripp
Centrales						
Coscinodiscaceae						
Melosira sp.	0.4	—	—	—	1.2	0.8
Pennales						
Diatomaceae						
Diatoma vulgare	0.4	—	1.5	—	—	—
Fragilariaceae	—	—	—	0.8	0.9	0.4
Synedra sp.						
Achnanthaceae						
Achanthes lanceolata	20.6	37.8	56.1	49.8	0.9	1.6
Rhoicosphenia curvata	0.4	—	—	1.2	—	—
Cocconeis pediculus	15.0	18.2	0.4	—	—	—
Cocconeis placentula	62.4	44.0	41.2	—	—	—
Naviculaceae						
Navicula spp.	—	—	—	—	10.5	23.8
Diploneis sp.	—	—	—	—	2.4	—
Frustulia rhomboides	—	—	—	—	0.4	—
Gyrosigma sp.	—	—	—	—	—	0.4
Gomphonenataceae						
Gomphonema sp.	—	—	—	2.8	6.9	0.8
Cybellaceae						
Cymbella sp.	0.8	—	—	—	2.0	0.4
Rhopalodia spp.	—	—	—	—	—	0.4
Nitzschiaceae						
Nitzachia sp.	—	—	0.8	43.4	67.5	70.6
Denticula elegans	—	—	—	—	2.4	0.4
Surirellaceae						
Cymatopleura solea	—	—	—	—	0.9	—
Surirella sp.	—	—	—	2.0	4.0	0.4
Total Number Frustules/mm^2	5545	4950	1874	4488	1189	4575

From Pitt, R. and M. Bozeman. *Sources of Urban Runoff Pollution and Its Effects on an Urban Creek*, EPA-600/S2-82-090, U.S. Environmental Protection Agency, Cincinnati, OH. 1982.

Figure 4.11 Artificial substrate diatometer sampler being loaded with glass microscope slides in Coyote Creek.

biological conditions. As such, many elements were considered in the site investigation. The project included field sampling over a period of 3 years, and more than 40 sampling sites were periodically visited. A broad list of biological, chemical, and physical measurements was obtained. Even though the project was comprehensive, several omissions seem obvious. The most notable is the lack of toxicity testing. Some limited laboratory fathead minnow 96-hour exposure tests were conducted as part of the study, but were inconclusive and therefore not reported. The project was also conducted before effective and less costly *in situ* toxicity tests were developed. Another element that was missing was comprehensive habitat surveys. Formalized habitat survey procedures detailed in this book (Chapter 6 and Appendix A) would have been very useful during the Coyote Creek study. Finally, because the study design did not have any precedence, it was probably inefficient in that it obtained more information than was actually needed, and at more locations than necessary.

The longitudinal study design is very helpful in that gradients of conditions can be examined. The Coyote Creek study examined a very large number of locations along the creek in an attempt to identify locations that were partially degraded, but still in acceptable condition. When these locations are identified, watershed modeling can be used to calculate the assimilative capacity of the stream, which can then be used to determine necessary stormwater controls to provide these conditions farther downstream. Unfortunately, Coyote Creek was found to degrade very rapidly at the edge of development. Additional monitoring locations were therefore added in an attempt to isolate the degradation gradient. The highly variable conditions in the creek at the edge of urbanization were likely due to major flow changes seasonally and from year-to-year, preventing identification of an acceptably degraded site.

In many cases, a longitudinal study design can be combined with the other two major types of designs (parallel and trend studies) to obtain additional information. The trend case study presented is for a trend with time, but a trend with distance can also be evaluated using similar statistical procedures described in Chapter 7.

Example of Parallel Creeks Experimental Design — Kelsey and Bear Creeks, Bellevue, WA, Receiving Water Study

Several separate urban stormwater projects (as part of the U.S. EPA's Nationwide Urban Runoff Program, or NURP) were conducted in Bellevue, WA, to address the three major phases in designing an urban runoff control program (quantifying the specific local urban runoff receiving water problems, determining the sources of the problem pollutants, and selecting the most appropriate control measures). These projects were conducted from 1977 through 1982 and constitute one of the most comprehensive urban runoff/receiving water impact research programs ever conducted.

The U.S. Geological Survey (USGS) through its Tacoma, WA, office, conducted one of the projects, which was funded by the USGS and the Water Planning Division of EPA. The USGS (Ebbert et al. 1983; Prych and Ebbert undated) intensively monitored urban runoff quality and quantity from three residential areas in Bellevue and evaluated the effectiveness of a detention facility. Wet and dry atmospheric sources were also monitored by the USGS.

The University of Washington's Civil Engineering Department and the College of Fisheries Research Institute prepared five reports based on their studies, which were funded by the Corvallis Environmental Research Laboratory of EPA (Pedersen 1981; Perkins 1982; Richey et al. 1981; Richey 1982; Scott et al. 1982). Generally, the University of Washington's projects evaluated the receiving water conditions for direct impairments of beneficial uses.

The Municipality of Metropolitan Seattle (METRO) research was funded by Region X of EPA and was prepared by Galvin and Moore (1982). METRO analyzed many source area, urban runoff, and creek samples for metallic and organic priority pollutants.

The City of Bellevue also conducted a study, which was funded by the Storm and Combined Sewer Section of EPA and the City of Bellevue. The Bellevue report was prepared by Pitt (1985) and Pitt and Bissonnette (1984). The City of Bellevue collected and analyzed urban runoff and baseflow samples using flow-weighted techniques for more than 300 storms from two residential areas, in addition to extensively evaluating street and sewerage cleaning as stormwater management practices.

Bellevue's moderate climate has a mean annual precipitation of about 1.1 m (44 in) which occurs mostly as rainfall from October through May. Most of the rainfall results from frontal storms formed over the Pacific Ocean. During fall and winter months, low to moderate rainfall intensities are common. Even though the runoff quality was found to be much cleaner than in other locations in the United States, the urban creek was significantly degraded when compared to the rural creek, but still supported a productive, but limited and unhealthy salmonid fishery. Many of the fish in the urban creek, however, had respiratory anomalies. The urban creek was not grossly polluted, but flooding from urban developments has increased dramatically in recent years. These increased

Figure 4.12 Rural Bear Creek, Bellevue, WA.

Figure 4.13 Rural Bear Creek, Bellevue, WA, in undeveloped area.

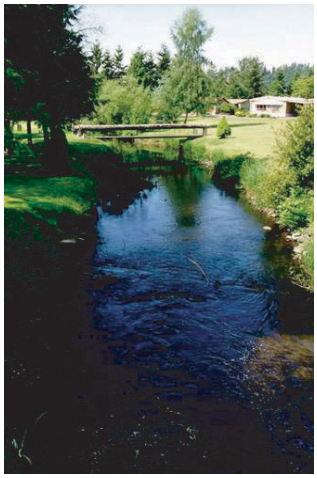

Figure 4.14 Rural Bear Creek, Bellevue, WA, passing through trailer park.

Figure 4.15 Urbanized Kelsey Creek, Bellevue, WA, in low-density residential area.

flows have dramatically changed the urban stream's channel, by causing unstable conditions with increased stream bed movement, and by altering the availability of food for the aquatic organisms. The aquatic organisms are very dependent on the few relatively undisturbed reaches. Dissolved oxygen concentrations in the sediments depressed embryo salmon survival in the urban creek. Various organic and metallic priority pollutants were discharged to the urban creek, but most of them were apparently carried through the creek system by the high storm flows to Lake Washington.

The in-stream studies were conducted in Bear Creek (Figures 4.12 through 4.14), a relatively undisturbed natural stream, and in Kelsey Creek (Figures 4.15 through 4.17), a heavily urbanized stream. The watershed studies were conducted in the Lake Hills and Surrey Downs neighborhoods (Figure 4.18).

Step 1. What's the Question?

Does urban runoff significantly affect Bellevue's receiving water uses; what are the sources of the urban runoff problem pollutants; and can public works practices (street cleaning and catchbasin cleaning) reduce the magnitude of these problems?

Figure 4.16 Urbanized Kelsey Creek, Bellevue, WA, in commercial area.

Figure 4.17 Kelsey Creek, Bellevue, WA, street crossing with sign.

Bellevue area waters have five designated beneficial uses:

1. Preservation of habitat suitable for aquatic organisms
2. Flood prevention by the conveyance of stormwater
3. Open space and resource preservation
4. Recreational uses (swimming and boating)
5. Aesthetics

Figure 4.18 Typical residential neighborhood in monitored Lake Hills and Surrey Downs watersheds, Bellevue, WA.

The Bellevue research projects (especially those conducted by the University of Washington team) investigated the potential impairments of these uses in the urbanized Kelsey Creek, compared to Bear Creek, the control stream.

Step 2. Decide on Problem Formulation

The basic problem formulation was to investigate parallel watersheds. Kelsey Creek is completely urbanized, while Bear Creek had only minor development and was used as a control stream. In addition, the street cleaning portions of the study compared parallel portions of the urban area (the Lake Hills and Surrey Downs catchments), with rotating street cleaning operations and outfall monitoring.

Step 3. Project Design, Step 4. Project Implementation, Step 5. Data Evaluation, and Step 6. Confirmatory Assessment

1. Qualitative Watershed Characterization

The Surrey Downs and Lake Hills test catchments are about 5 km apart and are each about 40 ha in size. They are both fully developed, mostly as single-family residential areas. The 148th Avenue dry detention basin study area is about 10 ha in area and is primarily a street arterial with

adjacent landscaping. The Surrey Downs area was developed in the late 1950s. Most of the slopes in the basin are moderate with some steeper slopes on the west side of the area. About 60% of the Surrey Downs area is pervious. Back and front yards make up most of the land surface area, while the streets make up 10%. There is relatively little automobile traffic in the Surrey Downs area and the on-street parking density is low. The storm drainage system discharges into an artificial pond located in an adjacent development. This pond discharges into Mercer Slough, which eventually drains to Lake Washington and Puget Sound. The Surrey Downs catchment ranges in elevation from about 3 to 55 m.

The Lake Hills catchment is about 41 ha in size and contains the St. Louise parish church and school in addition to single-family homes. These homes were also developed in the 1950s. Lake Hills has a slightly larger percentage of pervious area than Surrey Downs, but a slightly smaller typical lot size. The slopes in Lake Hills are also more moderate (with a few exceptions) than those found in Surrey Downs. Most of the streets in Lake Hills also carry low volumes of traffic and have low parking densities, except for two busy roads which cross through the area. The Lake Hills storm drainage system discharges into a short open channel which joins Kelsey Creek just downstream from Larsen Lake. Kelsey Creek also discharges into Mercer Slough and finally into Lake Washington and Puget Sound. The elevation of the Lake Hills study catchment ranges from 80 to 125 m.

The 148th Avenue S.E. catchment was used to investigate the effects of a dry detention facility on stormwater quality. The drainage area is about 10 ha. Slightly more than one fourth of this area is the actual street surface of 148th Avenue S.E., a divided, four-lane arterial. Other impervious areas include sidewalks, parking lots, office buildings, and parts of Robins Wood Elementary School.

The soils in all three of these test catchments are mostly the Arents-Alderwood variety, having 6 to 15% slopes. The surface soils are made up of gravelly sand loams with an estimated natural permeability of between 50 and 150 mm/hour. The total water capacity of this soil horizon is about 20 mm.

A demographic survey was conducted in the test catchments by the City of Bellevue at the beginning of the project (in 1977). Slightly more than three people per household were reported in both basins, while the population density per hectare was about 30 in Lake Hills and about 23 in Surrey Downs. More than half of the people in both basins had no dogs or cats, with the remainder of the households having one or more of each. Slightly more than two cars per household were reported, with about 10% of the households in each basin reporting four or more cars. Most of the automobile oil was disposed of properly in the household garbage or recycled, but between 5 and 10% of the households used oil to treat fence posts, dumped it onto the ground, or into the storm sewers. Most of the people carried their grass and leaves to the dump, or put them into the garbage, and about one third composted the organic debris on their lots.

2. Stream Characterization

Kelsey Creek flows through the City of Bellevue, while Bear Creek is about 30 km farther east. Kelsey Creek drains a watershed about 3200 ha in area, which is predominantly urban. About 54% of the Kelsey Creek watershed has single- and multiple-family residences, 24% has commercial or light industrial uses, and 22% has parks and undeveloped areas. A main channel of Kelsey Creek starts at Larson Lake and flows about 12 km through the City of Bellevue before discharging into Lake Washington. The USGS has continuously monitored Kelsey Creek flows since 1959 at a location about 2.5 km upstream from Lake Washington. Kelsey Creek is a relatively narrow stream with a mild slope. The mean channel slope is about 1.5% and the bank full width ranges from about 3.5 to 6.5 m in the study area. Along much of its length, Kelsey Creek appears disturbed. Channelization, riprapping, storm drain outfalls, scoured and eroded banks, and culverts are common. The stream bank (riparian) vegetation is mostly composed of low growing alder and vine maple with scattered big leaf maples and western red cedar trees. The understory is dominated by blackberry bushes.

Bear Creek starts at Paradise Lake and drains into Cottage Lake Creek. Its drainage area is about 3400 ha and is mostly rural in character. About 85% of the Bear Creek drainage is in pasture or woodlands with about 15% developed in single-family residences. Bear Creek also has a mild slope (about 0.6%) and is slightly wider than Kelsey Creek with a bank-full width ranging from about 7 to 11 m. Bear Creek has the appearance of a relatively undisturbed stream, especially when compared to Kelsey Creek. The vegetation along some reaches in Bear Creek has been modified, and there is some riprapping for bank stabilization. Most of these disturbances are quite small. Throughout most of the Bear Creek study reach, the creek is composed of alternating series of pools and riffles, frequent debris dams, side channels, and sloughs. The riparian vegetation along Bear Creek is mostly old growth alder, western red cedar, and douglas fir, with an understory of vine maple and salmonberry. Richey (1982) states that while Bear Creek receives no point source discharges, it is not pristine. Drainage from septic tanks, fertilizers, and livestock wastes has enriched the stream. Many homeowners have cut or modified the bank vegetation, installed small diversions, and created small waterfalls. These activities appear to have generated an increase in sediment transport. Building activity has also increased in the upper parts of the watershed since 1981. Much of the creek, however, remains in a natural condition and is typical of many of the gravel-bottomed streams in the Pacific Northwest.

3. Select Monitoring Parameters

The Bellevue city project included monitoring of the quality and quantity of stormwater runoff from two urban areas in the City of Bellevue. Street surface particulate samples were collected in these two basins along with storm drainage sediment samples. The City of Bellevue conducted various street cleaning operations in the two test basins and evaluated the effectiveness of various types of street cleaning programs and catchbasin cleaning activities in improving the quality of urban runoff. The USGS also monitored stormwater runoff quality and quantity in these two test basins. The USGS used different sampling techniques to monitor fewer storms but in much greater detail. The USGS monitored rainfall and dustfall quality and quantity along with the performance of a series of detention basins at a third Bellevue test site. The University of Washington's projects investigated urban runoff receiving water conditions and conditions in a control stream much less affected by urban runoff. The University's projects studied physical, chemical, and biological conditions to identify impacts associated with urban development on receiving water quality. The Seattle METRO project involved conducting trace metal and organic pollutant analyses for samples collected from these three other projects. The following list summarizes the major components of the Bellevue investigations:

- In-stream effects from urban stormwater (comparing test and control stream conditions over a 2-year period)
  - In-stream water quality (wet and dry weather observations) for conventional and nutrient constituents, plus some toxicants
  - Interstitial water quality in test and control streams for dissolved oxygen, nutrients, and metallic toxicants
  - Continuous stream flow rates
  - Aquatic organism food availability and utilization studies
  - Riparian vegetation, algae, benthic organisms, and fish
  - Creek sediment quality for conventional and toxic pollutants
  - Creek bank stability and stream bed erosion, and creek sedimentation and sediment transport
- Sources of urban runoff pollutants in two test catchments for 2-year period
  - Atmospheric particulate and rainfall contributions
  - Runoff monitoring from about 400 rain effects (91 to 99% of annual flow monitored during 2 years)

  - Stormwater quality from more than 200 events for conventional, nutrient, and toxic constituents (200 to 1000 analyses per constituent)
  - Baseflow quality from about 25 sampling periods for conventional, nutrient, and toxic constituents
  - Street dirt characteristics from about 600 samples (loading, particle size, washoff, and chemical quality)
  - Sewerage and catchbasin sediment accumulations over 2-year period (accumulation and quality) from about 200 inlets
- Effectiveness of urban runoff controls
  - Monitored street dirt loadings and runoff characteristics at two test catchments over 2-year period, comparing none with three times a week street cleaning effort
  - Measured changes in catchbasin sump accumulations of pollutants in about 200 inlets over 2 years in two catchments
  - Monitored influent and effluent from a dry detention pond for the 20 storms during the 2-year period when flows were sufficient to enter the pond system

Observations

Effects of Urban Runoff on Bellevue's Stream's Beneficial Uses — Richey (1982) summarizes some of the beneficial use impairments that the University of Washington study team addressed. Urbanization and stormwater runoff discharges to streams can have a wide variety of effects on these receiving waters. These include increased runoff, decreased surface storage, decreased transpiration, decreased infiltration, and a degradation in water quality. These effects may be either long term or intermittent. Changes in channel geomorphology caused by channelization in the clearing of stream bank vegetation may cause permanent stresses to the stream. Changes in the stream flows during runoff events, such as rapidly rising and falling hydrographs and increased total flows and peak discharges, are intermittent stress factors. The discharge and transport of pollutants can act as an intermittent stress factor, but the storage of these pollutants in the stream system (in the sediments or bioaccumulation) can act as a long-term or chronic stress factor. Therefore, it is necessary to identify not only the causative factor in impairing receiving water quality but also the times when these effects occur. Elevated concentrations of toxic materials in the runoff may affect receiving water organisms during a runoff event. However, they may also accumulate in the sediments and not affect the receiving water aquatic life until some time after they were discharged.

Richey points out the difficulty in identifying problem pollutants or their causes based upon their different destructive powers. She presents a hypothetical example where the gradual introduction of toxic pollutants in the receiving water results in a decline of fish species diversity and system productivity. Because the watershed has been urbanizing, increased flows have also occurred. If it is assumed that the increased flows causing flooding and scouring in the water body were the most important element restricting the fish populations, an abatement program incorporating detention facilities to reduce these flooding problems may be implemented. However, the input of toxic substances may not be reduced and significant improvements in the beneficial use may not occur. She concludes that it is very important to study all effects on a receiving body including hydrology, geomorphology, and pollutant inputs.

Scott et al. (1982) state that factors contributing to the instability of the physical receiving water system are relatively easy to identify but that their combined effect on the receiving water aquatic life is difficult to measure. They also mention that the Resource Planning Section of the King County Planning Division analyzed available data for 15 local streams in an attempt to establish a cause-and-effect relationship between urban development and stream degradation. They examined watershed variables such as the magnitude of the impervious areas, peak flows, water quality, aquatic insects, and salmonid escapement to rank the streams. Bear Creek ranked 12th in impervious surfaces, lowest in peak flow, 5th in water quality, 6th in aquatic insects, and 2nd in salmonid

escapement. Kelsey Creek ranked 2nd in impervious areas, 6th in peak flow, 15th in water quality, 50th in aquatic insects, and 8th in salmonid escapement.

1. Bellevue Receiving Water Beneficial Uses

Kelsey Creek, the urban receiving water studied during this project, has three primary functions: conveyance of stormwater from Bellevue to Lake Washington, providing a scenic resource for the area, and providing a habitat for fish. The most important beneficial use of Kelsey Creek is the conveyance of stormwater out of the city. The City of Bellevue, in its Storm Drainage Utility and support of projects such as these, has a commitment to provide the other beneficial uses. Richey (1982) states that Kelsey Creek can physically provide for all of these beneficial uses. The creek has been developed for the conveyance of stormwaters, but there are also areas in its lower reaches where the canopy cover is relatively intact and the stream banks and morphology are still quite natural. Dense growth of shrubbery and blackberry vines also provides cover and shade for stream aquatic life. The riprap allows the development of deep pools which can be a good habitat for fish. Perkins (1982) states that some of the upstream reaches and tributaries of Kelsey Creek are less disturbed and serve as a potential refuge area for aquatic life. The downstream reaches of Kelsey Creek, however, are less supportive of aquatic life due to channel instability and erosion, along with flashy flows and increasing floods.

2. Bear Creek and Kelsey Creek Water Quality

The University of Washington project monitored Kelsey Creek and Bear Creek water quality from May 1979 through April 1981. Table 4.16 (Richey 1982) summarizes these creek water quality observations. The values for the constituent concentrations were obtained during stable flow periods only when the creeks were not rising or falling rapidly. The major ion types are similar for both Bear and Kelsey Creeks: calcium/magnesium bicarbonate. The concentrations of these ions were typically lower in Bear Creek. Richey found that during the study period the average nutrient levels in Kelsey Creek were greater than those found in Bear Creek. Total phosphorus and soluble reactive phosphorus in Kelsey Creek were about 2.5 times higher than those found in Bear Creek. Both streams have ample supplies of both nitrogen and phosphorus for the aquatic organisms, and the nitrate plus nitrite concentrations had a distinct seasonal trend in Bear Creek, while they were essentially random in Kelsey Creek. High winter and low summer concentrations of nitrate plus nitrite have been observed in other rural streams and are thought to be controlled largely by the seasonal nitrogen uptake of terrestrial vegetation. Bear Creek has much more riparian vegetation than does Kelsey Creek. The high nitrogen concentrations in Bear Creek may also be caused by in-stream nitrification. In addition, the maximum ammonium concentrations in Bear Creek occurred during the autumn when there was decomposition of sockeye salmon bodies in the creek.

The observed low dissolved lead concentrations in Kelsey Creek and Bear Creek are not expected to exert a major impact on the aquatic life. However, other possible toxic compounds which may be washing into the stream system were not continuously monitored. Pedersen (1981) notes that massive fish kills in Kelsey Creek or its tributaries were observed on several occasions due to the dumping of toxic materials down storm drains. The resultant impact of this toxic material on the benthic organisms from these dumps was found to be substantial, but no permanent impact over long time periods was observed. The 5-day biochemical oxygen demand (BOD_5) concentrations were low in both streams. They found that the greatest differences in constituents between the two streams occurred in constituents that were in particulate forms.

Scott et al. (1982) listed the most important water quality differences between these two creeks:

- Kelsey Creek had higher nutrient concentrations than Bear Creek. /
- Kelsey Creek had one to two times the suspended particulate loads of Bear Creek. /

Table 4.16 Surface Water Quality (monthly average concentrations from May 1979 through April 1981)

		Kelsey Creek				Bear Creek				Ratio of Kelsey Creek Mean Values to Bear Creek Mean Values
	Units	Mean	SD*	Minimum	Maximum	Mean	SD	Minimum	Maximum	
Drainage area	ha	3109				3600				0.9
Instantaneous discharge	m^3/s			0.20	8.68			0.13	6.31	1.5/1.4 (min/max ratios)
Substrate size	mm	36.7	6.8			27.5	4.9			1.3
Summer temperature	°C				23.0				23.0	1.0 (ratio of max.)
Winter temperature	°C			5.0				3.2		1.6 (ratio of min.)
Total suspended solids	mg/L	11.0	7.4	2.5	32.9	4.7	3.0	0.8	11.9	2.3
Fine particulate organic carbon	mg C/L	0.87	0.53	0.10	2.51	0.75	0.36	0.32	1.51	1.2
Dissolved organic carbon	mg C/L	7.5	3.4	3.8	14.8	6.4	3.3	3.0	16.8	1.2
Total phosphorus	µg P/L	116	32	72	193	43	16	15	79	2.7
Soluble reactive phosphorus	µg P/L	82	27	54	167	24	16	8	63	3.4
Nitrate plus nitrite nitrogen	µg N/L	743	137	468	962	508	540	59	2350	1.5
Ammonia nitrogen	µg N/L	36	14	12	66	30	26	9	114	1.2
BOD_5	mg O_2/L	2.26	1.27	0.86	5.3	1.63	1.08	0.03	3.59	1.4
Dissolved lead	µg Pb/L	5	2	2	11	<4	—	<4	<4	>1.3

* SD = standard deviation.

Data from Richey, J. S. *Effects of Urbanization on a Lowland Stream in Western Washington*, Ph.D. dissertation, University of Washington, Seattle. 1982. With permission.

Table 4.17 Annual Kelsey and Bear Creek Discharges (June 1979 through May 1980, kg/ha/year)

Constituent	Kelsey Creek	Bear Creek	Ratio of Kelsey to Bear Creek Discharges
Total suspended solids	300	78	3.8
Fine particulate organic carbon (FPOC)	33	12	2.8
Dissolved organic carbon (DOC)	53	55	1.0
Soluble reactive phosphorus	0.56	0.17	3.3
Total phosphorus	0.87	0.33	2.6
Nitrate plus nitrite nitrogen	4.3	7.1	0.6

Data from Richey, J.S., et al. The effects of urbanization and stormwater runoff on the food quality in two salmonid streams. *Verh. Internat. Werein. Limnol.*, Vol. 21, pp. 812–818, Stuttgart. October 1981.

- Inorganic silt was the dominant fraction of the suspended particulate load in Kelsey Creek.
- The concentrations of potentially toxic materials in both study streams were quite low and possibly negligible.

Observed problems in Kelsey Creek included high water temperatures and elevated fecal coliform counts. The fecal coliform counts, however, varied considerably throughout the Kelsey Creek drainage system. Bear Creek also had high fecal coliform counts along with high inorganic nitrogen and total phosphorus concentrations.

The annual creek discharges of various water quality constituents are shown in Table 4.17 (Richey et al. 1981). The total solids concentrations were highest during the periods of high flows (late fall, winter, and early spring). Therefore, most of the solid material was transported during only a few months of the year. Thirty-three percent of the solids were transported out of Kelsey Creek and 35% out of Bear Creek during the high flow month of December alone. The annual yields of both particulate and soluble phosphorus were about three times greater in Kelsey Creek than in Bear Creek. The total suspended solids transport in Kelsey Creek was almost four times greater than Bear Creek. While the fine particulate organic matter in Kelsey Creek was almost three times more than in Bear Creek on an annual basis, the dissolved organic carbon transport was about the same. High phosphorus concentrations in the fall in Bear Creek may also be caused by decomposing sockeye salmon. Scott reported more than 1000 sockeye carcasses in the stream channel during the fall of 1979 and 1980.

Richey (1982) states that Kelsey Creek is surprisingly clean for a heavily urbanized stream. This might be because of the in-stream dilution of the contaminants, because some of the watershed is still relatively protected, or possibly the result of differences in the occurrence of the urban contaminants. She further states that Kelsey Creek is enriched but does not appear to be polluted in the classic sense. The rapid transport of water and materials appears to protect the stream by removing many of the potentially hazardous pollutants to downstream locations. In addition, the rapid transport of water also helps to maintain high levels of dissolved oxygen.

The City of Bellevue project (Pitt 1985) evaluated water quality with beneficial use criteria. Potential long-term problem pollutants are settleable solids, lead, and zinc. These long-term problems are caused by settled organic and inorganic debris and particulates. This material may silt up salmon spawning beds in the Bellevue streams and introduce high concentrations of potentially toxic materials directly to the sediments. Oxygen depletion caused by organic sediments may also occur under certain conditions, and the lead and zinc concentrations in the sediments may affect the benthic organisms. The discharge of particulate heavy metals, which settle out in the sediments, may be converted to more soluble forms through chemical or biological processes.

3. Creek Interstitial Water Quality

The University of Washington and the Seattle METRO project teams analyzed interstitial water for various constituents. These samples were obtained by inserting perforated aluminum standpipes

into the creek sediment. This water is most affected by the sediment quality and in turn affects the benthic organisms much more than the creek water column. Scott et al. (1982) found that the interstitial water pH ranged from 6.5 to 7.6 and did not significantly differ between the two streams but did tend to decrease during the spring months. The lower fall temperatures and pH levels contributed to reductions in ammonium concentrations. The total ammonia and ammonium concentrations were significantly greater in Kelsey Creek than in Bear Creek. They also found that the interstitial dissolved oxygen concentrations in Kelsey Creek were much below concentrations considered normal for undisturbed watersheds. These decreased interstitial oxygen concentrations were much less than the water column concentrations and indicated the possible impact of urban development. The dissolved oxygen concentrations in the interstitial waters and Bear Creek were also lower than expected, potentially suggesting deteriorating fish spawning conditions. During the winter and spring months, the interstitial oxygen concentrations appeared to be intermediate between those characteristic of disturbed and undisturbed watersheds.

The University of Washington (Richey 1982) also analyzed heavy metals in the interstitial waters. They found that copper and chromium concentrations were very low or undetectable, while lead and zinc were higher. Kelsey Creek interstitial water also had concentrations approximately twice those found in the Bear Creek interstitial water. They expect that most of the metals were loosely bound to fine sediment particles. Most of the lead was associated with the particulates and very little soluble lead was found in the interstitial waters. The interstitial samples taken from the standpipes were full of sediment particles that could be expected to release lead into solution following the mild acid digestion for exchangeable lead analyses. They also found that the metal concentrations in Kelsey Creek interstitial water decreased in a downstream direction. They felt that this might be caused by stream scouring of the benthic material in that part of the creek. The downstream Kelsey Creek sites were more prone to erosion and channel scouring, while the most upstream station was relatively stable.

Seattle METRO (Galvin and Moore 1982) also monitored heavy metals in the interstitial waters in Kelsey and Bear Creeks. They found large variations in heavy metal concentrations depending upon whether the sample was obtained during the wet or the dry season. During storm periods, the interstitial water and creek water heavy metal concentrations approached the stormwater values (200 µg/L for lead). During nonstorm periods, the interstitial lead concentrations were typically only about 1 µg/L. They also analyzed priority pollutant organics in interstitial waters. Only benzene was found and only in the urban stream. The observed benzene concentrations in two Kelsey Creek samples were 22 and 24 µg/L, while the reported concentrations were less than 1 µg/L in all other interstitial water samples analyzed for benzene.

4. Increased Kelsey Creek Water Flows

The increasing population of the City of Bellevue and the observed peak annual discharges have been studied by the University of Washington (Richey 1982). Bellevue was initially settled in 1883 but it grew slowly, reaching a population of only 400 by 1900. The Bellevue population density continued to be low until the 1940s. During this time, almost the entire Kelsey Creek drainage basin was undeveloped. In the late 1940s, the City of Bellevue's population was stimulated by the construction of the Lake Washington floating bridge connecting Bellevue to Seattle. From 1950 to 1970, low-density residential housing progressed rapidly, and the population of the greater Bellevue area increased by nearly 600%. By 1959, residential housing occupied a substantial portion of the Kelsey Creek watershed. The Bellevue population slowed during the 1970s due to the depressed local economy and the saturation of land development. In 1976, the population of the City of Bellevue was estimated to be 67,000 people. The peak annual discharges of Kelsey Creek almost doubled between the 1950s and the late 1970s. The frequency of flooding during this period of time also increased. Floods that used to return every 10 years in the early 1950s returned at least every other year during the late 1970s. The increase in the rate of runoff has also had a measurable effect on the channel stability in Kelsey Creek.

Figure 4.19 Stilling well at Bellevue flow monitoring station.

Figure 4.20 Level recorder at Bellevue flow monitoring station.

The University of Washington, in conjunction with the USGS, monitored flows from June 1979 through May 1980 (Perkins 1982; Richey et al. 1981) (Figures 4.19 and 4.20). The frequency of floods and the observed high flows have increased substantially in recent times. The peak flow for the same recurrence intervals have approximately doubled for recurrence intervals greater than 2 years. During the early period, a discharge of 7 m^3/s had a 10-year recurrence interval, while it had only a 1- to 2-year recurrence interval during the more recent period. Also, a 100-year recurrence interval storm had a peak flow of 8.4 m^3/s during the earlier period and was almost doubled to 16.7 m^3/s during the latter period.

The responses of the two streams during individual storms were also significantly different. Figure 4.21 shows how Kelsey Creek responded much more dramatically during two storms than did Bear Creek. The response of Kelsey Creek to these two example rains showed a very rapidly rising hydrograph, while Bear Creek responded relatively slowly. After peaking, the flows in Kelsey Creek typically returned to baseflow rates in less than 24 hours, while 48 hours or more were required in Bear Creek. The maximum annual discharges in Kelsey Creek during the study period were much greater than in Bear Creek (4.6 vs. 1.9 L/ha). The total annual runoff yields in both watersheds were similar; therefore, much more of the total runoff occurs during storms in Kelsey than in Bear Creek, while baseflows are much less in Kelsey than in Bear Creek.

Because of these increased flow rates, much of Kelsey Creek is characterized by unstable banks with much erosion and deposition of sediment. The amount of stream power available in Kelsey Creek is greater than in Bear Creek despite the slightly greater slope of Bear Creek. During peak flows, Kelsey Creek has more than twice the available power of Bear Creek. Kelsey Creek can therefore move and erode sediments much more effectively than Bear Creek.

Richey (1982) also summarized low flows observed in Kelsey and Bear Creeks. On a unit area basis, about 30% more water was flowing in Bear Creek during the summer of 1981 than in Kelsey Creek. The low flow summer discharge in Kelsey Creek was about 250 L/hour/ha while the Bear Creek flows were about 350 L/hour/ha.

5. Aquatic Organism Food Availability and Utilization

The University of Washington studied primary productivity and the availability of food in the two streams. Richey (1982) also examined primary productivity in both Kelsey and Bear Creeks. She found that on an annual basis, primary productivity per unit area (measured as carbon fixation)

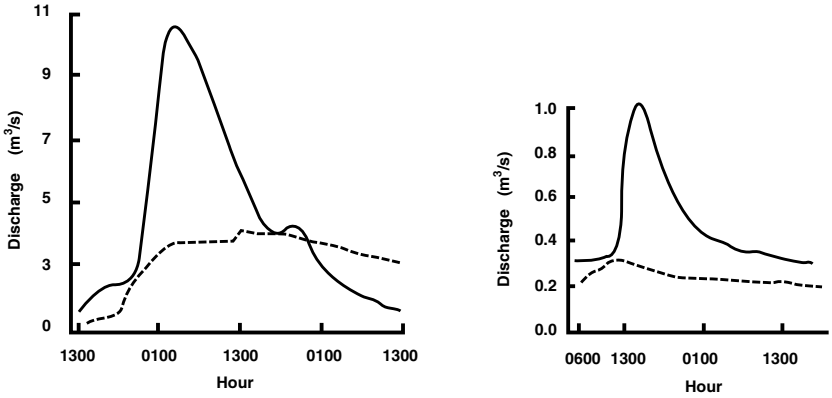

Figure 4.21 Hydrographs during winter and summer storms (December 14–16, 1979, and July 12–13, 1979) Note: solid line = urban Kelsey Creek; dashed line = rural Bear Creek. (From Richey, J.S. *Effects of Urbanization on a Lowland Stream in Western Washington*, Ph.D. dissertation, University of Washington, Seattle. 1982.)

was almost twice as large in Kelsey Creek (56 g C/m^2) than it was in Bear Creek (34 g C/m^2). She concluded that the scouring of the biomass during periods of high flows in Kelsey Creek limited the amount of primary production, even though there were sufficient nutrients available. The low levels of primary productivity measured in Bear Creek during October may have been the result of high turbidity, limiting the infiltration of sunlight in the water. Richey (1982) also examined the consumption of large organic material by grazing macroinvertebrates and microbes. The loss of leaf litter in both streams occurred at approximately equal rates. The causes for the loss of the leaf litter, however, were quite different. The microbial degradation and consumption by leaf shredding organisms are more important in Bear Creek while downstream transport of the leaf material in Kelsey Creek was most important. There was some macroinvertebrate consumption of leaf material in some of the Kelsey Creek locations, but this consumption occurred at a slower rate than in Bear Creek.

Richey (1982) also conducted experiments examining the toxicity of the periphyton in Kelsey Creek using mayflies. The adults emerged successfully in equal numbers, and the surviving larvae were indistinguishable in terms of activity levels from both Kelsey and Bear Creek periphyton.

The University of Washington's projects also examined the availability and quality of particulate organic matter as food in both creeks. They found no differences in the amount of particulate organic matter measured in the two creeks (about 100 g/m^2). There was significantly more particulate organic matter in Kelsey Creek during August and significantly less during November than in Bear Creek. The surface accumulations of material in Kelsey Creek had much more fine silts associated with them and had a lower carbohydrate content. They also analyzed the protein content of a particulate organic matter but with varying results.

Refuge areas seem to play an important role in Kelsey Creek. The more stable areas in Kelsey creek had aquatic life populations comparable to those found in Bear Creek. These refuge areas did not balance the lack of diversity observed in Kelsey Creek. The Kelsey Creek biota are relatively inefficient in utilizing food resources. The efficiency of utilization was only 3% in Kelsey Creek and about 20% in Bear Creek when the throughput of dissolved organic carbon was excluded (Perkins 1982).

6. Riparian Vegetation

Richey (1982) states that modifications to the vegetative cover have been very significant in Kelsey Creek. The riparian vegetation was relatively intact throughout the entire length of Bear Creek, while only the upper 800 m of Kelsey Creek had a significant amount of intact riparian

vegetation. Most of the riparian vegetation along Kelsey Creek was new growth alders less than 150 mm in diameter, vine maple, and blackberry vines. The riparian vegetation along most of Bear Creek was old growth fir, cedar, and alder, which are greater than 300 mm in diameter with an understory of salmonberry and vine maple. Riparian cover in the stream channel in both streams was common, however. Many sections of Kelsey Creek were overhung with dense blackberry vines, which did provide some shade and in-stream cover. Pedersen (1981) states that the vegetation along each watershed was possibly the major factor affecting species composition.

Scott et al. (1982) state that the most beneficial effect of stream alteration is the increase in solar energy reaching the stream surface as the result of the removal of a significant portion of the overhanging canopy. The current riparian vegetation along the middle and lower reaches of Kelsey Creek are only a small fraction of its former growth. The removal of this stream side cover, however, has not resulted in excessive water temperatures and appears to have indirectly benefited the trout populations in the urban stream. Bear Creek, which is heavily canopied along most of its length, can be considered light-limited. Maximum fish growth in Bear Creek occurs in the fall months after leaf fall when sunlight can reach the water. This is different from Kelsey Creek where fish growth is stimulated during the spring and early summer months when the periphyton and probably the benthic productions are greatest. Regardless of the relative production of the benthic invertebrates in each stream, it was found that the salmonids grew more rapidly in Kelsey Creek than in Bear Creek. The size of an age I migrant cutthroat trout from Kelsey Creek was near the length of age II outmigrants from Bear Creek.

7. Algae

University of Washington studies (Richey et al. 1981) found that periphyton algae were the predominant ingredient in the organic accumulation of material in Kelsey Creek. Algae was not nearly as important in Bear Creek. Richey (1982) conducted some algae bioassays with interstitial water, stormwater, and direct runoff water from the urban stream and its watershed. Only very low levels of inhibition to growth were found, and there were few instances where there were growth differences from samples taken from the two different streams. These tests indicated that the particulate-bound metals were mostly not available to the algae. She found that the stream interstitial water caused slight growth inhibition during the laboratory algal tests but that the indigenous algal cells were much less affected. Similar results were found with the stormwater and the runoff waters. She concludes that there is a potential for some toxic impacts of the stormwaters on the algae in Kelsey Creek, but it did not appear to be a dominant factor in limiting algae survival.

8. Benthic Organisms

Pedersen examined the benthic organisms in Kelsey and Bear Creeks as part of the University of Washington's project. He studied the relative occurrence of these bottom organisms in the two streams from about 350 samples. The variety of the organisms found was striking. Insects such as mayflies, stoneflies, caddisflies, and beetles were observed only rarely in Kelsey Creek and were usually of the same few families. Baetids, however, were found in large numbers in certain regions of Kelsey Creek (relatively undisturbed channel sections with riparian vegetation intact). Bear Creek demonstrated a much more diverse distribution of benthic organisms and usually showed more than one dominant family in each major grouping. However, the overall abundance of benthic organisms based on the average number of organisms per sample was not significantly different in Kelsey and Bear Creeks. Kelsey Creek had a mean abundance of about 53 organisms per sample, while Bear Creek had a value of about 48. A total of 179 samples were obtained at Kelsey Creek, while 127 samples were obtained from Bear Creek.

The worm category in Kelsey Creek was dominated by oligochaetes, which represented about 50% of benthic biota in Kelsey Creek. Amphipods, and occasional crayfish, made up about 36%

of the total benthos population. In Bear Creek, the worm category counted for only about 12% of the total benthos, while the amphipod and crayfish group accounted for less than 15% of the total. Chironomids showed up at about 10% in Kelsey Creek, demonstrating a fairly stable population over time except in late July when the population jumped to nearly 30% of the total benthos. The chironomids in Bear Creek made up closer to 20% of the total benthos population. In summary, the benthic life-forms dominating Kelsey Creek were of the collector-gatherer feeding types, which have a greater potential to survive in disturbed systems.

The benthos in Kelsey Creek generally showed a constantly changing composition with large variations in total numbers while the composition in Bear Creek did not change as much. The Bear Creek benthic organisms were also much more evenly distributed among the different taxa. Several of the Kelsey Creek stations can be considered polluted with some marginally unpolluted, while most of the Bear Creek stations were considered to be unpolluted.

The lack of the different representatives of the herbivores in Kelsey Creek (such as stoneflies or caddisflies) which were found in Bear Creek was probably due to the sensitive nature of Hemouridae and most trichoptera to environmental stress (Pederson 1981). Mayflies such as the baetids are more adaptable to minor disturbances. The lack of other herbivores could have allowed the baetids to increase their numbers due to a lack of competition and predators.

The violent flows and increased sedimentation in Kelsey Creek could be a problem for most benthic organisms, except those such as oligochaetes and chironomids, which are burrowers and filter feeders, and amphipods, which can burrow or swim and filter feed. Generally, filter feeders prefer areas of little sediment accumulation where they are exposed to maximum current. The fact that the chironomids maintain relatively stable populations in Kelsey Creek through storms and possible extreme water quality conditions as compared to other groups of insects could be due to their relatively short generation time and high recovery potential. Not all chironomids or oligochaetes, however, are limited to strictly polluted conditions; they can have dense populations where other insects are also found.

Richey (1982) found frequent dense beds of large clams (Unionidae) in Bear Creek, while they were not found in Kelsey Creek. The clams found in Bear Creek were large, indicating a stable and old population. These clams are very sensitive to heavy siltation and bed instability. They depend upon fine particulates carried in the water column for their diet. Therefore, it is not surprising that they were not found in Kelsey Creek. The high inorganic content in the suspended solids in Kelsey Creek and the unstable nature of the channel bed probably prevents their survival in Kelsey Creek. However, empty shells were found buried in the Kelsey Creek stream bed and no live organisms were observed. Therefore, they had probably existed in Kelsey Creek but have been gradually excluded by a shifting habitat and a gradual decrease in the quality of the available food and problems associated with channel instability.

9. Fish

Scott et al. (1982) reviewed two earlier studies that examined the fish populations in Bear and Kelsey Creeks. They stated that Kelsey Creek was a major producer of coho salmon and also supported significant numbers of cutthroat trout and kokanee salmon at one time. A 1956 survey, however, indicated that the Kelsey Creek salmon population was already in jeopardy due to increased urban development. Another study in 1972 found that the cutthroat were more abundant than the coho. Kokanee populations are noted to have declined throughout the Lake Washington drainage area because of the successful introduction of sockeye salmon in major tributaries. This 1972 study also observed occasional chinook salmon in Kelsey Creek. Food availability was determined not to be a limiting factor in the fish populations at that time. This earlier study did, however, find that a new culvert at the lower end of Kelsey Creek did block upstream fish passage under certain flow conditions. This problem was then corrected and the major factor impairing

salmon reproduction in the urban streams was thought to be siltation resulting from construction activities.

The University of Washington (Scott et al. 1982) examined fish life in Kelsey and Bear Creeks for 3 years ending in 1981. Figure 4.22 summarizes the fish biomass observed at these two creeks for the different species during an example month (August 1981). Coho was found to comprise only a small fraction of the salmon found in Kelsey Creek, but they frequently exceeded 50% of the total salmon population of Bear Creek. There was also a limited number of cutthroat trout older than age II inhabiting Kelsey Creek. Cutthroat of up to age III were found in Bear Creek, although in limited numbers. The Kelsey Creek salmon were reduced substantially in 1980 relative to both 1979 and 1981. The maximum salmon density in Kelsey Creek in 1981 was about 1 fish/3 m^3, which was less than 30% of what was observed in 1979 and 1981. The salmonid population of Bear Creek during this 3-year period was also unstable, as the density of salmon increased in each succeeding year.

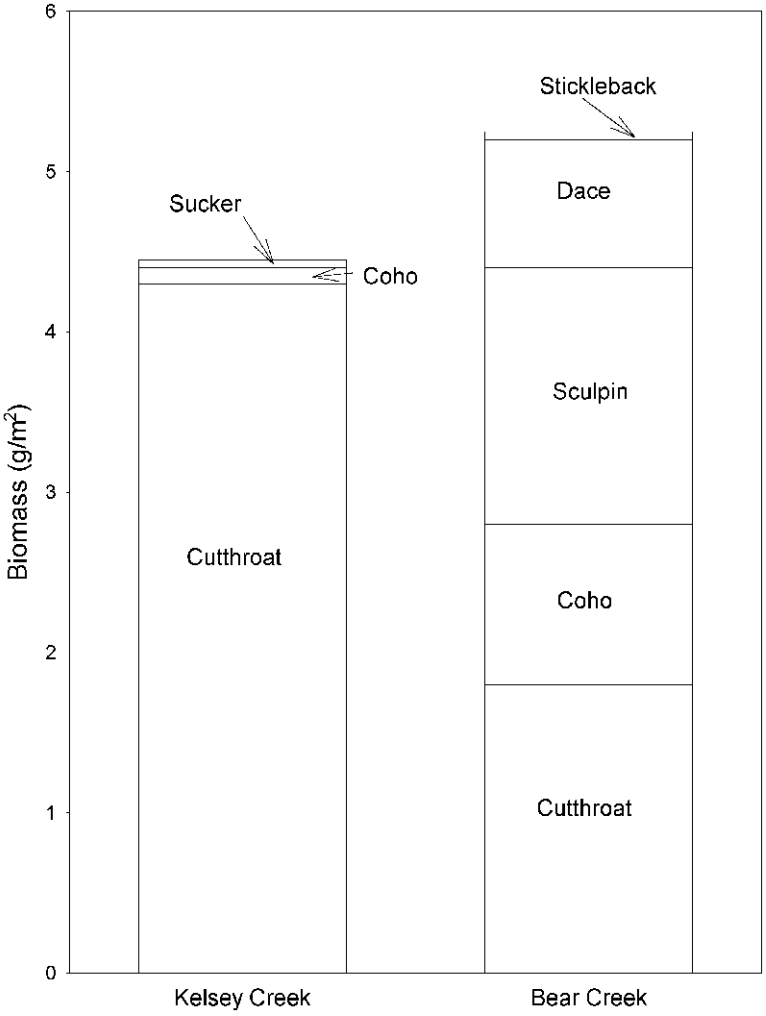

Figure 4.22 Average biomass of fish at sample sites in Bear (reference) and Kelsey (urbanized) Creeks, August 1981. (From Scott, J.B. et al. *Impacts of Urban Runoff on Fish Populations in Kelsey Creek, Washington*, Contract No. R806387020, U.S. Environmental Protection Agency, Corvallis Environmental Research Laboratory, Corvallis, OR. 1982.)

The dominant seasonal trends of fish biomass in Kelsey Creek showed a rapid buildup of biomass in the late winter and early spring followed by a sharp decline in early summer. The generally increasing trend of salmonid biomass in Bear Creek ended with a maximum of 3.7 g/m^2 in May of 1981. The maximum biomass in Kelsey Creek was about 6.5 g/m^2 in comparison. Non-salmon fish species were also quite abundant in Bear Creek, made up mostly of various species of sculpins and dace. Non-salmonids in Kelsey Creek were not very important, with only a few large-scale sucker found. Some dace stickleback and sculpin were also found in Kelsey Creek but in very small numbers. When all fish species were considered, it was found that Bear Creek supported only a slightly greater quantity of total fish biomass (5.2 g/m^2) compared to Kelsey Creek (4.5 g/m^2). Also, no single grouping of fish accounted for more than about 35% of the total fish biomass in Bear Creek. However, the salmonid biomass in Kelsey Creek was greater than the salmonid biomass in Bear Creek, with cutthroat trout comprising almost all of the salmon species found in Kelsey Creek, while large populations of coho salmon were found in Bear Creek along with cutthroat trout. In comparison to some standards, the salmonid production in Bear Creek is low, the direct consequence of a depressed standing crop.

Scott et al. (1982) state that perhaps the best measure of the relative health of a stream in the Pacific Northwest is the number of smolts it produces. The number of smolts in Kelsey Creek is approximately 40% less than that observed in other area creeks. The relative abundance of the cutthroat trout may explain the apparently poor salmonid smolt production of the Kelsey Creek watershed. Cutthroat trout require a larger territory than the typical coho smolt. Therefore, because of the large cutthroat population in Kelsey Creek, the smaller than normal smolt production may be expected.

The Kelsey Creek cutthroat appeared to grow considerably more rapidly than cutthroat observed previously in other streams. The average length of an age I cutthroat smolt in Kelsey Creek was close to the average length of an average age II cutthroat smolt in other streams. The Kelsey Creek age II smolts were typical of the lengths for other age III smolts. Also, most of the fish in Kelsey Creek outmigrated at age I. Typically, cutthroat smolts from other streams generally outmigrate from ages II through IV. It is believed that the cutthroat migrating from Kelsey Creek spend an additional year in Lake Washington before entering Puget Sound.

Scott et al. (1982) summarized the potential effects of sedimentation on stream-living salmon. These include the clogging and abrasion of gills, abrasion or adherence of sediment to the egg chorion, increasing susceptibility to diseases, modification of behavior, blocking emergence of alevins, reducing spawning habitat, changing intragravel permeability with reduced dissolved oxygen concentrations, introducing potentially toxic materials associated with the suspended material, and altering the structure and productivity of the food resources available to the fish. They studied the incidence of damaged gills on the fish in Kelsey and Bear Creeks (Scott et al. 1982). They found that from 0 to 77% of the fish sampled in Kelsey Creek were afflicted with respiratory anomalies. The season and location along the channel, as well as the age and species of the fish, affected these anomalies. Cutthroat, as an example, had afflictions that rapidly increased after mid-May. Older cutthroat also had less incidence of gill damage. Small coho salmon in Kelsey Creek had little gill damage. They also note that the incidence of damage to gills in the cutthroat trout in Kelsey Creek generally decreased in a downstream direction. No cutthroat trout and only two of the coho salmon sampled in Bear Creek had damaged gills.

In-stream embryo bioassays indicated that coho embryo salmon survival was significantly greater in Bear Creek but that no difference was found when using rainbow trout embryos. Streamside bioassays, however, indicated that the surface waters of Kelsey Creek did not significantly reduce the survival of the salmon embryos. The survival of the embryos during the winter bioassays was significantly greater in Bear Creek than in Kelsey Creek, but no difference in survival was noted during the spring bioassay tests. While the laboratory and field bioassays tended to indicate minimal toxic influences, other field observations suggested a stronger possibility of toxic problems. Coho salmon were absent in the more heavily developed areas, and the incidence of

cutthroat trout with gill damage increased in those areas. Higher levels of toxic pollutants, such as lead, were occurring with the increases of sediment transport in these more developed areas and may have contributed to the observed increase in gill damage.

Creek flows may also significantly affect the salmon fisheries. Scott et al. (1982) state that high creek flows may increase the sweeping of poorly swimming fish from the creeks. The highest flows where migration of fish from the creek were monitored was a little over 4 m^3/s which was less than one third of the peak flow recorded during the study period on Kelsey Creek. At these monitored flows, the species with relatively poor swimming ability were swept from the system, while the salmon were better able to withstand these increased flows. They estimated that a flood with a recurrence interval of about 5 years in Kelsey Creek having a peak instantaneous discharge of about 11 m^3/s may be expected to increase the coho embryo mortality by about 20%. This would increase the scour mortality during a 5-year flood to 10% or less. The lower summer flows may also limit the survival of some salmon populations (especially coho salmon) (Richey 1982).

Pedersen (1981) states that the salmon in Kelsey Creek seem to be adjusting their feeding to invertebrates that are present based upon fish stomach contents analyses. Their growth did not appear to be limited by the type of diet available in Kelsey Creek. The salmon fishery in Kelsey Creek seems to be surviving; the City of Bellevue and the Municipality of Metropolitan Seattle have supported the installation and maintenance of stream-side boxes for the incubation of sockeye salmon eggs. This program has provided direct involvement for the local school children and, therefore, also serves the educational aspects of the beneficial uses for these urban streams.

10. Creek Sediment Quality

Several of the University of Washington projects and the Seattle METRO project investigated physical and chemical characteristics of the Kelsey and Bear Creek sediments. Perkins (1982) stated that the size and composition of the sediments near the water interface tended to be more variable and of a larger median size in Kelsey Creek than in Bear Creek. These particle sizes varied in both streams on an annual cycle in response to runoff events. Larger particle sizes were more common during the winter months when the larger flows were probably more efficient in flushing through the finer materials. Pedersen (1981) also states that Kelsey Creek demonstrated a much greater accumulation of sandy sediments in the early spring. This decreases the suitability of the stream substrates for benthic colonization. Scott et al. (1982) state that the level of fines in the sediment samples appears to be a more sensitive measure of substrate quality than the geometric mean of the particle size distribution. Fines were defined as all material less than about 840 μm in diameter.

METRO (Galvin and Moore 1982) also analyzed organic priority pollutants in 17 creek sediments including several in Kelsey and Bear Creeks. Very few organic compounds were detected in either stream, with the most notable trend being the much more common occurrence of various PAHs in Kelsey Creek while none was detected in Bear Creek.

Scott et al. (1982) state that stream bed substrate quality can be an important factor in the survival of salmonid embryos. Richey (1982) describes sediment bioassay tests which were performed using Kelsey and Bear Creek sediments. She found that during the 4-day bioassay experiment, no mortalities or loss of activities were observed in any of the tests. She concluded that the chemical constituents in the sediment were not acutely toxic to the test organism. However, the chronic and/or low level toxicities of these materials were not tested.

11. Creek Bank and Stream Bed Erosion

Richey (1982) made some observations about bank stabilities in Kelsey and Bear Creeks. She notes that the Kelsey Creek channel width has been constrained during urban development. Thirty-five percent of the Kelsey Creek channel mapped during these projects was modified by the addition of some type of stabilization structure. Only 8% of Bear Creek's length was stabilized.

Most of the stabilization structures in Bear Creek were low walls in disrepair, while more than half the structures observed along Kelsey Creek were large riprap or concrete retention walls. The need for the stabilization structures was evident from the extent and severity of erosion cuts and the number of deposition bars observed along the Kelsey Creek stream banks. Bridges and culverts were also frequently found along Kelsey Creek; these structures further act to constrict the channel. As discharges increase and the channel width is constrained, the velocity increases, causing increases in erosion and sediment transport.

The use of heavy riprapping along the creek seems to worsen the flood problems. Storm flows are unable to spread out onto a floodplain, and the increased velocities are evident downstream along with increased sediment loads. This rapidly moving water has enough energy to erode unprotected banks downstream of riprap. Many erosion cuts along Kelsey Creek downstream of these riprap structures were found. Similar erosion of the banks did not occur in Bear Creek. Much of the Bear Creek channel had a wide floodplain with many side sloughs and back eddies. High flows in Bear Creek could spread onto the floodplains and drop much of their sediment load as the water velocities decreased.

12. Creek Sedimentation and Sediment Transport

The University of Washington studies also examined sediment transport in Kelsey and Bear Creeks. Richey (1982) found that the relative lack of debris dams and off-channel storage areas and sloughs in Kelsey Creek contributed to the rapid downstream transit of water and materials. The small size of the riparian vegetation and the increased stream power probably both contributed to the lack of debris in the channel. It is also possible that the channel debris may have been cleared from the stream to facilitate rapid drainage. The high flows from high velocities caused the sediments to be relatively coarse. The finer materials were more easily transported downstream. Larger boulders were also found in the sediment but were probably from failed riprap or gabion structures.

The effects of erosion and sediment deposition in Kelsey Creek were more severe than those found in Bear Creek. Kelsey Creek's channel was scoured to deeper depth, there was much more channel instability in Kelsey Creek, and the numbers of erosion cuts and deposition bars were much more frequent in Kelsey Creek. Richey (1982) reported that the sediment transport in Bear Creek during December 1979 was 27 kg/ha, while 98 kg/ha left Kelsey Creek. The suspended solids transport was almost exponentially related to discharge. On an annual basis, Kelsey Creek discharged almost four times as much suspended solids as did Bear Creek, but most of this material passed through the stream in a few hours or days. Richey (1982) found that much of the solids transport in Kelsey Creek occurred during the rapid rise of the hydrograph when the energy to move sediment material was increasing. The silts and associated pollutants were rapidly transported through the system during these periods. The scouring of the channel appeared to remove temporarily stored silts and the associated pollutants. The higher levels of particulate transport in Kelsey Creek are probably due to increased stream power rather than increased sources of sediment material in the watershed. However, there were substantial amounts of in-stream sources of sediment material in Kelsey Creek to augment the runoff discharged sediment. Because of the lack of debris dams in the downstream sections of Kelsey Creek, the transported materials are carried significant distances before deposition. The high stream power available to transport the materials and the erodable nature of the stream banks in the watershed areas along with the lack of storage sites along the stream all contributed to high particulate yields from Kelsey Creek. Because much of the suspended particulate material in Kelsey Creek was from the relatively unpolluted bank materials, the sediments and suspended loads in Kelsey Creek had much lower concentrations of many of the typical urban pollutants compared to the urban runoff that was discharged to the creek.

Sources of Urban Runoff Pollutants

1. Atmospheric Particulate and Rainfall Deposition of Pollutants

The USGS (Ebbert et al. 1983; Prych and Ebbert undated) studied dustfall quantity and quality along with rain quality at each of three locations in the test watersheds. Seattle METRO also examined the metallic and organic priority pollutant quality of atmospheric particulates. These data indicated that the airborne PAHs are combustion products, while the street dirt PAHs are from petroleum product spills. In August of 1980, ash from the eruption of Mt. St. Helens fell in the study area and substantially increased the dustfall measurements. These increased dustfall values were typically two to four times the average monthly values. During most months, dry atmospheric dustfall was much greater than the total solids associated with the rainwater.

2. Runoff Water Sources

The City of Bellevue study (Pitt 1985) monitored runoff and rainfall characteristics at the two main study locations (Surrey Downs and Lake Hills) during the 2 years of the project. Bellevue receives about 1 m of rain each year. Dry periods of more than a week are quite rare. Rains come on an average about once every 2 or 3 days throughout the year. Slightly more than 100 rains may occur each year, but the amount of rain associated with each is quite small. Most of the rains are less than 6 mm. The largest rains monitored during this project were about 100 mm.

The Lake Hills rain depths were about 12% more than the comparable Surrey Downs rains. The average duration of the Lake Hills rains was also about 10% longer than the Surrey Downs rains. The Lake Hills rains also started about a half hour before the rains in Surrey Downs began. Most of the rain events had less than 6 mm of rain, and less than 10% of the rain events had depths greater than 25 mm. Most of the rainfall quantities were associated with rain events greater than about 15 mm. The much more common small rains did not add up to much total depth. The rains that were smaller than 6 mm accounted for less than 25% of the total rainfall depth, while about 30% of the total rainfall depth was associated with rains greater than 25 mm.

Almost 400 runoff events were monitored at the Surrey Downs and Lake Hills monitoring stations during the 2-year study period. Almost 99% of the rains that occurred in Surrey Downs and 91% of the Lake Hills rains were monitored. The baseflow in the Surrey Downs basin accounted for about 23% of the total annual flow, while the baseflow was only about 13% of the total annual flow in Lake Hills. The stormwater flows in Lake Hills were about 35% greater than in Surrey Downs. Overall, the base plus stormwater urban flows from Lake Hills were about 18% greater than Surrey Downs on an equal area basis.

For both study years and test basins, only about 25% of the rain that fell in the test basins left the areas as runoff. The small rains typically had the smallest runoff factors, while the large rains had the largest factors. For very small rains, no runoff is expected to occur from the pervious areas nor from the impervious areas that drain to these pervious areas. Starting at about 2.5 mm of rain, however, the volumetric runoff coefficients (Rv) are about 0.3 to 0.5 times the maximum values that they are likely to obtain. The dry season runoff coefficients are less than the wet season values due to different soil moisture conditions. For all rains greater than about 2.5 mm, impervious surfaces contribute more than 60% of the total urban runoff flows. The remainder of the flows are approximately evenly divided between front and back yards, while vacant lots and parks contribute very little flow due to their limited presence in the area. Street surfaces contribute about 25% of the total urban flows for most rains causing runoff.

3. Stormwater and Baseflow Urban Runoff Quality and Pollutant Source Areas

Collecting stormwater runoff quality data was a major aspect of the City of Bellevue's and the USGS projects. In addition, Seattle METRO analyzed some of the samples collected by the City of Bellevue for metallic and organic priority pollutants. Most of the analytical effort was associated with a core list of important constituents. Tables 4.18 and 4.19 summarize USGS and City of Bellevue stormwater quality data for these core constituents. The USGS obtained many discrete

Table 4.18 Urban Runoff Quality Reported by the USGS (many discrete samples for a limited number of storms)

Constituent (mg/L, unless otherwise noted)	Maximum	Minimum	Approx. Median	No. of Discrete Samples Analyzed
Temperature, °C	14.8	2.6	8.0	49
Specific conductance, µmhos/cm	1480	12	41	1299
pH, pH units	7.9	3.4	6.7	1093
COD	780	8	60	681
BOD_5	40	<0.1	6.6	321
BOD ultimate	115	3.5	20	138
Particulate organic carbon	40	<0.1	2.1	638
Dissolved organic carbon	120	0.2	7.5	681
Fecal coliforms, No./100 mL	66,000	1	980	326
Suspended solids	2740	1	50	1180
Dissolved solids	788	8	35	241
Nitrate plus nitrite nitrogen	4.5	<0.01	0.21	691
Ammonia nitrogen	7.2	<0.01	0.14	689
Total Kjeldahl nitrogen	45	0.21	1.1	687
Dissolved Kjeldahl nitrogen	33	<0.01	0.63	686
Total phosphorus	9.2	0.01	0.15	686
Dissolved phosphorus	7.2	<0.01	0.06	685
Lead	1.8	0.004	0.14	693
Oil and grease	10	<1	2.5	16

Data from Ebbert, J.C. et al. *Data Collected by the U.S. Geological Survey During a Study of Urban Runoff in Bellevue, Washington, 1979–82*. Preliminary U.S. Geological Survey Open-File Report, Tacoma, WA. 1983.

Table 4.19 Urban Runoff Quality Reported by the City of Bellevue (total storm, flow-weighted composite samples for most runoff events, Surrey Downs and Lake Hills observations combined, 2/80–1/82) (mg/L, unless otherwise noted)

Constituent	Maximum	Minimum	Average	No. of Flow-Weighted Total Storm Samples Analyzed
Specific conductance, µmhos/cm	300	16	41	204
pH, pH units	7.4	5.2	6.3	204
Turbidity, NTU	150	4	19	204
Total solids	620	24	109	208
Total Kjeldahl nitrogen	5.9	<0.5	1.0	208
COD	150	13	46	208
Total phosphorus	3.6	0.002	0.26	208
Lead	0.82	<0.1	0.17	208
Zinc	0.37	0.03	0.12	208

From Pitt, R. *Characterizing and Controlling Urban Runoff through Street and Sewerage Cleaning*. U.S. Environmental Protection Agency, Storm and Combined Sewer Program, Risk Reduction Engineering Laboratory. EPA/600/S2-85/038. PB 85-186500. Cincinnati, OH. 467 pp. June 1985.

samples throughout individual storms but only analyzed data from a small percentage of the total runoff events that occurred during the study period. The City of Bellevue's sampling procedures involved collecting total storm flow-weighted composite samples throughout most of the events that occurred during the sampling period at the Surrey Downs and Lake Hills sites.

The USGS (Ebbert et al. 1983) found that when the stormwater runoff discharge was high, the concentrations of the constituents in particulate forms tended to be high, and the concentrations of the constituents in dissolved forms tended to be low. During periods of low discharge, particulate concentrations were low, and the dissolved concentrations were high. There was very little variation in most of the constituent concentrations for each of the three sites for most rains. The hardness of the stormwater was generally very low. About two thirds of the total solids and phosphorus loads, and one third of the total Kjeldahl nitrogen, total nitrogen, and organic carbon loads were associated with particulates. They also found that about 15% of the total nitrogen load was in the form of dissolved nitrate plus nitrite nitrogen, about 10% is as dissolved ammonia, 40% as dissolved organic nitrogen, and 35% was particulate Kjeldahl nitrogen.

Seattle METRO (Galvin and Moore 1982) analyzed about 21 of the total storm flow-weighted composite samples from Bellevue for 14 metallic priority pollutants. The stormwater metal concentrations were very low when compared to other urban runoff metal data for other locations (except for arsenic). They also found that the stormwater metal concentrations did not vary significantly between the study areas. METRO also analyzed many of the samples for dissolved concentrations of the different metals in addition to the total concentrations. Only copper and zinc showed significant dissolved concentrations, while the other metals were almost completely associated with the particulates in the stormwater. None of the organic priority pollutants detected by METRO was found in more than 25% of the samples submitted. Of the 111 organic priority pollutants, only 19 were detected at least once in the METRO stormwater sample analyses. Except for one value (a pentachlorophenol value of 115 µg/L), they were all very close to the detection limits.

The USGS also analyzed about 16 of their discrete samples for a long list of insecticides and herbicides. Lindane, Diazinon, Malathion, Dieldrin, and 2,4-D were detected in more than half the samples. Endosulfan, Silvex, and 2,4,5-T were found in about one third of the samples submitted. Many of the insecticides and herbicides analyzed were not detected in any of the samples.

The USGS (Prych and Ebbert undated) also examined stormwater-suspended sediment size distributions in four to seven samples. These analyses showed that 64% of the particulate material in stormwater was associated with particle sizes smaller than 62 µm. Only about 10% of the stormwater particles had sizes greater than 250 µm.

The City of Bellevue study (Pitt 1983) also examined the baseflow quality at Surrey Downs and Lake Hills. The runoff water quality at Bellevue was much better compared to most other locations. The baseflow quality, on the other hand, was found to be worse than expected. This was probably because the study basins were completely urbanized and the baseflows were percolated urban sheet flow waters from previous storms that were draining out of the surface soils. In basins with undeveloped upstream areas, the baseflow would originate mostly from the non-urbanized upper reaches and would have much better quality. The data shown in Table 4.20 were collected from 26 composite samples collected over 24-hour periods from both Surrey Downs and Lake Hills.

Table 4.21 shows the measured annual baseflow and stormwater runoff yields for the two test catchments. There was an apparent increase in storm runoff discharges at Lake Hills, while Surrey Downs had larger baseflow contributions. The baseflow contributions were much less than the storm-generated flows, but the phosphorus and TKN baseflow discharges comprised about 25 to 30% of the total Surrey Downs discharges.

Pitt (1985) made estimates of the pollutant contributions from the different source areas. Table 4.22 summarizes these estimates. During very small rains, most of the runoff, and therefore pollutant discharges, was associated with the directly connected impervious areas. As the rain total increased (greater than about 2.5 mm), the pervious areas became much more important. These

Table 4.20 Baseflow Water Quality Reported by the City of Bellevue (Surrey Downs and Lake Hills data combined) (mg/L, unless otherwise noted)

Constituent	Maximum	Minimum	Average	No. of 24-hr Composite Baseflow Samples Analyzed
Specific conductance, μmhos/cm	430	138	260	18
Total solids	326	108	202	26
COD	67	6.8	23	26
Total Kjeldahl nitrogen	2.4	0.20	0.8	26
Total phosphorus	1.2	0.027	0.16	26
Lead	0.1	<0.1	<0.1	26
Zinc	0.47	0.026	0.09	26

From Pitt, R. *Characterizing and Controlling Urban Runoff through Street and Sewerage Cleaning.* U.S. Environmental Protection Agency, Storm and Combined Sewer Program, Risk Reduction Engineering Laboratory. EPA/600/S2-85/038. PB 85-186500. Cincinnati, OH. 467 pp. June 1985.

Table 4.21 Annual Baseflow and Stormwater Runoff Mass Yields Reported by the City of Bellevue (kg/ha/yr)

	Surrey Downs			Lake Hills		
Constituent	Baseflow	Storm Runoff	Total	Baseflow	Storm Runoff	Total
Total solids	110	205	315	76	280	360
COD	11	90	100	9.9	110	120
Total Kjeldahl nitrogen	0.60	1.8	2.4	0.20	2.7	2.9
Total phosphorus	0.11	0.40	0.51	0.04	0.69	0.73
Lead	0.03	0.26	0.29	0.02	0.45	0.47
Zinc	0.060	0.24	0.30	0.027	0.31	0.34

From Pitt, R. *Characterizing and Controlling Urban Runoff through Street and Sewerage Cleaning.* U.S. Environmental Protection Agency, Storm and Combined Sewer Program, Risk Reduction Engineering Laboratory. EPA/600/S2-85/038. PB 85-186500. Cincinnati, OH. 467 pp. June 1985.

Table 4.22 Source Area Contributions for Runoff Pollutants from Bellevue Residential Areas (for 2.5 to 65 mm rains) (% contributions from source areas)

Source Area	Total Solids	COD	Phosphates	Total Kjeldahl Nitrogen	Lead	Zinc
Streets	9	45	32	31	60	44
Driveways and parking lots	6	27	21	20	37	28
Rooftops	<1	3	5	10	<1	24
Front yards	44	13	22	19	<1	2
Back yards	39	12	20	20	<1	2
Vacant lots and parks	2	<1	<1	<1	<1	<1

From Pitt, R. *Characterizing and Controlling Urban Runoff through Street and Sewerage Cleaning.* U.S. Environmental Protection Agency, Storm and Combined Sewer Program, Risk Reduction Engineering Laboratory. EPA/600/S2-85/038. PB 85-186500. Cincinnati, OH. 467 pp. June 1985.

patterns varied significantly for different areas depending on the rain characteristics and land uses. It was estimated that for most rain events, total solids originated mostly from the back and front yards in the test areas, and street surfaces contributed only a small fraction of the total solids urban runoff discharge. Street surfaces, however, were expected to make up most of the lead, zinc, and COD concentrations in urban runoff. Phosphates and total Kjeldahl nitrogen were mostly contributed from street surfaces, driveways, and parking lots combined. Front and back yards made up slightly less than half of these nutrient contributions to the outfall. It was noted that zinc contributions from rooftops made up about one fourth of the total zinc discharges. These zinc rooftop sources were expected to be associated with galvanized metal rain gutters and downspouts.

4. Street Dirt Contributions to Urban Runoff Discharges

The City of Bellevue examined street dirt loadings in the three urban runoff test areas during the 2-year period of study (Pitt 1985). By the end of January 1982, about 600 street surface accumulation samples were collected from the test areas in Bellevue. Each of these 600 street surface samples was separated into eight different particle sizes. The smallest particle sizes account for only a small fraction of the total material. This was especially true during the wet season when the rains were most effective in removing the smallest particles. During the dry season, the larger particle sizes accounted for relatively small fractions of the total solids weight. Most of the street surface particulates were associated with particles in the size range of 125 to 1000 μm.

The Bellevue street surfaces were relatively clean when compared to other locations throughout the country. This difference is expected to be mostly due to the frequent rains that occur in Bellevue. The initial accumulation rates (assumed to be equal to the deposition rates) in the test areas were estimated to vary between 1 and 6 (with an average of about 3) g/curb-meter/day. This is comparable to accumulation rates observed in other locations for smooth streets in good condition. However, the Bellevue streets never have an opportunity to become extremely dirty due to the relatively frequent rains.

The Bellevue study (Pitt 1985) also examined the chemical characteristics associated with the particulates in different size ranges. The chemical characteristics were not unusual when compared to other locations throughout the United States. The Seattle METRO project (Galvin and Moore 1982) also examined heavy metals in the street surface particulate samples collected by the Bellevue sampling team. All of the inorganic priority pollutants, except selenium, were detected in the street dirt. The most abundant metals were lead, zinc, chromium, copper, nickel, arsenic, cadmium, and beryllium. METRO did not find any clear differences between metal concentrations in the two residential basins nor when these residential basin street dirt characteristics were compared with commercial and industrial samples collected in Seattle. They also found that the concentrations of metals were greatest in the finer size particles, but these fine particles accounted for only a small portion of the total solids loadings on the street surfaces. When these metallic priority pollutant analyses were compared with similar analyses conducted elsewhere in the United States, the Bellevue concentrations tended to be quite low (except for arsenic).

Seattle METRO (Galvin and Moore 1982) also analyzed street dirt samples for organic priority pollutants. Of the 111 organic priority pollutants, only about 30 were detected in the street dirt samples. Two of the PAHs (fluoranthene and phenanthrene) were found in all of the street dirt samples. Several of the compounds had concentrations greater than 1 mg constituent/kg total solids, while one phthalate was recorded as great as 35 mg constituent/kg total solids. It was also noted that most of the organic priority pollutants were associated with the finest particle size fractions. The halogenated aliphatics, monocyclic aromatics, phenolics, and phthalate esters were very common in the residential samples but were only infrequently found in the other samples. The industrial sample, however, periodically had very high concentrations of some of the organic constituents.

Most of the material that washed off the street surfaces during rains occurred in particle sizes less than about 125 µm. Only about 10% of the washoff material was greater than about 500 µm in size. The largest street surface particulates were notably absent in the runoff water. For all of the sites combined, only about 14% of the total solids were removed by rains observed during the test period. The washoff percentage is substantially greater for lead (about 21%) because of the greater abundance of lead found in the smaller particle sizes.

5. Sewerage and Catchbasin Sediment Accumulations

Sewerage system sediment loadings were periodically observed in the Surrey Downs and Lake Hills study areas during the City of Bellevue project (Pitt 1985). The storm drainage system was cleaned before the start of the project and the accumulating sediment volumes in inlets and catchbasins were observed nine times during the 2 years. During the second year of observations, the amount of accumulated material remained relatively constant. Typically, there was about twice as much sediment in the storm drainage systems at any one time as there was on the streets. Table 4.23 shows the calculated sewerage accumulation rates in inlets and catchbasins in Surrey Downs and Lake Hills. These accumulation values were the rates observed after the initial cleaning and before the stable Year 2 volumes were obtained. During the second year (October 1981) a very large storm (about 100 mm) occurred. However, the loading observations before and after this event were not significantly different, indicating very little net removal due to flushing. The chemical quality of the catchbasin and inlet sump material was very similar to the street dirt materials, for similar particle sizes.

A survey of the pipe dimensions and slopes throughout each of the study areas was made during the early months of the project by the City of Bellevue (Pitt 1985). Very few pipes in either Surrey Downs or Lake Hills had slopes less than 1%, the slope assumed to be critical for sediment accumulation. Frequent observations of sediment accumulations in the pipes throughout the two study areas were also made. Generally, very small amounts of sediment were found in the sewerage in Lake Hills and Surrey Downs. The pipes that had significant quantities of sediment were sloped less than 1.5% and/or located close to a source of sediment. The characteristics of the sewerage sediment were also similar to the characteristics of the sediment in the close-by manholes and catchbasins and the street surface materials. The volume of sediment accumulated in the Lake Hills

Table 4.23 Stormwater Inlet Sediment Volumes and Accumulation Rates

	Total Inlets	Inlets per ha	Sediment per ha (L/month)	Sediment per Inlet (L/month)	Approximate Months Needed to Reach Steady-State Volume	Steady-State Volume per ha (L)	Steady-State Volume per Inlet (L)
Surrey Downs (38.0 ha)							
Catchbasins	43	1.1	5.3	4.8	13	68	62
Inlets	27	0.7	2.0	2.8	20	40	57
Manholes	6	0.2	0.8	4.0	19	15	76
Average	76 (total)	2.0 (total)	8.1	4.2	15	123	62
Lake Hills (40.7 ha)							
Catchbasins	71	1.7	2.4	1.4	18	43	25
Inlets	45	1.1	1.5	1.4	14	22	20
Manholes	15	0.4	1.6	4.0	23	36	90
Average	131 (total)	3.2 (total)	5.5	1.7	18	100	31

From Pitt, R. *Characterizing and Controlling Urban Runoff through Street and Sewerage Cleaning.* U.S. Environmental Protection Agency, Storm and Combined Sewer Program, Risk Reduction Engineering Laboratory. EPA/600/S2-85/038. PB 85-186500. Cincinnati, OH. 467 pp. June 1985.

pipes was about 0.04 m^3/ha (about 70 kg/ha). In Surrey Downs, the pipe sediment volume was estimated to be more than 0.5 m^3/ha (about 1000 kg/ha). Most of the sediment in Surrey Downs was located in silted-up pipes along 108th Street and Westwood Homes Road, which were not swept and had nearby major sediment sources. The pipe sediment volume estimated to be available for runoff transport in Surrey Downs was about 0.01 m^3/ha (about 15 kg/ha).

Urban Runoff Controls

1. The Effects of Street Cleaning in Controlling Urban Runoff Pollutant Discharges

The coordination of the street surface sampling, street cleaning operations, and runoff monitoring activities during the City of Bellevue project allowed many different data analysis procedures to be used to investigate possible effects of street cleaning on runoff water quality. The use of two test basins and the rotation of the street cleaning operations also allowed one basin to be compared against the other along with internal basin comparisons.

The design of an effective street cleaning program requires not only a determination of the accumulation rates, but also an assessment of the performance of specific street cleaning equipment for the actual conditions encountered. The street cleaning tests conducted by the City of Bellevue (Pitt 1985) utilized two different street cleaning frequencies. These two frequencies included no cleaning and intensive three times a week cleaning. Each cleaning frequency was employed in both the Surrey Downs and the Lake Hills test catchments for a several-month period and were then rotated. There was also a several-month period when no street cleaning was conducted in either test catchment. Runoff was simultaneously monitored for the two catchments during these varying street cleaning programs.

During the entire project period, street dirt loadings were about 115 g/curb-meter (with an extreme value of about 350) during the period of no street cleaning. The loadings were reduced to about 60 g/curb-meter shortly after the start of street cleaning. Median particle sizes decreased with the start of street cleaning because of the selective removal of the large particle sizes by street cleaners. The rain periods all reduced the street surface loadings appreciably, except for the largest rain observed during the study. The rains also increased the median particle sizes because they were most effective in removing the finer material. The largest rain had little effect on the net loading change, probably because of substantial erosion material carried onto the street during this major storm and the relative cleanliness of the street surface before the storm occurred.

Street loadings responded rapidly to initiation of street cleaning. Changes from periods of street cleaning to no street cleaning were not as rapid. The Bellevue study collected many street surface particulate samples in the two test basins immediately before and immediately after the streets were cleaned. Street cleaning equipment cannot remove particulates from the street surface unless the loadings are greater than a certain amount. This value was about 85 g/curb-meter in the test basins for the mechanical broom street cleaners and about 30 g/curb-meter for the regenerative air street cleaner. If the initial street surface loading values were smaller than this, the residual loadings typically were equal to the initial loadings.

Statistical analysis showed that the frequent rains in Bellevue were probably more effective than the street cleaning in keeping Bellevue streets clean. The street surface loadings after rains were usually about 50 g/curb-meter, and the mechanical street cleaning equipment could only remove the street surface particulates down to about 85 g/curb-meter. It was also found that typical mechanical street cleaning equipment is quite ineffective in removing the small particle sizes that are removed by rains. However, a modified street cleaner resulted in an almost constant residual loading value in the cleaning width after cleaning, irrespective of the initial loading. This indicates a very important advantage in the cleaning effectiveness for this street cleaner.

Much data analysis effort during the Bellevue City project was directed toward attempting to identify differences in runoff concentrations and yields caused by street cleaning operations (Pitt

1985). No significant differences in runoff yields or concentrations during periods of intensive street cleaning vs. no street cleaning were observed. Street surfaces contributed less than 25% of the runoff yield for most storms. Therefore, street cleaning would have to be extremely effective to cause stormwater yield improvements approaching 25%. For very small rains, street surface washoff is estimated to contribute more than 60% of most of the constituents to the runoff yield. For larger rains, however, the importance of street washoff diminishes. With intensive street cleaning, only the larger particle sizes are significantly reduced, while particle sizes most subject to washoff by rains are not effectively reduced. This may result in less than a 6% expected improvement in runoff water quality for intensive street cleaning. The modified regenerative air street cleaner is expected to have only slightly better effectiveness in reducing runoff yields. The modified street cleaner may reduce the runoff yields by as much as 10%.

2. Sewerage Inlet Cleaning Effects in Reducing Urban Runoff Yields

The City of Bellevue's project (Pitt 1985) also studied the potential benefits of cleaning sewerage inlet structures in controlling urban runoff discharges. The rains preferentially removed the finer, more heavily polluted, and more available materials during washoff. The sediments in the catchbasins and the sewerage were mostly the largest particles that were washed off the street. Catchbasin sump sediments can be relatively conveniently removed to eliminate this potential source of urban runoff pollutants. Because the catchbasin sediment accumulation rate is quite low, frequent cleaning of catchbasins is not necessary.

Only about 60% of the available sump volumes in the inlets were used for detention of particulates. The structures with large sump volumes required less frequent cleaning and held larger volumes of sediments. It is expected that cleaning these inlet sumps about twice a year could reduce the lead and total solids urban runoff discharges by between 10 and 25%. COD, total Kjeldahl nitrogen, total phosphorus, and zinc may be controlled by between 5 and 10% with semiannual catchbasin cleaning. Cleaning less frequently than this would reduce these expected improvements. If the catchbasin sumps are left full, the potential exists for dramatically increased runoff yields during rare events that may flush captured material. Some pollutants may also be chemically changed by oxidation-reduction reactions or other chemical or biological changes in the catchbasins.

3. The Use of Dry Detention Basins in Controlling Urban Runoff Discharges

The USGS (Ebbert et al. 1983) tested the effectiveness of a dry detention facility in the 148th Avenue S.E. test catchment. The detention basin system consisted of five normally dry grass-lined swales which were contoured into a small park adjacent to the road. The swales were about 300 m long and 30 m wide. There were five control structures used to regulate the flow and the storage along the 27-in trunk line running under the park. The original design of the detention system permitted the flow and storage to be regulated by weirs and valves. Runoff from low-intensity storms was originally allowed to pass through the system with little detention, while discharge from higher intensity storms was detained behind the weirs in the 27-in trunk line. During extreme events, the higher flows ran over the weirs when the detention basins were full.

During the study, the USGS (Ebbert et al. 1983) modified the control structures to permit the slow release of water stored in the detention basin, which was then monitored with a recorder installed behind the weir. Water was therefore stored during much smaller rains than in the original configuration. The detention time was about 30 min or less, which was sufficient time for settling of sand and some coarse silt. Much of the finer material, however, was probably transported directly through the detention system. Earlier data indicated that most of the suspended sediment in the storm runoff at this site was finer than 62 µm. The results of the monitoring (Prych and Ebbert undated) indicated that the detention of the storm runoff had little effect on the concentrations of the runoff constituents. The performance of the detention basins on the four to seven storms that

were tested seemed to depend mostly on the distribution of the constituents between the suspended and dissolved phases. The volume of the storm sewer behind the weir used to control the flow was adequate to store the runoff during about 70% of the storms that occurred during that phase of the study. For the other 30% of the storms, the volume of the sewer was insufficient to store all the detained water and some was backed up into the grass-lined depressed area. When the grassy area was inspected after a storm, only a trace of fine residual material was noted on the blades of grass.

Over the entire detention phase of the study, there were about 20 storms (about 10% of all storms) large enough to cause detention in the grassy swale. At the end of the study, only a small amount of suspended sediment was seen on the grass. It was estimated that less than one tenth of the total amount transported through the system was detained. The USGS (Prych and Ebbert undated) also examined the ability of the detention facility to affect the discharge rate of storms. The average ratio of peak discharge rates without detention to detention was 0.63.

Step 7. Project Conclusions

Degradation of Habitat and Biological Communities

- The urbanized Kelsey Creek environmental quality was much better than expected, but was degraded when compared to the less urbanized Bear Creek. Kelsey Creek apparently lacked gross contamination by pollutants. The direct toxic effects of pollutants during storms appeared to be small; the stream did support a small, unhealthy salmonid population. Kelsey Creek salmon did grow faster than Bear Creek salmon, however.
- The fish population in Kelsey Creek had adapted to its degrading environment by shifting the species composition from coho salmon to less sensitive cutthroat trout and by making extensive use of less disturbed refuge areas.
- Studies of damaged gills found that up to three fourths of the fish in Kelsey Creek were affected by respiratory anomalies, while no cutthroat trout and only two of the coho salmon sampled in Bear Creek had damaged gills.
- Massive fish kills in Kelsey Creek and its tributaries were observed on several occasions during the project due to the dumping of toxic materials into storm drains.
- There were significant differences in the numbers and types of benthic organisms found. Mayflies, stoneflies, caddisflies, and beetles were rarely observed in Kelsey Creek but were quite abundant in Bear Creek. These organisms are commonly regarded as sensitive indicators of environmental degradation. By comparison, Kelsey Creek fauna was dominated by oligochaetes, chironomids, and amphipods, commonly regarded as species more tolerant to environmental degradation.
- As an example of a degraded aquatic habitat in Kelsey Creek, a species of clams (Unionidae) was not found in Kelsey Creek, but was found in Bear Creek. These clams are very sensitive to heavy siltation and unstable sediments. Empty clam shells, however, were found buried in the Kelsey Creek sediments, indicating their previous presence in the creek and their inability to adjust to the changing conditions.
- The benthic organism composition in Kelsey Creek varied radically with time and place while the organisms were much more stable in Bear Creek.

Degradation of Habitat and Biological Conditions, Possible Causes

- These aquatic organism differences were probably mostly associated with the increased peak flows in Kelsey Creek caused by urbanization and the resultant increase in sediment-carrying capacity and channel instability of the creek.
- There was also the potential for accumulation of toxic materials in the stream system affecting aquatic organisms, but only low concentrations of toxic materials were found in the receiving waters.
- The concentrations of dissolved oxygen in the urban creek's gravel waters were quite low and may have decreased the survival of salmon embryos. In-stream embryo bioassays indicated that

coho embryo salmon survival was significantly greater in Bear Creek than in Kelsey Creek, but no difference was found when using rainbow trout embryos.
- Direct receiving water effects from urban runoff may not have been significant for most storms. Potential long-term problems, however, may be associated with settleable solids, lead, and zinc. These settled materials may have silted up spawning beds and introduced high concentrations of potentially toxic materials directly to the sediments. The oxygen depletion observed in the interstitial waters was probably caused by organic sediment buildup from runoff events.
- Kelsey Creek had much lower flows than Bear Creek during periods between storms. About 30% less water was available in Kelsey Creek during the summers, even though both creeks have drainage basins of similar size, rainfall characteristics, and soils. These low flows may also have significantly affected the aquatic habitat and the ability of the urban creek to flush toxic spills or other dry-weather pollutants from the creek system.
- Kelsey Creek had higher water temperatures (probably due to reduced shading) than Bear Creek. This probably caused the faster fish growth in Kelsey Creek.

Conveyance of Stormwater

- Kelsey Creek had extreme hydrologic responses to storms. Flooding substantially increased in Kelsey Creek during the period of urban development; the peak annual discharges have almost doubled in the last 30 years, and the flooding frequency has also increased due to urbanization.
- These increased flows in urbanized Kelsey Creek resulted in greatly increased sediment transport and channel instability.

Open Space and Resource Preservation Beneficial Uses

- The lack of adequate buffer zones and natural creek banks along much of the urban reaches of Kelsey Creek is balanced by extensive park system developments along selected reaches. Natural creek reaches are very important for the aquatic organisms in Kelsey Creek.
- Creek bank-side homeowners have made extensive channel and riparian vegetative changes, which significantly reduced the ability of the creek to support aquatic life.

Recreational Beneficial Uses

- The natural small size of Kelsey Creek restricts its usefulness for most water contact-related activities, although swimming does occur in the lower reaches of Kelsey Creek during the summer.
- The fecal coliform bacteria counts in Kelsey Creek were high and variable. These organisms indicate the potential presence of pathogenic bacteria and commonly exceeded water contact numeric criteria.

Aesthetics Beneficial Uses

- This use is related to most of the above uses; unsightly creeks are not utilized in educational field trips or as swimming areas, or desired as amenities to property.
- Dead fish from periodic toxic material spills significantly degrade this use.
- Debris and unstable channels also adversely affect the aesthetic quality of Kelsey Creek.

Sources of Increased Flows and Pollutants

- For all rains greater than about 2.5 mm (0.1 in), the impervious surfaces (streets, sidewalks, driveways, parking lots, and rooftops) were found to contribute more than 60% of the total urban runoff flows. The remainder of the flows were approximately evenly divided between front and back yards, while vacant lots and parks contributed very little to the flows due to their limited

presence in the test areas. For most of the rain events monitored, the street surfaces contributed about 25% of the total urban runoff flows.

- Most of the total solids in urban runoff originated from front and back yards in the test areas. The street surfaces contributed only a small fraction to the total solids of urban runoff discharges. Lead, zinc, and COD, however, were mostly contributed from street surfaces. Nutrients (phosphorus and total Kjeldahl nitrogen) were found to originate mostly from street surfaces, driveways, and parking lots combined.
- Pesticides were only found in the residential street dirt samples, and not in the arterial, commercial, or industrial street dirt samples. The arterial street dirt samples had much higher concentrations of lead, most likely due to increased automobile activity.
- Many organic priority pollutants were detected in the soil samples. The most important organics found were the polycyclic aromatic hydrocarbons (PAHs), which were frequently detected in the street dirt samples and the Kelsey Creek sediment samples.
- Motor vehicle activity was expected to be the primary contributor of most of the toxic organic and inorganic priority pollutants. Gasoline and diesel fuel combustion products, lubricant and fuel leakages, and wear of the vehicles affected the street dirt material most significantly.
- Almost as much of the street dirt was lost to the air, as suspended particulates, as was washed off during rain events.
- Only a small fraction of the total particulate loadings on the impervious surfaces was removed by the rains (about 15%). Large particles were not effectively removed, while about one half of the smallest particles (less than 50 μm) were washed off during rains. These small particles were not very abundant, but had very high heavy metal and nutrient concentrations.
- Most of the settled particulate material in the storm drainage inlets and sewerage pipes was not removed by the observed storms.

Control of Urban Runoff by Street and Storm Drainage Inlet Cleaning and by Dry Detention Ponds

- Intensive street cleaning (three times a week) resulted in rapid and significant decreases in street surface loadings; from about 110 g/curb-meter down to about 55 g/curb-meter. The median particle sizes also decreased significantly with intensive street cleaning. A regenerative air street cleaner showed substantially better performance in removing the finer street surface materials than the regular mechanical street cleaner.
- Extensive data analysis did not show any significant improvements in runoff water quality during periods of intensive street cleaning. The street cleaning operations tested are only expected to improve runoff quality by a maximum of about 10%. The street cleaning equipment preferentially removed the larger particle sizes, while the rain events preferentially removed the finer materials. Street cleaning was not very effective in removing the particulates available for washoff.
- Mechanical broom street cleaning was effective in removing the larger litter from the streets.
- Infrequent street cleaning may result in significant increases in fugitive dust losses to the atmosphere.
- After an initial cleaning, it required almost a full year for sediment to reach a stable volume in the inlet structures. Only about 60% of the total available sump volumes in inlets and catchbasins was used for detention of particulates. Cleaning the inlets and catchbasin sumps about twice a year was expected to reduce the lead and total solids urban runoff concentrations by between 10 and 25%. COD, the nutrients, and zinc might be controlled between 5 and 10%.
- The small detention basin tested (detention time of 30 min or less) did not have any significant effect on urban runoff quality.
- The small detention basin did have a significant effect on the peak flow rates. The peak flow rates were reduced by about 60%.

Summary

The Bellevue studies indicated the very significant interrelationships between the physical, biological, and chemical characteristics of the urbanized Kelsey Creek system. The aquatic life

beneficial uses were found to be impaired and stormwater conveyance was found to be significantly stressed by urbanization. These degradations were most likely associated with increased flows from the impervious areas in the urban area. Changes in the flow characteristics could radically alter the ability of the stream to carry the polluted sediments into the other receiving waters. If the stream power of Kelsey Creek was reduced, then these toxic materials could be expected to be settled into its sediment, with increased effects on the stream's aquatic life. Reducing peak flows would also reduce the flushing of smaller fish and other aquatic organisms from the system.

If detention basins were used to control peak flows, they would have to be carefully located and designed so that increased flow rates did not occur in downstream areas. The placement of flow-modifying structures throughout the watershed could significantly affect the response time of the watershed to rain events, with possible resultant increases in downstream peak flows.

It was found that substantial quantities of water originated from the impervious areas in the developed areas. More careful planning to increase the perviousness of these areas should also be considered.

Another recommendation is to preserve any of the refuge areas in Kelsey Creek and to carefully design any channelization project to include refuge areas for the aquatic life. Because of the larger potential for sedimentation of toxic pollutants in Kelsey Creek, increased awareness of the beneficial uses and undesirable discharges to the drainage system will be more important. The large assimilative capacity of the water bodies that currently receive most of these pollutants are currently masking this concern.

Many recommendations concerning the public works practices in the Bellevue area can also be made based on this project. However, their effects on improving the urban runoff quality would probably be quite small. If intensive street cleaning was implemented, along with semiannual catchbasin sediment cleaning, urban runoff discharges for most pollutants would be reduced by about 10%, while some of the heavy metal discharges may be reduced by as much as 25%. Even though these reductions are quite small, they may be important to reduce the accumulation of these highly polluted sediments in the smaller creek systems, especially if peak flushing flows are reduced.

Critique of Parallel Stream Analyses in Bellevue

The Bellevue, WA, NURP project included many in-stream measurements to compare the test Kelsey Creek with the control Bear Creek. The study included numerous physical and biological measurements. In addition, in-stream toxicity tests were conducted. This large research program included numerous components. As for the Coyote Creek study, this program was likely much larger than needed. Newer tools and the use of efficient indicators could have reduced the sampling and analytical effort. The very large number of storms evaluated and the long-term stream studies were extremely enlightening, but similar conclusions could have been obtained through less expensive means. Again, this was one of the first comprehensive receiving water studies conducted, and there was little guidance to indicate what to expect.

The numerous researchers and different institutions conducting this research program indicated numerous communication and coordination problems, especially concerning preliminary conclusions. Most of the researchers were reluctant to share their results with the other groups until they had completed their thorough evaluations. If better communications were practiced, efficient modifications to the field activities would have been possible. However, the many experts involved in this research program resulted in a very important multidisciplinary study that would not have been possible with a smaller team of researchers.

In general, parallel stream investigations can be expanded well beyond a two-stream comparison by including numerous streams having variable levels of development. This has been a common experimental design for recent receiving water investigations. However, it is still important to conduct the study over a long duration and in numerous locations to best understand the dynamics of the systems. In many cases, in-stream variations can easily mask differences between streams.

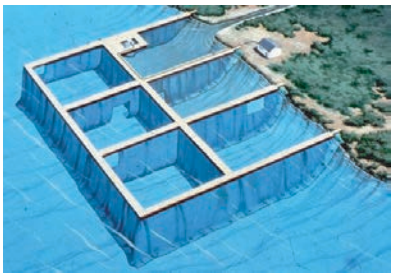

Figure 4.23 . Drawing showing underwater features of an FBM facility. (Used with permission of Fresh Creek Technologies, Inc.)

Figure 4.24 FBM installation located at Lake Trehormingen, Sweden. (Used with permission of Fresh Creek Technologies, Inc.)

Example of Long-Term Trend Experimental Design — Lake Rönningesjön, Sweden, Receiving Water Study

An example showing the use of trend analyses for investigating receiving water effects of stormwater is presented here, using a Swedish lake example that has undergone stormwater treatment (Pitt 1995a). The significant beneficial use impairment issue is related to decreasing transparency due to eutrophication. The nutrient enrichment was thought to have been aggravated by stormwater discharges of phosphorus. Stormwater treatment was shown to decrease the phosphorus discharges in the lake, with an associated increase in transparency. The data available include nutrient, chlorophyll *a*, transparency, and algal evaluations conducted over a 20- to 30-year period, plus treatment plant performance information for 10 years of operation. This trend evaluation was conducted by Pitt (1995a) using data collected by Swedish researchers, especially Enell and Henriksson-Fejes (1989–1992).

A full-scale plant, using the Karl Dunkers' system for treatment of separate stormwater (the Flow Balancing Method, or FBM) and lake water, has been operating since 1981 in Lake Rönningesjön, Taby (near Stockholm), Sweden. The FBM and the associated treatment system significantly improved lake water quality through direct treatment of stormwater and by pumping lake water through the treatment system during dry weather. Figure 4.23 is an illustration of an idealized FBM system showing how inflowing stormwater is routed though a series of interconnected compartments, before being discharged to the lake. A pump can also be used to withdraw water from the first compartment to a treatment facility. Figure 4.24 is a photograph of an FBM installation located at Lake Trehormingen, Sweden. Figure 4.25 shows wetland vegetation growing in one of the compartments of the FBM at Lake Rönningesjön, while Figure 4.26 shows the building containing the chemical treatment facility at the Lake Rönningesjön facility.

The annual average removal of phosphorus from stormwater and lake water by the ferric chloride precipitation and clarification treatment system was 66%, while the annual average total lake

Figure 4.25 . Wetland vegetation growing in FBM cell at Lake Rönningesjön, Sweden. (Used with permission of Fresh Creek Technologies, Inc.)

Figure 4.26 Chemical treatment facility at FBM installation at Lake Rönningesjön, Sweden. (Used with permission of Fresh Creek Technologies, Inc.)

phosphorus concentration reductions averaged about 36%. Excess flows are temporarily stored in the FBM before treatment. Stormwater is pumped to the treatment facility during rains, with excess flows stored inside in-lake flow-balancing tanks. The treatment system consists of a chemical treatment system designed for the removal of phosphorus and uses ferric chloride precipitation and crossflow lamella clarifiers. The stormwater is pumped from the flow-balancing storage tanks to the treatment facility. Lake water is also pumped to the treatment facility during dry periods, after any excess stormwater is treated.

Step 1. What's the Question?

The specific question to be addressed by this research was whether controlling phosphorus in stormwater discharges to a lake would result in improved lake water quality. Secondly, this evaluation was made to determine if the treatment system was designed and operated satisfactorily.

Step 2. Decide on Problem Formulation

The problem formulation employed for this project was a long-term trend analysis. Up to 30 years of data were available for some water quality parameters, including about 10 years of observations before the treatment system was implemented. Data were available for two sampling locations in the lake, plus at the stormwater discharge location. In addition, mass balance data were available for the treatment operation.

Monitored water quality in Lake Rönningesjön, near Stockholm, Sweden, was evaluated to determine the changes in transparency and nutrient concentrations associated with retrofitted stormwater controls. Statistical trend analyses were used to evaluate these changes. Several publications have excellent descriptions of statistical trend analyses for water quality data. In addition to containing detailed descriptions and examples of experimental design methods to determine required sampling effort, Gilbert (1987) devotes a large portion of his book to detecting trends in water quality data and includes the code for a comprehensive computer program for trend analysis. That information and other experimental design issues on conducting a trend investigation are briefly reviewed in Chapter 7 of this book.

Step 3. Project Design

Qualitative Watershed and Lake Characterization

Lake Rönningesjön is located in Taby, Sweden, near Stockholm. Figure 4.27 shows the lake location, the watershed, and the surrounding urban areas. The watershed area is 650 ha, including Lake Rönningesjön itself (about 60 ha) and the urban area that has its stormwater drainage bypassing the lake (about 175 ha). The effective total drainage area (including the lake surface) is therefore about 475 ha. Table 4.24 summarizes the land use of the lake watershed area. About one half of the drainage area (including the lake itself) is treated by the treatment and storage operation.

The lake volume is about 2,000,000 m^3 and the lake has an annual outflow of about 950,000 m^3. The estimated mean lake resident time is therefore slightly longer than 2 years. The average lake depth is 3.3 m. It is estimated that rain falling directly on the lake surface contributes about one half of the total lake outflow.

The treatment process consists of an in-lake flow-balancing storage tank system (the Flow Balancing Method, or FBM) to contain excess stormwater flows which are pumped to a treatment facility during dry weather. The treatment facility uses ferric chloride and polymer precipitation and crossflow lamella clarifiers. Figure 4.28 shows the cross section of the FBM in the lake. It is made of plastic curtains forming the cell walls, supported by floating pontoons and anchored to the lake bottom with weights.

Figure 4.29 shows that the FBM provides storage of contaminated water by displacing clean lake water that enters the storage facility during dry weather as the FBM water is pumped to the

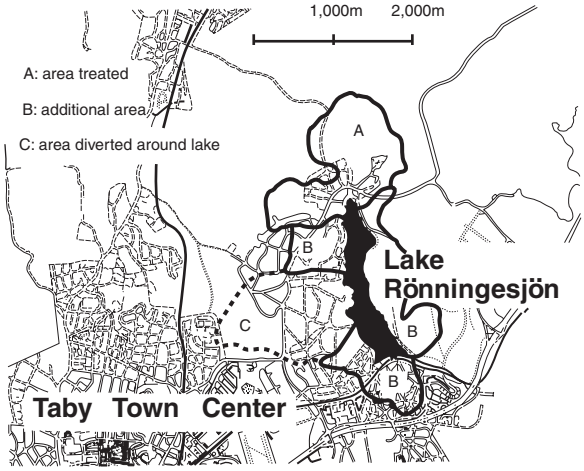

Figure 4.27 Lake Rönningesjön watershed in Taby, Sweden. (From Pitt 1995a. Used with permission of Fresh Creek Technologies, Inc.)

Table 4.24 Lake Rönningesjön Watershed Characteristics

	Area Treated, ha	Additional Area, ha	Total Area, ha
Urban	50	100	150 (32%)
Forest	75	80	155 (32%)
Agriculture	65	45	110 (23%)
Lake surface	60	0	60 (13%)
Total drainage	250	225	475 (100%)

From Pitt 1995a.

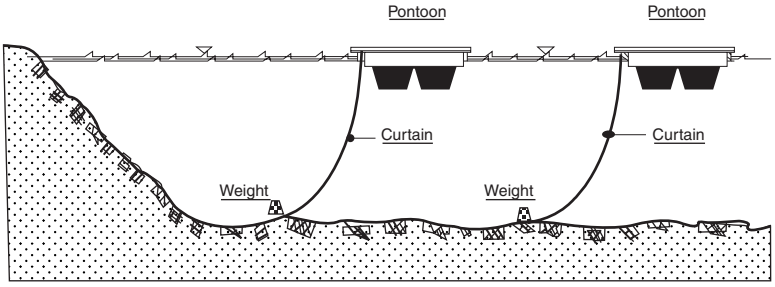

Figure 4.28 Cross section of FBM in-lake tanks. (From Pitt 1995a. Used with permission of Fresh Creek Technologies, Inc.)

treatment system. All stormwater enters the FBM directly (into cell A). The pump continuously pumps water from cell A to the chemical treatment area. If the stormwater enters cell A faster than the pump can remove it, portions of the stormwater flows through curtain openings (as a slug flow) into cells B, C, D, and finally E, displacing lake water (hence the term *flow balancing*). As the pump continues to operate, stormwater is drawn back into cell A and then to the treatment facility. The FBM is designed to capture the entire runoff volume of most storms. The Lake Rönningesjön treatment system is designed to treat water at a higher rate than normal to enable lake water to be pumped through the treatment system after all the runoff is treated.

The FBM is mainly intended to be a storage device, but it also operates as a wet detention pond, resulting in sedimentation of particulate pollutants within the storage device. The first two cells of the FBM facility at Lake Rönningesjön were dredged in 1991, after 10 years of operation, to remove about 1 m of polluted sediment.

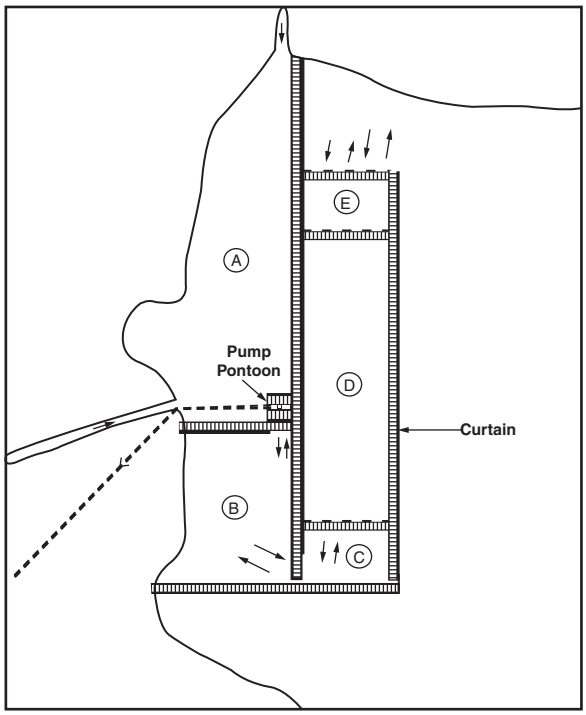

Figure 4.29 Flow pattern in FBM. (From Pitt 1995a. Used with permission of Fresh Creek Technologies, Inc.)

Table 4.25 Stormwater Treatment System Operating Cost Breakdown

Chemicals	26%
Electricity	8
Sludge transport	3
Labor	41
Sampling and analyses	22

From Pitt 1995a.

The treatment flow rate is 60 m^3/hour (about 0.4 MGD). The ferric chloride feed rate is about 20 to 35 g/m^3 of water. About 30 m^3 of thickened sludge is produced per day for co-disposal with sludge produced at the regional sanitary wastewater treatment facility. The annual operating costs are about $28,000 per year (or about $0.03 per 100 gallons of water treated), as shown in Table 4.25.

From 1981 through 1987, the FBM operated an average of about 5500 hours per year (about 7.6 months per year), treating an average of about 0.33 million m^3 per year. The treatment period ranged from 28 to 36 weeks (generally from April through November). The FBM treatment system treated stormwater about 40% of its operating time and lake water about 60% of its operating time. The FBM treatment system directly treated about one half of the waters flowing into the lake (at a level of about 70% phosphorus removal).

Lake Rönningesjön and Treatment System Phosphorus Budgets

Two tributaries flow directly to the treatment facility. Excess flows (exceeding the treatment plant flow capacity) are directed to the FBM in the lake. As the flows in the tributaries fall below the treatment plant capacity, pumps in the FBM deliver stored stormwater runoff for treatment. When all of the stormwater is pumped from the FBM, the pumps deliver lake water for treatment. Tables 4.26 and 4.27 summarize the runoff and lake volumes treated and phosphorus removals during the period of treatment.

Table 4.26 Water Balance for Treatment System (m^3)

	From Trib. A	From Trib. B	Total Stormwater	From Lake	Total Treated and Discharged	Stormwater, % of Total Treated
1981	185,100	101,100	286,200	121,600	407,700	70
1982	112,700	41,000	153,700	238,700	391,900	39
1983	14,400	6400	20,800	250,000	271,000	8
1984	122,000	53,000	175,000	95,000	270,000	65
1985	96,600	46,500	143,100	149,000	292,400	49
1986	216,000	86,000	302,000	48,000	350,000	86
1987	243,000	97,000	340,000	13,000	353,000	96
1988	26,200	19,300	45,500	186,300	231,800	20
1989	24,900	19,900	44,800	267,700	312,500	14
1990	12,160	8,330	20,490	201,270	221,760	9
1991	11,610	7780	19,390	121,730	141,120	14

From Pitt 1995a.

Table 4.27 Phosphorus Treatment Mass Balance (kg)

	From Trib. A	From Trib. B	From Lake	Total to Treatment	P Discharged to Lake	P Removal	% Removal
1981	20.3	16.8	10.2	47.3	13.6	33.7	71.2
1982	8.0	8.0	18.0	34.0	12.8	21.2	62.4
1983	1.5	2.5	20.0	24.0	11.0	13.0	54.2
1984	10.0	9.5	3.0	22.5	10.0	12.5	55.6
1985	7.1	5.9	2.1	15.1	4.3	10.8	71.5
1986	15.2	21.4	3.7	40.3	5.1	35.2	87.3
1987	18.6	7.5	1.7	27.8	4.3	23.5	84.5
1988	1.7	2.3	9.2	13.2	6.1	7.1	53.8
1989	1.7	1.4	14.1	17.2	7.6	9.6	55.8
1990	1.3	0.3	10.5	12.1	3.7	8.4	69.4
1991	7.7	9.8	5.6	23.1	8.9	14.2	61.5

From Pitt 1995a.

There have been highly variable levels of phosphorus treatment from stormwater during the period of operation. The years from 1988 through 1990 had low phosphorus removals. These years had relatively mild winters with substantial stormwater runoff occurring during the winter months when the treatment system was not operating. Normally, substantial phosphorus removal occurred with spring snowmelt during the early weeks of the treatment plant operation each year. The greatest phosphorus improvements in the lake occurred during the years when the largest amounts of stormwater were treated.

The overall phosphorus removal rate for the 11 years from 1981 through 1991 was about 17 kg/year. About 40% of the phosphorus removal occurred in the FBM from sedimentation processes, while the remainder occurred in the chemical treatment facility. This phosphorus removal would theoretically cause a reduction in phosphorus concentrations of about 10 µg/L per year in the lake, or a total phosphorus reduction of about 100 µg/L during the data period since the treatment system began operation. About 70% of this phosphorus removal was associated with the treatment of stormwater, while about 30% was associated with the treatment of lake water.

Select Monitoring Parameters

Lake Rönningesjön water quality has been monitored since 1967 by the Institute for Water and Air Pollution Research (IVL); the University of Technology, Stockholm; the Limnological Institute at the University of Uppsala; and by Hydroconsult Corp. Surface and subsurface samples were obtained at one or two lake locations about five times per year. In addition, the tributaries being

treated, incoming lake water, and discharged water were all monitored on all weekdays of treatment plant operation. The creek tributary flow rates were also monitored using overflow weirs. Phosphorus, nitrogen, chlorophyll *a*, and Secchi disk transparency were all monitored at the lake stations.

Step 4. Project Implementation, Step 5. Data Evaluation, and Step 6. Confirmatory Assessment

Observed Long-Term Lake Rönningesjön Water Quality Trends

The FBM started operation in 1981. Based on the hydraulic detention time of the lake, several years would be required before a new water quality equilibrium condition would be established. A new water quality equilibrium will eventually be reached after existing pollutants are reduced from the lake water and sediments. The new water quality conditions would be dependent on the lake flushing rate (or detention time, estimated to be about 2.1 years), and the new (reduced) pollutant discharge levels to the lake. Without lake water treatment, the equilibrium water quality would be worse and would take longer to obtain.

Figure 4.30 is a plot of all chlorophyll *a* data collected at both the south and north sampling stations. Very little trend is obvious, but the wide swings in chlorophyll *a* values appeared to have been reduced after the start of stormwater treatment. Figure 4.31 is a three-dimensional plot of

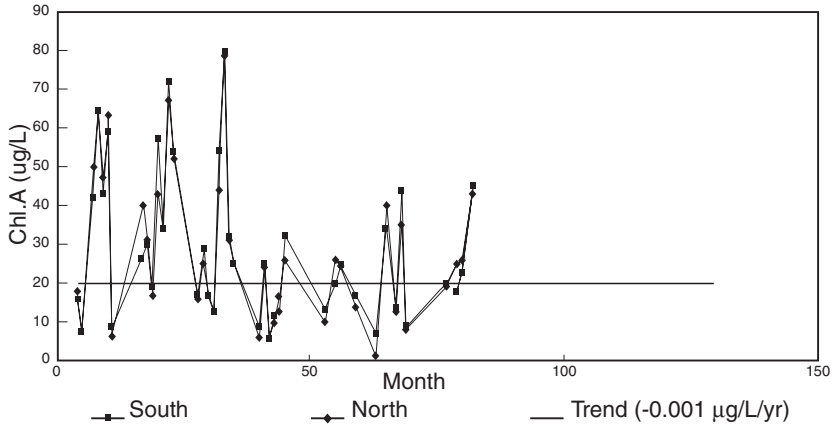

Figure 4.30 Chlorophyll *a* observations with time (µg/L). (From Pitt 1995a.)

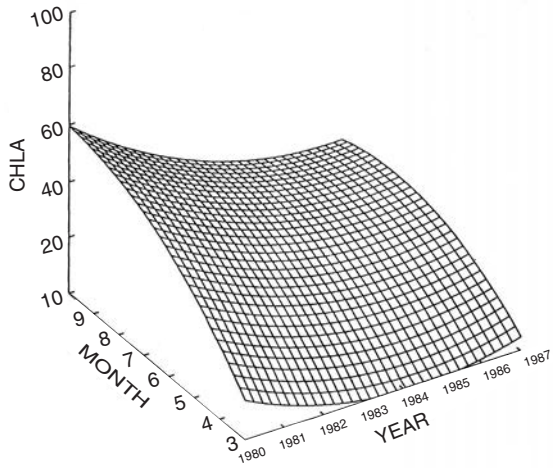

Figure 4.31 Chlorophyll *a* trends by season and year (µg/L). (From Pitt 1995a.)

smoothed chlorophyll *a* data, indicating significant trends by season. The values started out relatively low each early spring and dramatically increased as the summer progressed. This was expected and was a function of algal growth. Homogeneity, seasonal Kendall, and Mann–Kendall statistical tests (Gilbert 1987) were conducted using the chlorophyll *a* data. The homogeneity test was used to determine if any trends found at the north and south sampling stations were different. The probabilities that the trends at these two stations were the same were calculated as follows:

	χ^2	**Probability**
Season	14.19	0.223
Station	0.00001	1.000
Station–season	0.458	1.000
Trend	21.64	0.000

This test shows that the trend was very significant ($P < 0.001$) and was the same at both sampling stations ($P = 1.000$). The seasonal trend tests only compared data obtained for each season, such as comparing trends for June observations alone. The station-season interaction term shows that the chlorophyll *a* concentration trends at the two stations were also very similar for all months ($P = 1.000$). Therefore, the sampling data from both stations were combined for further analyses.

The seasonal Kendall test calculated the chlorophyll *a* concentration trends and determined the probabilities that they were not zero, for all months separately. This test and the Mann–Kendall tests found that both the north and south sampling locations had slight decreasing (but very significant) overall trends in concentrations with increasing years ($P \leq 0.001$). However, individual monthly trends were not very significant ($P \geq 0.05$). The trends do show an important decrease in the peak concentrations of chlorophyll *a* that occurred during the fall months during the years of the FBM operation. The 1980 peak values were about 60 µg/L, while the 1987 peak values were lower, at about 40 µg/L.

Swedish engineers (Söderlund 1981; Lundkvist and Söderlund 1988) summarized major changes in the algal species present and in the algal biomass in Lake Rönningesjön, corroborating the chlorophyll *a* and phosphorus-limiting nutrient observations. From 1977 through 1983, the lake was dominated by a stable population of thread-shaped blue-green algae species (especially *Oscillatoria* sp. and *Aphanizomenon flos aquae f. gracile*). Since 1985, the algae population has been unstable, with only a small amount of varying blue-green (*Gomphosphaeria*), silicon (*Melosira*, *Asterionella*, and *Synedra*), and gold (*Chrysochromulina*) algae species. They also found a substantial decrease in the algal biomass in the lake. From 1978 through 1981, the biomass concentration was commonly greater than 10 mg/L. The observed maximum was about 20 mg/L, with common annual maximums of 15 mg/L in July and August of each year. From 1982 through 1986, the algal biomass was usually less than 10 mg/L. The observed maximum was 14 mg/L and the typical annual maximum was about 6 mg/L each late summer. The lake showed an improvement in its eutrophication level since the start of stormwater treatment, going from hypotrophic to eutrophic.

Figure 4.32 is a plot of all Secchi disk transparency data obtained during the project period. A very large improvement in transparency is apparent from this plot, but large variations were observed in most years. A large improvement may have occurred in the first 5 years of stormwater treatment and then the trend may have decreased. The smoothed plot in Figure 4.33 shows significant improvement in Secchi disk transparency since 1980. This three-dimensional plot shows that the early years started off with clearer water (as high as 1 m transparency) in the spring and then degraded as the seasons progressed, with transparency levels decreasing to less than 0.5 m in the fall. The later years indicated a significant improvement, especially in the later months of the year.

Homogeneity, seasonal Kendall, and Mann–Kendall statistical tests (Gilbert 1987) were conducted using the Secchi disk transparency data. The homogeneity test was used to determine if any

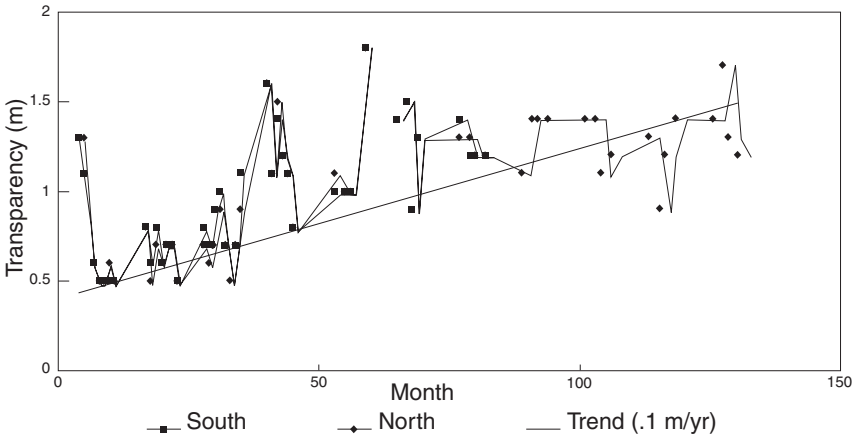

Figure 4.32 Secchi disk transparency observations with time (m). (From Pitt 1995a.)

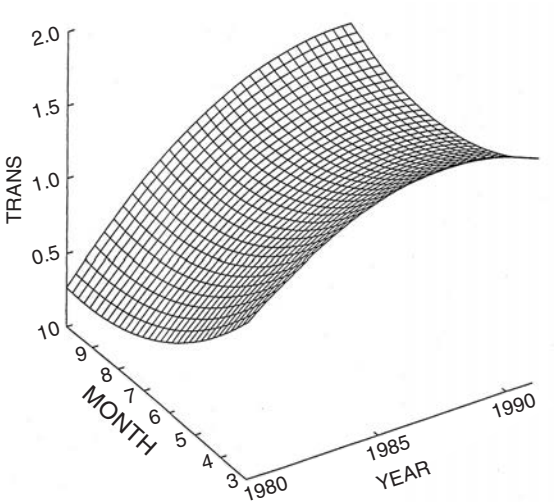

Figure 4.33 Secchi disk trends by season and year (m). (From Pitt 1995a.)

trends found at the north and south sampling stations were different. The probabilities that the trends at these two stations were the same were calculated as follows:

	χ^2	Probability
Season	17.15	0.103
Station	0.012	0.913
Station–season	3.03	0.990
Trend	29.44	0.000

These statistics show that the observed trend was very significant ($P < 0.001$) and was the same at both stations. The seasonal Kendall and Mann–Kendall tests found that both the north and south sampling locations had increasing transparency values (the average trend was about 0.11 m per year) with increasing years ($P < 0.001$). The trend in later years was found to be less than in the early years. The transparency has remained relatively stable since about 1987 (ranging from about 1 to 1.5 m), with less seasonal variation.

Figure 4.34 plots observed phosphorus concentrations with time, while Figure 4.35 is a smoothed plot showing seasonal and annual variations together. The initial steep decreases in

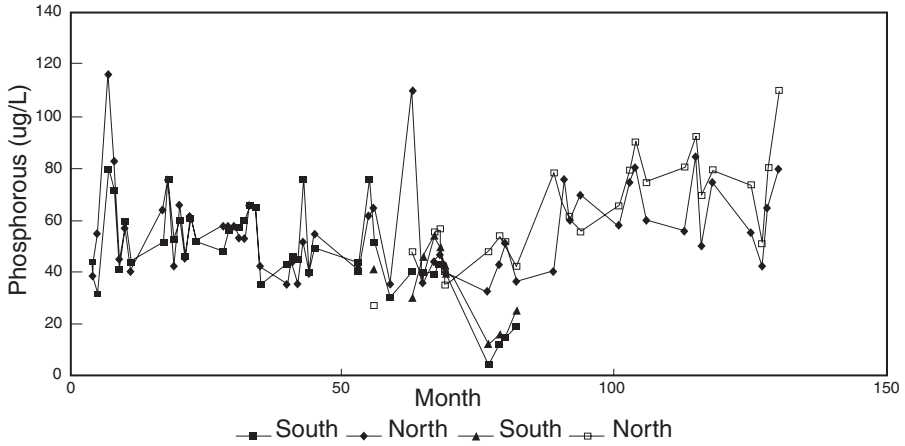

Figure 4.34 Total phosphorus observations with time (μg/L). (From Pitt 1995a.)

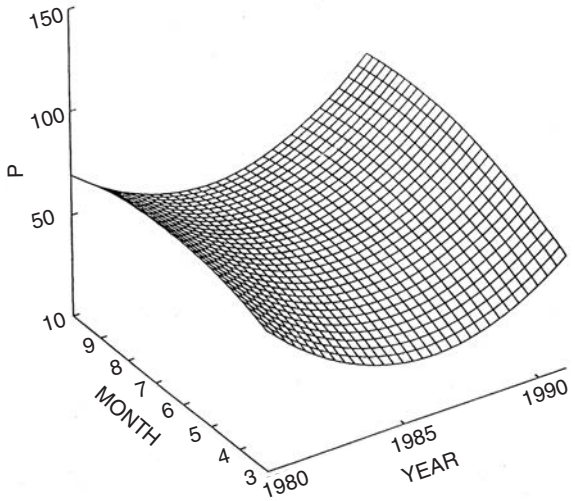

Figure 4.35 Total phosphorus trends by season and year (μg/L). (From Pitt 1995a.)

phosphorus concentration in the early years of the FBM operation were followed by a sharp increase during later years. The increase was likely associated with the decreased levels of stormwater treatment during the mild winters of 1988 through 1990 when the treatment system was not operating; large amounts of untreated stormwater were discharged into the lake instead of being tied up as snow to be treated in the spring as snowmelt runoff.

Individual year phosphorus concentrations leveled off in the summer (about July). These seasonal phosphorus trends were found to be very significant ($P \leq 0.002$), but were very small, using the seasonal Kendall test (Gilbert 1987). Homogeneity tests found no significant differences between lake sample phosphorus concentrations obtained at the different sampling locations, or depths, irrespective of season:

	χ^2	**Probability**
Season	15.38	0.166
Station	0.0033	0.954
Station–season	1.64	0.999
Trend	12.43	0.000

The overall lake phosphorus concentrations ranged from about 15 to 130 µg/L, with an average of about 65 µg/L. The monitored stormwater, before treatment, had phosphorus concentrations ranging from 40 to >1000 µg/L, with an average of about 200 µg/L.

An increase in nitrogen concentrations also occurred from the beginning of each year to the fall months. The overall annual trend decreased during the first few years of the FBM operation, but it then subsequently increased. These total nitrogen concentration variations were similar to the total phosphorus concentration variations. However, homogeneity, seasonal Kendall, and Mann–Kendall statistical tests (Gilbert 1987) conducted using the nitrogen data found that neither the north nor south sampling locations had significant concentration trends with increasing years ($P > 0.2$). However, lake Kjeldahl nitrogen concentration reductions were found to occur during years when the FBM system was treating the largest amounts of stormwater.

Lake Water Quality Model

A simple water quality model was used with the Lake Rönningesjön data to determine the total annual net phosphorus discharges into the lake and to estimate the relative magnitude of various in-lake phosphorus-controlling processes (associated with algal growth and sediment interactions, for example). These estimated total phosphorus discharges were compared to the phosphorus removed by the treatment system. The benefits of the treatment system on the lake water quality were then estimated by comparing the expected lake phosphorus concentrations (as if the treatment system was not operating) to the observed phosphorus concentrations.

Thomann and Mueller (1987) presented the following equation to estimate the resulting water pollutant concentrations associated with varying input loadings for a well-mixed lake:

$$S_t = (M/V)\exp(-T/Td) \tag{4.1}$$

where S_t = concentration associated with a step input at time t
M = mass discharge per time-step interval (kg)
V = volume of lake (2,000,000 m^3)
T = time since input (years)
Td = hydraulic residence time, or lake volume/lake outflow (2.1 years)

This equation was used to calculate the yearly total mass discharges of phosphorus to Lake Rönningesjön, based on observed lake concentrations and lake hydraulic flushing rates. It was assumed that the varying concentrations observed were mostly caused by varying mass discharges and much less by variations in the hydraulic flushing rate. The flushing rate was likely to vary, but by relatively small amounts. The lake volume was quite constant, and the outflow rate was expected to vary by less than 20% because of the relatively constant rainfall that occurred during the years of observation (average rainfall of about 600 mm, with a coefficient of variation of about 0.15).

The total mass of phosphorus discharged into the lake each year from 1972 to 1991 was calculated using the following equation (an expansion of Equation 4.1), solving for the M_{n-x} terms:

$$\begin{aligned} S_n = {} & M_n\left[\exp\left(-T_n/Td\right)/V\right] + M_{n-1}\left[\exp\left(-T_{n-1}/Td\right)/V\right] + M_{n-2}\left[\exp\left(-T_{n-2}/Td\right)/V\right] \\ & + M_{n-3}\left[\exp\left(-T_{n-3}/Td\right)/V\right] + \cdots \end{aligned} \tag{4.2}$$

where S_n is the annual average phosphorus concentration during the current year, M_n is the net phosphorus mass discharged into the lake during the current year, M_{n-1} is the phosphorus mass

discharged during the previous year, M_{n-2} is the phosphorus mass that was discharged 2 years previously, etc.

The effects of discharges into the lake many years earlier have little effect on the current year's observations. Similarly, more recent discharges have greater effects on the lake's concentrations. The magnitude of effect that each year's step discharge has on a more recent concentration observation is dependent on the $\exp(-T_n/Td)$ factors shown in Equation 4.2. A current year's discharge affects that year's concentration observations by about 40% of the steady-state theoretical value (M/V), and a discharge from 5 years earlier would affect the current year's concentration observations by less than 10% of the theoretical value for Lake Rönningesjön. Similarly, a new steady-state discharge would require about 4 years before 90% of its equilibrium concentration would be obtained. It would therefore require several years before the effects of a decrease in pollutant discharges would have a major effect on the lake pollutant concentrations.

The annual control of phosphorus ranged from about 10 to 50%, with an average lake-wide level of control of about 36%, during the years of treatment plant operation. It is estimated that there would have been about a 1.6 times increase in phosphorus discharges into Lake Rönningesjön if the treatment system was not operating. There was a substantial variation in the year-to-year phosphorus discharges, but several trends were evident. If there was no treatment, the phosphorus discharges would have increased over the 20-year period from about 50 to 75 kg/year, associated with increasing amounts of contaminated stormwater, in turn associated with increasing urbanization in the watershed. With treatment, the discharges were held relatively constant at about 50 kg/year (as evidenced by the lack of any observed phosphorus concentration trend in the lake). During 1984 through 1987, the phosphorus discharges were quite low compared to other years, but increased substantially in 1988 and 1989 because of the lack of stormwater treatment during the unusually mild winters.

Figure 4.36 is a plot of the annual average lake phosphorus concentrations with time. If there had been no treatment, the phosphorus concentrations in the lake would have shown a relatively

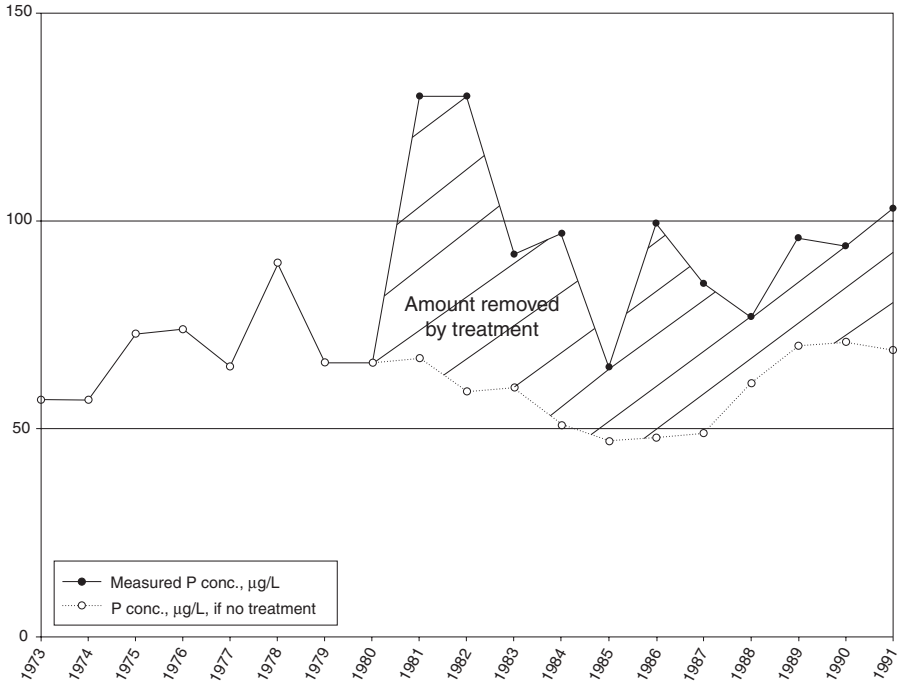

Figure 4.36 Effects of treatment on Lake Rönningesjön total phosphorus concentrations (µg/L). (From Pitt 1995a.)

steady increase from about 50 to about 100 µg/L over the 20-year period. With treatment, the lake phosphorus concentrations were held within a relatively narrower range (from about 50 to 75 µg/L). The lake phosphorus concentration improvements averaged about 50 µg/L over this period of time, compared to an expected theoretical improvement of about 100 µg/L. Therefore, only about one half of the theoretical improvement occurred, probably because of sediment-water interchange of phosphorus, or other unmeasured phosphorus sources.

Step 7. Project Conclusions

The in-lake flow-balancing method (FBM) for storage of excess stormwater during periods of high flows allowed for lower treatment flow rates, while still enabling a large fraction of the stormwater to be treated for phosphorus removal. The treatment system also enabled lake water to be treated during periods of low (or no) stormwater flow. The treatment of the stormwater before lake discharge accounted for about 70% of the total observed phosphorus discharge reductions, while the lake water treatment was responsible for the remaining 30% of the discharge reductions. The lake water was treated during 60% of the operating time, but resulted in less phosphorus removal, compared to stormwater treatment. The increased efficiency of phosphorus removal from stormwater compared to lake water was likely due to the more abundant particulate forms of phosphorus that were removed in the FBM by sedimentation and by the stormwater's higher dissolved phosphorus concentrations that were more efficiently removed during the chemical treatment process.

Lake transparency improved with treatment. Secchi disk transparencies were about 0.5 m before treatment began and improved to about 1 to 1.5 m after treatment. The total phosphorus concentrations ranged from about 65 to 90 µg/L during periods of low levels of stormwater treatment, to about 40 to 60 µg/L during periods of high levels of stormwater treatment.

The annual average removal of phosphorus by the ferric chloride precipitation and clarification treatment system was 66%, with a maximum of 87%. The observed phosphorus concentration improvements in the lake were strongly dependent on the fraction of the annual stormwater flow that was treated. The annual average total lake phosphorus discharge and concentration reductions averaged about 36%, or about one half the maximum expected benefit.

Critique of the Trend Analyses at Lake Rönningesjön

The water sampling for this project was irregular. Only a relatively few samples were obtained in any one year, but up to 30 years of data were obtained. In addition, no winter data were available due to icing of the lake. In general, statistically based trend analyses are more powerful with evenly spaced data over the entire period of time. However, this is typically unrealistic in environmental investigations because of an inability to control other important factors. If all samples were taken on the 15th of each month, for example, the samples would be taken under highly variable weather conditions. Weather is a significant factor in urban runoff studies, obviously, and this statistical methodology requirement would have severely confounded the results. The trend analyses presented by Gilbert (1987) enable a more reasonable sample collection effort, with some missing data. However, the procedure does require relatively complete data collected over an extended period of time. It would have been very difficult to conduct this analysis with only a few years of data, for example. The seasonal patterns were very obvious when multiple years of before and after treatment were monitored. In addition, the many years of data enabled unusual weather conditions (such as the years with unusually mild winters) to stand out from the more typical weather conditions.

The analytical effort only focused on a few parameters. This is acceptable for a well-designed and executed project, but prohibits further insights that a more expansive effort may obtain. Since this project was specifically investigating transparency-associated eutrophication, the parameters evaluated enabled the basic project objectives to be effectively evaluated. However, the cost of labor for the sampling effort is a major component of an investigation like this one, and some

additional supportive analyses may not have added much to the overall project cost while adding potentially valuable additional information.

In general, trend analyses require a large amount of data, typically obtained over a long period of time. These requirements cause potential problems. Experimental designs for a several-year (or several-decade) monitoring effort are difficult to carry out. Many uncontrolled changes may occur during a long period, such as changes in laboratory analysis methods. Laboratory method changes can affect the specific chemical species being measured, or at least have differing detection limit capabilities. This study examined basic measurements that have not undergone major historical changes, and very few "non-detectable" values were reported. In contrast, examining historical heavy metal data is very difficult because of changes in instrumentation and associated detection limits. The need for a typically long-duration study also requires a long period before statistically relevant conclusions can be obtained. Budget reductions in the future always threaten long-term efforts. In addition, personnel changes lead to inconsistent sampling and may also possibly lead to other errors. Basically, adequate trend analyses require a large amount of resources (including time) to be successful. The use of historical data not collected for a specific trend analysis objective is obvious and should be investigated to supplement an anticipated project. However, great care must be expended to ensure the quality of the data. In most cases, incorrect sampling locations and dates, let alone obvious errors in reported concentrations, will be found in historical data files. These problems, in conjunction with problems associated with changing laboratory methods during the monitoring period, require special attention and effort.

Case Studies of Current, Ongoing, Stormwater Projects

Los Angeles County Stormwater Monitoring Program to Support Its Stormwater Discharge Permit

Step 1. What's the Question?

Los Angeles County is currently conducting a comprehensive stormwater monitoring program in conjunction with its stormwater discharge permit. The Los Angeles region of the California Regional Water Quality Control Board (RWQCB) oversees the enforcement of the NPDES stormwater discharge permit for the Los Angeles area. The County of Los Angeles is the principal permittee of the municipal permit and is the permit coordinator responsible for administration for the 80 co-permittees (Rashedi and Liu 1996). The municipal permit had partitioned Los Angeles County and adjacent areas into five regional drainage basins: Santa Monica Bay, Upstream Los Angeles River, Upper San Gabriel River, Lower Los Angeles River, Lower San Gabriel River, and Santa Clarita Valley.

The originally proposed monitoring program was thought to be insufficient by local environmental groups and a suit was filed by the NRDC (*Natural Resources Defense Council v. County of Los Angeles,* CV 94-5978, C.D. Cal). After lengthy discussions between experts representing Los Angeles County and the NRDC, a settlement was reached between NRDC and Los Angeles County (with the approval of the California RWQCB) which specified the scope of work for the monitoring program needed to support the stormwater discharge permit. This program is described in the following paragraphs. Because of the importance and magnitude of the work involved, it is likely that changes to this program will be needed as information is collected and reviewed. Like all monitoring programs, it is necessary to retain a certain degree of flexibility and make slight changes in the monitoring program based on periodic comprehensive data reviews. In this case study, for example, certain monitoring parameters may be eliminated from the basic monitoring program if they are infrequently observed. However, they should still be periodically monitored on a less frequent schedule in case their initial absence was due to seasonal or unusual weather-related factors.

Figure 4.37 Santa Monica Bay/Beach.

Figure 4.38 Downtown Los Angeles.

Figure 4.39 Los Angeles River and roadway crossings.

Figure 4.40 Los Angeles River showing small central pilot channel containing perennial flow.

This monitoring program is multifaceted and will last for several years. The information to be obtained will enable the county to fulfill its permit obligations by conducting a stormwater management program based on local data and conditions. Without this local information, decisions that would have been made and stormwater management activities to be conducted would likely result in inadequate stormwater control and be very expensive for the benefits received. The comprehensive monitoring program being conducted will enable cost-effective management decisions to be made in the future. Figure 4.37 shows one of the major receiving waters addressed in the Los Angeles County stormwater management program (Santa Monica Bay), while Figure 4.38 shows the characteristics of the intensively developed ultra-urban area affecting local receiving waters. Figures 4.39 and 4.40 show the massive concrete-lined Los Angeles River draining much of the Los Angeles basin (discharges to Long Beach, not to the Santa Monica Bay).

Step 2. Decide on Problem Formulation

The Los Angeles County activities address the three main topics necessary in a comprehensive stormwater monitoring program: (1) measurements of the effects of stormwater on local receiving water beneficial uses, (2) identification of the sources of the problem pollutants responsible for these problems, and (3) local evaluations of candidate stormwater control practices to reduce the discharge of these problem pollutants and conditions.

This is a large effort and will include components of many of the sampling strategies available (such as comparing stormwater characteristics from multiple land use areas and evaluating trends in receiving water quality over time). Most of the monitoring activities will be conducted over a

3- to 5-year period and will include sampling during all seasons. Long-term evaluations are especially important in southern California because of the tremendous variability in precipitation from year to year. Some years have very little rain, while others, like the 1997–98 rain year affected by El Niño, are characterized by massive flooding. Under these conditions, it is very difficult to define what is "typical" and to design a comprehensive and effective stormwater management program without a monitoring program extending over several years and including many events.

Step 3. Project Design

The Los Angeles County stormwater permit (CA0061654) required the implementation of a monitoring program to control and eliminate the sources of stormwater pollution being discharged from the separate municipal stormwater drainage system. The California Regional Water Quality Control Board (Board Order No. 90-079) required the following actions in the monitoring program:

1. Initiate a monitoring network of initially nine stations to establish long-term trends in stormwater quality in the Santa Monica Drainage Basin.
2. Use a stormwater model in conjunction with the monitoring program to refine annual estimates of pollutant loads to Santa Monica Bay.
3. Implement targeted monitoring to identify sources of specific toxic pollutants in the local stormwater.
4. Implement a monitoring program to evaluate the effectiveness of specific stormwater controls.
5. Implement monitoring to identify locations of illegal practices and to eliminate pollutant sources.
6. Develop and implement a program to evaluate stormwater impacts on selected receiving waters including conducting toxicity studies in the Santa Monica Bay Drainage Basin.

The nine initial sampling locations were first separated into four "mass emission" stations to examine long-term water quality trends, and five land use stations that were relatively homogeneous to obtain unit area loadings and typical effluent concentrations. Critical source area locations will also be monitored to characterize stormwater from locations expected to contribute especially high loadings of toxicants. Thirteen "baseline" stormwater management practices will also be selected for evaluation. Public education (inlet sign painting, billboards, and radio messages) are of special interest.

1. Qualitative Watershed Characterization — The four mass emission sites currently being monitored are in large watersheds and are as follows (LACDPW 1995):

- Ballona Creek. 89 mi^2, representing much of the 127 mi^2 watershed that is not tidally influenced. The overall level of imperviousness is about 53%, and the land uses are approximately as follows: 19% open space, 30% single-family residential, 32% multiple-family residential, 14% commercial, and 4% industrial. The gauging/sampling station location is in a concrete-lined trapezoid channel, about 100 ft wide with a maximum depth of about 25 ft.
- Malibu Creek. 105 mi^2, representing almost all of the 110 mi^2 watershed. The overall imperviousness is about 13%, and the land uses are approximately as follows: 54% open space, 36% single-family residential, 5% multiple-family residential, and about 5% commercial and industrial combined. The monitoring station is located in a natural section of the creek, about 200 ft wide.
- Los Angeles River at Wardlow Rd. 815 mi^2, the largest watershed discharging into the Pacific Ocean in Los Angeles County. This site has been an active gauging station since 1931. The channel is concrete-lined and 400 ft wide. The maximum depth is 22 ft, while a shallow 28-ft-wide pilot channel carries dry-weather flows. This very large watershed contains all of the Los Angeles County land uses. Stream diversions, dams, and spreading areas are common in the watershed, all affecting the flows, especially from the upper foothill areas.
- San Gabriel River. 460 mi^2, also at an existing gauging station. Numerous flow regulation facilities also exist in this large watershed. The river is partially stabilized with concrete at the monitoring station and is 200 ft wide. The maximum depth is from 11 to 14 ft.

These stations represent the four major drainage points for the watersheds that discharge into the ocean from Los Angeles County. Up to 10 storms per year will be monitored at each of these locations. The purpose of monitoring at these drainages is to observe trends in stormwater quality over the period of monitoring. The data will also be useful in confirming the models calibrated from the land use specific monitoring stations. However, the large number of flow modification structures in the large watersheds will hinder some of the comparisons.

Besides the initial mass emission drainage monitoring stations listed above, initial land use monitoring stations were also established. These drainages represent relatively homogeneous (or simple combined) land uses and are as follows:

- Trancas Canyon. 7.45 mi^2, 97% open space (mostly in the Santa Monica Mountains National Recreation Area), and 3% low-density residential, with 1% imperviousness
- Palos Verdes Estates. 1.7 mi^2, 81% single-family residential, and 19% open space, with 40% imperviousness
- Manhattan Beach. 200 acres, 98% single-family residential and 2% commercial, with 42% imperviousness
- Downtown Los Angeles drain. 150 acres, 51% industrial and 49% commercial, with 91% imperviousness
- City of Santa Monica drain. 50 acres, 96% commercial (Santa Monica Mall) and 4% multifamily residential, with 92% imperviousness

A marginal benefit analysis was conducted by Woodward Clyde Consultants (WCC) and Psomas (1996), using the procedures described in Chapter 5, to identify additional land use monitoring sites to best represent the wide range of land uses in Los Angeles County. Table 4.28 lists the general land use categories for Los Angeles County, showing the percentage of each in the area covered by the NPDES stormwater discharge permit, plus the percentage of the total area total suspended solids (TSS) and copper loadings. Site surveys were conducted for the 12 most important land uses shown on this table (excluding vacant land). These 12 land uses comprised about 75% of the area of all land uses, excluding the vacant land. Seven to eight homogeneous areas representing each of these land use areas were surveyed during a 5-week period in the summer of 1996. Site survey information included detailed descriptions of the land use and age of the area, the nature and character of the buildings, the routing of on-site drainage (roof drainage and paved area drainage), the condition of the streets and other impervious areas, gutter types, the nature of the landscaping adjacent to the road, the presence of treated wood near the streets, and landscaping practices. In addition, measurements from maps and aerial photographs were made to determine the areas of each element of the development (roofs, streets, sidewalks, gutters, driveways, parking/storage areas, paved playgrounds, other paved areas, landscaped areas, and other pervious areas). Figure 4.41 shows box plots of the site-measured directly connected impervious areas for each of these 12 major land use areas.

The individual land use categories are also ranked in Table 4.28 according to their total area contributions of these attributes. The estimated contributions for each land use category were based on measured site characteristics (especially imperviousness) of the most important land uses, plus the best estimates of runoff characteristics for these land uses. Analyses using other expected critical pollutants (especially bacteria) would have been informative, but preliminary data were not available. Similar analyses using runoff volume, COD, and P were also conducted, with very similar results: the same land uses were always included in the group of the most important land uses.

Figure 4.42 is the plot from the marginal benefit analysis of all Los Angeles County land use areas, showing the decreasing marginal benefits associated with monitoring an increasing number of land use monitoring sites. From this analysis, a total of seven land uses were identified: high-density single-family residential, vacant land, light industrial, transportation, retail and commercial, multifamily residential, and educational facilities. Multifamily residential and educational facilities were therefore added to the five land use areas previously selected for monitoring. It must be noted that heavy industrial land use data are being collected by the industrial component of the NPDES

Table 4.28 Land Uses in Los Angeles County and Estimated Pollutant Discharge Rankings

Land Use Category	% of Area	Rank Based on Area	% of TSS Load	Rank Based on TSS Load	% of Copper Load	Rank Based on Copper Load
Vacant land	56.0	1	19.5	2	13.3	3
High-density single-family residential	18.6	2	22.9	1	32.5	1
Light industry	3.2	3	14.8	3	17.1	2
Multifamily residential	2.8	4	4.9	6	6.9	4
Retail and commercial	2.5	5	9.5	4	4.6	6
Transportation	1.7	6	5.6	5	6.5	5
Low-density SFR	1.6	7	1.6	11	2.2	8
Educational facilities	1.6	8	3.6	7	1.7	11
Receiving waters	1.4	9	0.0	34	0.0	34
Open space/recreation	1.2	10	1.6	13	0.54	19
Mixed residential	1.1	11	1.5	14	2.1	10
Utility facilities	1.1	12	1.2	15	0.69	16
Natural resources extraction	0.73	13	2.1	8	2.4	7
Institutions	0.66	14	1.6	12	0.76	14
Urban vacant	0.64	15	0.26	24	0.14	26
Golf courses	0.64	16	0.46	21	0.16	25
Rural residential	0.62	17	0.29	23	0.40	22
Floodways and structures	0.62	18	0.85	17	0.29	23
Heavy industry	0.51	19	1.9	9	2.2	9
General office use	0.49	20	1.8	10	0.86	12
Agriculture	0.45	21	0.21	25	0.11	29
Under construction	0.41	22	0.56	19	0.65	17
Other commercial	0.33	23	1.2	16	0.58	18
Nurseries and vineyards	0.33	24	0.10	29	0.27	24
Mobile homes and trailer parks	0.25	25	0.50	20	0.71	15
Mixed transportation and utility	0.14	26	0.66	18	0.77	13
Animal husbandry	0.11	27	0.09	30	0.09	31
Military installations	0.10	28	0.12	27	0.13	27
Maintenance yards	0.08	29	0.38	22	0.44	21
Mixed commercial and industrial	0.04	30	0.07	31	0.09	30
Harbor facilities	0.04	31	0.12	26	0.52	20
Marina facilities	0.03	32	0.03	33	0.07	32
Mixed urban	0.03	33	0.05	32	0.06	33
Communication facilities	0.02	34	0.11	28	0.13	28

program, and construction sites were not deemed an appropriate source to be included in this program by the county.

Further analyses were conducted to select smaller watershed areas for monitoring critical sources (WCC and Psomas Assoc. 1996). A list of industrial categories (by SIC codes), along with their ranking by their pollution potential and the number of the facilities, is shown in Table 4.29. The pollution potential rank was determined based on the number of sources in the area, the relative size of the paved areas at each source, the likelihood of specific toxic pollutants, and the exposure potential of the on-site sources. From this analysis, the following critical light industrial and commercial sources were selected for potential monitoring:

- Wholesale trade (including scrap yards and auto dismantlers)
- Automotive repair/parking (intend to stress repair facilities over parking areas in the monitoring program)
- Fabricated metal products (including electroplating)
- Motor freight (including trucking)
- Chemical manufacturing

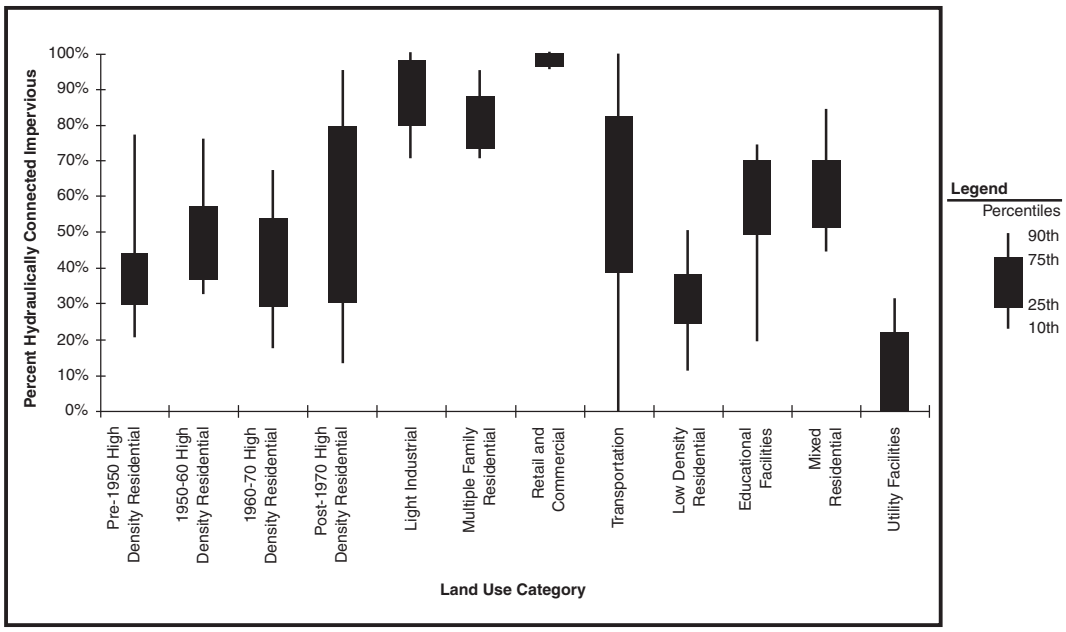

Figure 4.41 Box plots of hydraulically connected impervious areas of the most important Los Angeles County land use areas. (From Woodward Clyde Consultants and Psomas and Associates. *Evaluation of Land Use Monitoring Stations*. Prepared for the Los Angeles County Department of Public Works. August 1996.)

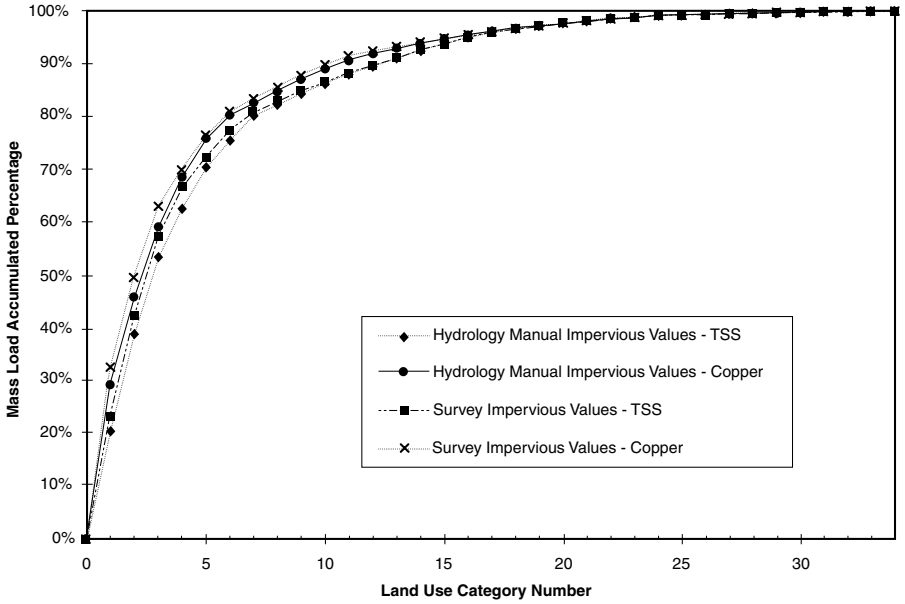

Figure 4.42 Marginal benefit analysis of all Los Angeles County land use areas. (From Woodward Clyde Consultants and Psomas and Associates. *Evaluation of Land Use Monitoring Stations*. Prepared for the Los Angeles County Department of Public Works. August 1996.)

Table 4.29 Ranking of Candidate Critical Sources in Los Angeles County

Industrial Category	SIC Code	No. of Facilities in Los Angeles County Study Area	Ranking Based on Pollution Potential
Wholesale trade (scrap, auto dismantling)	50	587	1
Automotive repair/parking	75	6067	2
Fabricated metal products	34	3283	3
Motor freight	42	872	4
Chemical manufacturing	28	1069	5
Automotive dealers/gas stations	55	2744	6
Primary metals products	33	703	7
Electric/gas/sanitary	49	2001	8
Air transportation	45	431	9
Rubbers/miscellaneous plastics	30	1034	10
Local/suburban transit	41	336	11
Railroad transportation	40	319	12
Oil and gas extraction	13	327	13
Lumber/wood products	24	905	14
Machinery manufacturing	35	4223	15
Transportation equipment	37	1838	16
Stone, clay, glass, concrete	32	733	17
Leather/leather products	31	163	18
Miscellaneous manufacturing	39	1144	19
Food and kindred products	20	1249	20
Petroleum refining	29	231	21
Mining of nonmetallic minerals	14	39	22
Printing and publishing	27	2432	23
Electric/electronic	36	1636	24
Paper and allied products	26	451	25
Furniture and fixtures	25	1368	26
Personal services (laundries)	72	2515	27
Instruments	38	1029	28
Textile mills products	22	440	29
Apparel	23	1900	30

From WCC and Psomas 1996.

These source categories were found to be poorly represented in past stormwater studies, with very little characterization data already available. Therefore, all of these categories were selected for further monitoring.

2. Receiving Water Characterization — The near-shore Pacific Ocean, local ocean beaches, and the large streams and major rivers are the receiving waters examined during this monitoring effort. As an example of the characteristics of the receiving waters, the Los Angeles River has a watershed of 827 mi^2, draining portions of the San Gabriel Mountains, the San Fernando Valley, and a large part of the metropolitan area of the city of Los Angeles. Lowe and Rashedi (1996) reviewed the historical flows in the Los Angeles River and reported an average runoff flow of about 235 million m^3/year, corresponding to about 4.4 in of runoff (a volumetric runoff coefficient of about 1/3, typical for large urban areas). The Los Angeles River also has a relatively small base flow, of about 14 million m^3/year, which is primarily treated wastewater discharged from upstream treatment facilities. Seasonal variations of flows are very large. Lowe and Rashedi (1996) reported that about 80% of the rainfall occurs in the winter, between November and March, with about 84% of the annual runoff also occurring during these months. January typically has the greatest flows and only about 2% of the annual runoff occurs in June through August. There is also a great variation in flows from year to year. They found about a 15 times difference in annual flows between the 10th percentile year and the 90th percentile year. These flow variations reported for the Los Angeles

River are likely similar to the variations that may be found in other urbanized rivers and streams of Los Angeles County. The physical nature of the Los Angeles River is greatly modified. It is completely channelized and concrete-lined for most of its length through the urban area toward the ocean. The river is very wide (about 400 ft) and relatively shallow (about 20 ft) in the downstream reaches. It has a shallow low-flow pilot channel about 25 ft wide and 2 ft deep. Many of the other major receiving waters in the county are also greatly modified, although all are smaller than the Los Angeles River.

A receiving waters study is also planned as part of the Los Angeles County monitoring program. This will be a joint effort between USC, UCSB, and the Southern California Coastal Water Research Project. An ongoing toxicity study conducted by UCLA will also be supported by the Los Angeles County Department of Public Works (LACDPW). The receiving water studies include a plume study to investigate the dispersion of stormwater flows and pollutants into the ocean from Malibu and Ballona Creeks. Marine benthic conditions near the outfalls of these two large creeks will also be investigated. The toxicity studies will investigate the stormwater flows from these two creeks, plus the affected sediments. The plume study will investigate discharges over 2 years from these creeks into Santa Monica Bay following strong winter storms. The spatial and temporal nature of the stormwater plumes will be mapped, and the interaction between the stormwater and the ocean water will be determined. The suspended particulate matter and dissolved organic material discharges will be of special interest. The benthic study will investigate water quality (DO, salinity, density, temperature, light transmissivity, and pH), sediment characteristics (grain size, organic and other constituent concentrations), and the structure of the benthic invertebrate community. The toxicity study will examine water column toxicity by using sea urchin fertilization tests and toxicity identification examinations (TIE). Sediment toxicity tests will include amphipod survival tests, sea urchin growth tests, chemical analyses of sea urchin tissue, and TIE tests. Two stormwater and one dry-weather flow sample will also be tested for toxicity (using sea urchin fertilization tests) at the Los Angeles River and the San Gabriel River monitoring stations in each of 2 years.

3. Select Monitoring Parameters and Magnitude of Sampling — The nine initial monitoring stations were instrumented with refrigerated automatic water samplers. Since the mass emission sampling locations required lifts greater than 15 ft and very long sample line lengths, auxiliary pumps were located in the stream channels that delivered a continuous flow of water close to the automatic samplers. The stormwater samples are being collected on a flow-proportionate basis, using existing flow monitoring facilities if available, or installing flow monitoring equipment, if needed. The samples were collected as discrete samples and then manually composited for analyses. Certain parameters (bacteria and VOCs) required manual sampling. The dry-weather sampling uses the same automatic samplers, but the samplers are reprogrammed to obtain samples on a time-weighted basis. At least one rain gauge capable of measuring rain intensity was also installed in the upper watersheds. The LACDPW operates many rain gauges throughout the Santa Monica Drainage Basin, and these were used to supplement the installed gauges.

Table 4.30 lists the priorities for the monitored constituents and the associated sample volumes needed to conduct the selected constituents. The total sample volume needed for the complete list of analyses to be collected from the automatically collected stormwater samples is about 8 L. As shown in Chapter 6, many of these analyses may be conducted using procedures requiring much smaller sample volumes. However, the use of alternative (but acceptable) methods can be more costly, especially if the laboratory needs to develop new methods. Only 40 mL of water is needed for the VOC analyses, but the samples must be manually collected because specialized automatic VOC samplers are not being used. Other analyses to be conducted on manually collected grab samples include total coliforms, fecal coliforms, fecal streptococcus, oil and grease, total phenols, cyanide, pH, and temperature. About 2.5 L of water is needed for these additional analyses.

Table 4.30 Analyses Priority and Sample Volumes Needed for Automatically Collected Stormwater Samples

Priority	Constituent	Method	Sample Volume Needed (mL)
1	Heavy metals (total and dissolved)	EPA[a] 200	500
2	Total petroleum hydrocarbons (TPH)	EPA 418.1	1000
3	Semivolatile organic compounds	EPA 8250	1000
4	Pesticides and PCBs	EPA 8250 or 608	1000
5	Total suspended solids (TSS)	EPA 160.1	100
6	Volatile suspended solids (VSS)	EPA 160.1	100
7	Total organic carbon (TOC)	EPA 415.1	25
8	Chemical oxygen demand (COD)	EPA 410.4	500
9	Specific conductance	EPA 120.1	100
10	Total dissolved solids (TDS)	EPA 160.1	100
11	Turbidity	EPA 180.1	100
12	Biochemical oxygen demand (BOD_5)	EPA 405.1	1000
13	Dissolved phosphorus	EPA 300	50
14	Total phosphorus	EPA 300	50
15	Total ammonia nitrogen	EPA 350.2	500
16	Total Kjeldahl nitrogen	EPA 351.3	100
17	Nitrate and nitrite nitrogen	SM[b] 4110	100
18	Alkalinity	EPA 310.1	100
19	Chloride	SM 4110	50
20	Fluoride	SM 4110	300
21	Sulfate	SM 4110	50
22	Herbicides	EPA 619	1000

[a] EPA published method.
[b] *Standard Methods for the Examination of Water and Wastewater.*

Sampling at the land use monitoring locations will include the complete list of constituents, unless the constituent is frequently not detected. If the constituent is not found at the method detection limit (MDL) in at least 25% of the samples, it will be eliminated from the list for routine analyses. However, the constituent will be analyzed at least once a year. In addition, once sufficient storms at a specific location have been sampled to allow the event mean concentration (EMC) of a constituent to be determined with an error rate of 25%, or less, that constituent will also be removed from the list of analyses to be conducted at that location. The land use station will remain in operation until the following constituent EMCs are determined at the 25% error level:

Total PAHs
Chlordane
Cd, Cu, Ni, Pb, Cr, Ag, Zn
TSS
Total nitrogen
Total phosphorus

A chain-of-custody record was prepared specifically for this project by the LACDPW. The sampling program also included routine QA/QC field activities, such as the use of field blanks for manual VOC sampling and field duplicates for all events. Before the sampling program began, a sampling instruction manual was prepared, detailing such things as specific sampling equipment features, sample handling, and field equipment lists. The *Quality Assurance Manual* from the local laboratory being used (Environmental Toxicology Laboratory of the County of Los Angeles Office of Agricultural Commissioner/Weights and Measures) was also included in the initial proposed stormwater monitoring program description prepared by the LACDPW.

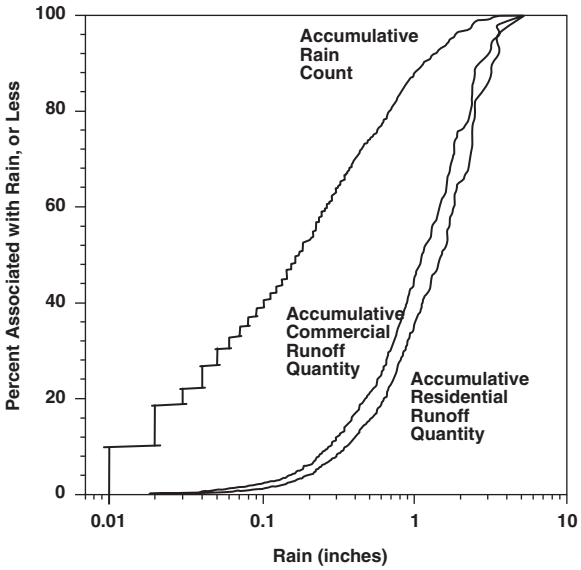

Figure 4.43 Probability plots of rain depth and runoff depths for 1969–1993 LAX conditions. (From Pitt et al. 1999.)

The initial monitoring design was to program the automatic samplers to obtain the needed sample volume for a 0.4-inch storm, with a maximum rain depth of 1.7 inches capable of filling the samplers. During the 45-year period from 1948 to 1993, about 1350 rains occurred at LAX (assuming a conventional 6-hour inter-event dry period), or about 30 rain events per year. Figure 4.43 shows a probability plot of rain event depths and estimated runoff depths for residential and commercial sites in the Los Angeles area for 1969 through 1993 rains. The median rain depth (by count and considering all rains) was about 0.2 inches, and about 70% of all recorded rains at LAX were less than 0.4 inches. About 5% of the rain events were greater than 1.7 inches in rain depth. Therefore, only about 25% of all rains (by occurrence) were in the range of 0.4 to 1.7 inches in depth. The 0.4-inch rain depth needed for complete analyses was therefore found to be relatively large, resulting in a significant number of events that would not be represented in the monitoring program. A special monitoring test was therefore conducted to determine the minimum rain event size that would produce significant runoff that could also be adequately sampled. The results of this special test indicated that the samplers could be programmed to capture runoff from at least a 0.25-inch rain, resulting in about 90% of the annual runoff volume being represented in the monitoring program.

Experimental design calculations also indicated the need for very large paired data sets to observe statistically significant differences in stormwater runoff quality from most public education and public works practices. With a coefficient of variation of 1 (common for most stormwater concentration data), plus a 20% likelihood of false negatives and 95% confidence, about 200 paired observations would be needed if the control program produces a change of about 25% in stormwater characteristics. If the change is about 50%, then about 50 paired observations would be needed. If the control program produced about 95% differences in stormwater characteristics (only possible for the most effective stormwater controls, such as well-designed and operated wet detention ponds or grass swales), then only 15 pairs of data would be needed. In an area having relatively few rain events per year, it could take many years to obtain adequate data for important decisions.

The sampling plan for the critical source areas includes monitoring at six sites in each of the five categories (WCC and Psomas Assoc. 1996). These monitoring activities will also include evaluations of site stormwater controls. The first year will include monitoring of the sites without controls, while the second year of monitoring will include the use of site controls at three of the sites in each category. These paired tests will enable site and rainfall differences to be identified to enable more accurate stormwater control evaluations. Five rain events will be monitored using manual grab sampling during the first year, and ten will be monitored during the second year. The

Table 4.31 Constituents to Be Monitored as Part of the Critical Source Area Monitoring Program

Constituent	Wholesale Trade	Automotive Repair/Parking	Fabricated Metal Products	Motor Freight	Chemical Manufacturing
pH	X	X	X	X	X
Specific conductance	X	X	X	X	X
Oil and grease	X	X	X	X	
Semivolatile organics	X	X	X	X	X
Total petroleum hydrocarbons (TPH)		X		X	
Chemical oxygen demand (COD)	X	X	X	X	X
Total suspended solids (TSS)	X	X	X	X	X
Total dissolved solids (TDS)	X	X	X	X	X
Total organic carbon (TOC)	X	X	X	X	X
MBAS (detergents)		X			
Heavy metals (Al, Cd, Cr, Cu, Fe, Pb, Ni, and Zn)	X	X	X	X	
Other (based on chemicals handled at facility)			X		X

From WCC and Psomas Assoc. 1996.

samples will be composited before analysis into test and control samples for each source area category. The samples will be analyzed for the constituents shown on Table 4.31.

The stormwater controls to be investigated will be selected from the following ranked listing:

Infiltration
Media filtration (sand filters and similar devices)
Oil/water separators
Water quality inlets (oil spill containment)
Biofiltration (vegetated swales or filter strips)
Wet or extended detention dry ponds
Constructed wetlands
Runoff quantity control ponds and vaults
Multiple systems

In addition, industrial and commercial source controls will also be considered, including preventive maintenance, spill containment, material handling, litter control, etc.

Step 4. Project Implementation (Routine Initial Semiquantitative Survey)

An important initial step in any monitoring program is to collect and review any existing data and information. LACDPW has been actively monitoring surface water quality since the late 1960s (Rashedi and Liu 1996). Since the mid-1980s, 28 sampling sites have been routinely monitored during both dry weather (monthly observations) and wet weather (three to four storms per year). Table 4.32 lists the constituents that have been included in these monitoring activities.

The available data were reported by LACDPW as part of its evaluation of existing stormwater quality monitoring data (task 5.2, *Report of Waste Discharge*, volume 8). This report included some of the stormwater data (TDS, chloride, pH, sulfate, nitrite, lead, fecal coliforms, enterococcus, and total coliforms) for several storms a year. The bacteria were generally high, as is typical for stormwater. Fecal coliforms averaged from 10,000 to 100,000 organisms per 100 mL, and the enterococci were only slightly lower. Similar monitoring was also conducted at these locations during dry weather. The dry weather fecal coliform observations were much lower, being about 1000 to 10,000 organisms per 100 mL, while the TDS and chlorides were higher. The "Basin Plan Objective" for fecal coliforms is only 200 organisms per 100 mL, with most observations greatly exceeding this value.

Table 4.32 Constituents Monitored at 28 Surface Water Sampling Locations since the Late 1980s

Constituent	Dry Weather	Wet Weather
Minerals	X	X
Pesticides	X	X
Total petroleum hydrocarbons	X	
Heavy metals	X	Total and filtered
Bacteria (total and fecal coliforms, streptococci, and enterococci)	X	X
Suspended solids (total and volatile)		X
Oil and grease		X
Biochemical oxygen demand		X
Total organic carbon		X
Volatile organic compounds	Semiannually	X

Rashedi and Liu (1996) reported that the top ten compounds with the highest numbers of exceedances of the water quality objectives were: fecal coliforms, enterococcus, TDS, ammonia, chloride, nitrite, pH, sulfate, total coliforms, and lead. The available data indicated very high variabilities in concentrations, with no obvious and consistent trends observed. However, most of the lower basin monitoring data showed higher concentrations of chloride, sulfate, lead, and TDS than the corresponding upper basin areas. Lead concentrations sharply decreased after 1990, and the most recent data were mostly below the water quality objective limits. The dry-weather flow lead concentrations were generally higher than the storm-generated flows in the Los Angeles River (Lowe and Rashedi 1996).

Rashedi and Liu (1996) also evaluated the available data for different land uses. They found higher concentrations of total and fecal coliforms, lead, TDS, chloride, and sulfate in drainages having large industrial areas. Higher chloride, sulfate, TDS, nitrate, ammonia, total coliforms, and lead concentrations were found in watersheds that were heavily urbanized.

Because of the observed high variability (typical for stormwater quality), a large number of samples (probably at least 50) will be needed to obtain event mean concentration values having errors of 25%, or less. If only five storms can be monitored per year at each of the monitoring locations, it may require at least a decade before enough data are collected for the necessary statistical analyses to satisfy the project objectives.

Several special studies were also conducted to investigate potential local monitoring problems. One included an investigation of reducing the smallest storm size that could be monitored, and another investigated problems associated with monitoring in very wide and shallow channels. As noted previously, the samplers were programmed to sample storms as small as 0.25 inches, reduced from the initial design of 0.4 inches. This reduction in the small storm size that could be sampled should increase the capture of the annual runoff significantly. About 15 to 20% of the annual runoff is associated with rains less than 0.4 inches, while less than 10% of the annual runoff is expected from storms less than 0.25 inches in depth (using a conventional interevent dry period of 6 hours and for the LAX rain history from 1969 to 1993). The larger range of storms to be monitored will enable the collection of most storms that occur and will allow analyses of concentration variations associated with rain depth. The design of many less expensive stormwater controls is based on the assumption that higher concentrations of pollutants occur with small rains, or with the first portion of rains. Therefore, this monitoring effort will enable this important characterization aspect to be investigated. The number of events associated with these small storms is also very large and is therefore important in relation to water quality objectives (especially bacteria). Characterizing these smaller events will therefore enable better evaluations of exceedance frequency and durations of water quality objectives.

A study was conducted at the monitoring station at Ballona Creek to investigate whether the single midstream sampling location was reasonably representative of the 100×25 ft channel (WCC and CDM 1996). Four surface samples (collected from locations evenly spaced along the width

of the channel) were compared to the single midchannel automatic sampling location at the channel bottom during three storms. Samples were obtained at 3-hour intervals during the storm durations and were analyzed for temperature, pH, specific conductivity, turbidity, TDS, TSS, copper (total and dissolved), zinc (total and dissolved), and nitrate. The three storms monitored were 1.8, 3.1, and 2.2 inches in depth, all quite large, but sufficient to create enough depth in the channel to enable sampling over a wide area. The flows were confined in a channel about 50 to 100 ft wide and from 2 to 8 ft deep, and the water velocities ranged from 0.2 to 0.3 ft/s during this study. The differences in constituent concentrations for the different sampling locations for any storm were found to be much less than the differences in concentrations between storms. As an example, the middle bottom sample was from 5 to 25% different from the overall average, with no clear bias, for suspended solids. Calculations were also made by LACDPW (1998) to determine the flow distances required for complete mixing in the channel during these events (to achieve less than a 10% variation in water quality). It may require from 600 to 2500 ft of channel length from a discharge to achieve this level of mixing for these storms. At the Ballona Creek monitoring station, three upstream outfalls are within 2500 ft. However, these outfalls only represent about 2% of the complete drainage area. The required flow distances for complete mixing at the other wide channel sites (200 to 400 ft in width) would likely be substantially longer, depending on the expected flow rates and water depths. However, problems associated with automating a multilocation sampling system are difficult, requiring multiple sampling pumps spread across the channel, instead of the single unit used here.

An important aspect of any monitoring program is the health and safety of the project personnel. The LACDPW requires all employees to identify the likely hazards that may be encountered on their jobs. For this project, these hazards included hazardous weather conditions, working in confined spaces, hazards associated with chemicals, snakes, poison ivy, traffic, falling, drowning, etc. The county requires field sampling personnel to undergo a minimum of 40 hours of Hazardous Materials Awareness training and other training to enable the personnel to evaluate potentially hazardous situations and safety concerns.

Step 5. Data Evaluation

This case study describes the development of a workplan for a large and comprehensive stormwater management program. Only preliminary data are currently available, as described above, which were used to modify and refine the initial workplan.

Step 6. Confirmatory Assessment (Optional Tier 2 Testing)

There are several additional stormwater monitoring programs being conducted in southern California that can be very useful for Los Angeles County. One of the most interesting is a unique epidemiological study conducted at Santa Monica Bay beaches to examine human health risks associated with swimming in water contaminated by stormwater. It is summarized in the following paragraphs and tables. This study was the first large-scale epidemiological study in the United States to investigate possible adverse health effects associated with swimming in ocean waters affected by discharges from separate storm drains (*Water Environment & Technology* 1996a,b; *Environmental Science & Technology* 1996; Haile et al. 1996).

During a 4-month period in the summer of 1995, about 15,000 ocean swimmers were interviewed on the beach and by telephone 1 to 2 weeks later. They were queried concerning illnesses since their beach outing. The incidence of illness (such as fever, chills, ear discharge, vomiting, coughing with phlegm, and credible gastrointestinal illness) was significantly greater (from 44 to 127% increased incidence) for oceangoers who swam directly off the outfalls, compared to those who swam 400 yards away, as shown on Table 4.33. As an example, the rate ratio (RR) for fever was 1.6, while it was 2.3 for ear discharges, and 2.2 for highly credible gastrointestinal illness

Table 4.33 Comparative Health Outcomes for Swimming in Front of Storm Drain Outfalls, Compared to Swimming at Least 400 Yards Away

Health Outcome	Relative Risk, %	Rate Ratio	Estimated Association	Estimated No. of Excess Cases per 10,000 Swimmers (rate difference)
Fever	57	1.57	Moderate	259
Chills	58	1.58	Moderate	138
Ear discharge	127	2.27	Moderate	88
Vomiting	61	1.61	Moderate	115
Coughing with phlegm	59	1.59	Moderate	175
Any of the above symptoms	44	1.44	Weak	373
HCGI-2	111	2.11	Moderate	95
SRD (significant respiratory disease)	66	1.66	Moderate	303
HCGI-2 or SRD	53	1.53	Moderate	314

From SMBRP (Santa Monica Bay Restoration Project). *A Health Effects Study of Swimmers in Santa Monica Bay.* Santa Monica Bay Restoration Project. Monterey Park, CA. October 1996.

comprised of vomiting and fever (HCGI). Disease incidence dropped significantly with distance from the storm drain. At 400 yards, and beyond, upcoast or downcoast, elevated disease risks were not found. The results did not change when adjusted for age, beach, gender, race, socioeconomic status, or worry about health risks associated with swimming at the beach.

These interviews were supplemented with indicator and pathogenic bacteria and virus analyses in the waters. The greatest health problems were associated with times of highest concentrations (*E. coli* > 320 cfu/100 mL, enterococcus > 106 cfu/100 mL, total coliforms >10,000 cfu/100 mL, and fecal coliforms > 400 cfu/100 mL). Bacteria populations greater than these are common in urban runoff and in urban receiving waters. Symptoms were found to be associated with swimming in areas where bacterial indicator levels were greater than these critical counts. Table 4.34 shows the health outcomes associated with swimming in areas having bacterial counts greater than these critical values. The association for enterococcus with bloody diarrhea was strong, and the association of total coliforms with skin rash was moderate, but nearly strong.

The ratio of total coliform to fecal coliform was found to be one of the better indicators for predicting health risks when swimming close to a storm drain. When the total coliforms were greater than 1000 cfu/100 mL, the strongest effects were generally observed when the total to fecal coliform ratio was 2. The risks decreased as the ratio increased. In addition, illnesses were more common on days when enteric viruses were found in the water.

The percentage of survey days exceeding the critical bacterial counts was high, especially when closest to the storm drains, as shown on Table 4.35. High densities of *E. coli*, fecal coliforms, and enterococcus were observed on more than 25% of the days; however, there was a significant amount

Table 4.34 Health Outcomes Associated with Swimming in Areas Having High Bacterial Counts

Indicator (and critical cutoff count)	Health Outcome	Increased Risk, %	Risk Ratio	Estimated Association	Excess Cases per 10,000 Swimmers
E. coli (>320 cfu/100 mL)	Ear ache and	46	1.46	Weak	149
	nasal congestion	24	1.24	Weak	211
Enterococcus (>106 cfu/100 mL)	Diarrhea w/blood	323	4.23	Strong	27
	and HCGI-1	44	1.44	Weak	130
Total coliform bacteria (>10,000 cfu/100 mL)	Skin rash	200	3.00	Moderate	165
Fecal coliform bacteria (>400 cfu/100 mL)	Skin rash	88	1.88	Moderate	74

From SMBRP (Santa Monica Bay Restoration Project). *A Health Effects Study of Swimmers in Santa Monica Bay.* Santa Monica Bay Restoration Project. Monterey Park, CA. October 1996.

Table 4.35 Percentages of Days When Samples Exceeded Critical Levels

Bacterial Indicator	0 yards	1 to 100 yards Upcoast	1 to 100 yards Downcoast	400+ yards Upcoast
E. coli (>320 cfu/100 mL)	25.0	3.5	6.7	0.6
Total coliforms (>10,000 cfu/100 mL)	8.6	0.4	0.9	0.0
Fecal coliforms (>400 cfu/100 mL)	29.7	3.0	8.6	0.9
Enterococcus (>106 cfu/100 mL)	28.7	6.0	9.6	1.3
Total/Fecal coliform ratio ≤5 (and total coliforms >1000 cfu/100 mL)	12.0	0.5	3.9	0.4

From SMBRP (Santa Monica Bay Restoration Project). *A Health Effects Study of Swimmers in Santa Monica Bay.* Santa Monica Bay Restoration Project. Monterey Park, CA. October 1996.

of variability in observed counts in the water samples obtained directly in front of the drains. The variability and the frequency of high counts dropped considerably with distance from the storm drains. Upcoast bacteria densities were less than downcoast densities probably because of prevailing near-shore currents.

The SMBRP (1996) concluded that less than 2 miles of Santa Monica Bay's 50-mile coastline had problematic health concerns due to the storm drains flowing into the bay. They also concluded that the bacterial indicators currently being monitored do help predict risk. In addition, the total to fecal coliform ratio was found to be a useful additional indicator of illness. As an outcome of this study, the Los Angeles County Department of Health Services will post new warning signs advising against swimming near the outfalls ("Warning! Storm drain water may cause illness. No swimming"). These signs will be posted on both sides of all flowing storm drains in Los Angeles County. In addition, county lifeguards will attempt to warn and advise swimmers to stay away from areas directly in front of storm drain outlets, especially in ponded areas. The county is also accelerating its studies on sources of pathogens in stormwater.

Step 7. Project Conclusions

It was necessary to modify the original workplan for conducting this large and comprehensive stormwater management study in support of the local stormwater discharge permit. Los Angeles County is probably the largest and most complex urban area that has ever attempted to conduct such a comprehensive study needed for the permit and to direct its future stormwater management decisions. In addition to its unique complexity and size, highly variable and sometimes violent rain conditions also occur. These have all contributed to produce a study that is examining many scales of the stormwater problem. Even though there will still exist some deficiencies in this project (such as not examining beneficial use problems in the smaller urban drainages that have informal human contact recreation), the results of this work will be very important for many years to come.

Birmingham Separate Sewer Overflow Program Monitoring

The Department of Civil and Environmental Engineering at the University of Alabama at Birmingham (Lalor and Pitt 1998) participated in a multiyear research project funded by the U.S. Environmental Protection Agency to develop a protocol to enable municipalities to assess local problems associated with sanitary sewer overflows (SSOs). SSOs and receiving waters are highly variable, resulting in highly variable conclusions pertaining to local problems. If SSOs occur frequently and affect small streams having substantial human contact, the problem is likely serious. However, if the receiving water is relatively large, the SSOs infrequent, and human contact rare, the problems associated with these discharges may be insignificant. This project therefore developed and demonstrated a preliminary protocol to enable municipalities to understand their specific local SSO-related problems and to plan better for their control.

Step 1. What's the Question?

Identify and quantify the human and environmental risks associated with SSOs in urban streams. Need to quantify the sources, fates, and exposure mechanisms of pathogens and toxicants in SSOs. Human exposure ranges from informal human contact associated with children playing in urban receiving waters to consumption of water and fish contaminated by upstream SSOs.

Step 2. Decide on Problem Formulation

As in most environmental research projects, this project was designed as a series of overlapping individual experiments, some of short duration and some long, some examining specific individual processes and some examining many processes interacting together. The conventional stream monitoring activities associated with this project involve longitudinal "above" and "below" monitoring following the stream path as it flows past several known SSO locations. The project test sites have different characteristics to test the sensitivity of the monitoring program in identifying the known SSO discharges and to determine if the SSO discharges were causing measurable beneficial use impairments. Initial monitoring during the first project phase only included specific tracer analyses that were thought to be the most sensitive in detecting SSO discharges. Later project phases could include more comprehensive chemical and biological monitoring at the locations along the streams that were found to have a variety of SSO effects. From this sequence of tests, the ability of these different parameters to detect SSO discharges and their effects for different stream conditions will be determined. The initial test locations include:

- A local hillside where a low-volume, but constant SSO is occurring, flowing into a moderate-sized stream
- A moderate-sized stream (Five-Mile Creek), having a watershed area of about 100 mi^2 with a large intermittent SSO and a small continuous SSO
- A small, completely urbanized stream (Griffin Brook), having a watershed area of about 10 mi^2 with numerous small SSOs

A sampling strategy examining the individual streams as they flowed past the SSO locations (longitudinal sampling along the flow path) was used for most of the field studies. The variable conditions that these test sites provide enabled us to investigate a range of discharge and receiving water conditions, and different resulting problems. The hillside site was used to investigate changes in the SSO's characteristics as it flowed toward the creek. The moderate- and small-sized receiving waters also used longitudinal sampling, with samples collected above and below the known discharge locations, and for an extended distance downstream. The moderate-sized stream also included small-scale up- and downgradient analyses of sediment conditions. The field studies were also conducted during different seasons and flow patterns, contrasting wet- and dry-weather conditions and warm and cold weather.

Another important aspect of this research was to determine suitable risk assessment approaches and tools to enable municipalities to determine the magnitude of local SSO-related problems. Therefore, various experiments were conducted to enable receiving water models to be calibrated for expected local SSO characteristics. The experiments conducted and planned include:

- *In situ* bacteria and other pathogen die-off tests
- Photosynthesis and respiration (P/R) of sewage-contaminated waters
- Interaction of water column pollutants and contaminated sediments and interstitial waters
- Interstitial water measurements
- Measurement of frequency, duration, and magnitude of WWF events
- Sediment oxygen demand (SOD) and sediment P/R tests
- Settleability of SSO-related bacteria and toxicants

Step 3. Project Design

Qualitative Watershed Characterization and Stream Characterization — There are several sites where samples were taken. The sites were located in and along two urban streams in the Birmingham, AL, area. These sites were chosen to allow for overland, upstream, in-stream, and downstream samples near known SSO locations.

Five-Mile Creek — The Five-Mile Creek area has ten sampling sites along an approximately 3-mile reach from Five-Mile Creek Road to Highway 79. Five-Mile Creek is located in the northern part of Birmingham and is surrounded by industrial and suburban development. This series of sampling locations includes sites from 500 ft upstream to 1000 ft downstream from known SSO discharge points.

Overland Flow Sampling Site — The small-volume, overland flow/continuous discharge SSO site is located on Five-Mile Creek, and in-stream sampling points are above and below its location. In order to evaluate the effects of overland flow on SSO characteristics (especially pathogen die-off and particulate toxicant settling), several hillside locations were sampled as the discharge flowed overland toward the stream.

Griffin Brook — Griffin Brook is within a small, fully developed watershed, and is a first-order stream. Griffin Brook is located within Homewood, a suburb located in the southern Birmingham area, and discharges into Shades Creek. The Griffin Brook test reach is approximately 2.5 miles in length, bracketing several known small SSO discharges.

Select Monitoring Parameters — The stream sampling locations were tested during the first project phase using a brief set of chemical and microbiological parameters. These parameters were thought to be the most sensitive to enable the identification of SSO discharges. These parameters (mostly based on earlier work on identifying inappropriate discharges into storm drainage systems; Pitt et al. 1993; Lalor 1994) were:

- Indicators of sewage (detergents, ammonia, potassium, fluoride, color, and odor)
- Other conventional parameters (pH, turbidity, and conductivity)
- Rapid microbiological analyses for *E. coli*., enterococci, and total coliforms (using IDEXX Quan-titrays)

The later phase of the project could involve more comprehensive analyses at the sites found to have detectable SSO discharges. These analyses will be used to quantify the receiving water effects of SSOs on beneficial uses (contact and noncontact recreation, water supply, consumptive fishing, and aquatic life uses). These analyses may include the following parameters:

Primary list (for routine analysis of most samples):

- Pathogens, including protozoa (*Giardia* and *Cryptosporidium*), *Pseudomonas aeruginosa*, and *Shigella*, along with *E. coli*. Viruses, if possible, will also be investigated.
- Trash and other debris along the streams.
- Toxicants, including partitioned metals (lead, copper, cadmium, and zinc, using graphite furnace atomic adsorption spectrophotometer, or other methods having comparable detection limits), partitioned organics (PAHs, phenols, and phthalate esters using GC/MSD with SIM, or HPLC), herbicides, and insecticides (using GC/ECD or immunoassays); suggest routinely using toxicant screening method, such as Azur's Microtox™, for possible guidance in modifying specific list of toxicants.
- Nutrients, including phosphates, total phosphorus, ammonia, total Kjeldahl nitrogen, nitrate plus nitrite, and partitioned TOC (or at least COD).

- Additional conventional parameters affecting fates and effects of pollutants in receiving waters, including hardness, alkalinity, pH, specific conductivity, particle size analyses, turbidity, suspended solids (SS), volatile suspended solids (VSS), and dissolved solids (TDS).

Secondary list (in addition to the above-listed analyses at selected critical locations at least seasonally):

- Selected additional metallic toxicants (such as arsenic and mercury and possible screening using mass spec/mass spec) and selected additional organic toxicants (such as VOCs)
- Long-term NBOD and CBOD (for k rates and ultimate BOD)
- Particulate organic carbon (POC)
- Major cations and anions
- Continuous pH, ORP, specific conductivity, temperature, and turbidity should also be conducted using an *in situ* water quality sonde.

Sediment analyses (seasonal analyses):

- Particle size distributions of sediment
- Acid volatile sulfides (AVS) in sediments
- Toxicants and nutrients by particle size
- BOD and COD (and possibly POC) by particle size
- Interstitial water analyses for key parameters, especially pathogens, nutrients, pH, and ORP, plus others, volume permitting

Numerous seasonal biological attributes should also be included at each sampling reach, including:

- Benthic macroinvertebrates (natural and artificial substrates)
- Algae (natural and artificial substrates) and macrophytes
- *In situ* toxicity test assays

Partitioned analyses of the toxicants in runoff and in the receiving water is very important, as the form of the pollutants will have great effects on their fate and treatability. Conventional assumptions that only filterable toxicants have a toxic effect on receiving water organisms is not always correct.

The sampling requirements will vary for each primary parameter, based on the concentration variations observed. In most cases, 1 year of data (including about 15 to 35 events) will likely be sufficient. For most parameters (assuming a COV of 0.75 to 1.0), this number of samples will result in an event-mean concentration (EMC) value estimate with about 25% levels of error, and will enable effective comparisons to be made between paired upstream and downstream locations. The secondary parameters will only be analyzed about four times (seasonally) and at fewer locations. The likely errors in their EMCs will therefore be quite large. However, the purpose of these measurements is for screening: to identify the presence of additional significant parameters. The seasonal sediment and biological analyses should be sufficient because their variability is much less than for the water parameters.

An important aspect of this research project is to develop an approach useful for municipalities to determine the local risks and the role that SSOs play in TMDL calculations. As such, this project will develop several alternative field program recommendations that should result in different levels of confidence. The above list of parameters will therefore be narrowed considerably for these alternative approaches.

Step 4. Project Implementation (Routine Initial Semiquantitative Survey) and Step 5, Data Evaluation

A series of initial tests was conducted during the first project period to investigate methods to measure the fates of the critical pathogens and toxicants associated with SSO events. This initial effort includes the following experiments:

Initial Steam Surveys in Five-Mile Creek and in Griffin Brook — A number of SSO discharge points were observed along Five-Mile Creek. Figure 4.44 shows a large, intermittent, SSO discharge

Figure 4.44 Five-Mile Creek SSO discharge during large flow.

Figure 4.45 Five-Mile Creek under normal flow conditions.

Figure 4.46 Typical SSO discharge point along banks of Five-Mile Creek.

Figure 4.47 Unusual continuous SSO discharge from surcharged/broken sewerage along Five-Mile Creek.

during a large rain event, Figure 4.45 shows Five-Mile Creek under normal flow conditions, while Color Figure 4.1* shows this discharge mixing with the creek during this large overflow. Figure 4.46 shows another intermittent SSO discharge location at a poorly sealed sanitary sewer manhole in the creek right-of-way. Moderate rains causing surcharging conditions in the sewerage would obviously cause a large SSO at this location. Figure 4.47 shows an unusual continuous (but relatively low volume) SSO discharge that was caused by a leaking sewer on a hillside discharging to Five-Mile Creek.

The initial stream surveys in Five-Mile Creek found no significant SSO discharge effects in the stream during wet or dry weather in the proximity of the small continuous hillside discharge shown in Figure 4.47, except within a few feet of the discharge location. No samples were obtained during high creek flows when the large intermittent SSO was discharging. However, visual observations were obtained during one large discharge event, indicating very large amounts of SSO being discharged into Five-Mile Creek (Figures 4.44 and Color Figure 4.1). During this event, the SSO discharge was likely about 10% of the creek flow and was visually obvious for several hundred feet downstream of the discharge location. This SSO discharge is scheduled to be corrected by Jefferson County in the near future.

The stream surveys in Griffin Brook indicated significant effects from continuous SSO discharges during dry weather, but no noticeable SSO effects during wet weather. The numerous SSOs were all individually quite small, but were responsible for a significant portion of the dry-weather

* Color figures follow page 370.

Figure 4.48 Griffin Brook during wet weather conditions. (Courtesy of Robin Chapman.)

flow in the stream during the summer. During rains, the much higher flows and the moderate to high concentrations of most pollutants in the urban runoff masked the continuous SSO discharges, effectively diluting the SSOs below detection (Figure 4.48).

In Situ Bacteria and Other Pathogen Die-off Tests — Dialysis bags were initially used to measure *in situ* die-off of pathogens (Figures 4.49 and 4.50). *In situ* die-off tests are more accurate indicators of pathogen die-off compared to laboratory tests, as actual environmental conditions are allowed to affect the test organisms. The dialysis bags allow water, nutrients, and gases to enter the bags, but restrain the test organisms. Samples of raw sewage collected from known SSO discharge locations were diluted with stream water and placed in sealed bags. The bags were fitted into large-diameter plastic pipes (with coarse screening on the ends) for protection and anchored in the streams. Bags were then periodically removed and the pathogen populations determined and compared to the initial conditions. In later, extended tests lasting several weeks, we found that the dialysis bag material decomposed, allowing substantial leakage. We have since replaced these initial chamber designs with ones using plastic tubing with membrane filter ports. These new designs and test results are described in Chapter 6.

Photosynthesis and Respiration of Sewage-Contaminated Waters — The aim of this experiment was to examine the acclimation period of the effects of a sewage discharge to a receiving water's dissolved oxygen, and to measure the photosynthesis and respiration (P/R) rates for several mixtures of sewage and receiving waters. The P/R discussion in Chapter 6 describes the test results and summarizes the specific procedures used. The acclimation period of an intermittent discharge into a receiving water may be relatively long, requiring extended observations to obtain an understanding of the likely dissolved oxygen effects. The use of continuously recording water quality sondes enables the collection of water quality data over an extended period (14 days during this

Figure 4.49 Placement of *in situ* pathogen die-off test chambers in Five-Mile Creek. (Courtesy of John Easton.)

Figure 4.50 In place pathogen die-off test chambers. (Courtesy of John Easton.)

field study). Traditional measurements of P/R rates are performed using light and dark bottles over a short period of time, usually several hours, and with little replication. These short period data are then used to construct a dissolved oxygen curve for a 1-day cycle, for the light and dark bottles, from which P/R calculations are made. With the continuously recording sondes, several curves can be constructed over multiple days having variable weather, providing far more useful results than the traditional method. In addition, the acclimation period can be accurately determined and considered in DO calculations.

The net effect of the P/R processes is that the dissolved oxygen level in the water rises during the daylight and falls at night. In addition, the pH of typical receiving waters is governed by the carbonic acid/bicarbonate/carbonate buffering system. Increases in the dissolved CO_2 concentration cause corresponding decreases in pH, and vice versa. Therefore, the pH increases during the daytime hours because CO_2 is being fixed by photosynthetic organisms and is thereby removed from the water. Then, at night, pH drops because atmospheric CO_2 and CO_2 being produced by respiration increase the concentration of CO_2 in the water. The DO and pH sonde probes measured these changes directly. In addition, changes in temperature, ORP, and specific conductance were also observed.

The site for this experiment was a small lake on private property located in Shelby County, AL, to ensure security for the sondes. This lake rarely, if ever, received sanitary sewage, producing a likely worst case for acclimation. YSI 6000 sondes were used to measure the following parameters during these experiments: depth, specific conductance, dissolved oxygen, turbidity, pH, oxidation-reduction potential, and temperature. The sondes were programmed to acquire data in unattended mode for 2 weeks at 15-min intervals. Raw sewage was obtained at the Riverview Sewage Treatment Plant. Lake water was used for diluting the sewage in the following ratios: 0/100%, 33/67%, 67/33%, and 100/0% (sewage/lake water). The test chambers were 5-gallon clear plastic bags containing 15 L of the test water mixtures. The measurement ends of the sondes were placed into the test chamber bags and sealed with tape after as much air as possible was removed. The test chambers and sondes were placed on the lake bottom in approximately 1 to 2 ft of water near the shore and in full sun.

The 0% sewage test chamber indicated a 5-day biochemical oxygen demand, BOD_5, of approximately 2.5 mg/L. The 33% sewage chamber had initial anoxic conditions, but after acclimating for approximately 5 days, there was a diurnal photosynthesis/respiration variation observed: the DO levels in this chamber were supersaturated during the daylight hours. When this chamber was pulled at the experiment's end, there was a large amount of green biomass present, indicating large amounts of photosynthesizing material. The 67% and the 100% sewage test chambers stayed at anoxic DO levels throughout the test period.

Plots of DO were then created using the 0 and 33% sewage results for the last 5-day period in order to calculate the P/R rates, corrected for the experimental photoperiod. The net photosynthesis rates for the 33% sewage were very high, ranging from 12 to 30 mg/L/day for the 5 days of useful data, indicating variations associated with different cloud cover. The net photosynthesis rates for the 0% sewage/100% lake water mixture were typical for local lake waters, being approximately 1 to 2 mg/L/day.

The use of the YSI 6000 sonde, with the rapid-pulse DO sensor, allowed these simple experiments to be conducted. Conventional P/R measurements using light and dark bottles would not be sensitive to the relatively long acclimation period noted for raw sewage discharges into waters that rarely receive SSOs. In areas having more consistent SSOs, the acclimation period would not be as long. In addition, the long-duration experiment enabled us to observe variations in the P/R rates corresponding to different weather conditions and other factors. The use of only a single random P/R value (which would be obtained using conventional *in situ* light/dark bottle tests) could result in large errors.

Interaction of Water Column Pollutants and Contaminated Sediments and Interstitial Waters — There are five processes that affect the pollutant exchange between the water column and the sediment interstitial water and that affect the fates of SSO discharged pollutants: (1)

hydrodynamics, currents, and wave action; (2) resuspension/erosion of sediments; (3) flocculation, settling speeds, and deposition; (4) sorption of chemicals to sediments; and (5) flux/diffusion of chemicals from the water column to interstitial water, and vice versa. The most important processes, or those that contribute most to short-period chemical exchange, in a stream such as Five-Mile Creek, are those that promote turbulent mixing of the water column and the interstitial water. Therefore, experiments were conducted to measure the relative exchange rates between the water column and interstitial water for coarse and fine stream bed sediments. Results of these tests are presented in Chapter 6.

This study examined the exchange of water and the degradation of interstitial water due to poor water quality flowing over its surface. It was expected that differences in sediment particle size between the monitored sites will impact exchange, i.e., sites having larger, well-graded sediment particles will allow more rapid and complete exchange between the interstitial water and the stream water than will smaller sediment particle sizes.

The test locations for this experiment on Five-Mile Creek were near a site of a continuous SSO. At this site, raw sewage, at a rate of several liters per minute, flows over about 300 ft of ground before discharging into the creek. The flow in the creek ranged from approximately 2 to 10 m^3/s during the experiment. Four sondes were deployed: two were located upstream and two were located downstream of the SSO discharge point. At each upstream and downstream site, one sonde was located on the creek bottom and the second sonde was buried under approximately 6 in of sediment. The sondes were protected from large particles by placing them inside 75 µm aperture nylon mesh bags.

The YSI 6000 sondes enabled direct measurements of the lag time and magnitude response from the surface to the interstitial water for several parameters. There were no detectable differences between the upstream and downstream water quality data, in relation to the continuous SSO location. The background levels of pollutants in the creek masked the smaller SSO discharge effects. The differences in the flow rates of the SSO discharge and the creek were high, causing great dilution. However, the data from the buried sondes were used to compare interstitial water characteristics at the two sites based upon different sediment characteristics.

At the fine sediment site, the temperature plots indicated a definite lag time between changes in the water column and the sediment interstitial water of approximately 6 hours from peak to peak at the fine sediment site and approximately 2 hours at the coarse sediment site. The data at the coarse sediment site showed a much closer correlation between the water and the interstitial water than for the fine sediment site. The interstitial water at the coarse sediment site changed with the water column, albeit at a reduced magnitude, while the interstitial water at the fine sediment site showed no change.

Specific conductance was selected as the best parameter for monitoring chemical exchange between the water column and sediment interstitial water. The rate of relative chemical exchange was much higher and more variable in the coarse sediment than in the fine sediment. In the coarse sediment, the much more rapid process of turbulent mixing was occurring, as opposed to the slower process of diffusion, which is the driving force in the fine sediment.

The use of the continuously recording sondes, especially with the rapid-pulse DO sensors, enabled real-time interstitial water quality changes to be made. These measurements are especially important for sensitive parameters that are not possible to accurately measure in collected samples (especially ORP). The continuous measurements showed that interstitial water within fine sediments was basically isolated from the overlying water column, and the quality of the interstitial water was therefore affected by sediment quality. The coarse sediments, however, allowed a relatively free exchange of water between the overlying water and the interstitial water, with much less of an influence of sediment quality on interstitial water quality.

Interstitial Water Measurements — Peepers (described in Chapter 5) were used to contrast interstitial water conditions in sediments having different textures and levels of contamination. The

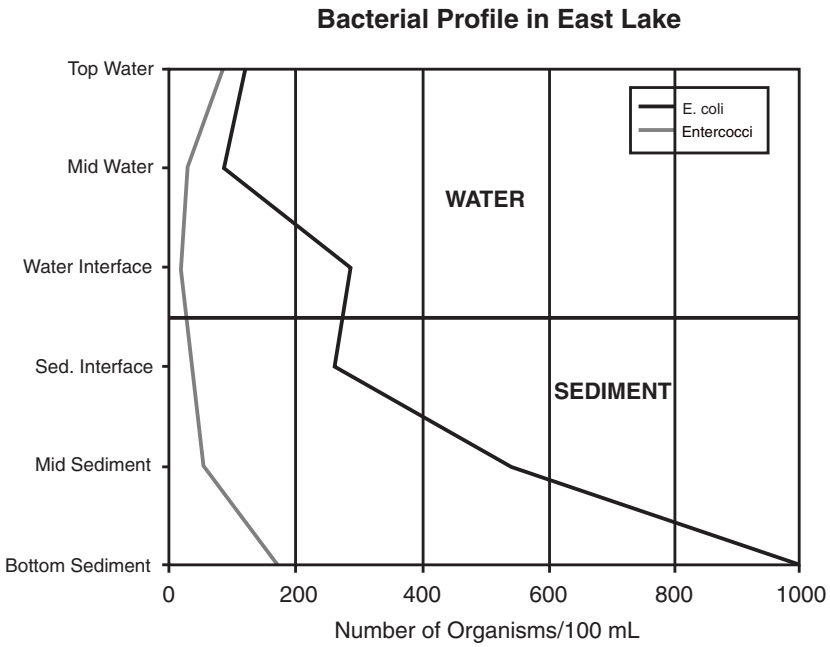

Figure 4.51 Interstitial water bacteria populations contrasted to overlying water conditions.

fine vertical spatial resolution enables measurements close to the sediment–water interface and at deeper depths. Initial experiments were conducted to examine bacteria population variations with depth. Figure 4.51 presents bacteria observations using the peepers. Very high bacteria populations were observed in the sediments, much greater than the overlying water column observations. These data indicate that the deposition of particulates, with associated bacteria, is likely an important fate mechanism for wet-weather flow bacteria. These bacteria may also be readily scoured during periods of high flows, as shown during monitoring on the Rideau River in Ottawa (Pitt 1983b).

Ten peepers were constructed for monitoring vertical variations in interstitial water quality. The peepers are machined from Delrin™ and have 46 (8 mL) cells, 1 cm apart. For use, the cells are covered with a 74-μm nylon screen, which will hold water, but allow diffusion of most pollutants, bacteria, and silts. The peepers are washed with concentrated nitric acid, rinsed with deionized water, and all cells are filled with Reverse Osmosis quality water (18 Mohms resistivity). The cells are then sealed with the nylon screen membrane, and the slotted covers are bolted on. Special stainless steel covers slide over the peepers, protecting the membranes during transport and placement. The prepared peepers are then brought to the field (keeping them horizontal to minimize water loss) and carefully pushed into the soft deposits of the stream bed, leaving at least a few of the uppermost cells above the sediment surface. After installation, the stainless steel covers are then carefully removed by sliding them off, leaving the membranes relatively unimpacted by sediments.

The array of cells allows investigations of the effects of depth on interstitial water chemistry and microbiology. The peeper is placed in the sediment and allowed to equilibrate for a period of time, usually at least 2 hours with the relatively coarse screen. After this period, the stainless steel covers are pushed over the peepers, and the units are removed from the sediment when they are carefully rinsed with clean water to remove any superficial sediment from the cell coverings. In order to extract the water samples from the cells, a small hole is made in the mesh covering with a sharp object, allowing a 10-mL plastic syringe to withdraw the sample water. The water is then transferred to a small storage vial and sealed and brought to the laboratory for analysis. pH and conductivity are measured on site using a micro probe.

Measurement of Frequency, Duration, and Magnitude of WWF Events — This experiment was conducted to examine the utility of the continuous recording YSI 6000 sondes as a tool for monitoring the duration, frequency, and magnitude of wet-weather flow events. Short-term, or runoff-induced, pollution effects can be studied in detail using these instruments. The long deployment time and continuous monitoring capability of the YSI 6000 enables acquisition of data for multiple events, i.e., as many as occur during the time of deployment. The sonde can be programmed to record stream depth, turbidity, and specific conductivity, all found to be all good indicators of wet-weather flows. Chapter 6 contains illustrations of the data obtained during these experiments.

Depth and turbidity values both increased, and the specific conductivity values decreased simultaneously at the beginning of a WWF event. The rise period for all of the parameters was very rapid, and the peaks occurred very early in the runoff event. They then returned to the previous levels within 1 to 2 days, depending upon the parameter. The data set acquired for water depth is obviously the parameter that best correlates to the runoff hydrographs.

The flow in Five-Mile Creek rapidly changes with rain conditions, especially considering that the watershed is relatively large (many square miles). However, the water quality remained degraded long after the water levels decreased to baseflow conditions. The turbidity remained elevated for about 30 hours, and the specific conductivity remained depressed for about 40 hours, although the hydrograph response was completed in about 12 hours. Because of the common rains in Alabama (rains occurring about every 3 to 5 days, and moderate rains similar to that which was monitored occurring about every 10 to 15 days), the degraded water quality associated with the WWF could affect the creek about 10 to 20% of the time. In addition, several days of exposure to degraded conditions may be common, instead of the several hours of exposure to degraded conditions typically assumed for WWF effects.

Continuously recording sondes, especially those capable of long-term monitoring of depth, turbidity, and specific conductivity, are therefore very useful in indicating the frequency, magnitude, and duration of WWF degradation on in-stream water quality. If located upstream and downstream from a major SSO discharge point, these devices can also continuously measure the magnitude of the SSO flows in relation to the receiving water flow. The SSO location where the sondes were located for this demonstration did not cause any measurable difference in the sonde parameters (DO, temperature, specific conductivity, pH, ORP, turbidity, or water depth) because of its relatively small flow in relation to the large creek flow.

Additional Tests for Sediment Oxygen Demand (SOD), Sediment P/R, and Settleability of Bacteria and Toxicants — A series of tests is also planned to more fully examine the role that sediments play with SSO pathogens, oxygen-demanding material, and toxicants. These tests will be necessary to calibrate receiving water models and estimate the fates and risks associated with SSO discharges. Four clear plastic bottomless boxes are being constructed as SOD chambers. A flange opening on one side of the boxes will hold the YSI 6000 continuously by recording sondes. During short-term use, two of the test chambers will be covered with opaque material (such as aluminum foil) to act as a dark chamber for respiration analyses, while two will remain clear for respiration plus photosynthesis measurements. During extended tests, the chambers will remain clear, measuring respiration during the night and photosynthesis plus respiration during the day. The chambers will also have temporary bottoms available for background water P/R analyses. This will enable the SOD to be directly measured over a period of several days, as in the previously described *in situ* water column P/R tests. Deployment of the test chambers over a several-day period above and in the vicinity of an SSO discharge will result in sufficient data to indicate SSO-impacted SOD under various weather conditions.

It is possible that much of the reported die-off of bacteria in natural waters is actually associated with settling. Very high bacteria populations have been noted near the sediment–water interface and these can be easily resuspended during periods of high flow or other turbulence (Pitt 1983b). These settling experiments will therefore supplement the *in situ* peeper tests and the *in situ* die-off tests to

distinguish settling and die-off of bacteria and biochemical changes of the pollutants. Conventional laboratory settling columns (30 cm in diameter and 1.3 m in height) will be used to measure the settling rate of SSO pollutants, especially bacteria and toxicants. Control tests (using a continuously stirred chamber) will indicate die-off of the bacteria and biochemical modifications of the chemicals.

Step 6. Confirmatory Assessment

Being a multiyear project, numerous project elements should be conducted during later project phases. An important element of this later work will be confirmation of the recommended approach developed during the earlier phases, based on actual receiving water beneficial use impairment measurements. The recommended approach will present several options, having increasingly complex and expensive activities, but with increasing confidence in the conclusions. It is expected that a moderate level of activity will be the most cost-effective approach. However, the costs associated with correcting SSOs in an area are extremely high and additional information and associated higher confidence in the assessment studies will result in a greater degree of success of the control program.

Step 7. Project Conclusions

The preliminary results confirmed several obvious hypotheses: small SSOs discharging into large receiving waters produce little measurable effects, while large intermittent SSOs discharging into smaller water bodies can be serious. However, many small, continuous SSOs in small urbanized waterways can dominate dry-weather conditions, producing hazardous situations, while they are completely obscured during most wet-weather events by the larger flows and pollutants associated with urban runoff.

The small experiments demonstrated useful tools needed for calibrating receiving water models used for estimating fates and exposures of SSO pollutants. Without site and SSO specific tests, modeling estimates could be very misleading.

It is expected that the extensive list of chemical and biological parameters being investigated during this project can be effectively reduced to result in cost-effective investigations of local SSO problems, especially considering the very high cost of reducing SSO discharges. The information obtained can also be used in a TMDL evaluation to determine the role of SSOs in relation to other discharges in a watershed.

Outlines of Hypothetical Case Studies

The following hypothetical case studies represent commonly encountered situations where the effects of stormwater runoff may need to be determined. These brief examples are based on similar studies and reflect integrated, weight-of-evidence study designs (as described previously). As always, available resources will determine how comprehensive a design is feasible. The following designs assume relatively limited resources, yet address the essential components that allow for reliable weight-of-evidence-based conclusions and decision making. Additional resources are needed for Tier 2 level "confirmatory" assessments that identify specific stressors, their relative contribution to degradation, and their sources. These test designs can easily fit into the EPA Ecological Risk Assessment paradigm or Stressor Identification Evaluation Process. For additional information on useful multistressor assessment methods see Baird and Burton (2001).

Effect of Outfall on Algal Growth

Case Situation: A permitted industrial effluent contains low levels of nitrogen and phosphorus and is discharged into a small urban stream. The upstream watershed is predominantly older residential neighborhoods. Stormwater runoff is discharged directly into the stream. Upstream of the

outfall the stream is intermittent, with occasional no-flow conditions occurring during dry, summer periods. However, the industry provides continual flow from its outfall, providing aquatic habitat downstream throughout the year. The receiving stream has excessive algal growth downstream of the outfall. The state environmental agency is concerned over the role of the effluent on the algal growth and suggests additional wastewater treatment should be added to reduce nutrient levels.

Step 1. What's the Question?

Does the outfall degrade water quality and cause excessive algal growth in the receiving stream?

Step 2. Problem Formulation

There are basically two separate issues that should be addressed. First, if there were no industrial outfall, what would be the quality of the downstream reach? Given the intermittent nature of the stream, it is likely that both the benthic macroinvertebrate and fish populations would be very limited and only of a brief seasonal nature. However, the environmental agency may argue that since the outfall does occur, it must be free of excess nutrients. The second issue is whether nutrients from the outfall are degrading downstream conditions. These two issues dictate that upstream and downstream sampling be conducted during low and high flow conditions, monitoring the relationship of flow and outfall loadings with both biological communities and nutrient concentrations.

Steps 3 and 4. Project Design and Implementation

A site reconnaissance found >90% of the upstream watershed was an older, middle-income residential neighborhood with no septic systems. There were no continual discharges or combined sewers evident; however, stormwater discharges emptied directly to the stream. This suggests that runoff would include nutrients (from lawn fertilizers and small mammal feces), pathogens (from small mammal feces), pesticides (from lawn/garden care chemicals and agrichemicals in rainfall), and some metals and petroleum products (from automobiles and roadways). The stream habitat was relatively good throughout, with a good riparian zone, some stream canopy, and sand to cobble substrates with little siltation or embeddedness. However, there were no pools of depths greater than 1 ft, indicating a susceptibility to drought conditions. Excessive algal growth occurred near the outfall, but decreased downstream. Various fish species and benthic macroinvertebrates were observed downstream, but not upstream of the outfall.

A weight-of-evidence, multicomponent assessment design was used. This included physicochemical monitoring of key parameters (ammonia, nitrate, nitrite, total and orthophosphorus, turbidity, temperature, pH, conductivity, flow) during a low and high flow event. The outfall was sampled with an ISCO automatic sampler during each event. Composited samples were collected during low flow by grab sampling and during high flow with a flow-activated ISCO automatic sampler. ISCO samples were separated into 15-min intervals. Flow was measured using a Marsh-McBirney flow meter. Effluent flow was monitored continuously by the plant and did not vary during the low and high flow sampling events. During high flow, flow was measured during pre-crest and post-crest for comparisons to ISCO samples and stage graphs. In addition, the benthic macroinvertebrate and fish communities were assessed at two sites upstream and downstream of the outfall during low flow conditions in late summer using the EPA's Rapid Bioassessment Protocol I (EPA 1987, Appendices B and C). This process includes a Qualitative Habitat Evaluation Index assessment at the same sites (Appendix A). Finally, the EPA algal 96-hour growth test using *Selenastrum capricornutum* (Appendix D) was conducted during the low and high flow events on three samples (upstream, outfall, and downstream).

Steps 5 and 6. Data Evaluation and Confirmatory Assessment

The data showed significant water quality differences between high and low flow conditions in the stream. The outfall contributed nondetectable levels of phosphorus, nitrite, and ammonia and approximately 1 mg/L nitrate. The habitat downstream was better than the upstream habitat. The upstream reach was only isolated pools during late summer. The downstream habitat had flowing water and greater canopy cover. During low flow, nitrogen and phosphorus were nondetectable in both upstream and downstream water samples. During high flow conditions nutrient levels were highly elevated and did not differ significantly between upstream and downstream samples. A conversion to mass loading based on flow conditions showed the outfall contribution less than 1% of the nitrogen to the stream on an annual basis, as compared to one high flow event. No fish or benthic macroinvertebrates were recovered from the upstream isolated pools during the summer low flow sampling. Several pollution-tolerant species were recovered downstream. The algal growth test showed increased growth in the outfall sample. The upstream and downstream samples showed similar low levels of growth. No confirmatory assessment was deemed necessary.

Step 7. Conclusions

The weight of evidence clearly established that while the outfall does contribute nitrogen to the stream, it is insignificant in comparison to the nutrient loading during high flow conditions from the upstream residential area. The pollution-tolerant species found downstream of the outfall are typical of an urban waterway and likely reflect the stressor loadings from the upstream watershed. Stormwater controls should be installed to yield the greatest improvement to water quality.

Effect of On-Site Runoff from an Industry

Case Situation: A manufacturer has site runoff discharging into a drain which empties directly into a small stream. The manufacturer has a large amount of on-site vehicular traffic and uses a variety of inorganics (e.g., caustics, metals) and petroleum products in the production process. The upstream watershed is mixed urban and agricultural. As part of the stormwater permitting process, the company must determine whether its runoff is contaminated.

Step 1. What's the Question?

Does the on-site runoff degrade receiving water quality?

Step 2. Problem Formulation

A potential for stormwater contamination exists since there is a large amount of impervious area being drained that is susceptible to spills from industrial processes, chemical accidents, and diesel-gasoline-powered vehicles. The watershed upstream of the stormwater outfall is approximately 50% commercial and industrial sites and 50% agriculture (crops and pasture). The brief survey of the stream showed primarily pollution-tolerant species with occasional sensitive species both upstream and downstream of the outfall.

Steps 3 and 4. Project Design and Implementation

The stormwater from the test site had the potential to be contaminated with a wide range of compounds, which may or may not have water quality standards. Given the changing nature of the stormwater quality and the sporadic discharges, it is unlikely that any chemical data could be

logically interpreted using water quality standards. The uncertain and changing exposures that organisms would undergo in the stream would not allow for reliable predictions of ecological effects using chemical data only. To achieve an adequate database would require extensive inorganic and organic monitoring during many runoff events. Therefore, to improve data interpretation in a cost-effective manner, a tiered approach was chosen, whereby biological effects were first monitored to determine if detrimental impacts were occurring.

Tier 1 of the study involved a stream survey of benthic macroinvertebrates upstream and downstream of the stormwater outfall using the Ohio EPA's Invertebrate Community Index approach (Appendix B). This was conducted for 30 days during the summer, during which two storm events occurred. During those storm events, flow-activated ISCO samplers collected samples from the outfall, upstream and downstream. Short-term chronic toxicity testing was conducted on the water samples using *Ceriodaphnia dubia* (Appendix D). In addition, toxicity testing was conducted on upstream and downstream samples during low flow conditions.

In the event that toxicity or biological impairment was suspected due to the outfall, a Tier 2 study was designed that focused on identification of the stressor. This involved both laboratory and field testing, using EPA's Toxicity Identification Evaluation (TIE) procedure and *in situ* exposures of caged organisms (Appendix D). The Phase 1 TIE was conducted on a fresh composited outfall sample using *C. dubia* acute exposures. The *in situ* exposures were conducted during low and high flow events (4 days each), upstream and downstream of the outfall. Two species were used: *Daphnia magna* (a zooplankton similar to *C. dubia*) and *Hyalella azteca* (a benthic macroinvertebrate recommended by EPA for sediment toxicity testing). These organisms were exposed in different treatments to better identify potential stressors: (1) light vs. dark cages to identify whether photo-induced toxicity from polycyclic aromatic hydrocarbons (PAHs) exists, and (2) small vs. large mesh cages to identify whether suspended solids contribute to mortality. Basic water quality measures monitored during the exposures were DO, pH, temperature, conductivity, turbidity, ammonia, alkalinity, and hardness.

Steps 5 and 6. Data Evaluation and Confirmatory Assessment

Tier 1 testing found the benthic invertebrate populations upstream and downstream of the outfall were of fair quality; however, those downstream scored lower. This suggested that the outfall may be contributing stressors to the stream; however, given the variable nature of benthic invertebrate communities and stormwater, these results were not conclusive. The toxicity testing results were mixed as shown in Table 4.36.

These data suggest that toxicity from the outfall is variable, but does exist. Its effect on the receiving water is uncertain, as the upstream and downstream samples were not significantly different statistically. It is also apparent that storm events are toxic in the stream, but baseflow conditions are not. The results of the ICI showed both upstream and downstream communities were of poor quality.

Table 4.36 *C. dubia* Survival and Reproduction at Manufacturing Site

	Upstream	Outfall	Downstream
Storm event no. 1	60% 15 neonates	70% 13 neonates	62% 10 neonates
Storm event no. 2	75% 20 neonates	20% 0 neonates	65% 10 neonates
Baseflow event	90% 28 neonates	NA	95% 32 neonates

Tier 2 testing was then initiated. The laboratory-based TIE Phase 1 suggested metals as a primary toxicant and nonpolar organics as a possible toxicant. The in-stream *in situ* exposures showed no significant differences between upstream and downstream, at low or high flows. High turbidity existed during high flow events, and hardness values, while lower during high flow events, were still >300 mg/L $CaCO_3$. The suspended solids exposure treatment during high flow showed relatively high survival when solids were removed. During low flows there was greater toxicity in the light treatments suggesting PAH-photoinduced toxicity exists.

Step 7. Conclusions

These results show the outfall is toxic, primarily due to metals. The concentrations of metals found, however, are not at a level that is likely to cause toxicity in the receiving water due to its high hardness. The nonpolar organic toxicity observed in the effluent may be contributing to the photoinduced toxicity observed during baseflows. However, since these effects were also noted upstream, there are likely additional sources of PAHs upstream. The high levels of suspended solids appear to be contributing to the poor benthic community quality also and will require watershed-based controls to mitigate the problem. These studies did not ascertain whether or not chemicals associated with the suspended solids are contributing to mortality, nor did they rule out other stressors in the receiving stream, such as pesticides. The conclusion is that the outfall does contribute some toxicity to the receiving water, but not at a significant level that could be detected in the stream.

Effect of a Dry Detention Pond

Case Situation: A shopping center has many acres of property that drain into a dry detention pond. The detention pond outfall empties into a stream. A local citizens group expresses concern that water quality is poor downstream of the outfall. A study is initiated to determine whether the dry detention pond drainage is contributing to stream degradation.

Step 1. What's the Question?

Does the dry detention pond outfall degrade water quality in the stream?

Step 2. Problem Formulation

Four different situations are likely to be encountered in urban watersheds where dry detention ponds are used that will affect the study design. First, the outfall discharges into the headwaters of a stream so that the upstream–downstream sampling design is not possible. In this case, a nearby ecoregion reference site may be used that has a similar sized drainage area and the habitat is similar. If habitat modification is a possible cause of impairment (stress), the reference site should have a reasonably good habitat that is unmodified. Since this is a headwater area, fish and benthic communities are likely to be limited by stream size, available habitat, and food availability. Therefore, monitoring should focus on toxicity and loadings of pollutants (chemical and physical) to downstream areas.

The second situation often encountered is that the upstream reach is also degraded, so the upstream–downstream sampling design is somewhat problematic. Again, a nearby reference site is useful, but mainly as a control site to ensure method validity. The key approach in this situation is to assess the outfall quality and its loading of pollutants to the stream during high flow conditions.

An upstream–downstream sampling approach may show increased toxicity and contamination downstream or dilution of upstream contamination.

The third situation encountered is that the upstream area is relatively unimpacted, so traditional upstream–downstream sampling designs as described above may be used.

Finally, the fourth situation is the use of "side-stream" detention ponds where the detention pond is located adjacent to the stream or drainage and captures water only during unusually high flow periods (possibly only a couple of times a year). In small drainages, a dry detention pond may have a lined channel passing through the excavated area that carries the stormwater. Only when the stormwater flow exceeds the capacity of a downstream culvert does the water back up into the adjacent area (like an artificial floodplain). Side-stream dry ponds can also be located adjacent to larger receiving waters, and can fill with excessive flows when the stream stage exceeds a side overflow weir. In many cases, these larger side-stream dry ponds are used as recreation areas. It is difficult to monitor the benefits of these ponds during events where the pond is in operation, as their operation is commonly so intermittent that they rarely divert water.

The primary benefit of a dry detention pond is the reduction in peak stormwater runoff flow rates and associated energy. The increased flow and energy resulting from greater runoff across impervious areas and loss of infiltration basins can cause flooding and/or destroy stream habitat, resulting in beneficial use impairments. Unfortunately, many of the detention ponds in use do not reduce flow enough, still resulting in habitat alteration. In addition, monitoring dry detention ponds rarely has shown significant and important pollutant concentration and mass yield reductions. Some dry ponds partially may act as percolation ponds where some of the runoff is infiltrated.

Steps 3 and 4. Project Design and Implementation

Since many detention pond outfalls discharge into small headwaters or tributaries, the first situation described in Step 2 will be addressed. A site reconnaissance showed that the watershed that drains into the dry detention pond is >90% impervious parking lots. This suggests that runoff may contain suspended soils, salt (during periods of snowmelt and possibly for a few additional months, depending on the levels of deicing salt applications), petroleum products and metals (from automobiles), and perhaps low levels of pesticides associated with precipitation events. The stream into which the pond discharges is a first-order tributary and is intermittent in flow; however, it joins a small, high-quality, perennial stream approximately 200 yards from the pond.

As in the previous case study examples, this site should be studied at both low and high flow conditions. There should be a minimum of four stations, two on the tributary (near outfall and near mouth) and two on the perennial stream just upstream and downstream of the tributary confluence. In addition, it would be useful to have a similar ecoregion reference site for comparison. At each site, qualitative habitat evaluation indices (Appendix A) would be evaluated, along with rapid bioassessments of the benthic macroinvertebrate communities (Appendix B) on one occasion during the summer. Toxicity testing (*Pimephales promelas* 7-day survival and growth assay, Appendix D) was conducted on grab water samples collected during first flush conditions and at low flow. In addition, toxicity of depositional sediments (*Hyalella azteca* 10-day assay, Appendix D) was conducted at three sites (near mouth of tributary, and upstream and downstream of confluence at the first depositional sites). General water quality measures were also made during low and high flow collection periods.

If toxicity was observed, confirmatory assessments would consist of *in situ* toxicity exposures on the tributary and two sites on the perennial stream. These exposures would include treatments to evaluate whether toxicity was associated with water or sediments, suspended solid or dissolved fractions, and whether PAH-photoinduced toxicity was a stressor (as described in the preceding Case Study Example). Extensive chemical analyses were not warranted as the only source was a parking lot. If advanced treatment was recommended, then identification of the dominant chemical stressors might be needed.

Steps 5 and 6. Data Evaluation and Confirmatory Assessment

It was apparent that the tributary had received substantial loadings of eroded soils during the construction of the shopping center, as the natural large-grained sediments were embedded with clays and silts. The habitat quality of the two tributary sites was very different due to the change in gradient, which precluded comparisons of station impairment. The perennial stream habitats did not vary appreciably from each other.

The laboratory toxicity tests showed growth impairment in both of the tributary high flow samples, but not in any other water samples (high or low flow). The amphipod *H. azteca* had poor survival in the tributary and downstream perennial stream sediments. The benthic community results showed only a fair community in the intermittent tributary, but a good community in the perennial stream.

Confirmatory Tier 2 studies revealed that most of the toxicity was associated with the suspended solids; however, some toxicity was also observed in the small mesh (50 μm) chambers. No water column treatment difference were observed in the light–dark treatments. However, the sediment light treatments showed increased toxicity during baseflow conditions in the tributary and downstream samples.

Step 7. Conclusions

The dry detention pond outfall was toxic during the first flush of the events. Since the drainage area was mostly a large paved area, with simple drainage, high concentrations are more common near the beginning of storms than later. However, if short periods of high rain intensity occur later in the storm, an additional surge of high concentrations would likely occur due to the increased storm energy. If the drainage area was a typical mixed urban area, the drainage system would be more complex and the different surfaces would cause flows coming from different areas to be much more mixed, significantly reducing any first-flush effect.

Most of the toxicity was associated with suspended solids and likely contributed to the toxic sediments observed downstream. It is uncertain whether this toxicity from the pond is significantly impacting the perennial stream without more extensive studies. Improved reduction of suspended solids, possibly by retrofitting the pond to an extended detention pond or a wet pond, would likely result in improved downstream aquatic communities.

Effect of a Wet Detention Pond

Case Situation: A wet detention pond is located on-line, in a creek that drains a developing watershed of approximately 3 mi^2. The pond was created by constructing a small dam across the creek. The creek begins in farmland and drains into the residential development containing expensive homes before reaching the detention pond. The detention pond water quality has degraded, with eutrophic conditions such as algal blooms and occasional fish kills. The state environmental protection agency suspects additional downstream problems may be due to the pond and conducts an assessment.

Step 1. What's the Question?

Is the wet detention pond impairing water quality downstream?

Step 2. Problem Formulation

Wet detention ponds typically are located on or off a stream. On-line ponds are constructed in the existing waterway and capture all upstream flows. Adjacent ponds are located next to the stream,

before the outfall, and only treat water originating from the smaller drainage, and not the complete receiving watershed. The advantage of these ponds is as for dry detention ponds, in that they can reduce the power associated with high flow events, thereby reducing habitat destruction and loss of aquatic organisms. If large enough, they can also capture appreciable amounts of the stormwater particulates and associated pollutants. Since on-line ponds may treat much larger areas, they need to be correspondingly larger for similar levels of treatment. In addition, the low head dams across the stream result in a loss of flowing stream reach, block fish migration, degrade the habitat needed for more pollution-sensitive species, and allow accumulation of depositional sediments that contain toxicants. This study will not focus on the water quality of the pond, but whether the outflow from the pond degrades downstream beneficial uses.

Steps 3 and 4. Project Design and Implementation

Water quality was evaluated during both low and high flow conditions. There were three stations, two downstream of the pond and one upstream. An ecoregion reference site was also selected with which to compare fish and benthic community results. At each site, qualitative habitat evaluation indices (Appendix A) were evaluated, along with rapid bioassessments of the fish and benthic macroinvertebrate communities (Appendices B and C). Toxicity was assessed using *in situ* exposures of caged organisms (Appendix D). The *in situ* exposures were conducted during low and high flow events (4 days each), upstream and downstream of the outfall. Two species were used: *Daphnia magna* and *H. azteca*. Contrary to earlier case studies, PAH-photoinduced toxicity was not suspected as a potential stressor in this watershed. So *in situ* treatments were limited to water and sediment exposures. Basic water quality measures monitored during the exposures were DO, pH, temperature, conductivity, turbidity, ammonia, alkalinity, and hardness. Testing was conducted during the spring and late summer to investigate critical time periods of pesticide application, fish spawning, and low flow conditions.

If toxicity was observed in the water column during high flow, a TIE would be conducted as described above. This would help identify the source of the toxicity. If sediment toxicity or community impairment was observed, confirmatory assessments would consist of additional sediment toxicity testing and bioaccumulation testing. Toxicity of depositional sediments (*H. azteca* and *Chironomus tentans* 10-day assay, Appendix D) would be conducted at all sites where depositional sediments occurred. Since pesticides were suspected from both the farming and residential areas, bioaccumulation of organochlorines (such as DDT, chlordane) was also investigated by looking at fish tissue samples. If upper trophic level fish could not be captured, then semipermeable membrane devices (SPMDs) would be used to collect bioaccumulable substances (see Chapter 6).

Steps 5 and 6. Data Evaluation and Confirmatory Assessment

High levels of turbidity were observed during high flow events. The majority of this turbidity appeared to originate from upstream farmland and erodable stream banks. Toxicity was observed during high flow conditions in the water column. Slight toxicity was observed in stream sediment exposures. Habitat conditions did not vary appreciably among sites. The benthic communities were of fair quality at all sites and were not significantly different. The fish community was poor upstream of the detention pond and fair to poor below. Ammonia was found at elevated levels during the late summer period at all sites.

Follow-up confirmatory assessments showed significant sediment toxicity in laboratory exposures. A TIE evaluation suggested pesticides may be present during the spring high flow periods. Fish tissue residues showed detectable levels of chlordane and DDE.

Step 7. Conclusions

The wet detention pond affected downstream water quality appreciably. The upstream and downstream portions appeared to be impacted by elevated levels of pesticides and nutrients from the farming and residential drainage. The poor water quality observed in the pond was likely due to the buildup of nutrients in the sediments and water, allowing for excessive productivity and occasional anoxia. The widespread toxicity and detection of pesticides in the fish suggest upstream stormwater controls are needed.

SUMMARY: TYPICAL RECOMMENDED STUDY PLANS

Components of Typical Receiving Water Investigations

The specifics for any receiving monitoring program would be determined by the study objectives and the site conditions. As an example, Table 4.37 summarizes some general parameters that should be included in an urban water use evaluation study, depending on the specific beneficial uses of interest. Of course, the final parameters selected for study would vary for specific site conditions and historical information. As expected, an investigation of drainage uses (the primary use for an urban waterway) would be relatively straightforward compared to studies of other use impairments. However, investigations of drainage problems can be expensive and time-consuming. When the other uses are added to the list of potential objectives, the necessary data collection effort can become very comprehensive and expensive. Therefore, a staged approach is usually recommended, with a fairly simple initial effort used to obtain basic information. This information can then be used to develop specific experimental designs for later study stages.

Example Receiving Water Investigations

The following scenarios are brief examples of simple to complex receiving water investigations that incorporate many of the elements shown in Table 4.37. The first example, budgeted in Table 4.38, is the least expensive and would be appropriate for a single monitoring condition, such as a small lake or pond, or a short segment of a relatively small and homogeneous stream, having a single stormwater outfall. The proposed sampling effort is:

Water quality:	1 location × 1 season × 2 phases × 5 events/periods = 10 samples for analyses
Bacteria:	With above water samples, lab to analyze (*E. coli* and enterococci)
YSI sondes:	Rental for first/single deployment, $1000 per month
Inappropriate discharge screens:	1 outfall × 2 replicates = 2 samples
Habitat:	1 season × 2 phases × 2 locations = 4 station tests
Rapid bioassessment (RBP):	1 season × 2 locations × 3 replicates = 6 site visits
Toxicity:	1 season × 2 phases × 2 locations = 4 station tests

Twenty sets of outfall water samples during both wet- and dry-weather phases would be needed to obtain an allowable error of 40% for typical levels of variation (as described in Chapter 5). However, since this is a single season sampling effort, not many wet-weather events are likely to occur. Therefore, it is assumed that five wet-weather events would be monitored during about a 1- to 3-month period, and the error in estimating the event mean concentration (EMC) could therefore be larger than 40%. A laboratory budget of $225 per sample should cover both *E. coli* and enterococci bacteria analyses, and selected total heavy metals and nutrients, plus COD and suspended solids

Table 4.37 Parameters of Concern When Evaluating Different Receiving Water Uses

	Drainage	Biological Life and Integrity	Noncontact Recreation	Swimming and Other Contact Recreation	Water Supply	Shellfish Harvesting and Other Consumptive Fishing Uses
Debris and obstructions (channel conveyance capacity)	X					
Habitat destruction (channel stability, sediment scour, and deposition)		X				X
High/low flows (rates and durations)		X	X	X		X
Aesthetics, odors, and trash			X	X		
Safety (bank condition, garbage)			X	X		
Public access			X	X		
Inappropriate discharges		X	X	X	X	X
Benthic macroinvertebrate species present		X				X
Fish species present		X				X
Polluted sediment (SOD and toxicants[a])		X				X
Toxicity and bioaccumulation of toxicants[a]		X				X
Health-related water quality standards (especially microorganisms[b] and toxicants[a])				X	X	X
Wet-weather quality (toxicants[a], nutrients[c], DO, temperature, alkalinity, and hardness)		X				X

Primary constituents are indicated in bold/underlined and should be analyzed for most all samples. Others can be analyzed less often as screening tests. In all cases, the common constituents should also be analyzed for all samples.

[a] Toxicants (organic toxicants such as pesticides, herbicides, and PAHs; metallic toxicants such as **zinc**, **copper**, **lead**, cadmium, arsenic, and mercury) and toxicity tests (such as **Microtox screening test,** plus other *in situ* and laboratory toxicity tests).

[b] Microorganisms (indicator bacteria and selected pathogens such as: **fecal coliforms**, ***E. coli***, **enterococci**, and *Pseudomonas aeruginosa*).

[c] Nutrients (**ammonia**, TKN, **nitrates**, TP, **phosphates**).

Common constituents, added to all water quality investigations (**pH**, **conductivity**, **turbidity**, **suspended solids**, **COD**).

analyses. These data would be supplemented with field screening in the drainage system during two dry-weather flow periods (assuming water was found during both visits) to identify inappropriate sources of wet-weather flows. It is recommended that a YSI 6000 probe be rented for a 1 month to measure flow (depth values) and water quality variations (DO, temperature, conductivity, turbidity, and pH) during several runoff events and periods of dry weather in the receiving water. This would indicate the duration and severity of the runoff events and the associated recovery periods. Diurnal DO and temperature fluctuations would also be measured. This water quality data would be supplemented with habitat, rapid bioassessment (RBP), and limited *in situ* and laboratory toxicity testing above and below the outfall (two locations). This collective information should give a good indication of the presence of receiving water problems at the site. Of course, because it is a single season analysis, an appropriate sampling schedule needs to be carefully selected, probably based on critical biological conditions in the receiving water (likely early spring or late summer, depending on the expected organisms present and the local weather patterns). Besides being a minimum sampling

Table 4.38 Monitoring Cost Estimate for Single Outfall in a Single Receiving Water Segment of Interest

	Equipment Cost			Analytical Cost			Labor Cost				
	Unit Cost	No. Needed	Total Cost	Unit Cost	No. Needed	Total Cost	Labor (hrs)	No. Needed	Total hrs	Costs at $35/hr	Total Costs
Field screening				$50	2	$100	1.5	2	3	$105	$205
Habitat				na	4	na	0.35	4	1.4	49	49
Toxicity				500	4	2000	na	na	na	na	2000
RBP				na	na	na	2	6	12	420	420
YSI probe (rental)	$1000	1	$1000	na	na	na	8	1	8	280	1280
Water and bacteria (lab)				225	10	2250	na	na	na	na	2250
Site costs			1000						100	3500	4500
Total			$2000			$4350			124.4	$4354	$10,704

effort incorporating all recommended phases of a monitoring program, this scheme could be used as the initial effort for a longer-duration and more complex study.

The next scenario is for a more complex situation where there are 25 outfalls in a moderately sized (first-order) receiving water about 2 miles long in a completely urbanized watershed, 3 mi^2 in area. This is also presented as a first step in a possible recurring effort to cover more seasons or several years. The main purpose of this program is to identify possible serious receiving-water problems that would warrant more extensive evaluations. This scenario could be repeated at other similarly sized receiving waters in an area. In many ways, this scenario is very similar to the previously described program, except that the water sampling for bacteriological and chemical analyses would be conducted in the receiving water with some outfall samples. Outfall screening (using purchased test kits) during dry weather would also be conducted to identify inappropriate discharges. Table 4.39 shows the estimated costs, and the following lists the proposed effort for this program:

Water quality:	1 location × 1 season × 2 phases × 20 sets = 40
Bacteria:	With above water samples, lab to analyze (*E. coli* and enterococci)
YSI sondes:	Rental for first/single deployment, $1000 per month
Inappropriate discharge screens:	25 outfalls × 2 replicates = 50 samples
Habitat:	1 season × 2 phases × 25 locations = 50 station tests
RBP:	1 season × 4 locations × 3 replicates = 12 site visits
Toxicity:	1 season × 2 phases × 4 locations = 8 station tests

The last option shown is a relatively complete approach, covering all seasons, and is reasonably comprehensive and, therefore, relatively expensive. Again, the components are similar to the above programs, but the number of samples is greatly increased to cover the two critical seasons (RBP and sondes during four seasons) and to collect both outfall and receiving water samples. Because of the study duration, it would likely be more economical to purchase the YSI 6000 sondes and the bacteriological test equipment. The other water quality analyses would be conducted by a commercial laboratory. It may be appropriate to add selected immunoassay tests for pesticides and PAHs for some of the water samples (at about $25 each). Much greater site costs are shown because flow monitoring and rainfall monitoring will also be conducted during this effort. The sampling effort is shown below, while the estimated cost is shown in Table 4.40:

Water quality:	4 locations × 2 seasons × 2 phases × 20 sets = 320
Bacteria:	4 locations × 2 seasons × 2 phases × 20 sets = 320
YSI sondes:	4 locations × 4 seasons = 16 deployments
Inappropriate discharge screens:	25 outfalls × 2 seasons × 3 replicates = 150 samples
Habitat:	4 seasons × 2 phases × 25 locations = 200 station tests
RBP:	2 seasons × 4 locations × 5 replicates = 40 site visits
Toxicity:	4 seasons × 2 phases × 4 locations = 32 station tests

In all cases, major modifications are expected to be made to the above scenarios for real situations. In addition, the initial analyses will provide information that should be used to reexamine the complete workplan. Obviously, the above costs are only crude approximations, depending on local labor costs, site access, the availability of equipment, etc.

This chapter outlined an approach for designing appropriate multicomponent assessment projects for various conditions and objectives. As will be stressed throughout this book, it is critical that potential problems be examined using complementary and supportive procedures. It is inefficient, and subject to significant evaluation errors, to rely on simplistic single parameter/media approaches. Typical urban receiving waters are likely most affected by habitat degradation, frequent

Table 4.39 First Evaluation for 2-Mile Stream Segment Having 25 Outfalls

	Equipment Cost			Analytical Cost			Labor Cost				
	Unit Cost	No. Needed	Total Cost	Unit Cost	No. Needed	Total Cost	Labor (hrs)	No. Needed	Total hrs	Costs at $35/hr	Total Costs
Field screening	$1600	1	$1600	20	50	$1000	1.5	50	75	$2625	$5225
Habitat				na	50	na	0.35	50	17.5	612	612
Toxicity				500	8	4000	na	na	na	na	4000
RBP				na	na	na	2	12	24	840	840
YSI probe (rental)	1000	1	1000	na	na	na	8	1	8	280	1280
Water and bacteria (lab)				225	40	9000	na	na	na	na	9000
Site costs			2500						200	7000	9500
Total			$5100			$14,000			324	$11,357	$30,457

Table 4.40 Annual Sampling Effort for a Moderately Sized, Completely Urbanized Watershed Having 25 Outfalls

	Equipment Cost			Analytical Cost			Labor Cost				
	Unit Cost	No. Needed	Total Cost	Unit Cost	No. Needed	Total Cost	Labor (hrs)	No. Needed	Total hrs	Costs at $35/hr	Total Costs
Field screening	$1600	1	$1600	20	150	$3000	1.5	150	225	$7875	$12,475
Habitat				na	200	na	0.35	200	70	2450	2450
Toxicity				500	32	16,000	na	na	na	na	16,000
RBP				na	na	na	2	40	80	2800	2800
Bacteria	3000	1	3000	15	320	4800	0.05	320	16	560	8360
YSI probe	7000	4	28,000	na	na	na	8	16	128	4480	32,480
Water quality				175	320	56,000	na	na	na	na	56,000
Site costs			15,000						640	22,400	37,400
Total			$47,600			$79,800			1159	$40,565	$167,965

high flows, and contaminated sediment. While water and sediment chemical analyses can be expensive, they should not necessarily be rejected outright. Some of these more expensive analyses may be critical when evaluating biological and habitat information, for example. The number of needed data observations (as discussed in Chapter 5) and the sampling methods (described in Chapters 5 and 6) are critical for a successful assessment, in addition to the selection of the most appropriate assessment endpoints and overall assessment strategy.

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CHAPTER 5

Sampling Effort and Collection Methods

"A little experience often upsets a lot of theory."

Cadman

CONTENTS

INTRODUCTION

This chapter begins by describing experimental design methods enabling the user to determine the sampling effort needed to accomplish project objectives. The statistical basis for this approach is required to justify the allocation of scarce resources. In many cases, certain elements of a multifaceted study program, as required for practically all receiving water studies, require much more time and money than other elements of the program. The approach and tools given in this chapter enable one to balance project resources and scope with expected outcomes. It can be devastating to project conclusions if needed numbers of samples are not obtained at the appropriate time. The tools in this chapter enable one to better plan and conduct a sampling program to minimize this possibility. Of course, all projects conclude with some unresolved issues that were not considered at the outset. This can only be minimized with increased experience and subject knowledge, and by retaining some flexibility during project execution.

The tools presented here assume some prior knowledge of the situation (especially expected variation in a variable to be measured) in order to determine the sampling effort. This is initially obtained through professional judgment (based on one's experience in similar situations and from the literature), and is generally followed up with a multistaged sampling effort where an initial experimental design sampling effort is conducted to obtain a better estimate of parameter variability. That estimate can then be used to help foresee and estimate the needed sampling effort during later sampling periods. In all cases, the tools presented here enable one to obtain a level of confidence concerning the significance of the project conclusions. As an example, if it is necessary to compare two sampling location conditions (a very common objective), the sampling effort will determine the sensitivity of the study. Depending on the variability of the parameter of interest, a few samples collected may be useful to identify only very large differences in conditions between two sampling locations. Of course, the objective of the study may be only to confirm large differences (such as between reference and grossly contaminated sites, or between influent and effluent conditions for a stormwater measure known to be very effective). Unfortunately, in most cases involving nonpoint source discharges, the differences are likely to be much more subtle, requiring numerous samples and careful allocations of project resources. The tools presented in this chapter enable one to predict the statistical sensitivity of different sampling schemes, allowing informed decisions and sound budget requests to be made.

The other elements of this chapter involve specific options for collecting samples from the many ecosystem components of interest. Quality control/quality assurance (QA/QC) sampling requirements are described along with basic considerations for safe sample collection (selecting sampling locations, preventing sample contamination, sample volumes needed, sample shipping, personnel requirements, etc.). Water sampling (manual sampling, automatic samplers, sampler setup options, sampler modifications, bedload samples, suspended sediment samples, floatable material sampling, source area sheetflow sampling, etc.) are also described and discussed. This chapter also includes important considerations pertaining to sediment sampling and interstitial (pore water) sampling. The material included in this chapter, therefore, describes how to collect basic water and sediment samples for receiving water studies. Chapter 6, in turn, discusses measurement methods, including the collection of biological samples.

EXPERIMENTAL DESIGN: SAMPLING NUMBER AND FREQUENCY

The first task in any study is to formulate the questions being addressed. The expected statistical analysis tools (described in Chapter 7) that are expected to be used for evaluating the data should also be an early part of the experimental design. Alternative study plans can then be examined, and finally, the sampling effort can be estimated.

Sampling Plans

All sampling plans attempt to obtain certain information (usually average values, totals, ranges, etc.) about a large population by sampling and analyzing a much smaller sample. The first step in this process is to select the sampling plan and then to determine the number of samples needed. Many sampling plans have been well described in the environmental literature. The following are the four main categories, plus subcategories, of sampling plans (Gilbert 1987):

- Haphazard sampling. Samples are taken in a haphazard (not random) manner, usually at the convenience of the sampler when time permits. Especially common when the weather is pleasant. This is only possible with a very homogeneous condition over time and space; otherwise biases are introduced in the measured population parameters. It is therefore not recommended because of the difficulty in verifying the homogeneous assumption. This is the most common sampling strategy when volunteers are used for sampling, unless the grateful agency is able to spend sufficient time to educate the volunteer samplers about the problems of this type of sampling and to specify a more appropriate strategy.
- Judgment sampling. This strategy is used when only a specific subset of the total population is to be evaluated, with no desire to obtain "universal" characteristics. The target population must be clearly defined (such as during wet-weather conditions only) and sampling is conducted appropriately. This could be the first stage of later, more comprehensive sampling of other target population groups (multistage sampling).
- Probability sampling. Several subcategories of probability sampling have been described:
  - Simple random sampling. Samples are taken randomly from the complete population. This usually results in total population information, but it is usually inefficient as a greater sampling effort may be required than if the population was subdivided into distinct groups. Simple random sampling doesn't allow information to be obtained for trends or patterns in the population. This method is used when there is no reason to believe that the sample variation is dependent on any known or measurable factor.
  - Stratified random sampling. This may be the most appropriate sampling strategy for most receiving water studies, especially if combined with an initial limited field effort as part of a multistage sampling effort. The goal is to define strata that result in little variation within any one strata, and great variation between different strata. Samples are randomly obtained from several population groups that are assumed to be internally more homogeneous than the population as a whole, such as separating an annual sampling effort by season, lake depth, site location, habitat category, rainfall depth, land use, etc. This results in the individual groups having smaller variations in the characteristics of interest than in the population as a whole. Therefore, sample efforts within each group will vary, depending on the variability of characteristics for each group, and the total sum of the sampling effort may be less than if the complete population was sampled as a whole. Also, much additional useful information is likely if the groups are shown to actually be different.
  - Multistage sampling. One type of multistage sampling commonly used is associated with the required subsampling of samples obtained in the field and brought to the laboratory for subsequent splitting for several different analyses. Another type of multistage sampling is when an initial sampling effort is used to examine major categories of the population that may be divided into separate clusters during later sampling activities. This is especially useful when reasonable estimates of variability within a potential cluster are needed for the determination of the sampling effort for composite sampling. These variability measurements may need to be periodically reverified during the monitoring program.
  - Cluster sampling. Gilbert (1987) illustrates this sampling plan by specifically targeting specific population units that cluster together, such as a school of fish or clump of plants. Every unit in each randomly selected cluster can then be monitored.
  - Systematic sampling. This approach is most useful for basic trend analyses, where evenly spaced samples are collected for an extended time. Evenly spaced sampling is also most efficient when trying to find localized hot spots that randomly occur over an area. Gilbert (1987) presents

guidelines for spacing of sampling locations for specific project objectives relating to the size of the hot spot to be found. Spatial gradient sampling is a systematic sampling strategy that may be worthy of consideration when historical information implies an aerial variation of conditions in a river or other receiving water. One example would be to examine the effects of a point source discharge on receiving-sediment quality. A grid would be described in the receiving water in the discharge vicinity whose spacing would be determined by preliminary investigations.

- Search sampling. This sampling plan is used to find specific conditions where prior knowledge is available, such as the location of a historical (but now absent) waste discharger affecting a receiving water. Therefore, the sampling pattern is not systematic or random over an area, but stresses areas thought to have a greater probability of success.

Box et al. (1978) contains much information concerning sampling strategies, specifically addressing problems associated with randomizing the experiments and blocking the sampling experiments. Blocking (such as in paired analyses to determine the effectiveness of a control device, or to compare upstream and downstream locations) eliminates unwanted sources of variability. Another way of blocking is to conduct repeated analyses (such as for different seasons) at the same locations. Most of the above probability sampling strategies should include randomization and blocking within the final sampling plans (as demonstrated in the following example and in the use of factorial experiments).

Albert and Horwitz (1988) warn that the user of statistics should be critical and alert in making decisions based on sample estimates, and they list the following as essential aspects of statistical sampling:

- Sampling should not be undertaken until the questions have been determined and properly framed. The expense of conducting a survey can only be justified if the questions answered have a value. Vague or unstructured exploratory surveys are wasteful.
- The individuals included in the sample must be chosen at random, specifically from a population that is well defined.

Example Use of Stratified Random Sampling Plan

Street dirt samples were collected in San Jose, CA, during an early EPA project to identify sources of urban runoff pollutants (Pitt 1979). The samples were collected from narrow strips, from curb to curb, using an industrial vacuum. Many of these strips were to be collected in each area and combined to determine the dust and dirt loadings and their associated characteristics (particle size and pollutant concentrations). Each area (stratum) was to be sampled frequently to determine the changes in loadings with time and to measure the effects of street cleaning and rains in reducing the loadings. The analytical procedure used to determine the number of subsamples needed for each composite sample involved weighing individual subsamples in each study area to calculate the coefficient of variation (COV = standard deviation/mean) of the street surface loading. The number of subsamples necessary (N), depending on the allowable error (L), was then determined. An allowable error value of about 25%, or less, was needed to keep the precision and sampling effort at reasonable levels. The formula used (after Cochran 1963) was:

$$N = 4\sigma^2/L^2$$

With 95% confidence, this equation estimates the number of subsamples necessary to determine the true mean value for the loading within a range of ±L. As will be shown in the following discussions, more samples are required for a specific allowable error as the COV increases. Similarly, as the allowable error decreases for a specific COV, more samples are also required. Therefore, with an allowable error of 25%, the required number of subsamples for a study area with a COV of 0.8 would be 36.

Initially, individual samples were taken at 49 locations in the three study areas to determine the loading variabilities. The loadings averaged about 2700 lb/curb-mile in the Downtown and Keyes Street areas, but were found to vary greatly within these two areas. The Tropicana area loadings were not as high, and averaged 310 lb/curb-mile. The Cochran (1963) equation was then used to determine the required number of subsamples in each test area. The data were then examined to determine if the study areas should be divided into meaningful test area groups.

The purpose of these divisions was to identify a small number of meaningful test area-groupings (strata) that would require a reasonable number of subsamples and to increase the usefulness of the test data by identifying important groupings. Five different strata were identified for this research: two of the areas were divided by street texture conditions into two separate strata each (good vs. poor), while the other area was left undivided. The total number of individual subsamples for all five areas combined was 111, and the number of subsamples per strata ranged from 10 to 35. In contrast, 150 subsamples would have been needed if the individual areas were not subdivided. Subdividing the main sampling areas into separate strata not only resulted in a savings of about 25% in the sampling effort, but also resulted in much more useful information concerning the factors affecting the values measured. The loading variations in each strata were reexamined seasonally, and the sampling effort was readjusted accordingly.

Factorial Experimental Designs

Factorial experiments are described in Box et al. (1978) and in Berthouex and Brown (1994). Both of these books include many alternative experimental designs and examples of this method. Berthouex and Brown (1994) state that "experiments are done to:

1. Screen a set of factors (independent variables) and learn which produce an effect
2. Estimate the magnitude of effects produced by experimental factors
3. Develop an empirical model
4. Develop a mechanistic model."

They concluded that factorial experiments are efficient tools in meeting the first two objectives and are also excellent for meeting the third objective in many cases. Information obtained during the experiments can also be very helpful in planning the strategy for developing mechanistic models. The main feature of factorial experimental designs is that they enable a large number of possible factors that may influence the experimental outcome to be simultaneously evaluated.

Box et al. (1978) presents a comprehensive description of many variations of factorial experimental designs. A simple 2^3 design (three factors: temperature, catalyst, and concentrations at two levels each) is shown in Figure 5.1 (Box et al. 1978). All possible combinations of these three factors are tested, representing each corner of the cube. The experimental results are placed at the appropriate corners. Significant main effects can usually be easily seen by comparing the values on opposite faces of the cube. If the values on one face are consistently larger than on the opposite face, then the experimental factor separating the faces likely has a significant effect on the outcome of the experiments. Figure 5.2 (Box et al. 1978) shows how these main effects are represented, along with all possible two-factor interactions and the one three-factor interaction. The analysis of the results to identify the significant factors is straightforward.

One of the major advantages of factorial experimental designs is that the main effect of each factor, plus the effects of all possible interactions of all of the factors can be examined with relatively few experiments. The initial experiments are usually conducted with each factor tested at two levels (a high and a low level). All possible combinations of these factors are then tested. Table 5.1 shows an experimental design for testing four factors. This experiment therefore requires 2^4 (=16) separate experiments to examine the main effects and all possible interactions of these four factors. The signs signify the experimental conditions for each main factor during each of the 16 experiments.

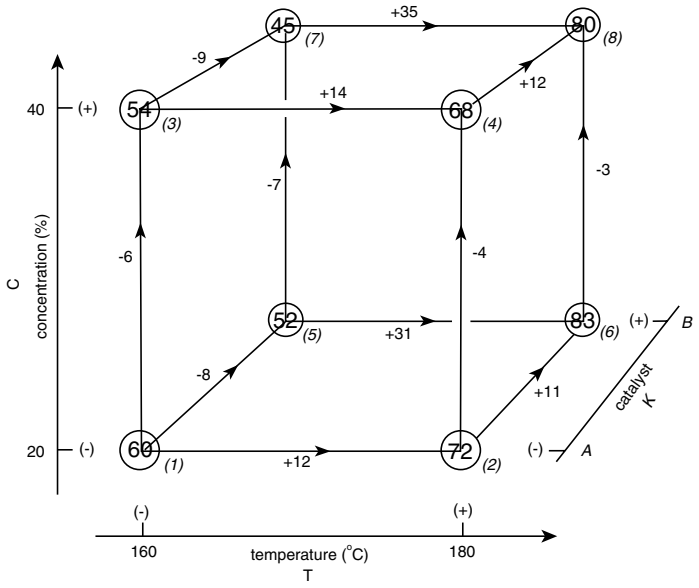


Figure 5.1 Basic cubic design of 2^3 factorial test. (From Box, G.E.P., W.G. Hunter, and J.S. Hunter. *Statistics for Experimenters*. Copyright 1978. This material used by permission of John Wiley & Sons, Inc., New York.)

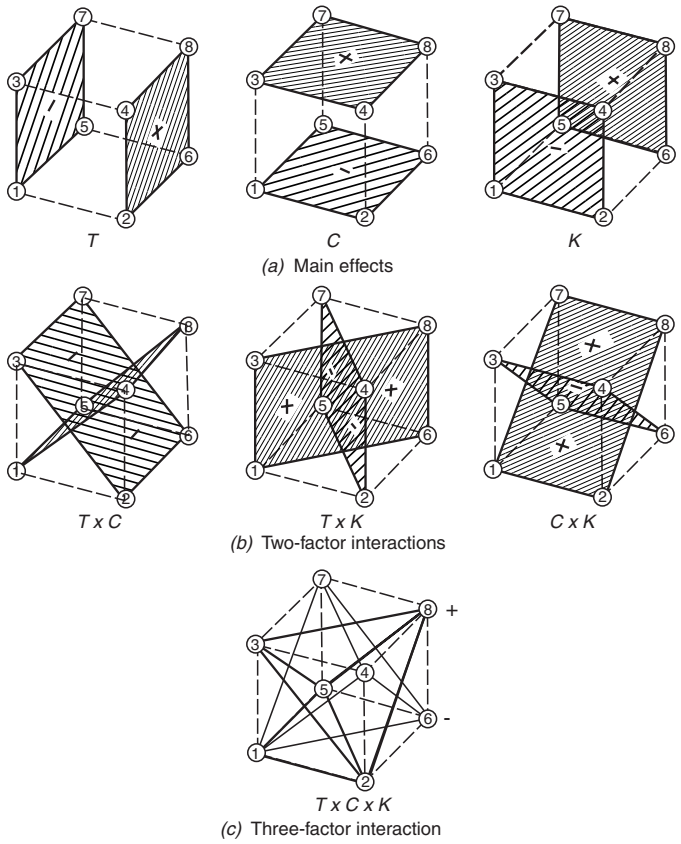


Figure 5.2 Main effects and interactions for 2^3 factorial test. (From Box, G.E.P., W.G. Hunter, and J.S. Hunter. *Statistics for Experimenters*. Copyright 1978. This material used by permission of John Wiley & Sons, Inc., New York.)

Table 5.1 Factorial Experimental Design for Four Factors and 16 Experiments

Experiment No.	A	B	C	D	AB	AC	AD	BC	BD	CD	ABC	ABD	BCD	ABCD
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	−	+	+	+	−	−	−	+	+	+	−	−	+	−
3	+	−	+	+	−	+	+	−	−	+	−	−	−	−
4	−	−	+	+	+	−	−	−	−	+	+	+	−	+
5	+	+	−	+	+	−	+	−	+	−	−	+	−	−
6	−	+	−	+	−	+	−	−	+	−	+	−	−	+
7	+	−	−	+	−	−	+	+	−	−	+	−	+	+
8	−	−	−	+	+	+	−	+	−	−	−	+	+	−
9	+	+	+	−	+	+	−	+	−	−	+	−	−	−
10	−	+	+	−	−	−	+	+	−	−	−	+	−	+
11	+	−	+	−	−	+	−	−	+	−	−	+	+	+
12	−	−	+	−	+	−	+	−	+	−	+	−	+	−
13	+	+	−	−	+	−	−	−	−	+	−	−	+	+
14	−	+	−	−	−	+	+	−	−	+	+	+	+	−
15	+	−	−	−	−	−	−	+	+	+	+	+	−	−
16	−	−	−	−	+	+	+	+	+	+	−	−	−	+

The shaded main factors are the experimental conditions, while the other columns specify the data reduction procedures for the other interactions. A plus sign shows when the factor is to be held at the high level, while a minus sign shows when the factor is to be held at the low level for the main experimental conditions (A through D). This table also shows all possible two-way, three-way, and four-way interactions, in addition to the main factors. Simple analysis of the experimental results allows the significance of each of these factors and interactions to be determined. As an example, the following list shows the four factors and the associated levels for tests conducted to identify factors affecting runoff quality:

A: Season (plus: winter; minus: summer)
B: Land use (plus: industrial; minus: residential)
C: Age of development (plus: old; minus: new)
D: Rain depth (plus: >1 in; minus: <1 in)

These factors would require the selection of four sampling locations:

1. Old industrial area
2. New industrial area
3. Old residential area
4. New residential area

The above experiments are designed to collect stormwater runoff data from four test locations. Obviously, both winter and summer seasons must be monitored, and rainfall events of varying depths will be sampled. Rains both less than 1 inch and greater than 1 inch will need to be sampled at all monitoring stations in both seasons in order to obtain the needed information.

Even though factorial experiments are best suited in controlled laboratory settings, they have been very useful in organizing environmental data for analysis. Table 5.2 shows an example where environmental data were organized using a simple factorial design. The design called for a 2^3 experiment to investigate the effects of soil moisture, soil texture, and soil compaction on observed soil infiltration rates (Pitt et al. 1999a). This table shows the calculations from 152 double-ring infiltration tests for the Horton (1939) equation final infiltration rate coefficient (f_c).

Replicate observations enhance the data analysis efforts, and grouped standard error values can be calculated (Box et al. 1978) to identify the significant factors affecting runoff quality. In Table 5.2, at least 12 replicates were conducted for each test condition to improve the statistical basis

Table 5.2 Example Factorial Experiment Analysis for Field Project Investigating Infiltration into Disturbed Urban Soils

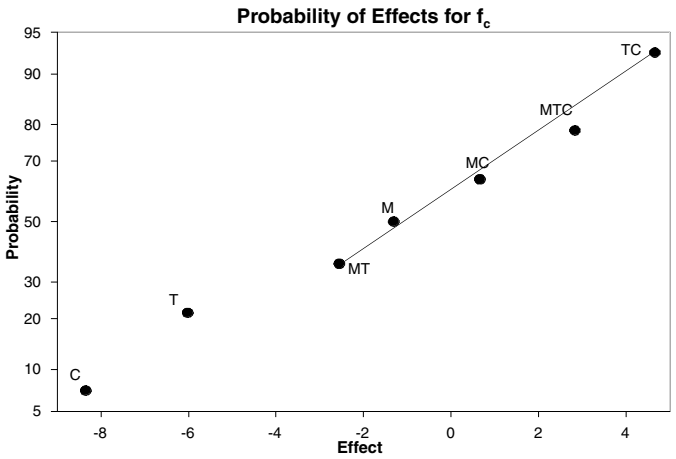
Moisture (Wet = +/Dry = –)	Texture (Clay = +/Sand = –)	Compacted (Yes = +/No = –)	Factorial Group	Average	Standard Error	Number
+	+	+	1	0.23	0.13	18
+	+	–	2	0.43	0.50	27
+	–	+	3	1.31	1.13	18
+	–	–	4	16.49	1.40	12
–	+	+	5	0.59	0.35	15
–	+	–	6	7.78	4.00	17
–	–	+	7	2.25	0.98	21
–	–	–	8	13.08	2.78	24
			Overall average	5.27		
			Calculated polled S.E.	1.90		

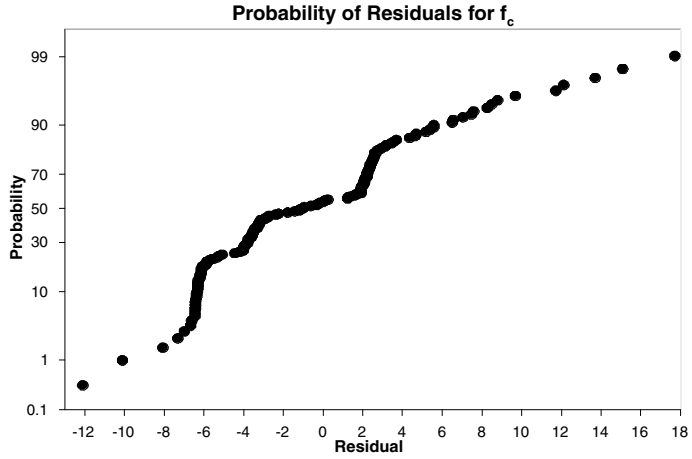
Factorial Group	Effects	Rank	Prob.
C	–8.35	1	7.14
T	–6.02	2	21.43
MT	–2.55	3	35.71
M	–1.31	4	50.00
MC	0.66	5	64.29
MTC	2.83	6	78.57
TC	4.66	7	92.86

$f_c = 5.27 \pm (T/2) \pm (C/2)$

$f_c = 5.27 \pm (-6.02/2) \pm (-8.35/2)$

T	C	Calculated Values
+	+	–1.92
+	–	6.43
–	+	4.10
–	–	12.45





From Pitt, R., J. Lantrip, R. Harrison, C. Henry, and D. Hue. *Infiltration through Disturbed Urban Soils and Compost-Amended Soil Effects on Runoff Quality and Quantity.* U.S. Environmental Protection Agency, Water Supply and Water Resources Division, National Risk Management Research Laboratory. EPA 600/R-00/016. Cincinnati, OH. 231 pp. December 1999a.

for the conclusions. These unusually large numbers of replicates were needed because of the inherently large variability within each test category. If the variability was less, then the number of required replicates could have been much less (as described later in this chapter). In addition, the site test conditions were not known with certainty when the field tests were run, as some field estimates required confirmation with later laboratory tests that resulted in the reclassification of some of the data.

If observations are not available for some of the needed conditions (such as the monitoring equipment failing during the only large event that occurred at the old industrial site during the summer), then a fractional factorial design can still be used to organize the data and calculate the effects for all of the main factors, and for most of the interactions (as noted in the above experiment). Once the initial experiments are completed, follow-up experiments can be efficiently designed to examine the linearity of the effects of the significant factors by conducting response surface experimental designs. In addition, further experiments can be conducted and merged with these initial experiments to examine other factors that were not considered in the first experiments. Because of the usefulness and adaptability of factorial experimental designs, Berthouex and Brown (1994) recommend that they "should be the backbone of an experimenter's design strategy."

Number of Samples Needed to Characterize Conditions

An important aspect of any research is the assurance that the samples collected represent the conditions to be tested and that the number of samples to be collected is sufficient to provide statistically relevant conclusions. Unfortunately, sample numbers are most often not based on a statistical process and follow traditional "best professional judgments," or are resource driven. The sample numbers should be equal between sampling locations if comparing station data (EPA 1983b) and paired sampling should be conducted, if at all possible (the samples at the two comparison sites should be collected at the "same" time, for example), allowing for much more powerful paired statistical comparison tests (see Chapter 7). In addition, replicate subsamples must also be collected and then combined to provide a single sample for analysis for many types of ecosystem sampling. Cairns and Dickson (1971) observed from many years of experience that at least three artificial substrate samplers, 3 to 10 dredge hauls, and three Surber square foot samples were the minimum number of samples required to describe benthic macroinvertebrates at a given station. These are then combined (to reduce analysis expenses) or kept as separate samples (more costly, but provides a legitimate measure of variation/precision).

Receiving water studies frequently include objectives to characterize various chemical, biological, and physical parameters of the water body itself, or influencing features (meteorological, discharges, watershed, etc.). An experimental design process can be used that estimates the number of needed samples based on the allowable error, the variance of the observations, and the degree of confidence and power needed for each parameter. A basic equation that can be used is as follows:

$$n = [COV(Z_{1-\alpha} + Z_{1-\beta})/(error)]^2$$

where

- n = number of samples needed
- α = false positive rate ($1 - \alpha$ is the degree of confidence. A value of α of 0.05 is usually considered statistically significant, corresponding to a $1 - \alpha$ degree of confidence of 0.95, or 95%)
- β = false negative rate ($1 - \beta$ is the power. If used, a value of β of 0.2 is common, but it is frequently ignored, corresponding to a β of 0.5)
- $Z_{1-\alpha}$ = Z score (associated with area under normal curve) corresponding to $1 - \alpha$. If α is 0.05 (95% degree of confidence), then the corresponding $Z_{1-\alpha}$ score is 1.645 (from

standard statistical tables).

$Z_{1-\beta}$ = Z score corresponding to 1 – β value. If β is 0.2 (power of 80%), then the corresponding $Z_{1-\beta}$ score is 0.85 (from standard statistical tables). However, if power is ignored and β is 0.5, then the corresponding $Z_{1-\beta}$ score is 0.

error = allowable error, as a fraction of the true value of the mean

COV = coefficient of variation (sometimes noted as CV), the standard deviation divided by the mean. (Data set assumed to be normally distributed.)

This equation is only approximate, as it requires that the data set be normally distributed. However, if the coefficient of variation (COV) values are low (less than about 0.4), then there is probably no significant difference in the predicted sampling effort. This equation is only appropriate as an approximation in many cases, as normal distributions are rare (log-normal distributions are appropriate for most water quality parameters) and the COV values are typically relatively large (closer to 1). The presentation of the results and the statistical procedures used to evaluate the data, however, should calculate the exact degree of confidence of the measured values.

Figure 5.3 (Pitt and Parmer 1995) is a plot of this equation, showing the approximate number of samples needed for an α of 0.05 (degree of confidence of 95%), and a β of 0.2 (power of 80%). As an example, if an allowable error of about 25% is desired and the COV is estimated to be 0.4, then about 20 samples would have to be analyzed. The samples could be composited and a single analysis conducted, but this would not allow the COV assumption to be confirmed, or the actual confidence range of the concentration to be determined. The use of stratified random sampling can usually be used to advantage by significantly reducing the COV of the subpopulation in the strata, requiring fewer samples for characterization, as illustrated above.

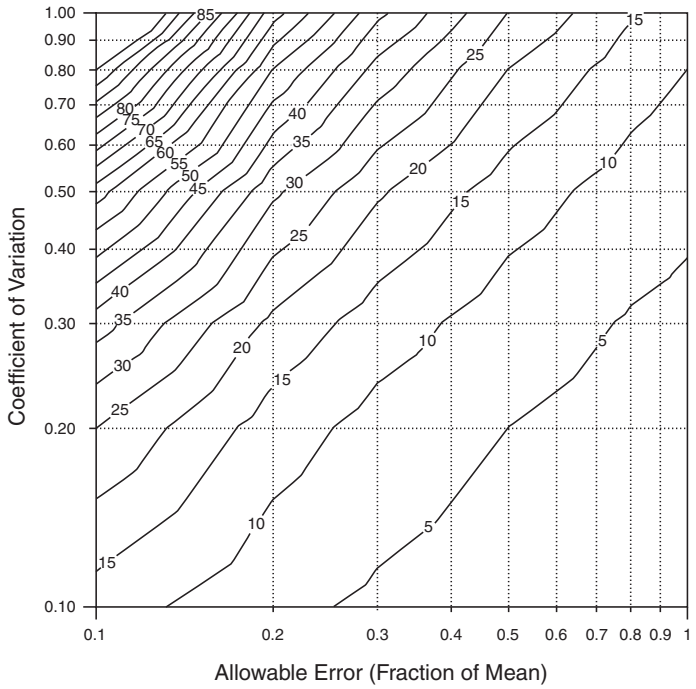


Figure 5.3 Sample requirements for confidence of 95% (α = 0.05) and power of 80% (β = 0.20). (From Pitt, R. and K. Parmer. *Quality Assurance Project Plan: Effects, Sources, and Treatability of Stormwater Toxicants*. Contract No. CR819573. U.S. Environmental Protection Agency, Storm and Combined Sewer Program, Risk Reduction Engineering Laboratory. Cincinnati, OH. February 1995.)

Gilbert (1987) presents variations of this basic equation that consider the number of samples needed to determine the probability of occurrence within a specified range (such as to calculate the frequency of standard violations). He also presents equations that consider correlated data, such as when the observations are not truly independent, as when very high pollutant concentrations affect values in close spatial or temporal proximity. As expected, correlated data necessitate more samples than indicated from the basic equations. Additional sample size equations are presented in experimental design texts and in listings from government agencies (such as Table 5.3 from Environment Canada 1994).

Types of Errors Associated with Sampling

Unfortunately, there are many errors associated with a receiving water study. Errors associated with too few (or too many) samples for a parameter of interest is only one category. Sampling and analytical errors may also be significant and could add to these other errors. Hopefully, the collective sum of all errors is known (through QA/QC activities and adequate experimental design) and manageable. An important aspect of a monitoring program is recognizing the levels of errors and considering the uncertainties in developing recommendations and conclusions.

Generally, errors can be divided into precision and bias problems. Both of these errors, either together or separately, have dramatic effects on the final conclusions of a study. Figure 5.4 (Gilbert 1987) shows the effects of these errors. Bias is a measure of how close the measured median value is to the true median value, while precision is a measure of how "fuzzy" the median estimate is (the repeatability of the analyses; used to determine the confidence of the measurements).

Errors in decision making are usually divided into Type 1 (α: alpha) and Type 2 (β: beta) errors:

α (alpha) (Type 1 error) — a false positive, or assuming something is true when it is actually false. An example would be concluding that a tested water was adversely contaminated, when it actually was clean. The most common value of α is 0.05 (accepting a 5% risk of having a Type 1 error). Confidence is $1 - \alpha$, or the confidence of not having a false positive.

β (beta) (Type 2 error) — a false negative, or assuming something is false when it is actually true. An example would be concluding that a tested water was clean when it actually was contaminated. If this was an effluent, it would therefore be an illegal discharge with the possible imposition of severe penalties from the regulatory agency. In most statistical tests, β is usually ignored (if ignored, β is 0.5). If it is considered, a typical value is 0.2, implying accepting a 20% risk of having a Type 2 error. Power is $1 - \beta$, or the certainty of not having a false negative.

It is important that power and confidence be balanced for an effective monitoring program. Most studies ignore power, while providing a high value (typically 95%) for the level of confidence. This is an unrealistic approach because both false negatives and false positives are important. In many environmental programs, power (false negative problems) may actually be more critical than confidence. If a tested water had a Type 2 error (false negative), inappropriate discharges would occur. Typical fines imposed by regulatory agencies are $10,000 per day for nonpermitted discharges. Future liability for wastes discharged due to an error in measurement or negligence can easily reach into millions of dollars for cleanup and mitigation of health effects. Clearly, one wants to minimize costs, yet have the assurance that the correct decision is being made. However, errors will always be present in any analysis, and some uncertainty in the conclusions must be accepted. Obviously, it can become prohibitively expensive to attempt to reduce monitoring errors to extremely low levels, especially when the monitoring program is affected by uncontrollable environmental factors.

Chapter 7 describes statistical analysis procedures that can be used for data analyses. It is always important to report the statistical significance (and importance) of the test results. The "importance" of the test results relates to the magnitude of the difference between two alternatives, for example,

Table 5.3 Typical Listing of Sample Size Equations That Are Useful for Environmental Research

Objective	Formula	Ref.
To determine the sample size required to detect an effect in an impacted area vs. a control area over time: a)- Resampling same sites before and after impact and testing if the mean change in the control area is the same as that in the impacted area	$n = 2(t_\alpha + t_\beta)^2\left(\frac{S}{\Delta}\right)^2$	Green 1989
b)- Sampling different sites before and after impact and testing if the mean change in the control area is the same as that in impacted area	$n = 4(t_\alpha + t_\beta)^2\left(\frac{S}{\Delta}\right)^2$ where: n = number of samples for each of the control and impact areas S = standard deviation Δ = magnitude of change required to be a real effect with specified power (1 – b) t_α = t statistic given a Type I error probability t_β = t statistic given a Type II error probability	Green 1989
To determine if the mean value for an impacted area differs significantly from a standard value (e.g., sediment quality criterion)	$n \geq \frac{(Z_\alpha + Z_\beta)^2}{d^2} + 0.5Z_\alpha^2$ where: n = sample size Z_α = Z statistic for Type I error probability (e.g., x = 0.05) Z_β = Z statistical for Type II error probability (e.g., B – 0.90) d = magnitude of the difference to be detected (i.e., effect level)	Alldredge 1987
To determine if the mean value for an impacted area differs significantly from the mean of a control site	$n \geq \frac{2(Z_\alpha + Z_\beta)^2}{d^2} + 0.25Z_\alpha^2$ where: n = sample size Z_α = Z statistic for Type I error probability (e.g. x = 0.05) Z_β = Z statistical for Type II error probability (e.g., B – 0.90) d = magnitude of the difference to be detected (i.e., effect level)	Alldredge 1987
To determine the number of samples that would be required to determine a mean value (representative of the area) with a given statistical certainty	$y\bar{x} = t_c\left[\frac{S_x}{(N-1)^{0.5}}\right]$ where: y = accepted error in the percent of the mean value (e.g., y = 10%) $\bar{x}$ = mean value of x_i (i = 1…n) S_x = standard deviation t_c = confidence coefficient (e.g., 90% or $t_{0.95}$) N = number of samples	Håkanson 1984

Table 5.3 Typical Listing of Sample Size Equations That Are Useful for Environmental Research *(Continued)*

Objective	Formula	Ref.
To determine the number of samples required to give a result with a specific confidence limit	$N = \frac{(t_1 + t_2)^2}{d^2}\ S$ where: t_1 = one-tailed t value with $N - 1$ d.f. corresponding to a level of confidence t_2 = one-tailed t value with $N - 1$ degrees of freedom corresponding to the probability that the sample size will be adequate to achieve the desired precision S = sample standard deviation d = the acceptable range of variation for the variable being measured	Gad and Weil 1988
To determine the number of samples required to achieve a maximum acceptable error	$n = \frac{Z^2\sigma^2}{E^2}$ where: n = number of samples Z = Z statistic E = maximum acceptable error	Gilbert 1981
To determine the number of samples required to estimate a mean	$n = \frac{(Z_{\alpha/2})\sigma^2}{d^2}$ where: n = number of samples Z = Z statistic (standard normal curve) σ^2 = variance $\alpha/2$ = probability of a 95% confidence level d = the distance between the center of the lower confidence and the upper confidence bound	Milton et al. 1986
To determine the number of samples required for a particular power for: a) A normal distribution (i.e., $x > s^2$) b) A Poisson distribution (i.e., $x - S^2$) c)- A negative binomial distribution (i.e., $s < S^2$)	a) $N = \frac{10^4(t^2 s^2)}{(R^2 \bar{x}^2)}$ b) $N = \frac{10^4 t^2}{(R^2 \bar{x}^2)}$ c) $N = 10^4\left(\frac{t^2}{R^2}\right)\left[\left(\frac{1}{x}\right) + \left(\frac{1}{K}\right)\right]$ where: N = number of samples t = t statistic for a desired confidence level $\bar{x}$ = mean value from preliminary sampling or historical data s = standard deviation of mean R^2 = percentage coefficient of variation K = index of clumping	Kratochvil and Taylor 1981

Data from EC (Environment Canada). *Guidance Document on Collection and Preparation of Sediments for Physicochemical Characterization and Biological Testing.* Environmental Protection Series Report, EPS 1/RM/29. Ottawa, Canada. pp. 111–113, December, 1994.)

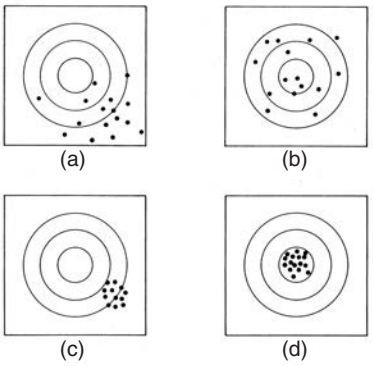


Figure 5.4 Accuracy definitions: (a) low precision, large bias, (b) low precision, small bias, (c) high precision, large bias, and (d) high precision, small bias (the only "accurate" case). (From Gilbert, R.O. *Statistical Methods for Environmental Pollution Monitoring*. Van Nostrand Reinhold. New York. Copyright 1987. This material is used by permission of John Wiley & Sons.)

and determines if a decision should be changed. In some cases, statistically significant results may occur simultaneously with small data differences (usually if low variations and/or large data sets are available). In this case, it may not be worthwhile, or feasible, to change a process or make other major changes.

Determining Sample Concentration Variations

An important requirement for using the above sampling effort equation is estimating the COV of the parameter of interest. In many cases, the approximate range of likely concentrations can be estimated for a parameter of interest. Figure 5.5 (Pitt and Lalor 2001) can be used to estimate the COV value for a parameter by knowing the 10th and 90th percentile ratios (the "range ratio"), assuming a log-normal distribution. Extreme values are usually not well known, but the approximate 10th and 90th percentile values can be estimated with better confidence. As an example, assume that the 10th and 90th percentile values of a water quality constituent of interest was estimated to be about 0.7 and 1.5 mg/L, respectively. The resulting range ratio is therefore 1.5/0.7 = 2.1 and the estimated COV value is 0.25.

Also shown in Figure 5.5 is an indication of the median value, compared to the 10th percentile value and the range ratio, assuming a log-normal distribution. As the range ratio decreases, the median comes close to the midpoint between the 10th and 90th percentile values. Therefore, at low COV values, the differences between normal distributions and log-normal distributions diminish, as stated previously. As the COV values increase, the mean values are located much closer to the 10th percentile value. In log-normal distributions, no negative concentration values are

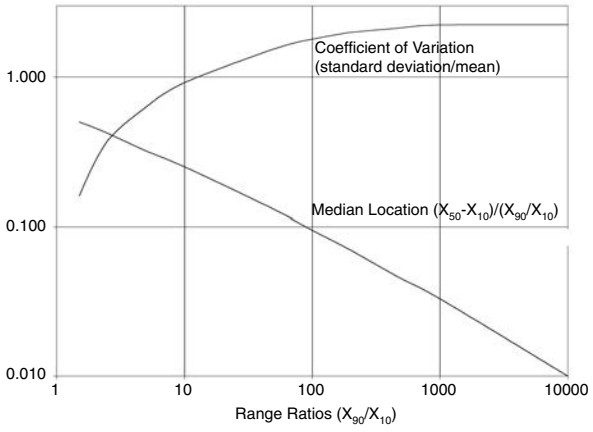


Figure 5.5 Determination of coefficient of variation from range of observa° tions (Pitt, R. and M. Lalor. *Identification and Control of Non-Stormwater Discharges into Separate Storm Drainage Systems. Development of Methodology for a Manual of Practice*. U.S. Environmental Protection Agency, Water Supply and Water Resources Division, National Risk Management Research Laboratory, Cincinnati, OH. 451 pp. To be published in 2001.)

allowed, but very large positive "outliers" can occur. In the previous example, the median location is about 0.4 for the range ratio of 2.1. The following calculation shows how the median value can be estimated using this "median location" value:

median location = 0.4 = $(X_{50} - X_{10})/(X_{90} - X_{10})$
therefore $X_{50} - X_{10} = 0.4(X_{90} - X_{10})$.
$(X_{90} - X_{10})$ = 1.5 mg/L – 0.7 mg/L = 0.8 mg/L.
Therefore $X_{50} - X_{10}$ = 0.4 (0.8) = 0.32 mg/L,
and X_{10} = 0.7 mg/L, X_{50} = 0.32 mg/L + 0.7 mg/L = 1.0 mg/L.

For comparison, the average of the 10th and 90th percentile values is 1.1 mg/L. Therefore, the concentration distribution is likely close to being normally distributed and the equation shown previously can be used to estimate the required number of samples needed because these two values are within about 10% of each other. The following paragraphs (from Pitt and Lalor 2001) show how log transformations of real-space data descriptors (COV and median) can be used in modifications of these equations.

Example of Log_{10} Transformations for Experimental Design Calculations

For relatively large COV values, it may be necessary to transform the data from known log-normal distributions (checked using log-normal probability paper, for example) before calculating the actual error associated with the collected data. Much urban receiving water quality data from the 10th to 90th percentile can typically be described as a normal probability distribution, after $\log_{10}$ transformations of the data. However, values less than the 10th percentile value are usually less than predicted from the log-normal probability plot, while values greater than the 90th percentile value are usually greater than predicted from the log-normal probability plot. Nontransformed water quality data do not typically fit normal probability distributions very well, except for pH (which are log transformed, by definition).

Figure 5.6 (Pitt and Lalor 2001) presents a relationship between the COV value in real space (nontransformed) and the standard deviation of $\log_{10}$ transformed data. Knowing the $\log_{10}$ transformed standard deviation values enables certain statistical experimental design features to be determined. The most significant feature is determining the number of observations needed to enable the data to be described with a specific error level. It can also be used to calculate the error associated with any observation, based on the assumed population distribution characteristics and the number of observations. As an example, consider a pollutant having a COV of 0.23 and a median value of 0.14. The resulting $\log_{10}$ transformed standard deviation would be about 0.12. One

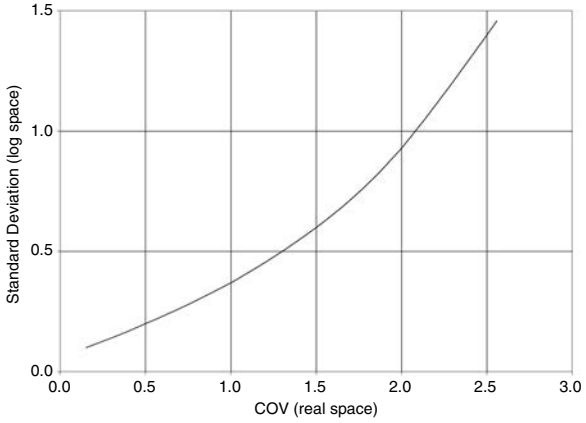


Figure 5.6 Relationship between COV (real space) and standard deviation ($\log_{10}$ space) (From Pitt, R. and M. Lalor. *Identification and Control of Non-Stormwater Discharges into Separate Storm Drainage Systems*. Development of Methodology for a Manual of Practice. U.S. Environmental Protection Agency, Water Supply and Water Resources Division, National Risk Management Research Laboratory, Cincinnati, OH. 451 pp. To be published in 2001.)

equation that has been historically used to calculate the number of analyses needed, based on the allowable error is (Cochran 1963):

$$\text{Number of samples} = 4(\text{standard deviation})^2/(\text{allowable error})^2$$

With an approximate 95% level of confidence ($1.96^2 \doteq 4$), this relationship determines the number of samples needed to obtain a value within the range of the sample mean, plus and minus the error. This equation can be rearranged to obtain the error, based on the number of samples obtained and the standard deviation. As an example, for 10 samples and the above standard deviation (0.12), the resulting approximate 95% confidence range (ignoring false negatives) of the median observation (0.14 mg/L) is:

$$\text{Error} = 2(0.12)/(10)^{0.5} = 0.076 \text{ in } \log_{10} \text{ space}$$

The confidence interval is therefore $\log_{10}(0.14) \pm 0.076$, which is –0.778 to –0.930 in $\log_{10}$ space. This results in an approximate 95% confidence range of $10^{-0.930}$ (= 0.12) to $10^{-0.778}$ (= 0.17). The absolute value for the error in the estimate of the median value is therefore between 14% ($100 \times (0.14 - 0.12)/0.14$) and 21% ($100 \times (0.17 - 0.14)/0.14$) for 10 samples. If the original untransformed data were used, the error associated with 10 samples is about 15%, within the range of the estimate after log transformations. These results are close because of the low COV value (0.23). If the COV value is large (>0.4), the need for log transformations increases.

Example Showing Improvement of Mean Concentrations with Increasing Sampling Effort

Many stormwater discharge samples were obtained from two study areas during the Bellevue, WA, Urban Runoff Program (Pitt 1985). The runoff from each drainage area was affected by different public works stormwater control practices, and the outfall data were compared to identify if any runoff quality improvements were associated with this effort. These data offer an opportunity to examine how increasing numbers of outfall data decreased the uncertainty of the overall average concentrations of the stormwater pollutants. Table 5.4 shows how the accumulative average of the observed concentrations eventually becomes reasonable steady, but only after a significant sampling effort. As an example: the average on the first three observations results in an EMC (event-mean concentration) that is in error by about 40%. It would require more than 15 samples before the average value would be consistently less than 10% from the seasonal average value, which only had a total population of 25 storm events, even with the relatively small COV value of 0.65.

Albert and Horwitz (1988) point out that taking averages leads to a tighter distribution. As shown above, the extreme values have little effect on the overall average, even with a relatively few observations (for a Gaussian distribution). The reduction in the standard deviation is proportional to $1/n^{0.5}$, for n observations. Even if the population is not Gaussian, the averages tend to be Gaussian-like. In addition, the larger the sample size, the more Gaussian-like is the population of averages.

Determining the Number of Sampling Locations (or Land Uses) Needed to Be Represented in a Monitoring Program

The above example for characterizing a parameter briefly examined a method to determine the appropriate number of samples to be collected and analyzed at a specific location. However, another aspect of sample design is determining how many components (specifically sampling locations) need to be characterized. The following example uses a marginal benefit analysis to help identify a basic characterization monitoring program. The sampling effort procedure discussed previously applies to the number of samples needed for each sampling location, while this analysis identifies

Table 5.4 Event-Mean Concentrations for Series of Storm Samples in Bellevue, WA

Storm No.	Lead Concentration (mg/L)	Moving Average Concentration (EMC)	Error from Seasonal Average (percent)
1	0.53	0.53	119
2	0.10	0.32	30
3	0.38	0.34	39
4	0.15	0.29	20
5	0.12	0.26	6
6	0.12	0.23	–3
7	0.56	0.28	16
8	0.19	0.27	11
9	0.38	0.28	16
10	0.23	0.28	14
11	0.20	0.27	11
12	0.39	0.28	16
13	0.53	0.30	24
14	0.05	0.28	16
15	0.26	0.28	16
16	0.05	0.27	10
17	0.05	0.25	5
18	0.39	0.26	8
19	0.28	0.26	8
20	0.10	0.25	5
21	0.29	0.25	6
22	0.18	0.25	4
23	0.31	0.25	5
24	0.10	0.25	2
25	0.10	0.24	0

From Pitt, R. *Characterizing and Controlling Urban Runoff through Street and Sewerage Cleaning*. U.S. Environmental Protection Agency, Storm and Combined Sewer Program, Risk Reduction Engineering Laboratory. EPA/600/S2-85/038. PB 85-186500. Cincinnati, OH. 467 pp. June 1985.

the number of sampling locations that should be monitored. This example specifically examines which land use categories should be included in a city-wide monitoring program when the total city's stormwater discharges need to be quantified with a reasonable error.

Land Use Monitoring for Wet-Weather Discharge Characteristics

The following paragraphs outline the steps needed to select the specific land uses that need to be included in a monitoring program to characterize stormwater runoff from an urban area to a specific receiving water. This method was also shown earlier in Chapter 4 for the Los Angeles County monitoring effort case study. The following example is loosely based on analyses of data for the Waller Creek drainage in Austin, TX.

Step 1 — This step identifies the land use categories that exist in the area of study. The information compiled during site selection activities will enable effective monitoring sites to be selected. In addition, this information will be very useful in extrapolating the monitoring results across the whole drainage area (by understanding the locations of similar areas represented by the land use-specific monitoring stations) in helping to identify the retrofit control programs that may be suitable for these types of areas, and in understanding the benefits of the most cost-effective controls for new development.

The initial list of land use areas to be considered for monitoring should be based on available land use maps, but they will have to be modified by overlaying additional information that should

have an obvious effect on stormwater quality and quantity. The most obvious overlays would be the age of development (an "easy" surrogate for directly connected imperviousness, maturity of vegetation, width of streets, conditions of streets, etc., that all affect runoff conditions and control measure applications) and the presence of grass swale drainage (which has a major effect on mass emissions and runoff frequency). Some of these areas may not be important (very small area represented in study area, especially with known very low concentrations or runoff mass) and may be eliminated at this step. After this initial list (with overlays) is developed, locations that are representative of each potential category need to be identified for preliminary surveys. About 10 representative neighborhoods in each category that reflect the full range of development conditions for each category should be identified. The 10 locations in each land use would be relatively small areas, such as a square block for residential areas, a single school or church, a few blocks of strip commercial, etc. The 10 sites would be selected over a wide geographical area of the study area to include topographical effects, distance from ocean, etc.

Step 2 — This step includes preliminary surveys of the land uses identified above. For each of the 10 neighborhoods identified in each category, simple field sheets are filled out with information that may affect runoff quality or quantity, including type of roof connections, type of drainage, age of development, housing density, socioeconomic conditions, quantity and maintenance of landscaping, condition of pavement, soils, inspections of storm drainage to ensure no inappropriate discharges, and existing stormwater control practices. These are simple field surveys that can be completed by a team of two people at the rate of about 10 locations a day, depending on navigation problems, traffic, and how spread out the sites are. Several photographs can also be made of each site and be archived with the field sheets for future reference.

Step 3 — In this step, measurements of important surface area components are made for each of the neighborhoods surveyed above. These measurements are made using aerial photographs of each of the 10 areas in each land use category. Measurements will include areas of rooftops, streets, driveways, sidewalks, parking areas, storage areas, front grass strips, sidewalks and streets, playgrounds, backyards, front yards, large turf areas, undeveloped areas, decks and sheds, pools, railroad rows, alleyways, and other paved and nonpaved areas. This step requires the use of good aerial photography in order to resolve the elements of interest for measurement. Print scales of about 100 ft per 1 inch are probably adequate, if the photographs are sharp. Photographic prints for each of the homogeneous neighborhoods examined on the ground in step 2 are needed. The actual measurements require about an hour per site.

Step 4 — In this step, the site survey and measurement information are used to confirm the groupings of the individual examples for each land use category. This step finalizes the categories to be examined, based on the actual measured values. As an example, some of the sites selected for field measurement may actually belong in another category (based on actual housing density, for example) and would then be reassigned before the final data evaluation. More important, the development characteristics (especially drainage paths) and areas of important elements (especially directly connected pavement) may indicate greater variability within an initial category than between other categories in the same land use (such as for differently aged residential areas, or high-density residential and duplex home areas). A simple ANOVA test would indicate if differences exist, and additional statistical tests can be used to identify the specific areas that are similar. If there is no other reason to suspect differences that would affect drainage quality or quantity (such as landscaping maintenance for golf courses vs. undeveloped areas), these areas could be combined to reduce the total number of individual land use categories/subcategories used in subsequent evaluations.

Step 5 — This step includes the ranking of the selected land use categories according to their predominance and pollutant generation. A marginal benefit analysis can be used to identify which

land use categories should be monitored. Each land use category has a known area in the drainage area and an estimated pollutant mass discharge. This step involves estimating the total annual mass discharges associated with each land use category for the complete study area. These sums are then ranked, from largest to smallest, and an accumulated percentage contribution is produced. These accumulated percentage values are plotted against the number of land use categories. The curve will be relatively steep initially and then level off as it approaches 100%. A marginal benefit analysis can then be used to select the most effective number of land uses that should be monitored.

The following is an example of this marginal benefit analysis to help select the most appropriate number of land uses to monitor. The numbers and categories are based on the Waller Creek, Austin, TX, watershed. Table 5.5 shows 16 initial land use categories, their land cover (as a percentage), and the estimated unit area loadings for each category for a critical pollutant. These loading numbers will have to be obtained using best judgment and prior knowledge. This table then shows the relative masses of the pollutant for each land use category (simply the % area times the unit area loading). The land uses are shown ranked by their relative mass discharges and a summed total is shown. This sum is then used to calculate the percentage of the pollutant associated with each land use category. These are then accumulated. The "straight-line model" is the straight line from 0 mass at 0 stations to 100% of the mass at 16 stations. The final column is the difference between these two lines (the marginal benefit).

Figure 5.7 is a marginal benefit plot of these values. The most effective monitoring strategy is to monitor seven land uses in this example. After this number, the marginal benefit starts to decrease. Seven (out of 16) land uses will also account for about 75% of the total annual emissions from these land uses in this area. A basic examination of the plot shows a strong leveling of the curve at 12 land uses, where the marginal benefit dramatically decreases and where there is little doubt of additional benefit for additional effort. The interpretation of these data should include the following issues that may expand the basic monitoring effort:

- The marginal benefit (as shown to include 7 of the 16 land uses for monitoring in this example)
- Land uses that have expected high unit area mass discharges that may not be included in the above list because of relatively low abundance, such as shopping malls in this example
- Land uses that are expected to become a significant component (such as the new medium-density residential area in this example)
- Land uses that have special conditions, such as a grass swale site in this example, that may need to be demonstrated/evaluated.

Step 6 — Final selection of monitoring locations. The top-ranked land uses will then be selected for monitoring. In most cases, a maximum of about 10 sites would be initiated each year. The remaining top-ranked land uses will then be monitored starting in future years because of the time needed to establish monitoring stations. In selecting sites for monitoring, sites draining homogeneous areas need to be found. In addition, monitoring locations will need to be selected that have sampling access, no safety problems, etc. To save laboratory resources, three categories of land uses can be identified. The top group would have the most comprehensive monitoring efforts (including most of the critical source area monitoring activities), while the lowest group may only have flow monitoring (with possibly some manual sampling). The middle group would have a shorter list of constituents routinely monitored, with periodic checks for all constituents being investigated.

Step 7 — The monitoring facilities need to be installed. The monitoring equipment should be comprised of automatic water samplers and flow sensors (velocity and depth of flow in areas expected to have surcharging flow problems), plus a tipping bucket rain gauge. The samples should all be obtained as flow-weighted composites, requiring only one sample to be analyzed per event at each monitoring station.

The sampler should initiate sampling after three tips (about 0.03 inches of rain) of the tipping bucket rain gauge at the sampling site. Another sample initiation method is to use an offset of the flow

Table 5.5 Example Marginal Benefit Analysis

	Land Use (ranked by % mass per category)	% of Area	Critical Unit Area Loading	Relative Mass	% Mass per Category	Accum. (% mass)	Straight-line Model	Marginal Benefit
1	Older medium-density residential	24	200	4800	22.8	22.8	6.25	16.5
2	High-density residential	7	300	2100	10.0	32.7	12.5	20.2
3	Office	7	300	2100	10.0	42.7	18.8	24.0
4	Strip commercial	8	250	2000	9.5	52.2	25.0	27.2
5	Multiple-family	8	200	1600	7.6	59.8	31.3	28.5
6	Manufacturing industrial	3	500	1500	7.1	66.9	37.5	29.4
7	Warehousing	5	300	1500	7.1	74.0	43.8	30.3
8	New medium-density residential	5	250	1250	5.9	80.0	50.0	30.0
9	Light industrial	5	200	1000	4.7	84.7	56.3	28.4
10	Major roadways	5	200	1000	4.7	89.4	62.5	26.9
11	Civic/educational	10	100	1000	4.7	94.2	68.8	25.4
12	Shopping malls	3	250	750	3.6	97.7	75.0	22.7
13	Utilities	1	150	150	0.7	98.5	81.3	17.2
14	Low-density residential with swales	5	25	125	0.6	99.1	87.5	11.6
15	Vacant	2	50	100	0.5	99.5	93.8	5.8
16	Park	2	50	100	0.5	100.0	100.0	0.0
	Total	100		21,075	100			

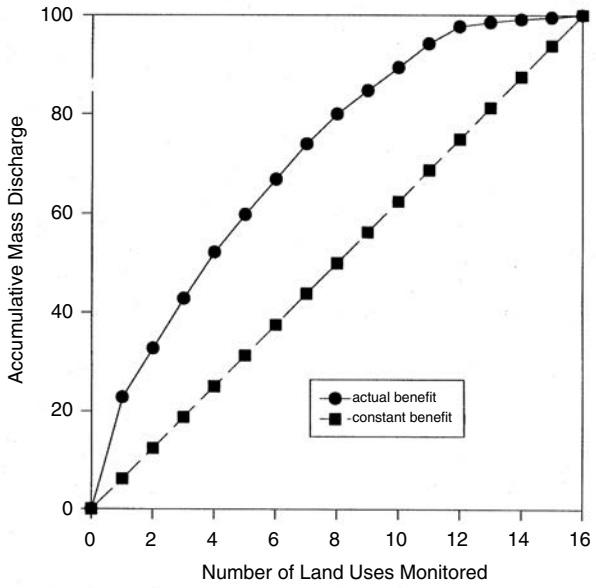


Figure 5.7 Marginal benefit associated with increasing sampling effort.

stage recorder to cause the sampler to begin sampling after a predetermined rise in flow conditions. False starts are then possible, caused by inappropriate discharges in the watershed above the sampling station. Frequent querying of sampler, flow, and rain conditions (using a data logger with phone connections) will detect this condition to enable retrieval of these dry-weather samples for analyses and to clean and reset the sampler. Both methods can be used simultaneously to ensure that only wet-weather samples are obtained. Of course, periodic (on random days about a month apart) dry-weather sampling (on a time composite basis over 24 hours) is also likely to be needed.

The base of the automatic sampler will need to be modified for a larger sample bottle (as much as a 100 L Teflon®-lined drum, with a 10 L glass bottle suspended for small events) in order to automatically sample a wide range of rain conditions without problems. A refrigerated base may also be needed, depending on ambient air conditions and sample holding requirements. The large drum will need to be located in a small freezer, with a hole in the lid where the sample line from the automatic sampler passes through.

Each sampler should also be connected to a telephone so the sampler status (including the temperature of the sample) and rainfall and flow conditions can be observed remotely. This significantly reduces personnel time and enables sampler problems to be identified quickly. Each sampler site will also need to be visited periodically (about weekly) to ensure that everything is ready to sample.

Step 8 — The monitoring initiation should continue down the list of ranked land use categories and repeat steps 6 and 7 for each category. At some point the marginal benefit from monitoring an additional land use category will not be sufficient to justify the additional cost.

While it is difficult to state how long this eight-step process should take, as a very rough estimate, it could take the following times to complete each step for a large city: Steps l to 3, 1 month each; Steps 4 and 5, 1 month combined; Step 6, 3 months; Step 7, 3 months; Step 8, continuous, for a total of about 10 months. This process was totally completed by Los Angeles County, for the unincorporated areas, in just a few months (see Chapter 4 case study).

Determining the Number of Samples Needed to Identify Unusual Conditions

An important aspect of receiving water effects studies is investigating unusual conditions. The methods presented by Gilbert (1987) (“Locating Hot Spots”) can be used to select sampling

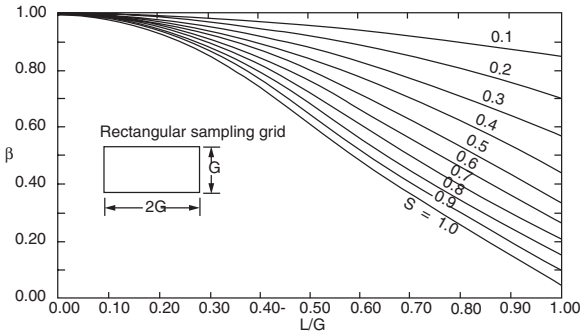


Figure 5.8 Sample spacing needed to identify unusual conditions. (From Gilbert, R.O. *Statistical Methods for Environmental Pollution Monitoring*. Van Nostrand Reinhold, New York. Copyright 1987. This material used with permission of John Wiley & Sons.)

locations that have acceptable probabilities of locating these unusual conditions. These methods are probably most applicable for lake or large stream sediment investigations in two dimensions. One-dimensional (longitudinal) studies can also be designed using a similar approach. Gilbert concluded that the use of a regular spacing of samples over an area was more effective when the contamination pattern was irregular, and an irregular pattern was best if the contamination existed in a repeating pattern. In almost all cases, unusual contamination has an irregular pattern and a regular grid is recommended. Gilbert presents square, rectangular, and triangular grid patterns to help locate sampling locations over an area. The sampling locations are located at the nodes of the resulting grids. Figure 5.8 (Gilbert 1987) is for the rectangular grid pattern, where the grid has a 2-to-1 aspect ratio. The figure relates the ratio of the size of a circular hot spot to the rectangular grid dimensions (sampling spacing) to the probability of detection. β is the probability of not finding the spot, while S is the shape factor for the hot spot (S = 1 for a circular spot; S = 0.5 for an elliptical spot). For example, if a semi-elliptical spot was to be targeted (S = 0.7) and the probability of not finding the spot was set at 25% (β = 0.25), the required L/G ratio would be 0.95+, with the rectangular width (G) about equal to the minor radius of the target.

Number of Samples Needed for Comparisons between Different Sites or Times

The comparison of paired data sets is commonly used when evaluating the differences between two situations (locations, times, practices, etc.). An equation related to the one given previously can be used to estimate the needed samples for a paired comparison:

$$n = 2\,[(Z_{1-\alpha} + Z_{1-\beta})/(\mu_1 - \mu_2)]^2\sigma^2$$

where α = false positive rate ($1 - \alpha$ is the degree of confidence. A value of α of 0.05 is usually considered statistically significant, corresponding to a $1 - \alpha$ degree of confidence of 0.95, or 95%)
β = false negative rate ($1 - \beta$ is the power. If used, a value of β of 0.2 is common, but it is frequently ignored, corresponding to a β of 0.5)
$Z_{1-\alpha}$ = Z score (associated with area under normal curve) corresponding to $1 - \alpha$
$Z_{1-\beta}$ = Z score corresponding to $1 - \beta$ value
μ_1 = mean of data set one
μ_2 = mean of data set two
σ = standard deviation (same for both data sets, same units as μ; both data sets are assumed to be normally distributed)

This equation is also only approximate, as it requires that the two data sets be normally distributed and have the same standard deviations. As noted previously, many parameters of interest in receiving water studies are likely closer to being log-normally distributed. Again, if the coefficient of variation

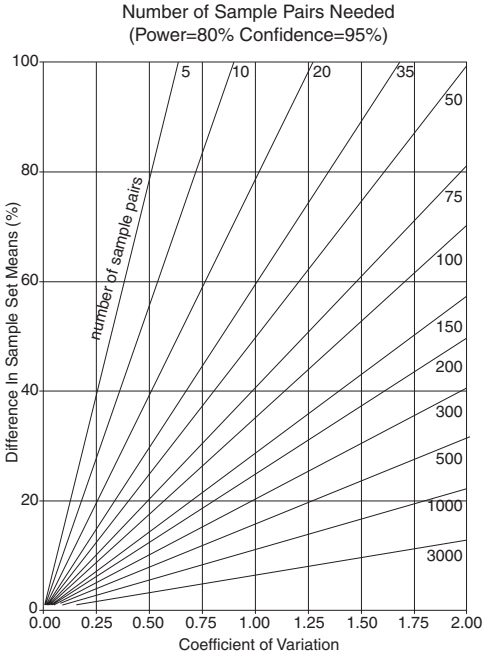


Figure 5.9- Sample effort needed for paired testing (power of 80% and confidence of 95%). (From Pitt, R. and K. Parmer. *Quality Assurance Project Plan: Effects, Sources, and Treatability of Stormwater Toxicants*. Con° tract No. CR819573. U.S. Environmental Protection Agency, Storm and Combined Sewer Program, Risk Reduction Engineer° ing Laboratory. Cincinnati, OH. February 1995.)

(COV) values are low (less than about 0.4), then there is probably no real difference in the predicted sampling effort. Also, variations after treatment are commonly lower than before treatment.

Figure 5.9 (Pitt and Parmer 1995) is a plot of this equation (normalized using COV and differences of sample means) showing the approximate number of sample pairs needed for an α of 0.05 (degree of confidence of 95%), and a β of 0.2 (power of 80%). As an example, 12 sample pairs will be sufficient to detect significant differences (with at least a 50% difference in the parameter value) for two locations, if the coefficient of variation is no more than about 0.5. Appendix A (Pitt and Parmer 1995) contains similar plots for many combinations of other levels of power, confidence, and expected differences.

Need for Probability Information and Confidence Intervals

The above discussions have presented information mostly pertaining to a simple characteristic of the population being sampled: the "central tendency," usually presented as the average, or mean, of the observations. However, much greater information is typically needed, especially when conducting statistical analyses of the information. Information concerning the probability distribution of the data (especially variance) was used previously as it affected the sampling effort. However, many more uses of the probability distributions exist. Albert and Horwitz (1988) state that the researcher must be aware of how misleading an average value alone can be, because the average tells nothing about the underlying spread of values. Berthouex and Brown (1994) also point out the importance of knowing the confidence interval (and the probability) of a statistical conclusion. It can be misleading to state simply that the results of an analysis are significant (implying that the null hypothesis, the difference between the means of two sets of data is zero, is rejected at the 0.05 level), for example, when the difference may not be very important. It is much more informative to present the 95% confidence interval of the difference between the means of the two sets of data.

One important example of how probability affects decisions concerns the selection of critical and infrequent conditions. In hydrology analyses, the selection of a "design" rainfall dramatically affects the design of a drainage system. Similarly, the likelihood of extreme events is also important for receiving water analyses (such as the frequency of high flushing flows vs. needed recovery

periods). The probability that a high flow rate in a stream (or any other factor of interest having a recurrence interval of "T" years) will occur during "n" years is:

$$P = 1 - (1 - 1/T)^n$$

As an example, the probability of a 5-year rain occurring at least once in a 5-year period is not 1, but is:

$$P = 1 - (1 - 1/5)^5 = 1 - (0.8)^5 = 1 - 0.328 = 0.67 \text{ (or 67\%)}$$

In another example, a flow having a recurrence interval of 20 years is assumed to cause substantial damage to critical biological species in a stream. That flow is likely to have the following probability of occurrence during a 100-year period:

$$P = 1 - (1 - 1/20)^{100} = 1 - (0.95)^{100} = 1 - 0.0059 = 0.994 \text{ (99.4\%)}$$

but only the following probability of occurrence during a 5-year period:

$$P = 1 - (1 - 1/20)^5 = 1 - (0.95)^5 = 1 - 0.774 = 0.227 \text{ (22.7\%)}$$

Figure 5.10 (McGee 1991) illustrates this equation. If a construction site is undergoing development for 2 years and the erosion control practices had to be certain of survival at least at the 95% level, then a 40-year design storm condition must be used! Similarly, a 1000-year design flow (one having only a 0.1% chance of occurring in any 1 year) would be needed if one needed to be 90% certain that it would not be exceeded during a 100-year period.

An entertaining example presented by Albert and Horwitz (1988) illustrates an interesting case concerning the upper limits of a confidence interval. In their example, an investigator wishes to

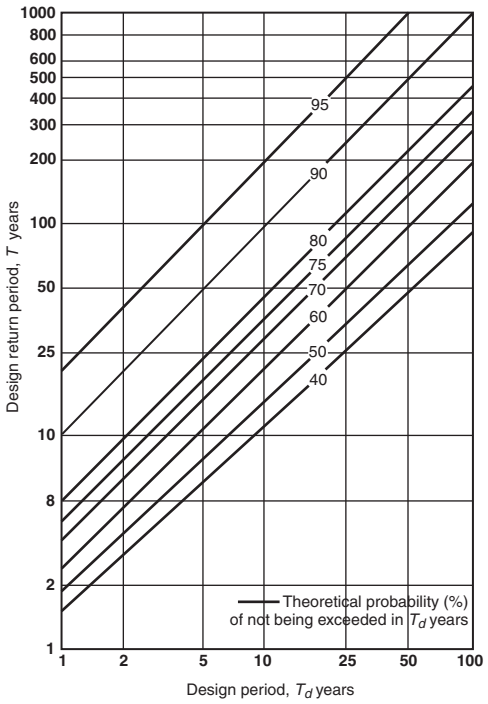


Figure 5.10 Design period and return period. (From McGee, T.J. *Water Supply and Sewerage*. McGraw-Hill, Inc., New York. 1991. With permission.)

determine if purple cows really exist. While traveling through a farming district, 20 cows are spotted, but none is purple. What is the actual percentage of cows that are purple (at a 95% confidence level), based on this sampling? The following formula can be used to calculate the upper limit of the 95% confidence interval:

$$(1 - 0)^n - (1 - x)^n = 0.95$$

or

$$1 - (1 - x)^n = 0.95$$

where n is the number of absolute negative observations and x is the upper limit of the 95% confidence interval. Therefore, for a sampling of 20 cows (n = 20), the actual percentage of cows that are purple is between 0.0% and 13.9% (x = 0.139). If the sample was extended to 40 cows (n = 40), the actual percentage of cows that are purple would be between 0.0% and 7.2% (x = 0.072). The upper limit of both of these cases is well above zero and, for most people, these results generally conflict with common sense. Obviously, the main problem with the above purple cow example is the violation of the need for random sampling throughout the whole population.

DATA QUALITY OBJECTIVES (DQO) AND ASSOCIATED QA/QC REQUIREMENTS

As noted in Chapter 4, the precision and accuracy necessary to meet the project objectives should be defined. After this is accomplished, the procedures for monitoring and controlling data quality must be specific and incorporated within all aspects of the assessment, including sample collection, processing, analysis, data management, and statistical procedures.

- When designing a plan, one should look at the study objectives and ask:
  - How will the data be used to arrive at conclusions?
  - What will the resulting actions be?
  - What are the allowable errors?

The first stage in developing DQOs requires the decision makers to determine what information in needed, reasons for the need, how the information will be used, and to specify time and resource limits. During the second stage, the problem is clarified and constraints on data collection identified. The third stage develops alternative approaches to data selection, selecting the optimal approach, and establishing the DQOs (EPA 1984, 1986).

Quality Control and Quality Assurance to Identify Sampling and Analysis Problems

Quality assurance and quality control (QA/QC) have been used in laboratories for many years to ensure the accuracy of analytical results. Unfortunately, similar formal QA/QC programs have been lacking in field collection and field analysis programs. Without carefully planned and executed sample collection activities, the best laboratory results are meaningless. Previous sections of this chapter have discussed the necessary experimental design aspects that enable the magnitude of the sampling effort to be determined. They specifically showed how the sample collection and data analysis efforts need to be balanced with experimental objectives. These sections stressed the need for a well-conceived experimental design to enable the questions at hand to be answered. This section presents additional information for conducting a water sampling

program. These two discussions therefore contain information pertaining to "good practice" in conducting a field investigation and are therefore fundamental components of a QA/QC program for field activities.

This section reviews some of the aspects of conventional laboratory QA/QC programs that must also be used in field investigations of receiving water problems. This is not a comprehensive presentation of these topics suitable for conventional laboratory use. It is intended only as a description of many of the components that should be used in field or screening analyses. It is also suitable as a description of the QA/QC efforts that supporting analytical laboratories should be using and can help the scientist or engineer interpret the analytical reports.

Use of Blanks to Minimize and Identify Errors

Blanks are the most effective tools for assessing and controlling contamination, which is a common source of error in environmental measurements. Contamination can occur from many sources, including during sample collection, sample transport and storage, sample preparation, and sample analysis. Proper cleaning of sampling equipment and sample containers, as previously described, is critical in reducing contamination. The use of appropriate materials that contact the sample (sampling equipment and sample containers especially) was also previously noted as being critical in reducing sample contamination. Field handling of samples (such as adding preservatives) may also cause sample contamination. During the Castro Valley urban runoff study, Pitt and Shawley (1982) found very high, but inconsistent, concentrations of lead in the samples. This was especially critical because the several months' delay between sending the samples to the laboratory and receiving the results prevented repeating the collection and analysis of the suspect samples. After many months of investigation, the use of trip blanks identified the source of contamination. The glass vials containing the HNO_3 used for sample preservation were color-coded with a painted strip. The paint apparently had a high heavy metal content. When the acid was poured into the sample container in the field, some of it flowed across the paint strip, leaching lead into the sample. About 1 year of runoff data for heavy metals had to be discarded.

There are many types of blanks that should be used in monitoring programs. The following are typical blanks and their purpose:

- Instrument blank (system blank). Used to establish the baseline response of an instrument in the absence of the analyte. This is a blank analysis using only the minimal reagents needed for instrument operation (doesn't include reagents needed to prepare the sample); could be only ultrapure water.
- Calibration blank (solvent blank). Used to detect and measure solvent impurities. Similar to the above blank but only contains the solvent used to dilute the sample. This typically is the zero concentration in a calibration series.
- Method blank (reagent blank). Used to detect and measure contamination from all of the reagents used in sample preparation. A blank sample (using ultrapure water) with all reagents needed in sample preparation is processed and analyzed. This value is commonly subtracted from the analytical results for the samples prepared in the same way during the same analytical run. This blank is carried through the complete sample preparation procedures, in contrast to the calibration blank which doesn't require any preparation, but is injected directly into the instrument.
- Trip blank (sampling media blank). Used to detect contamination associated with field filtration apparatus and sample bottles. A known water (similar to sample) is carried from the laboratory and processed in the field in an identical manner as a sample.
- Equipment blank. Used to detect contamination associated with the sampling equipment. Also used to verify the effectiveness of cleaning the sampling equipment. A known water (similar to sample) is pumped through the sampling equipment and analyzed. Rinse water (or solvent) after the final equipment cleaning can also be collected and analyzed for comparison with a sample of the fluid before rinsing.

Quality Control

Standard Methods for the Examination of Water and Wastewater (1995) lists seven elements of a good quality control program: certification of operator competence, recovery of known additions, analysis of externally supplied standards, analysis of reagent blanks, calibration with standards, analysis of duplicates, and the use of control charts. These elements are briefly described below.

Certification of Operators

Adequate training and suitable experience of analysts are necessary for good laboratory work. Periodic tests of analytical skill are needed. A test proposed by *Standard Methods* (1995) is to use at least four replicate analyses of a check sample that is between 5 and 50 times the method detection limit (MDL) of the procedure. The precision of the results should be within the values shown in Table 5.6.

Recovery of Known Additions

The use of known additions should be a standard component of regular laboratory procedures. A known concentration is added to periodic samples before sample processing. This increase should be detected compared to a split of the same sample that did not receive the known addition. Matrix interferences are detected if the concentration increase is outside the tolerance limit, as shown in Table 5.6. The known addition concentration should be between 5 and 50 times the MDL (or 1 to 10 times the expected sample concentration). Care should be taken to ensure that the total concentration is within the linear response of the method. *Standard Methods* (1995) suggests that known additions be added to 10% of the samples analyzed.

Analysis of External Standards

These standards are periodically analyzed to check the performance of the instrument and the calibration procedure. The concentrations should be between 5 and 50 times the MDL, or close to the sample concentrations (whichever is greater). *Standard Methods* (1995) prefers the use of certified standards, which are traceable to National Institute of Standards and Technology (NIST) standard reference materials, at least once a day. Do not confuse these external standards with the standards used to calibrate the instrument.

Table 5.6 Acceptance Limits for Replicate Samples and Known Additions

Parameter	Recovery of Known Additions (%)	Precision of Low-Level (<20 × MDL) Duplicates (±%)	Precision of High-Level (>20 × MDL) Duplicates (±%)
Metals, anions, nutrients, other inorganics, and TOC	80–120	25	10
Volatile and base/neutral organics	70–130	40	20
Acid extractable organics	60–140	40	20
Herbicides	40–160	40	20
Organochlorine pesticides	50–140	40	20
Organophosphate pesticides	50–200	40	20
Carbamate pesticides	50–150	40	20

Data from *Standard Methods for the Examination of Water and Wastewater. 19th edition.* Water Environment Federation. Washington, D.C. 1995.

Analysis of Reagent Blanks

Reagent blanks must also be analyzed periodically. *Standard Methods* (1995) suggests that at least 5% of the total analytical effort be reagent blanks. These blanks should be randomly spaced between samples in the analytical run order, and after samples having very high concentrations. These samples will measure sample carry-over, baseline drift of the instrument, and impurity of the reagents.

Calibration with Standards

Obviously, the instrument must be calibrated with known standards according to specific guidelines for the instrument and the method. However, at least three known concentrations of the parameter should be analyzed at the beginning of the instrument run, according to *Standard Methods* (1995). It is also preferable to repeat these analyses at least at the end of the analytical run to check for instrument drift.

Analysis of Duplicates

Standard Methods (1995) suggests that at least 5% of the samples have duplicate analyses, including those used for matrix interferences (known additions), while other guidance may suggest more duplicate analyses. Table 5.6 presents the acceptable limits of the precision of the duplicate analyses for different parameters.

Control Charts

The use of control charts enables rapid and visual indications of QA/QC problems, which can then be corrected in a timely manner, especially while it may still be possible to reanalyze samples. However, many laboratories are slow to upgrade the charts, losing their main benefit. Most automated instrument procedures and laboratory information management systems (LIMs) have control charting capabilities built in. *Standard Methods* (1995) describes a "means" chart for standards, blanks, and recoveries. A means chart is simply a display of the results of analyses in run order, with the ±2 (warning level) and ±3 (control level) standard deviation limits shown. At least five means charts should be prepared (and kept updated) for each analyte: one for each of the three standards analyzed at the beginning (and at least at the end) of each analytical run, one for the blank samples, and one for the recoveries. Figure 5.11 is an example of a means chart. The pattern of observations should be random and most within the warning limits. Drift, or sudden change, should also be cause for concern, needing immediate investigation. Of course, if the warning levels are at the 95% confidence limit (approximate ±2 standard deviations), then approximately 1 out of 20 samples will exceed the limits, on average. Only 1 out of 100 should exceed the control limits (if at the 99% confidence limit, or approximate ±3 standard deviations).

Standard Methods (1995) suggests that if one measurement exceeds the control limits, the sample should be immediately reanalyzed. If the repeat is within acceptable limits, then continue. If the repeat analysis is again outside the control limits, the analyses must be discontinued and the problem identified and corrected. If two out of three successive analyses exceed the warning limits, another replicate analysis is made. If the replicate is within the warning limits, then continue. However, if the third analysis is also outside the warning limits, the analyses must be discontinued and the problem identified and corrected. If four out of five successive analyses are greater than ±1 standard deviation of the expected value, or are in decreasing or increasing order, another sample is to be analyzed. If the trend continues, or if the sample is still greater than ±1 standard deviation of the expected value, then the analyses must be discontinued and the problem identified and corrected. If six successive samples are all on one side of the average concentration line, and the

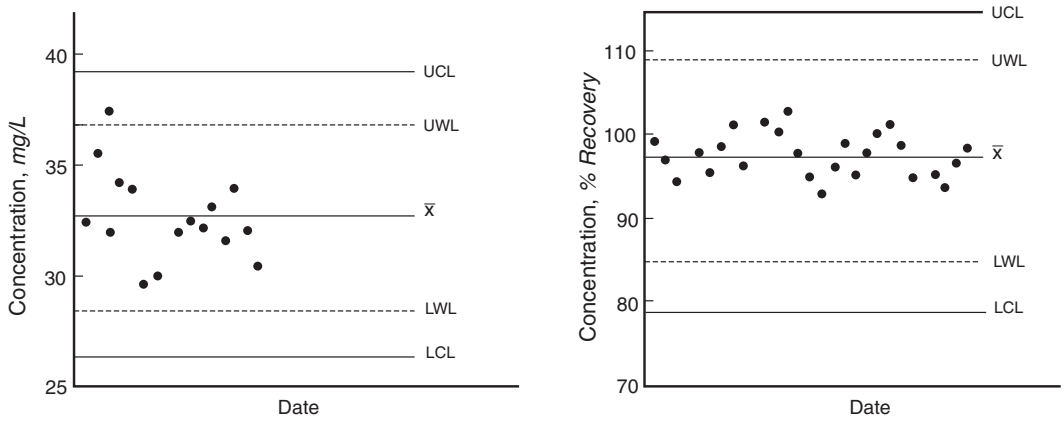


Figure 5.11 Means quality control chart (From *Standard Methods for the Examination of Water and Waste water. 20th edition*. Water Environment Federation. Washington, D.C. Copyright 1998. APHA. With permission.)

next is also on the same side as the others, the analyses must be discontinued and the problem identified and corrected. After correcting the problem, *Standard Methods* (1995) recommends that at least half the samples analyzed between the last in-control measurement and the out-of-control measurement be reanalyzed.

Standard Methods (1995) also points out that another major function of control charts is to identify changes in detection limits. Recalculate the warning and control limits (based on the standard deviations of the results) for every 20 samples. Running averages of these limits can be used to easily detect trends in precision (and therefore detection limits).

Carrying out a QA/QC program in the laboratory is not inexpensive. It can significantly add to the analytical effort. ASTM (1995) summarizes these typical extra sample analyses:

- Three or more standards to develop or check a calibration curve per run
- One method blank per run
- One field blank per set of samples
- At least one duplicate analysis for precision calculations for every 20 samples
- One standard sample to check the calibration for every 20 samples
- One spiked sample for matrix interference analyses for every 20 samples.

This can total at least eight additional analyses for every run of up to 20 samples.

Checking Results

Good sense is very important and should be used in reviewing analytical results. Extreme values should be questioned, for example, not routinely discarded. With a complete QA/QC program, including laboratory and field blanks, there should be little question if a problem has occurred and what the source of the problem may be. Unfortunately, few monitoring efforts actually carry out adequate or complete QA/QC programs. Especially lacking is timely updating of control charts and other tools that can easily detect problems. The reasons for this may be cost, ignorance, or insufficient time. However, the cost of discarded results may be very high, such as for resampling. In many cases, resampling is not possible, and much associated data may be worth much less without necessary supporting analytical information. In all cases, unusual analytical results should be reported to the field sampling crew and other personnel as soon as possible to solicit their assistance in verifying that the results are valid and not associated with labeling or sampling error.

Standard Methods (1995) presents several ways to check analytical results for basic measurements, based on a paper by Rossum (1975). The total dissolved solids concentration can be estimated using the following calculation:

$$TDS \cong 0.6\ (alkalinity) + Na + K + Ca + Mg + Cl + SO_4 + SiO_3 + NO_3 + F$$

where the ions are measured in mg/L (alkalinity as $CaCO_3$, SO_4 as SO_4, and NO_3 as NO_3). The measured TDS should be higher than the calculated value because of likely missing important components in the calculation. If the measured value is smaller than the calculated TDS value, the sample should be reanalyzed. If the measured TDS is more than 20% higher than the calculated value, the sample should also be reanalyzed.

The anion–cation balance should also be checked. The milliequivalents per liter (meq/L) sums of the anions and the cations should be close to 1.0. The percentage difference is calculated by (*Standard Methods* 1995):

$$\%\ \text{difference} = 100\ (\Sigma\ \text{cations} - \Sigma\ \text{anions}) / (\Sigma\ \text{cations} + \Sigma\ \text{anions})$$

with the following acceptance criteria:

Anion Sum (meq/L)	Acceptable Difference
0 to 3.0	±0.2 meq/L
3.1 to 10.0	±2%
10.1 to 800	±2 to 5%

In addition, *Standard Methods* (1995) states that both the anion and cation sums (in meq/L) should be 1/100 of the measured electrical conductivity value (measured as µS/cm). If either of the sums is more than 10% different from this criterion, the sample should be reanalyzed. The ratio of the measured TDS (in mg/L) and measured electrical conductivity (as µS/cm) values should also be within the range of 0.55 to 0.70.

Identifying the Needed Detection Limits and Selecting the Appropriate Analytical Method

The selection of the analytical procedure depends on a number of factors, including (in order of general importance):

- Appropriate detection limits
- Freedom from interferences
- Good analytical precision (repeatability)
- Minimal cost
- Reasonable operator training and needed expertise

One of the most critical and obvious determinants used for selecting an appropriate analytical method is the identification of the needed analytical detection limit. It is possible to select available analytical methods that have extremely low detection limits. Unfortunately, these very sensitive methods are typically costly and difficult to utilize. However, in many cases, these extremely sensitive methods are not needed. The basic method of selecting an appropriate analytical method is to ensure that it can identify samples that exceed appropriate criteria for the parameter being measured. If detection limits are smaller than a critical water quality criterion or standard, then analytical results that may indicate interference with a beneficial use can be selected directly. Appendix G presents water quality criteria for many constituents of concern in receiving water

studies, while Chapter 6 and Appendix E describe typical levels of performance for different analytical methods.

There are several different detection limits that are used in laboratory analyses. *Standard Methods* (1995) states that the common definition of a detection limit is that it is the smallest concentration that can be detected above background noise, using a specific procedure and with a specific confidence. The instrument detection limit (IDL) is the concentration that produces a signal that is three standard deviations of the noise level. This would result in about a 99% confidence that the signal was different from background noise. This is the simplest measure of detection and is solely a function of the instrument and is not dependent on sample preparation. The MDL accounts for sample preparation in addition to the instrument sensitivity. The MDL is about four times greater than the IDL because sample preparation increases the variability in the analytical results. Automated methods have MDLs much closer to the IDLs than manual sample preparation methods. An MDL is determined by spiking reagent water with a known concentration of the analyte of interest at a concentration close to the expected MDL. Seven portions of this solution are then analyzed (with complete sample preparation) and the standard deviation is calculated. The MDL is 3.14 times this measured standard deviation (at the 99% confidence level). The practical quantification limit (PQL) is a more conservative detection limit and considers the variability between laboratories using the same methods on a routine basis. The PQL is estimated in *Standard Methods* to be about five times the MDL.

A quick estimate of the needed detection limit can be made by assuming the likely concentration of the compound necessary for detection and the associated coefficient of variation (the COV, or the standard deviation divided by the mean) of the distribution of the analytical results, and applying a multiplier. If an estimated COV is not available, an alternative is to use the expected ratio of the 90th and 10th percentile values (the "range ratio") of the data and using Figure 5.5, assuming a log-normal probability distribution of the data (Pitt and Lalor 2001). Log-normal probability distributions are commonly used to describe the concentration distributions of water quality data, including stormwater data (EPA 1983a,b). The data ranging from the 10th to the 90th percentile can typically be suitably described as a log-normal probability distribution. However, values less than the 10th percentile value are usually less than predicted from the log-normal probability plot, while values greater than the 90th percentile value are usually greater than predicted from the log-normal probability plot. The range ratio can generally be selected easily based on the expected concentrations to be encountered, ignoring the most extreme values. As the range ratio increases, the COV also increases, up to a maximum value of about 2.5 for the set of conditions studied by Pitt and Lalor 2001.

Pitt and Lalor (2001) conducted numerous Monte Carlo analyses using mixtures having broad ranges of concentrations. Using these data, they developed guidelines for estimating the needed detection limits to characterize water samples. If the analyte has an expected narrow range of concentrations (a low COV), then the detection limit can be greater than if the analyte has a wider range of expected concentrations (a high COV). These guidelines are as follows:

- If the analyte has a low level of variation (a 90th to 10th percentile range ratio of 1.5, or a COV of <0.5), then the estimated required detection limit is about 0.8 times the expected median concentration.
- If the analyte has a medium level of variation (a 90th to 10th percentile range ratio of 10, or a COV of about 0.5 to 1.25), then the estimated required detection limit is about 0.23 times the expected median concentration.
- Finally, if the analyte has a high level of variation (a 90th to 10th percentile range ratio of 100, or a COV of about >1.25), then the estimated required detection limit is about 0.12 times the expected median concentration.

Reporting Results Affected by Detection Limits

Reporting chemical analysis results should be clear, based on the measured detection limits and QA/QC program. Concentrations below the IDL are not present with sufficient confidence to

detect them as significantly different from the baseline random noise of the instrument. These should be reported as not detected (generally given a "U" qualifier in organic compound analytical reports). Concentrations of a parameter above the IDL, but below the MDL, are present, but the confidence in the concentration value is less than 99% (can be given a "J" qualifier in organic analytical reports). Concentrations above the MDL indicate that the parameter is present in the sample and that the reported concentration is certain, at the 99% confidence level, or greater. Many other conditions may be present that degrade the confidence of the analytical results. These should all be carefully noted in the analytical report.

As noted in Chapter 7, nondetected ("left-censored") values present special problems in analyzing data. If only a few (or most) of the observations are below the detection limit, these problems are not very serious. However, if the detection limit available results in many left-censored data (say, between 25 and 75% of the observations), statistical analyses are severely limited. It may not be possible to statistically evaluate the effectiveness of a treatment process completely, for example, if many of the effluent concentrations of a critical pollutant are below the detection limit, even if the influent concentrations are well above the MDL. The removal of the pollutant is obviously important and effective, but it is not possible to calculate the significance of the differences in the observed concentrations. From a statistical (and engineering) viewpoint, it would be better if all concentrations determined by the analytical procedure be reported, even if they are below the designated "formal" detection limit, set using (extreme) 99% confidence limits. The use of the qualifiers (such as U and J as used in reporting GC/MS data) along with the numeric values and obvious reporting of the MDL should serve as a warning for the limited use of these values. However, analytical chemists are justifiably concerned about the misuse of "nondetected" values, and the availability of these values for statistical analyses will likely remain elusive. Unfortunately, nondetected values can be legally reported as "zero" in NPDES discharge reports, likely skewing mass calculations needed for TMDL, and other, evaluations.

GENERAL CONSIDERATIONS FOR SAMPLE COLLECTION

Sample collection and processing methods are dictated in part by the study objectives, regulatory requirements/recommendations, and proper QA/QC practice. The typical stormwater effects assessment will be comprised of in-stream water, sediment, and benthic invertebrate sampling. More intensive surveys may also sample other biological communities (e.g., fish, periphyton, zooplankton, phytoplankton, rooted macrophytes), watershed soils, interstitial sediment pore waters, dry- and wet-weather outfall effluents, and possibly sheet flows during rains. A number of publications have reviewed sampling methods which are applicable to stormwater assessments (Håkanson and Jansson 1983; EPA 1982, 1990c; ASTM 1991a).

It is important when sampling dynamic ecosystem components that there be an understanding that once the sample is collected and removed from the ecosystem, it no longer is a part of that ecosystem. It no longer will interact with the other ecosystem components spatially and temporally. A new ecosystem (the sample container) is created with different microenvironments, patch dynamics, and chemical transformations. For many sample constituents and parameters of concern, such as pesticides, suspended solids, and conductivity, the sampling process may do little to alter their levels from those present *in situ*. However, for other sample constituents and parameters, such as dissolved oxygen, un-ionized ammonia, metal speciation/solubility, microbial activity, pathogen survival, acid volatile sulfides, contaminant bioavailability, and toxicity, changes in the sample may be significant after sample collection. These changes cannot be predicted and are sample specific. Since the laboratory results of sample analyses are extrapolated to field conditions, these changes can potentially lead to erroneous conclusions on receiving water effects. Despite this bleak reality, accurate and precise studies have and can be conducted, provided proper sampling and processing practices are followed and there is an understanding

of method limitations, procedurally induced artifacts, and constituents interactions. There is no one optimal method by which to sample all streams and lakes. The major types of sampling activities are discussed in this chapter.

The discussion of the selection of analytical methods in Chapter 6 also includes information on field determinations. These may lessen these sample disturbance problems, but the typically less precise and less sensitive field methods may not offer a great advantage over the generally superior laboratory methods. Combinations or replicates of methods are therefore usually used (such as conducting both field and lab pH determinations and toxicity surveys), along with special tests to examine the effects of sample storage, to quantify possible sample modifications that may affect the analyte concentrations.

Discrete samples are needed for defining minimum and maximum values, for statistical analyses of point-in-time using replicates rather than composite samples, when constituents are labile, or when spatial variance at a site is to be measured. Continuous *in situ* monitors (discussed in Chapter 6) are also available to indicate real-time variations for key parameters (such as DO, temperature, conductivity, turbidity, pH, and ORP). These can be used to supplement composite analyses for a cost-effective solution compared to conducting only discrete analyses. Composites provide an estimate of the mean of the constituent (population) from which the individual samples are drawn. They should only be collected on an individual event or subevent basis, or for a defined time interval. Variance of the mean and precision cannot be obtained from a composite. Proper QA/QC requires that accuracy and precision be determined, which is usually not possible with compositing. Compositing reduces maximum and increases minimum values and thus is a better indicator of chronic, long-term exposure values (EPA 1990a). Coefficients of variation and errors can be based on EMCs (event mean concentrations) (EPA 1983a,b). There are much greater variations observed between different events than within events for most in-stream or outfall chemical conditions. Collecting discrete samples greatly increases the laboratory analytical costs, reducing the number of events represented. Clearly, the best sampling plan must be carefully selected based on the specific study requirements and usually includes components of several different basic approaches.

Samplers should be constructed of inert, nonreactive materials and capable of collecting the necessary sample volume. They must also be capable of programming to meet the specific sampling schedule and protocol needed for the specific study. There are many automatic water samplers that are relatively inexpensive and have a great deal of flexibility to meet many different project needs. However, some modifications may be needed, as described later in this chapter. Metal, low-density polyethylene, or polyvinyl chloride (PVC) samplers may slightly contaminate water samples with metals and organics, respectively. Sampler material is not as critical when sampling sediments because the quantity of contaminant contributed to the edge of the sample is not significant.

Basic Safety Considerations When Sampling

The most important factor when conducting a field monitoring program is personnel safety. If an adequate program cannot be carried out in a reasonably safe manner, an alternative to the monitoring program must be used. Similarly, an inadequate monitoring program would be hard to justify. Most of the hazards reflect site selection and sampling times. The use of automatic samplers and well-trained crews (more than one) will reduce many of the hazards.

Water and sediment sampling may expose field personnel to hazardous conditions. Obviously, water hazards (high flows, deep pools, soft sediments, etc.) are usually of initial concern. In many stormwater assessment studies, sampling during rainy weather in streams that may undergo rapid velocity and depth changes is necessary. Great care must be taken when approaching a stream in wet weather, as steep and slippery banks may cause one to slide into the water. Always sample in pairs and have adequate safety equipment available. At a minimum, this will include:

- Throw rope
- Inflatable life vests
- Nylon-covered neoprene waders (that offer some flotation, even when swamped)
- 2-way radio or cellular phone
- Weather radio

If the conditions warrant (such as with steep and slippery stream banks), the sampler personnel should be tied together, with an attachment to a rigid shore object. In all cases, only go into the stream if absolutely necessary. Try to collect all samples from shore, especially during heavy rains. Be extremely cautious of changing weather and stream conditions and cancel sampling when hazardous conditions warrant. Never enter a stream where your footing is unstable or if the water is too deep (probably more than 2 ft deep) or fast (probably more than 2.5 ft/s). Always enter the water cautiously and be prepared to make an efficient retreat if you feel insecure.

Other hazardous conditions may also occur when working near urban streams. Sharp debris in the water and along the banks require that protective waders be worn at all times while in the stream. No one should enter the water barefooted. Poison ivy, poison oak, and ticks thrive along many stream banks, requiring long pants and shirts. When in the field during sunny weather, sun screen and a hat are necessities. In many parts of the country, especially in the South, special caution is also required concerning snakes. Water moccasins are very common, and coral snakes and copperheads may also be present along streams. Again, waders offer some protection, but be careful when moving through thick underbrush where visibility is limited.

These cautions are necessary and are basically common sense. However, the greatest dangers associated with field sampling, especially in urban areas, are likely associated with dogs running loose, odd people, automobiles/trucks, and eating greasy fast food (dangers which are not restricted to stream sampling).

Selecting the Sampling Locations

Specific sampling locations are determined based on the objectives of the study and site-specific conditions. Obviously, safety is a prime consideration, along with statistical requirements expressed in the experimental design. In all cases, the sample must represent the conditions being characterized.

The process of selecting a sampling site is often given minimal thought when designing an assessment study. Site selections are driven by two basic criteria: accessibility/safety and upstream–downstream locations of pollutant discharges. However, given the ecosystem complexities and statistical concerns, the importance of this process in achieving representative samples and one's study objectives cannot be overemphasized. Stormwater runoff effects may not be detected unless the proper samples are obtained from the affected site during the critical time periods and compared to baseline conditions.

As described earlier in this chapter, random or nonrandom sampling plans are used to determine *within*-site sampling locations. Few studies follow a random selection process, but it is the preferred method allowing for quantitative analyses which meet statistical assumptions (EPA 1990c). Only by knowing the probability (from random selection) of selecting a specific sample can one extrapolate from the sample to the population in an objective way. Only by using a grid-random number approach may one consciously select sample locations without subconscious bias (EPA 1990c). This process only occurs after the measurements, station locations, and number of samples have been determined. (See Gilbert 1987 and EPA guidance for grid sampling and stratified random sampling for hot spots, as summarized earlier in this chapter.)

Because benthic community spatial distributions are related to habitat conditions, a simple random approach is not optimal. Rather, it is best to stratify the habitat types based on known physical differences and then select subsampling units in which randomization is used. See Ford and Turina (1985). Sampling increases precision and most likely accuracy. Strata which may be

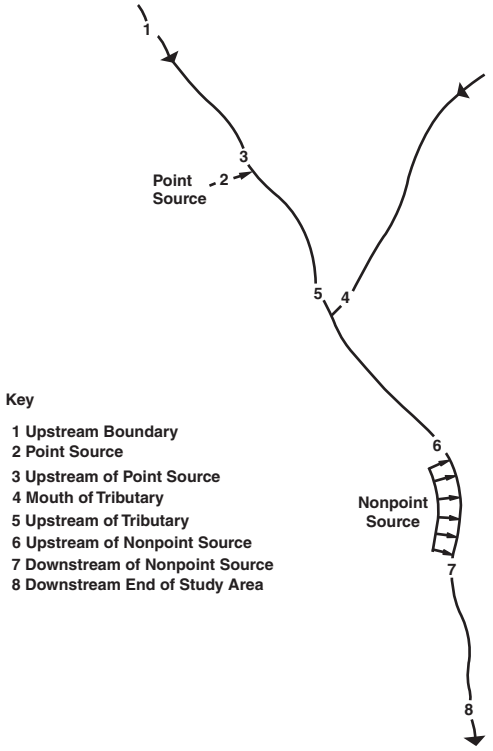


Figure 5.12 Recommended station locations for a minimal sampling program. (From EPA. *Handbook for Stream Sampling for Waste Load Allocation Applications*, Office of Research and Development, U.S. Environmental Protection Agency, Washington, D.C. EPA/625/6-86/013. 1986.)

used to define sampling units include habitat (pool vs. riffle), flow, temperature, sediment types, and others whose presence or effects may correlate to the parameter of interest. When locating sampling subunits in a nonrandom manner, one must consider samples semiquantitative for data extrapolation purposes (EPA 1990c).

Systematic sampling is often used in reconnaissance surveys and produces qualitative data. Samples are usually collected at key locations (e.g., a river bend) or at discrete intervals along a transect. This allows one to revisit fixed stations but ignores physical changes and disallows probability analyses. Kriging and other contaminant mapping techniques may be used when lake samples are collected using a systematic grid approach.

It is often more efficient and precise to have varying types of random sampling approaches for different parameters, such as: plankton — grid; macrophytes — shoreline transect; periphyton — shoreline transect. In small streams, fish and benthic macrobenthic sampling may be nonrandom, encompassing a total sub-reach section with true replication being impossible. This, of course, will violate some statistical assumptions.

Sites for sampling in a typical stream assessment are shown in Figure 5.12. Basic guidance for site location is as follows (modified from Cairns and Dickson 1971):

1. Two upstream reference stations are preferred, one immediately upstream of stressor inputs and one in upper reaches unimpacted by any anthropogenic influence. In addition, a nearby reference stream in the same ecoregion, which has similar watershed, flow, and habitat characteristics, is useful (EPA 1989).
2. Sample principal impact station, immediately below stressor inputs.
3. Note mixing patterns for point source inputs during subsampling.
4. Locate subsequent downstream stations based on pollutant loading, stream flow, sensitive areas, and suspected recovery–impact gradient. The maximum flow travel time between stations for conservative pollutants should be less than 2 days, and 5 to 8 km for reactive toxicants (EPA 1986). Sample station intervals are often about 0.5 day time-of-passage below a pollutant input for the

first 3 days, and 1 day thereafter (Kittrell 1969). In many urban streams, the sample locations are much more closely spaced, possibly only a few hundred meters apart, because of the large number of outfalls and frequent stream character variations due to artificial stream modifications. If a sample design is investigating the effects of a reach containing numerous outfalls on downstream waters, or possibly even an entire community, instead of a single discharge, wider spaced sampling locations below these areas would be needed.

5. Sample above and below tributaries.
6. Stations should have similar habitat and flow conditions, which typify the stream reach.
7. Samples should be replicated and collected in 1 day. Time of sampling must be noted, as many constituents have obvious natural diurnal cycles, e.g., dissolved oxygen (DO) and temperature. Sampling of indigenous communities such as periphyton, benthic invertebrates, and fish should occur as near as possible to the time that water quality samples are collected. In addition, weather conditions (air and water temperature, cloud cover, precipitation) during the sampling effort also should be noted. Riparian vegetation condition (especially seasonal growth) may also affect in-stream observations and also needs to be routinely noted.
8. Sampling should occur during each annual season in long-term studies to observe temporal cycles, seasonal stresses, and different organism groups and life stages.
9. Sampling should occur during a wide range of flow conditions.

Channel, flow, and stratification characteristics are particularly important when locating sample sites in streams, rivers, lakes, and reservoirs. Sampling near shore is seldom satisfactory except in small, upper reach streams. Whether using a random or systematic approach, one should carefully note the channel, flow, or stratification (lakes and reservoirs) conditions. In reservoirs, it is common for the principal flow to follow the old river channel and at a depth similar to the temperature (density) of the feeder stream. This area thus often contains the highest pollutant concentrations (e.g., suspended solids, fecal pathogens). Depositional zones, such as river bends and mouths, pools, and impoundment structures, should be sampled for sediment contamination and toxicity. For additional guidance on factors to consider in selecting station locations see below and Håkanson and Jansson (1983), and EPA (1983b, 1985, 1986, 1988, 1990 a,b,c).

As noted in Chapter 7, paired analyses are the most efficient sampling strategy. This can be simply sampling the influent and effluent of a control structure, outfalls of test and control watersheds, comparable stream habitats in test and control streams, or even the same stream sampling location, but at different seasons. Paired sampling can eliminate much variability, as many influencing factors are assumed to remain constant, enabling effects to be more easily seen. Obviously, if the differences between the two elements in the pair are expected to be large, and the background random variability is small, many fewer sampling pairs are needed to identify a statistically significant difference in the observations. Great care must be taken to select correct pairs, as the random variability can easily be greater than expected. Earlier sections of this chapter presented methods to determine the sampling effort for paired testing.

One example of likely inefficient paired sampling is sampling above and below an outfall in a stream. In almost all cases, the stream pollutant loads and flows are much greater than a single outfall discharge. Therefore, the differences expected in stream water quality upstream vs. downstream of an outfall would be very small and very difficult to detect. Exceptions may occur with large point source outfalls discharging during very low flow conditions. Otherwise, one large number is basically subtracted from another large number (with both having uncertainty) to determine the effects of a relatively small discharge. If this sampling strategy needs to be employed, make sure that the outfall discharge is also well characterized.

If loadings or stormwater concentrations of runoff from different land uses in a watershed are needed, then a sufficient number of examples need to be monitored. Many watersheds have several distinct land uses in their drainage area. It is important that a sufficient number of the land uses be adequately monitored in order to make an adequate mass balance. Examples of marginal benefits for increasing sampling locations was given earlier in this chapter and in Chapter 4.

The actual location of sampling is somewhat dependent on the type of sampler to be used. However, in all cases, the sample taken must be representative of the flow to be characterized. Permanently mounted automatic or semiautomatic samplers are most restricted in their placement, as security and better access is needed with them than with manual grab sampling. With manual sampling, less equipment is generally being carried to the sampling location (some type of manual dipper sampler, plus sample bottles, for example), while automatic samplers require a relatively large sample container, a multi-bottle sampler base, and batteries and other maintenance and cleaning supplies to be periodically carried to the sampler. Weekly visits to automatic samplers, at least, are needed for maintenance. In all cases, access during rains must be provided to all stormwater sampler locations. Manual stormwater sampling takes place during rains, of course, while automatic samplers may need to have their bottles switched during rains, or other checks made. Therefore, dangerous locations, such as those requiring steep ascents down clayey stream banks obviously must be avoided.

Permanently mounted samplers must have their intakes located to represent flow conditions. This is much easier with relatively small urban streams or outfalls compared to larger receiving waters. Wide, shallow, and fast-flowing streams are the most difficult to sample adequately. Great distances may be required before flows from individual discharges are completely mixed in these situations. Thomann and Mueller (1987) present the following USGS equation that can be used to estimate the distance needed before complete mixing occurs (for a side-stream discharge):

$$L_m = (2.6\ UB^2)/H$$

where U = the stream velocity in ft/s
B = the average stream width in feet
H = average stream width in feet

As an example, about 2000 m (6700 ft) may be required before complete mixing occurs for a stream that is 12 m (40 ft) wide, 1.5 m (5 ft) deep, and flowing at 2.4 m/s (8 ft/s). For a more typical urban stream with a 3 m (10 ft) width, 0.6 m (2 ft) depth, and flowing at 0.9 m/s (3 ft/s), the mixing length would be about 120 m (390 ft). Half of these distances would be needed if the discharge is located at the centerline of the stream (such as may occur for a diffuser for an industrial outfall). ASTM (1995) in standard D 3370 states that a distance of 1 to 3 miles below a tributary is usually sufficient to obtain complete mixing. It also suggests that samples be taken at least one half mile below dams or waterfalls to allow entrained air to escape.

These distances may be too great for many practical reasons, including the typical presence of numerous and fairly closely spaced outfalls along an urban creek (every several hundred feet). If it is not possible to site the sampler intake where the water will be well mixed, several sample intakes may be needed to obtain a composite sample across the stream. This can be accomplished by using several submerged pumps at different locations feeding a central large container located near the samplers. Automatic samplers are also restricted to a vertical height from the water surface to the sampler pump of about 7 m (since most use a peristaltic pump located on the sampler and therefore pull the water sample using vacuum suction). If the sampler height is greater than this critical height, a submerged pump can also be used to solve this problem. The automatic sampler would then sample from the large container that the submerged pumps are discharging into. In most cases, the submerged pumps would run continuously (needing on-site AC power or solar-charged batteries) and the flow-weighted sampler would be programmed to appropriately sample from the composite container, based on measured flows in the stream. The excess flow from the multiple pumps would overflow the composite container. Chapter 4 presented a case study for Los Angeles County, where this was an important consideration. The sample velocity in the sampler lines must be at least 100 cm/s to minimize particulate settling in the sampling lines. Care must also be taken to select a pump and sampler line that will not contaminate the samples (require stainless steel, Teflon, or appropriate

plastic) and be easy to clean in the field. Manual pump samplers, discussed later, may be suitable when sampling wide or deep streams or rivers from a bridge or boat.

Obviously, care must be taken to locate the sampler intakes to minimize induced scour of sediments and to prevent clogging from debris. All submerged pumps can quickly fail if the pump draws coarse particles into the pump, but doesn't have enough velocity in the sample line to discharge most of them completely through the sample line. If the intake is located on a creek bottom, the water entering the sampler intake will likely scour sediment from the surrounding area. Locating the sampler intake on top of a small anchored concrete slab in the creek minimizes scour. Elevating the sampler intake above the creek bottom also minimizes scour, but presents an obstruction to flows and catches debris easily. Elevating the intake slightly is important in obtaining a better sample if the flow is vertically stratified. In some cases, sampler intakes can be successfully located on the downstream side of a bridge piling or pier. Do not locate the sample intake near any treated wood structure if heavy metals or organics are to be sampled. Bedload sampling is discussed later.

Locating a sampler intake in an outfall pipe presents other problems. Because the pipe is likely to be smaller than a receiving water, horizontal differences in water quality should not be a problem. However, vertical differences may occur. The sampler intake also presents a greater obstruction to the pipe flow and therefore has a greater tendency to catch debris. To ensure a well-mixed water sample, the intake can be placed in an area that has turbulent flow. This may decrease volatile components in the water sample, but typical automatic samplers are inappropriate for collecting samples for volatile analyses anyway. Locating the intake on the downstream side of a flow monitoring flume would help obtain a mixed sample. In addition, added obstructions (bricks and concrete blocks) can be cemented to the pipe above the sampling location to induce well-mixed conditions during low to moderate flows, being careful not to cause pooling of water and sedimentation. Obviously, flow measurements would not be taken where obstructions are used to mix the flow.

Manual sampling is much more flexible and can be modified to better represent the flow conditions at the time of sampling. Obviously, multiple dips across a stream, and at multiple depths, will result in a better representation of the stream than a single sampling location. Special manual samplers (described later) are needed to collect depth-integrated samples that may be needed for sediment transport studies.

The advantages of manual sampling compared to automatic sampling are offset by the time frame that is represented in the sample. A grab sample taken at a single time will not be as representative of a storm event as an automatic sampler taking subsamples from many time periods during the event, even considering multiple vs. single sampling points. A single sampling location will be subjected to varying conditions during the storm, including horizontal and vertical variations. However, if a single sampling location is consistently biased compared to the cross section of the stream, that needs to be recognized and corrected. Therefore, it is necessary to observe conditions in the stream during the sampling times as much as possible to detect any potential bias. A bias may be caused by currents or nearby discharges, for example, and may be visually observed if colored or turbid water is indicating current conditions near the sampler. A hand-held *in situ* probe that can measure turbidity (such as sold by YSI, Solomat, or Horiba) is extremely helpful in checking flow variations near the sampler intake. These probes can also be very helpful during manual grab sampling to measure the likely flow variabilities during the time of sampling. Other parameters are usually available on these probes (such as conductivity, temperature, DO, pH, and specific ions) that would also be helpful in these field checks.

Sampler and Other Test Apparatus Materials

A major concern when samples are analyzed for trace contaminants is the need to use sampling equipment that will have minimal effect on the sample characteristics. Most modern automatic water samplers have been continuously improved over the years, and current models are designed

Table 5.7 Potential Sample Contamination from Sampler Material

Material	Contaminant
PVC – threaded joints	Chloroform
PVC – cemented joints	Methylethyl ketone, toluene, acetone, methylene chloride, benzene, ethyl acetate, tetrahydrofuran, cyclohexanone, organic tin compounds, and vinyl chloride
Teflon	Nothing
Polypropylene and polyethylene	Plasticizers and phthalates
Fiberglass-reinforced epoxy material (FRE)	Nothing
Stainless steel	Chromium, iron, nickel, and molybdenum
Glass	Boron and silica

Data from Cowgill, U.M. Sampling waters, the impact of sample variability on planning and confidence levels, in *Principles of Environmental Sampling*. Edited by L.H. Keith. ACS Professional Reference Book. American Chemical Society. pp. 171–189. 1988.

to have little effect on sample quality. Teflon-lined sample tubing, special silicon peristaltic pump tubing, and glass sample bottles are all that contact the sample for automatic water samplers designed for monitoring toxicants and most other stormwater pollutants.

Careful selection of materials for manual samplers is just as important as for automatic samplers. Sediment samplers made with stainless steel are available to minimize sample contamination. Cole Parmer includes an extensive table in its standard catalog that lists chemical compatibility with different materials, including many plastics, elastomers, metals, and nonmetals. The effects listed include "no effect," "minor effect," "moderate effect," and "severe effect, not recommended." This guidance is mostly for material degradation and high concentrations of the chemicals, but it is useful when considering potential contamination problems.

Table 5.7 lists potential contaminants from some sampler materials (Cowgill 1988). It was found that extensive steam cleaning (at least five washings using steam produced from distilled water) practically eliminated all contamination problems. Cemented materials should probably be avoided, as is evident from Table 5.7. Threaded or bolted-together sampler components are preferable. ASTM (1995), in standard E 1391, recommends preconditioning samplers (plus test chambers and sample containers) before their first use. ASTM summarized research that found that all plastics (including Teflon) leached elements, but that this could be minimized with a 7-day leaching using a 1:1 solution of HCl and deionized water and then another 7 days in a 1:1 solution of HNO_3 in deionized water. Overnight soaking in these solutions was found to be adequate for glassware. Care should be taken, however, when soaking material for long periods in relatively strong acids. We have destroyed some plastic sampler components (including Delrin) after several days. Therefore, always conduct a soaking test to ensure compatibility and use the least aggressive cleaning method suitable.

Pitt et al. (1999) tested leaching potentials for many other materials that may be used in sampling apparatus and also pilot-scale treatment units (Table 5.8). The most serious problems occurred with plywood, including untreated wood. Attempting to seal the wood with Formica™ and caulking was partially successful, but toxicants were still leached. Lining large wooden boxes with cleaned plastic sheeting is probably more suitable than using the Formica lining. Fiberglass screening material, especially before cleaning, also causes a potential problem with plasticizers and other organics. PVC and aluminum may be acceptable sampling apparatus material, if phthalate esters and aluminum contamination can be tolerated. Pitt et al. (1999) used aggressive water (18 megohm water, prepared using ion exchange) when conducting their leaching tests. They were also conducted over a 3-day period (for worst-case conditions during treatability tests). The much shorter contact times associated with sampling (especially after the sampler has been rigorously cleaned) should result in minimal contamination problems when using sampling equipment that has been reasonably selected to avoid contamination of compounds of major interest.

These tables indicate that care must be taken when selecting and cleaning sampling equipment. The use of Teflon reduces most of the problems, but it is quite expensive. Delrin is almost

Table 5.8- Potential Sample Contamination from Materials Used in Sampler and Pilot-Scale Treatability Test Apparatus

Material	Contaminant
Untreated plywood	Toxicity, chloride, sulfate, sodium, potassium, calcium, 2,4-dimethylphenol, benzylbutyl phthalate, bis(2-ethylhexyl) phthalate, phenol, *N*-nitro-so-di-*n*° propylamine, 4-chloro-3-methylphenol, 2,4-dinitrotoluene, 4-nitrophenol, alpha BHC, gamma BHC, 4,4′-DDE, endosulfan II, methoxychlor, and endrin ketone
Treated plywood (CCA)	Toxicity, chloride, sulfate, sodium, potassium, hexachloroethane, 2,4° dimethylphenol, bis(2-chloroethoxyl) methane, 2,4-dichlorophenol, benzylbutyl phthalate, bis(2-ethylhexyl) phthalate, phenol, 4-chloro-3° methylphenol, acenaphthene, 2,4-dinitrotoluene, 4-nitrophenol, alpha BHC, gamma BHC, beta BHC, 4,4′-DDE, 4,4′-DDD, endosulfan II, endosulfan sulfate, methoxychlor, endrin ketone, and copper (likely), chromium (likely), arsenic (likely)
Treated plywood (CCA) and Formica	Toxicity, chloride, sulfate, sodium, potassium, bis(2-chloroethyl) ether,* diethylphthalate, phenanthrene, anthracene, benzylbutyl phthalate, bis(2° ethylhexyl) phthalate, phenol,* *N*-nitro-so-di-*n*-propylamine, 4-chloro-3° methylphenol,* 4-nitrophenol, pentachlorophenol, alpha BHC, 4,4′-DDE, endosulfan II, methoxychlor, endrin ketone, and copper (likely), chromium (likely), arsenic (likely)
Treated plywood (CCA), Formica, and silica caulk	Lowered pH, toxicity, bis(2-chloroethyl) ether,* hexachlorocyclopentadiene, diethylphthalate, bis(2-ethylhexyl) phthalate, phenol,* *N*-nitro-so-di-*n*° propylamine, 4-chloro-3-methylphenol,* alpha BHC, heptachlor epoxide, 4,4′-DDE, endosulfan II, and copper (likely), chromium (likely), arsenic (likely)
Formica and silica caulk	Lowered pH, toxicity, 4-chloro-3-methylphenol, aldrin, and endosulfan 1
Silica caulk	Lowered pH, toxicity, and heptachlor epoxide
PVC pipe	*N*-nitrosodiphenylamine, and 2,4-dinitrotoluene
PVC pipe with cemented joint	Bis(2-ethylhexyl) phthalate,* acenaphthene, and endosulfan sulfate
Plexiglas and Plexiglas cement	Naphthalene, benzylbutyl phthalate, bis(2-ethylhexyl) phthalate, and endosulfan II
Aluminum	Toxicity and aluminum (likely)
Plastic aeration balls	2,6-Dinitrotoluene
Filter fabric material	Acenaphthylene, diethylphthalate, benzylbutyl phthalate, bis(2-ethylhexyl) phthalate, and pentachlorophenol
Sorbent pillows	Diethylphthalate and bis(2-ethylhexyl) phthalate
Black plastic fittings	Pentachlorophenol
Reinforced PVC tubing	Diethylphthalate, and benzylbutyl phthalate
Fiberglass window screening	Toxicity, dimethylphthalate, diethylphthalate,* bis(2-ethylhexyl) phthalate, di° *n*-octyl phthalate, phenol, 4-nitrophenol, pentachlorophenol, and 4,4′-DDD
Delrin	Benzylbutyl phthalate
Teflon	Nothing (likely)
Glass	Zinc (likely)

* Signifies that the observed concentrations in the leaching solution were very large compared to the other materials. Not all of the heavy metals had been verified.

From Pitt, R. et al. *Stormwater Treatment at Critical Areas: The Multi-Chambered Treatment Train (MCTT)*. U.S. Environmental Protection Agency, Wet Weather Flow Management Program, National Risk Management Research Laboratory. EPA/600/R-99/017. Cincinnati, OH. 505 pp. March 1999.

as effective, is somewhat less expensive, and is much easier to machine when manufacturing custom equipment. Both of these materials are fragile and cannot withstand rough handling. They are therefore not appropriate for sediment sampling, but can be used to advantage in water samplers. Glass is not usable for most sampling equipment, but is commonly used in bench-scale tests and when storing and preparing samples. Glass presents a problem with heavy metals attaching to the glass walls, and zinc leaching out of the glass. It is a necessary material when analyzing organics, however. Stainless steel is preferred for most sediment samplers and for hardware for water samplers. Plastics should not be used if contamination by phthalate esters is to be avoided. Many adequate and inexpensive sampler apparatus can be made of plastics, especially if cements are not used. In all cases, careful cleaning and preconditioning has been

shown to significantly reduce the concentrations of the contaminants in the leach water, stressing the need to thoroughly clean and condition the sampling equipment.

Volumes to Be Collected, Container Types, Preservatives to Be Used, and Shipping of Samples

The specific sample volume, bottle type, and preservative requirements should be specified by the analytical laboratory used. *Standard Methods* (1995) lists the basic container requirements, minimum sample sizes, required preservative, and the maximum storage period before the analyses need to be conducted. Table 5.9 shows these guidelines for water samples, while Table 5.10 lists the guidelines for sediment and pore water samples. Care must be taken to handle the samples properly to ensure the best analytical results. Numerous losses, transformations, and increases in pollutant concentrations may occur if these guidelines are not followed. Some analyses should be conducted as soon as possible (within a few hours of sample collection, or preferably on-site or *in situ*). These include CO_2, chlorine residual, DO (unless fixed), iodine, nitrite, ozone, pH, and temperature. ORP (oxidation-reduction potential) is also in this category of required on-site analyses, even though not included in this table. Parameters that need to be analyzed within 24 hours of sample collection (same day) include acidity, alkalinity, BOD, cyanide, chromium VI (and other specific ionic forms of metals), taste and odor, and turbidity. Microorganisms are not shown on this table either, and need to be analyzed within 24 hours of sample collection. Most of the nutrients need to be analyzed within 2 days. Many parameters can be stored for long periods of time, after preservation, specifically total forms of most heavy metals (6 months) and extracted organic compounds (30 days). In some cases, it may be possible to deviate from these guidelines if site-specific testing is conducted to demonstrate acceptable pollutant stability. The most important guidelines are the bottle type and preservative. Some parameters may be able to undergo longer storage periods, but this must be tested for specific conditions. The required sample volumes are all much greater than needed for most modern laboratory procedures and may be reduced (with permission from the laboratory) if shipping costs or sample storage facilities are a concern. Make sure that extra sample is available to redo critical analyses if problems develop, however. Be sure to verify these guidelines with the newest version of *Standard Methods*.

Sample Volumes

The volume of water or sediment needed depends on the types of toxicity assays, physical and chemical analyses, and level of precision (replicate numbers) needed. Usually 1 to 2 L is adequate for physical and chemical analyses. For static (daily) renewal toxicity assays, the quantities needed vary with the assay (Table 5.11). Volumes listed for sediments may be excessive if the sediment contains little interstitial water, such as found in sand, gravel, or compacted sediments, and few interstitial water chemical analyses are to be conducted. It is recommended that un-ionized ammonia generally be determined on interstitial water of sediments. If using the ion-selective electrode method, about 100 mL of aqueous solution is needed.

The following example for determining the water volume needed for laboratory analyses is based on the requirements of the UAB Environmental Engineering Laboratory. We have developed analytical modifications that require minimal amounts of sample in order to decrease shipping costs and storage problems, plus enabling small-scale treatability tests. Obviously, it is critical that the laboratory specify the sample volume requirements to ensure enough sample is available. Table 5.12 summarizes the sample quantities collected for each set of analysis. Also shown in this table is whether the sample is filtered or unfiltered (for constituent partitioning analyses). As an example, the metallic and organic toxicants are analyzed in both unfiltered and filtered sample portions in order to determine the amount of the pollutants associated with particulates and the amount that are considered "soluble." Filtering is through 0.45 μm membrane filters (using all-glass filtering apparatus and membrane filters that are found to have minimal effects

Table 5.9- Summary of Special Sampling and Handling Requirements for Water and Wastewater Samples[a]

Determination	Container[b]	Minimum Sample Size (mL)	Sample Type[c]	Preservation[d]	Maximum Storage Recommended/ Regulatory[e]
Acidity	P, G(B)	100	g	Refrigerate	24h/14d
Alkalinity	P, G	200	g	Refrigerate	24h/14d
BOD	P, G	1000	g, c	Refrigerate	6h/48h
Boron	P (PTFE) or quartz	100	g, c	None required	28d/6months
Bromide	P, G	100	g, c	None required	28d/28d
Carbon, organic, total	G	100	g, c	Analyze immediately; or refrigerate and add H_3PO_4 or H_2SO_4 to pH<2	7d/28d
Carbon dioxide	P, G	100	g	Analyze immediately	0.25h/N.S.
COD	P, G	100	g, c	Analyze as soon as possible, or add H_2SO_4 to pH<2; refrigerate	7d/28d
Chloride	P, G	50	g, c	None required	28d
Chlorine, total, residual	P, G	500	g	Analyze immediately	0.25h/0.25h
Chlorine, dioxide	P, G	500	g	Analyze immediately	0.5 h/N.S.
Chlorophyll	P, G	500	g, c	Unfiltered, dark, 4°C Filtered, dark, –20°C (Do not store in frost-free refrigerator)	28d/–
Color	P, G	500	g, c	Refrigerate	48h/48h
Conductivity	P, G	500	g, c	Refrigerate	28d/28d
Cyanide: Total	P, G	1000	g, c	Add NaOH to pH>12, refrigerate in dark	24h/14d;24h if sulfide present
Fluoride	P	100	g, c	None required	28d/28d
Hardness	P, G	100	g, c	Add HNO_3 to pH<2	6 months/6months
Iodine	P, G	500	g, c	Analyze immediately	0.5h/N.S.
Metals, general	P(A), G(A)	1000	g, c	For dissolved metals filter immediately, add HNO_3 to pH<2	6months/6months
Chromium VI	P(A), G(A)	1000	g	Refrigerate	24h/24h
Mercury	P(A), G(A)	1000	g, c	Add HNO_3 to pH<2, 4°C, refrigerate	28d/28d
Nitrogen:					
Ammonia	P, G	500	g, c	Analyze as soon as possible or add H_2SO_4 to pH<2, refrigerate	7d/28d
Nitrate	P, G	100	g, c	Analyze as soon as possible or refrigerate	48h/48h (28d for chlorinated samples)
Nitrate + nitrite	P, G	200	g, c	Add H_2SO_4 to pH<2, refrigerate	1–2d/28d
Nitrite	P, G	100	g, c	Analyze as soon as possible refrigerate	None /48h
Organic, Kjeldahl	P, G	500	g, c	Refrigerate; add H_2SO_4 to pH<2	7d/28d
Oil and grease	G,wide-mouth calibrated	1000	g, c	Add HCl to pH<2, refrigerate	28d/28d
Organic compounds:		200			
MBAS	P, G	250	g, c	Refrigerate	48h/N.S.
Pesticides	G(S), PTFE-lined cap	1000	g, c	Refrigerate; add 1000 mg ascorbic acid/L if residual chlorine present	7d/7d until extraction 40d after extraction
Phenols	P, G PTFE-lined cap	500	g, c	Refrigerate add H_2SO_4 to pH<2	*/28d until extraction

Table 5.9- Summary of Special Sampling and Handling Requirements for Water and Wastewater Samples[a] *(Continued)*

Determination	Container[b]	Minimum Sample Size (mL)	Sample Type[c]	Preservation[d]	Maximum Storage Recommended/ Regulatory[e]
Purgeables* by purge and trap	G, PTFE-lined cap	2×40	g	Refrigerate; add HCl to pH<2; add 1000 mg ascorbic acid/L if residual chlorine present	7d/14d
Base/neutrals and acids	G (S), amber	1000	g, c	Refrigerate	7d/7d until extraction; 40d after extraction
Oxygen, dissolved:	G, BOD bottle	300	g		
Electrode				Analyze immediately	0.25h/0.25h
Winkler				Titration may be delayed after acidification	8h/8h
Ozone	G	1000	g	Analyze immediately	0.25h/N.S.
pH	P, G	50	g	Analyze immediately	0.25h/0.25h
Phosphate	G(A)	100	g	For dissolved phosphate filter immediately; refrigerate	48h/N.S.
Phosphorus, total	P, G	100	g, c	Add H_2SO_4 to pH<2 and refrigerate	28d/–
Salinity	G, wax seal	240	g	Analyze immediately or use wax seal	6 months/N.S.
Silica	P (PTFE) or quartz	200	g, c	Refrigerate, do not freeze	28d/28d
Solids	P, G	200	g, c	Refrigerate	7d/2-7d
Sulfate	P, G	100	g, c	Refrigerate	28 /28d
Sulfide	P, G	100	g, c	Refrigerate; add 4 drops 2N zinc acetate/100 mL; add NaOH to pH>9	28d/7d
Temperature	P, G	—	g	Analyze immediately	0.25h
Turbidity	P, G	100	g, c	Analyze same day; store in dark up to 24 h, refrigerate	24/h48h

[a] See *Standard Methods* for additional details. For determination not listed, use glass or plastic containers; preferably refrigerate during storage and analyze as soon as possible.
[b] P = plastic (polyethylene or equivalent); G = glass; G (A) or P(A) = rinsed with 1 + 1 HNO; G(B) = glass, borosilicate; G(S) = glass, rinsed with organic solvents or baked.
[c] g = grab; c = composite
[d] Refrigerate = storage at 4°C ± 2 °C, in the dark; analyze immediately = analyze usually within 15 min of sample collection.
[e] Environmental Protection Agency, Rules and Regulation, 40 CFR Parts 100-149, July 1, 1992. See this citation for possible differences regarding container and preservation requirements.
Note: N.S. = not stated in cited reference; stat = no storage allowed; analyze immediately.

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on constituent concentrations). The sample volumes that need to be delivered to the laboratory (where further filtering, splitting, and chemical preservation will be performed) and the required containers are as follows:

- Three 500 mL amber glass containers with Teflon-lined screw caps
- Three 500 mL HDPE (high-density polyethylene) plastic containers with screw caps

A total of 3 L of each water sample is therefore needed for comprehensive analyses. In addition to the water samples, collected sediment must be shipped in the following sample bottles:

- One 500 mL amber glass wide-mouth container with Teflon-lined screw cap
- One 500 mL HDPE (high-density polyethylene) wide-mouth plastic container

Table 5.10 Type of Container and Conditions Recommended for Storing Samples of Sediment or Pore Water

End Use	Container Type	Wet Weight or Volume of Sample	Temperature	Holding Time
	Sediment			
Particle size distribution	1 Teflon 2 Glass 3 High-density polyethylene containers or bags	250 g	4 to 40°C Do not freeze	<6 mo
Major ions and elements: Al, C, Ca, Cl, Cr, Fe, Fl, H, K, Mn, Na, P, S, Si, Ti (oxides and total)	1 Teflon 2 High-density polyethylene containers or bags	250 g	<2°C	<2 wk
Nutrients: NH_4-N, NO_2-N, NO_3-N, TKN, TC, TOC	1 Teflon 2 Glass with Teflon or polyethylene-lined cap	100 g	<2°C	<48 h
Trace elements: Ag, Ba, Be, Cd, Co, Cr, Cu, Hg, Li, Mn, Mo, Ni, Pb, Sb, Sr, Va, Zn	1 Teflon 2 High-density polyethylene containers or bags	250 to 500 g	<2°C or –20°C	<2 wk <6 mo
Organic contaminants	1 Stainless steel canisters 2 Aluminum canisters 3 Amber glass with aluminum-lined cap	250 to 500 g	<2°C or –20°C	<2 wk <6 mo
Sediments for toxicity tests where the suspected contaminants are metals	1 Teflon 2 Glass 3 High-density polyethylene bags or containers	1 to 3 L	<2°C	<8 wk preferably <2 wk
Sediments for toxicity tests where the suspected contaminants are organic(s)	1 Glass with Al- or polyethylene-lined caps 2 Teflon 3 Stainless steel 4 High-density polyethylene bags or containers	1 to 3 L	<2°C	<8 wk preferably <2 wk
Control and reference sediment for toxicity tests	1 Teflon 2 Glass 3 High-density polyethylene bags or containers	>15 L	<2°C	<12 mo[a]
	Pore Water			
Major ions and elements: Ca, Mg, Cl, Si, Fl, Na, SO_4, K, Al, Fe, acidity, alkalinity	1 Teflon 2 Amber glass with Teflon-lined lids 3 High-density polyethylene containers	40 mL	–20°C	<6 wk
Nutrients in pore water: NH_4-N, NO_2-N, NO_3-N, C (total organic), P (soluble reactive), DIC, DOC	4 Amber glass with Teflon-lined lids	40 mL	–20°C	<6 mo
P (total)	1 Amber glass with Teflon-lined lids	40 mL	–20°C or <2°C with 1 mL of 30% H_2SO_4 per 100 mL	<6 wk <2 wk
Trace elements (total) in pore water: Ba, Be, Cd, Cr, Cu, Co, Li, Mn, Mo, Ni, Pb, Sb, Sr, Va, Zn	1 Teflon 2 Polyethylene	10 to 250 g	–20°C or <2°C with 2 mL of 1 *M* HNO_3 per 1000 mL pore water	<6 mo <6 wk

Table 5.10 Type of Container and Conditions Recommended for Storing Samples of Sediment or Pore Water *(Continued)*

End Use	Container Type	Wet Weight or Volume of Sample	Temperature	Holding Time
Ag	1 Amber Polyethylene	250 mL	<2°C with 1 g Na_2 EDTA per 250 mL pore water	<6 wk
Hg	1 Teflon 2 Glass (Soviral/Wheaton)	100 mL	<2°C with 1 mL H_2SO_4 per 100 mL of pore water	<6 wk
Organic contaminants in pore water[b]	1 Amber glass with Al-lined caps 2 Amber glass with Teflon-lined caps	1000 mL	–20°C or <2°C acidified with H_2SO_4 or with the addition of 10 g Na_2SO_4 per L of pore water	<6 mo <6 wk
Organochlorine and PCBs	1 Amber glass with Al-lined caps 2 Amber glass with Teflon-lined caps	1000 mL	–20°C or <2°C	<6 mo <6 wk
Organophosphates	1 Amber glass with Al-lined caps 2 Amber glass with Teflon-lined caps	1000 mL	–20°C or <2°C acidified with HCl to pH 4.4	<6 mo <6 wk
PCP	1 Amber glass with Al-lined caps 2 Amber glass with Teflon-lined caps	1000 mL	–20°C or <2°C acidified with H_2SO_4 to pH <4 or preserved with 0.5 g $CuSO_4$ per liter or pore water	<6 mo <6 wk
Phenoxy acid herbicides	1 Amber glass with Al-lined caps 2 Amber glass with Teflon-lined caps	1000 mL	–20°C or <2°C with acidification to pH <2 with H_2SO_4	<6 mo <6 wk
PAHs	1 Amber glass with Al-lined caps 2 Amber glass with Teflon-lined caps	1000 mL	–20°C or <2°C	<6 mo <6 wk
Pore water[c] or elutriate for toxicity tests	1 Amber glass with Teflon-lined caps	1 to 3 L	2°C	<72 h

[a] These sediments should be monitored over this period of time to ensure that changes that might occur to the physicochemical characteristics are acceptable.

[b] It is very difficult to collect sufficient pore water for analyses of volatile organic compounds and aromatic organic compounds.

[c] It is very difficult to collect sufficient pore water for standard toxicity testing; however, smaller quantities will suffice if the experimental design of the test accommodates extraction of successive samples of sediment and/or compositing of within-station replicate samples. It should be recognized that once pore water that has been collected in situ is exposed to oxygen (e.g., air) it becomes geochemically distinct (Mudroch 1992). The Microtox toxicity test only requires a few mL of sample and could be used as an indicator of pore water toxicity.

Table 5.11 Sample Volumes Needed for Toxicity Testing[a]

	Aqueous Phase[b] (L)		Solid Phase[c] (g wet weight)	
Assay	Acute	Short-Term Chronic[d]	Acute	Short-Term Chronic
Fish	2.5	2.5	400	600
Zooplankton				
Daphnia magna or *pulex*	0.2	0.3	200	100
Ceriodaphnia dubia	0.2	0.3	200	100
Amphipod				
Hyalella azteca	2.5	—	1000[d]	1500
Midge				
Chironomus tentans or *C. riparius*	2.5	—	1000[d]	1500
Phytoplankton				
Selenastrum capricornutum	—	0.4	—	—
Microtox[e]	0.1	—	—	—
Chemical analyses[f]	2.0		1000	

[a] Screening only. Definitive assays to produce effect levels (e.g., LC50, NOEL) require testing of five concentrations (e.g., 100%, 50%, 25%, 12.5%, 6.25%).
[b] Surface or interstitial waters, elutriates, or effluents.
[c] Whole sediment or soil, overlain with site, reference, or reconstituted water.
[d] Exposure periods of 10 days.
[e] Definitive test.
[f] Routine chemical analyses of alkalinity, hardness, conductivity, pH, turbidity, temperature, and dissolved oxygen. For sediment samples, interstitial waters may be used for most analyses. Volume of sediment needed will depend on sediments water content. Ammonia and particle size measurements recommended when testing sediments.

Table 5.12 Example Water Volume Requirements for Different Analytes When Using Special Low-Volume Analytical Methods

Constituent	Volume (mL)	Filtered?	Unfiltered?
Total solids	100		Yes
Dissolved solids	100	Yes	
Turbidity	30	Yes	Yes
Particle size (by Coulter Counter MultiSizer IIe)	20		Yes
Conductivity	70		Yes
pH (also on-site or *in situ*)	25		Yes
Color	25		Yes
Hardness	100		Yes
Alkalinity	50		Yes
Anions (F^-, Cl^-, NO_2^-, NO_3^{2-}, SO_4^{2-}, and PO_4^{2-})	25	Yes	
Cations (Li^+, Na^+, NH_4^+, K^+, Ca^{2+}, and Mg^{2+})	25	Yes	
COD	10	Yes	Yes
Metals (Pb, Cr, Cd, Cu, and Zn)	70	Yes	Yes
Semivolatile compounds (by GC/MSD)	315	Yes	Yes
Pesticides (by GC/ECD)	315	Yes	Yes
Microtox toxicity screen	10	Yes	Yes

The following list shows the amounts of sediment sample generally required for different chemical and physical analyses:

Inorganic chemicals	90–1000 mL
Organic chemicals	50–2000 mL
TOC, moisture	100–300 mL
Particle size	230–500 mL

Petroleum hydrocarbons	250–1000 mL
Acute toxicity tests	1–3 L
Bioaccumulation tests	3–4 L
Pore water extraction	2 L (sediment and assay dependent)
Elutriate preparation	1 L (assay dependent)

Sample Containers

Aqueous samples for toxicity testing may be collected and shipped in plastic containers, e.g., Cubitainers®. Dark borosilicate glass with Teflon-lined caps is recommended for samples to be used for organics analyses. High-density polyethylene containers are needed when metals are to be analyzed. Metals can sorb to glass, and new glassware may have zinc contaminants. Polyethylene is not recommended when samples are contaminated with oil, grease, or creosote.

All containers have been shown to adsorb various organic contaminants (Batley 1989; Batley and Gardner 1977; Schults et al. 1992). Polytetrafluoroethylene (PTF), e.g., Teflon, glass, and stainless steel have been shown to adsorb metals and organic compounds, acting as ion exchangers. However, sediments have many more binding sites than the container walls, and likely decrease the significance of container-associated loss for short-term exposures.

Wide-mouth containers made of either Teflon or high-density polyethylene, with Teflon-lined or polypropylene screw caps, are available in a variety of sizes from any scientific supply company and are considered the optimal all-purpose choice for sediment samples collected for both chemical and toxicity testing. Wide-mouth, screw-capped containers made of clear or amber borosilicate glass are also suitable for most types of analyses, with the notable exception of sediment metals, where polyethylene or Teflon is preferred. In addition, if a sediment or pore water sample is to be analyzed for organic contaminants, amber glass bottles are recommended over plastic. It should be noted that glass containers have several disadvantages, such as greater weight and volume and susceptibility to breakage, particularly when they are filled with sediment and frozen. Plastic bags made of high-density polyethylene can also be used for storing wet or dry sediment samples for certain end uses. Generally, when the end use of the sample is known, Tables 5.9 and 5.10 (and the primary references) should be consulted for specific recommendations regarding type of container, volume, and storage times.

Precleaned sample containers can be obtained from I-Chem (through Fisher Scientific at 800-766-7000) or Eagle Picher (at 800-331-7425). Fisher's catalog numbers and prices are as follows:

I-Chem #	Fisher #	Approx. Cost	Description
241-0500	05-719-74	$35/case of 12	Wide-mouth amber 0.5 L glass jars with Teflon-lined lids and labels
311-0500	05-719-242	$68/case of 24	Wide-mouth 0.5 L HDPE jars with Teflon-lined lids and labels

Eagle Picher sample containers are as follows:

122-16A	$25/case of 12	Wide-mouth amber 0.5 L glass jars with Teflon-lined lids
151500WWM	$46/case of 24	Wide-mouth 0.5 L HDPE jar with Teflon-lined lids

Cleaning Sample Bottles

ASTM (1995) has listed bottle cleaning/conditioning requirements in standard D 3370. New glass bottles (unless purchased precleaned) must be preconditioned before use by filling them with water for several days. This conditioning time can be shortened by using a dilute solution

of HCl. ASTM also points out that polyethylene is the only suitable material for sample containers when low concentrations of hardness, silica, sodium, or potassium are to be determined (in conflict with the above recommendation that warned of using polyethylene for samples containing creosote, oils, or greases). All sample containers must also be sealed with Teflon (preferred) or aluminum-lined caps. The bottles must be washed using a protocol similar to that described below for sampling equipment. ASTM (1995), in standard E 1391, also recommended more stringent preconditioning of sample containers before their first use in critical toxicological testing, as noted above (7-day leaching using a 1:1 solution of HCl and deionized water and then another 7 days in a 1:1 solution of HNO_3 in deionized water for plastics. Overnight soaking in these solutions was found to be adequate for glassware. Again, take care, and test for damage before soaking equipment in strong acid solutions).

Minimum cleaning includes cleaning the samplers, including sampling lines, with domestic tap water immediately after sample retrieval. Components that can be taken to the laboratory (such as the containers in the automatic samplers) are washed using warm tap water and laboratory detergent (phosphate free), rinsed with tap water, then distilled water, and finally laboratory grade (18 megohm) water.

ASTM (1995) presents standard D 5088-90 covering the cleaning of sampling equipment and sample bottles. This guidance varies from the above ASTM standard. It recommends a series of washings, depending on the analyses to be performed. The first wash is with a phosphate-free detergent solution (with a scrub brush, if possible), followed by a rinse of clean (known characteristics) water, such as tap water. If inorganic analyses are to be performed (especially trace heavy metals), then the sample-contacting components of the equipment and the sample bottles need to be rinsed with a 10% solution of reagent grade nitric or hydrochloric acid and deionized water. The equipment is rinsed again. If organic analyses are to be performed (especially trace organic compounds by GC/MSD), then the sample-contacting components of the equipment and sample bottles must be rinsed with pesticide-grade isopropanol alcohol, acetone, or methanol. The equipment and bottles are then rinsed with deionized water and allowed to air dry. The cleaned equipment needs to be wrapped with suitable inert material (such as aluminum foil or plastic wrap) for storage and transport. If sample components, such as tubing, cannot be reached with a brush, the cleaning solutions need to be recirculated through the equipment. Be careful of potentially explosive conditions when using alcohol or acetone. Intrinsically safe sampling equipment that does not produce sparks with electronic contacts or from motors, or friction heat, should be used whenever possible. Obviously, work in a well-ventilated area and wear protective garments, including eye protection, when cleaning the sampling equipment with the acid or solvents.

ASTM also recommends that the equipment components that do not contact the sample be cleaned with a portable power washer or steam-cleaning machine. If these are not available, a hand brush must be used with the detergent solution.

Containers can be a potential source of contamination and must be cleaned before receiving a field sample of sediment or pore water. New glass and most plastics should be cleaned to remove residues and/or leachable compounds, and to minimize potential sites of adsorption (Environment Canada 1994). A recommended sequence of cleaning activities for sediment samples is detailed in Table 5.13. It should be noted that precleaned containers for water and sediment samples are commercially available and are used with increasing frequency in many sampling programs.

Different general cleaning procedures are recommended for inorganic vs. organic analyses of sediment and pore water samples (Table 5.13). However, it should be noted that there is no universal procedure for all projects; a specific cleaning method can be very effective for one element, but not sufficient for another (Mudroch and Azcue 1995). Special attention must be paid in cases where sediment samples are collected in one type of container and subsequently analyzed for different types of organic and inorganic compounds. In such cases, the cleaning procedure can be a source of contamination for some of the parameters of interest. For example, contamination problems have been reported in the determination of chromium when sodium dichromate solution was used to

Table 5.13 Cleaning Procedures for Containers Destined to Hold Sediment Samples

For determination of inorganic constituents in the sediment samples:
1. Scrub containers with phosphate-free soap and hot water
2. Wash in high-pressure tap water
3. Degrease with Versa Clean (Fisher) or similar soap bath for 24 hours
4. Soak in a 72-hour acid bath with reagent grade 6 *M* nitric acid; drain off acid and rinse with hot water
5. Rinse with double-distilled water and allow to dry in a particle-free environment
6. Place containers in heavy polyethylene bags
For determination of organic constituents in the sediment samples:
1. Scrub containers with phosphate-free soap and hot water
2. Wash with high-pressure tap water
3. Clean with detergent such as Versa Clean (Fisher) or similar
4. Rinse three times with organic-free water
5. Rinse twice with methyl alcohol
6. Rinse twice with dichloromethane
7. Dry in an oven at 360°C for at least 6 hours

clean glass containers, or nitrate contamination was introduced by washing the containers with nitric acid, and phosphate contamination was introduced by washing the containers with phosphate-containing detergents (Mudroch and Azcue 1995). In these situations, it is usually advisable to use separate containers made of appropriate material and cleaned following applicable procedures for the different types of analyses to be performed. Finally, the rigorous cleaning procedures outlined in Table 5.13 may not always be necessary, especially if the chemicals of interest in the samples are expected to be present at high concentrations. Thus, the choice of cleaning procedure often must be left to the professional judgment of principal scientists based on study objectives and expected levels of the parameters of interest.

Field Processing of Samples and Preparation for Shipping

Water Samples

If the samples are to be analyzed locally, the field collection bottles (such as the automatic sampler base with bottles) can be delivered directly to the laboratory for processing. We generally conduct all filtering and preservation in the laboratory if at all possible, as this lessens the severe problems associated with field filtration and acid handling. Critical parameters (pH, DO, ORP, temperature) are analyzed *in situ* or on-site. If samples cannot be delivered to the laboratory quickly, field filtration and preservation will be necessary. Samples need to be split and individually preserved, as described in *Standard Methods*. A commercial sample splitter is available from Markson Scientific (800-858-2243) (catalog # 6614K1455 at about $265 for a 14 L polyethylene churn sample splitter, with 4 and 8 L splitters also available, Figure 5.13). Cone splitters are much more effective than churn splitters when suspended solids and particle size analyses are critical. A sample splitter is also useful if numerous individual sampler bottles are to be combined as a composite. The appropriate sample volumes are poured into the splitter from the individual bottles; the composite sample is then agitated and drained into individual bottles for shipping or further processing.

Personnel should wear latex gloves and safety glasses when handling the samples. Sample containers should be filled with no remaining headspace to reduce the loss of volatile components. Samples collected for microbiological analyses or suspended solids, however, should have air space to allow for sample mixing prior to testing. The caps must be screwed on securely and taped shut to reduce the possibility of losing some of the sample. The chain-of-custody seal can then be applied over the sealing tape. The paper chain-of-custody seals are not adequate to seal the lids on the jars. Do not let the water samples freeze.

Figure 5.13 Churn splitter used to divide sample into individual bottles for separate preservative treatments and storage conditions, plus for preparing QA/QC split samples for independent analyses.

Sediment Samples

In the field, sediment samples can be stored temporarily in refrigerated units on board the sampling vessel, placed into insulated containers containing ice or frozen ice packs, or taken immediately to a local storage facility where they can be placed either in a freezer or a refrigerator. Dry ice can be used to freeze samples for temporary storage or transport, as long as its efficacy is known and the user is aware of the regulations regarding the transportation of samples stored in this manner.

Sediment samples for toxicity or particle size testing must not be frozen. While in transit to a storage facility or laboratory, frozen samples must not be thawed. Samples that have a recommended storage temperature of 4°C should be cooled to that temperature using ice or refrigeration prior to placement in the transport container. The transport container should be refrigerated to 4°C or contain sufficient ice or frozen gel packs to keep the samples at 4 (±3)°C during transport to the laboratory. Depending on the logistics of the operation, field personnel may either transport samples to the laboratory themselves or utilize an overnight courier service. Samples must not freeze during transport, and light should be excluded from the transport container.

If a container with a sediment sample is to be frozen, it should be filled to only two thirds of its volume. For studies in which it is critical to maintain the collected sediment under anoxic conditions, the headspace in the container should be purged with an inert gas (e.g., nitrogen) before capping tightly. If samples are to be stored at 4°C, containers can be filled to the rim and air excluded during capping. Clear glass containers are often wrapped tightly with an opaque material (e.g., clean aluminum foil) to eliminate light and reduce accidental breakage (Environment Canada 1994).

Shipping Samples

Once the samples are split/divided into the appropriate shipping bottles (and preserved, if needed), the sample container label should be filled out completely and logged onto a shipping list for each shipping container. Shipping containers are usually plastic coolers. There needs to be adequate packing (preferably as many “ice” packs as can fit, plus bubble wrap) inside the shipping container to ensure that the sample bottles do not rub or bang against each other en route. Newspapers (flat, not wadded) can be placed on top of the samples and ice packs, directly under the lid, to further fill up any extra volume. Do not use packing peanuts (especially the water-soluble type) to fill up space. Wrap glass bottles with bubble wrap. Use sufficient “blue ice” or other cooling packs to ensure the coolers stay cool during shipment. Do not use water ice. The coolers must also be securely taped shut (seal the seams) to minimize leakage if a bottle breaks during shipment.

The samples should be sent via overnight courier so they arrive while laboratory personnel are present and sufficient time is available to initiate the critical analyses immediately (unless special arrangements have been made with the laboratory). Always call to schedule a sample shipment and fax a confirmation of the sample shipping information. Always keep a copy of any sample identification sheets and send the originals (by mail, not in the coolers). Include a shipping list (and copy of appropriate sampling forms) in an envelope taped to the outside of the cooler.

Chain-of-Custody and Other Documentation

When the sample is collected, the bottle labels and chain-of-custody forms must be filled out. In many cases, additional field sheets containing site or sample information are also completed. Documentation of collection and analysis of samples requires all the information necessary to: (1) trace a sample from the field to the final result of analysis; (2) describe the sampling and analytical methodology; and (3) describe the QA/QC program (Mudroch and Azcue 1995; Keith et al. 1983).

Correct and complete field notes are absolutely necessary in any sampling program. Poor or incomplete documentation of sample collection can make analytical results impossible to interpret. The following items should be recorded at the time of sediment sampling (Mudroch and Azcue 1995):

1. Project or client number
2. Name of sampling site and sample number
3. Time and date of sample collection
4. Weather conditions (particularly wind strength and direction, air and water temperature)
5. Sample collection information
6. Type of vessel used (size, power, engine type)
7. Type of sampler used (grab, corer, automatic, etc.) and any modifications made to the sampler during sampling
8. Names of sampling personnel
9. Notes on any unusual events that occurred during sampling (e.g., problems with recovered samples or sampling equipment, observations of possible contamination)
10. Sample physical description including texture and consistency, color, odor, estimate of quantity of recovered samples by a grab sampler, length and appearance of recovered sediment cores
11. Notes on further processing of samples in the field, particularly subsampling methods, type of containers, and temperature used for sample storage
12. Record any measurements made in the field, such as pH and ORP

Bound notebooks are preferred to the loose-leaf type and should be kept in a room or container that will protect against fire or water damage. Whenever legal or regulatory objectives are involved, notebook data should be entered in ink, each page should be signed and witnessed, and all errors or changes should be struck through one time and initialed (Keith 1991).

When samples are transported to a laboratory, an inventory list of each individual sample should be included in the shipment, and a separate copy sent to the laboratory. The inventory list should indicate the required analyses for each enclosed sample. The transport container should be labeled properly, including a description of the contents, the destination, any special handling instructions, and phone numbers to call on arrival or in case of an emergency. It is highly recommended that laboratories receiving samples be alerted to their impending arrival, particularly if samples will arrive on a weekend or holiday, so that appropriate arrangements can be made for their receipt.

Samples collected for legal purposes typically require the use of strict chain-of-custody procedures during handling and transport. This includes preparing detailed documentation regarding sample collection, preparation, and handling. All transport containers must remain locked during transport to and from the sampling site. The name and signature of the person who collected the sample should be placed on each sample container and witnessed, and the label should be securely fastened to the container after the sample has been placed in it and the lid tightly secured.

Appropriate chain-of-custody forms must be filled out for each transport container, including a complete listing and description of the enclosed samples. Each transport should be locked during pickup, transit, and delivery and should have a tape seal to demonstrate that it has not been opened during transport. The chain-of-custody documentation must accompany the transport container, and every time the package changes hands, the transfer of responsibilities must be documented with names and signatures. A file of all documentation (e.g., signed package slips, waybills, chain-of-custody forms) should be established, and all samples must be kept in a locked area of the laboratory with restricted access. All documentation of the analytical procedures and results should be kept on file and in control of the laboratory and/or project QA/QC officer (EC 1994).

The typical information provided on a chain-of-custody form includes:

- The sampling location
- The sample identification number
- The type of test or analytical procedure
- The name of the person who relinquishes the samples
- The date and time of sample collection
- The date and time when samples are relinquished
- The name of the person who should receive the sampling results

Sample Preservation and Storage at the Laboratory

Once the samples arrive in the laboratory, they must be logged in, sorted for further processing, and filtered and preserved, as needed. In addition, the sample temperatures and the presence of ice in the coolers should be checked upon arrival in the laboratory to verify that the samples were kept below critical temperatures during shipping. A reading of pH and temperature is conducted as soon as the samples arrive, and bacteria analyses need to be started as soon as possible.

Within a day, chilled samples must be filtered. Glass filters used for suspended solids analyses typically contain large amounts of zinc that easily contaminates samples, therefore, membrane filters need to be used for filtered (dissolved) metal analyses. The filtered and unfiltered sample portions are then divided and preserved. The following is an example from the UAB environmental engineering laboratories:

- Unfiltered sample in two 250 mL amber glass bottles (Teflon-lined lids) (no preservatives) for total forms of toxicity, COD, and GC analyses (using MSD and ECD detectors)
- Filtered sample in one 250 mL amber glass bottle (Teflon-lined lids) (no preservative) for filtered forms of toxicity, COD, and GC analyses (using MSD and ECD detectors)
- Unfiltered sample in one 250 mL high-density polyethylene (no preservatives) for solids, turbidity, color, particle size, and conductivity
- Filtered sample in one 250 mL high-density polyethylene (no preservatives) for anion and cation analyses (using ion chromatography), hardness, dissolved solids, and alkalinity
- Unfiltered sample in one 250 mL high-density polyethylene (HNO_3 preservative to $pH < 2$) for total forms of heavy metal, using the graphite furnace atomic adsorption spectrophotometer
- Filtered sample in one 125 mL high-density polyethylene (HNO_3 preservative to $pH < 2$) for filtered forms of heavy metal, using the graphite furnace atomic adsorption spectrophotometer

All samples are chilled on ice or in a refrigerator at 4°C (except for the HNO_3-preserved samples for heavy metal analyses) and analyzed within the holding times shown below:

- Immediately after sample collection or upon arrival in the laboratory: pH and microorganisms
- Within 24 hours: toxicity, ions, color, and turbidity
- Within 7 days: GC extractions, solids, and conductivity
- Within 40 days: GC analyses
- Within 6 months: heavy metal digestions and analyses

Drying, freezing, and storage temperature all affect toxicity (ASTM 1991a). Significant changes in metal toxicity to cladocerans and microbial activity have been observed in stored sediments (Stemmer et al. 1990b). Recommended limits for storage of metal-spiked sediments have ranged from less than 2 to 5 days (Swartz et al. 1985), less than 2 weeks (ASTM 1991a; Nebeker et al. 1984), to 2 to 8 weeks (EPA 2000). Cadmium toxicity in sediments has been shown to be related to acid volatile sulfide (AVS) complexation (DiToro et al. 1991). AVS is a reactive solid phase sulfide pool that apparently binds some metals, thus reducing toxicity (DiToro et al. 1991). When anoxic sediments were exposed to air, AVS was volatilized. If a study intends to investigate metal toxicity and the sediment environment is anoxic, then exposure to air might reduce or increase toxicity due to oxidation and precipitation of the metal species or loss of AVS complexation. It is generally agreed that sediments used for toxicity testing should not be frozen (Schuytema et al. 1989; ASTM 1991), should be stored at 4°C with no air space or under nitrogen, and analyzed as soon as possible (Reynoldson 1987).

Samples should be handled and manipulated as little as possible to reduce artifact formation and constituent alteration. It is sometimes necessary to remove debris and predatory organisms from samples to be used for toxicity testing. As large a filter pore size as possible should be used to prevent removal of suspended solids, which affect toxicity. Dredge (grab) collected sediment samples (for toxicity testing) should be placed in wide-mouth containers which allow the sample to be gently stirred. The sediment should be stirred until it is a slurry or any overlying water is mixed into the sediment matrix. If necessary, the sample may be sieved to remove large debris and homogenize the particle size distribution. It may not be possible to remove all predatory or nontest organisms from whole sediment toxicity assays. Caution should be exercised when sieved samples are used for testing, as the particle size distribution, redox gradients, and other alterations have occurred which may affect toxicity responses and the accuracy of lab-to-field extrapolations. Sieving is recommended for macroinvertebrate analyses because it increases counting efficiency (see EPA 1990c for additional information).

Elutriate testing was developed by the U.S. Army Corps of Engineers to simulate a condition that occurs during a dredging operation. When dredging effects are a study objective, elutriate analysis should be included in the test design. Elutriate samples are prepared by mixing (shaking) a 1 to 4 ratio of sediment to water for 30 minutes. The mixture is allowed to settle for 1 hour, and the supernatant is used for testing. There are modified methods which mix for longer periods, mix by aeration, or filter the supernatant. It is important that the method used be consistent because any modification may alter the elutriate's characteristics. TCLP tests are also sometimes conducted to determine the leaching potential of sediments under more severe conditions.

Personnel Requirements

Personnel needed to carry out an effective monitoring program fall into several classifications. Obviously, project directors need to design the program to fulfill the project objectives while staying within the available resources. In many cases, a calculated monitoring program may be impossible to carry out because of insufficient monitoring opportunities (necessary length of monitoring period available, number of rain events expected, etc.). Obviously, the project personnel therefore need to understand the local conditions. The project directors also need a varied understanding of many components of the ecosystem being investigated (hydrology, biology, chemistry, land use, etc.). Project field staff must be able to collect samples in an efficient and safe manner and be capable of working under changing and uncomfortable conditions. In all cases, at least two people need to go into the field together. Selection of laboratory personnel depends on the analyses to be conducted, and candidates will likely need to have substantial wet-weather sample analysis experience. Statistical experts are also needed to assist in the project design and to help analyze the data. Some of this effort could be handled by volunteers, but most comprehensive monitoring programs will also require a substantial effort by highly trained

technical personnel. Obviously, volunteer support can be very successful from an economical and educational viewpoint. This is especially important in nonpoint source/watershed studies where local residents need to have a greater role in decision making and in taking responsibility for the watershed.

Uses of Monitoring Data and the Appropriate Use of Volunteers in Monitoring Programs

An increasingly common method to obtain water quality data in receiving waters affected by stormwater is through the use of volunteer programs. Typically, a group of interested people is recruited by a local environmental organization. These people are trained in the use of relatively simple field test kits and carry out relatively broad-based observations. Usually, these people obtain relatively frequent data from local waters that supplement regulatory agency monitoring efforts. Historically, the most common volunteer efforts have been conducted mostly by lake-shore property owners who take Secchi disk readings of lake water transparency. However, with decreasing budgets for regulatory agencies and decreasing formal monitoring efforts conducted by state agencies, volunteer monitoring programs are increasing. The objectives for the use of these data must still define the parameters to be measured and other aspects of the experimental designs (sampling locations, frequencies, etc.). All too often, volunteer monitoring programs are relatively unstructured and are restricted to parameters that are relatively simple to measure. They therefore cannot truly replace most professional monitoring programs, but can be good supplements. Recent evaluations of simple field test kits have also identified their limitations, along with their advantages (Day 1996).

Volunteer monitoring programs are currently being conducted by several hundred groups throughout the U.S. The following list shows the number of volunteer monitoring programs having specific objectives for the use of the data (EPA 1994):

Education	439
Problem identification	333
Local decisions	288
Research	226
Nonpoint source assessment	225
Watershed planning	213
Habitat restoration	160
Water classification and standards	127
Enforcement	120
Legislation	84
305b compliance	53

Most of these uses require accurate information, because the data may have profound effects on regulatory agency decisions. In many states, however, water quality monitoring data collected by anyone who is not an employee of the state regulatory agency is not admissible as evidence in court. The lack of adequate quality assurance and quality control plus legal chain-of-custody procedures (including proof that samples or observations were obtained where claimed) are the most obvious problems with volunteer collected data.

The users of volunteer-collected data are also varied. The following list indicates the numbers of volunteer monitoring programs collecting data used by various groups (EPA 1994):

State governments	319
Local governments	315
Advocacy groups	288
Federal government	156
University scientists	142

The types of data being collected by volunteer monitoring groups have greatly expanded since the early days of Secchi disk surveys. The following list shows the number of volunteer monitoring programs that are collecting specific information/data (EPA 1994):

Water temperature	377
pH	313
Dissolved oxygen	296
Macroinvertebrates	259
Debris cleanups	218
Habitat assessments	211
Nitrogen	205
Phosphorus	202
Turbidity	192
Coliform bacteria	184
Secchi disk transparency	177
Aquatic vegetation	173
Flow	157
Birds and wildlife	152
Fish	150
Watershed mapping	138
Rainfall	131
Photographic surveys	129
Salinity	101
Sediment assessments	100
Alkalinity	98
Pipe surveys	96
TSS/TDS	91
Construction site inspections	81
BOD	75
Hardness	71
Chlorides	62
Chlorophyll *a*	60
Metals	56
Pesticides	24
Other bacteria	24
Hydrocarbons	14

Many of these parameters are well suited for trained volunteers. They can conduct relatively low-cost observations, which require minimal sampling or analytical equipment costs, for temperature, salinity, debris cleanup, habitat assessments, Secchi disk transparency, watershed mapping, photographic surveys, pipe surveys, and construction site inspections. Most of the other parameters (including most of the chemical analyses) would require the use of analytical equipment.

Relatively simple field test kits have been marketed in the United States for the past 30 years that can evaluate many of these parameters. However, few of these kits are suitable substitutes for conventional laboratory procedures. With care, good "screening" observations can be obtained from many of these kits. The sample collector, kit user, and data user must be aware of the limitations and hazards associated with many of these kits. The main concerns include:

- Safety (safe and correctly labeled reagents and clear instructions, including disposal guidance)
- Adequate sensitivity for required use of data
- Problems with interferences
- Ease of use and level of training needed
- Cost

Tests recently conducted at the University of Alabama at Birmingham have evaluated numerous field test kits for these criteria (Day 1996). The results are summarized in Chapter 6.

RECEIVING WATER, POINT SOURCE DISCHARGE, AND SOURCE AREA SAMPLING

Samples can be collected by manual grab or automatic samplers, the latter being more expensive but often superior when conditions fluctuate rapidly or sporadically, or when available personnel are lacking. Automatic samplers are essential for the NPDES program when effluents are monitored for permit requirements. Many types of automatic samplers exist (e.g., see EPA 1982) and none is ideal for all situations. The following variables must be considered when selecting a sampler (EPA 1982):

- Water or effluent variation (flow and constituents)
- Suspended solids concentration, dissolved gases, and specific gravity of effluent
- Vertical lift required
- Maintenance

Commonly used water samplers are listed in Table 5.14 and are discussed later in this section.

Automatic Water Sampling Equipment

Automatic water samplers that are commonly used for stormwater monitoring are available from ISCO and American Sigma, among others (Figures 5.14 to 5.22). These manufactures have samplers that have very flexible programming capabilities specifically designed for stormwater sampling and designed for priority pollutant sampling. A simpler automatic sampler is the Masterflex self-contained composite sampler (from Forestry Suppliers, Inc., for about $1500). This sampler is restricted to composite sampling only on a time-increment basis, and there is little control over the sample volumes that can be obtained. However, it may be a worthwhile option for simple sampling needs.

The American Sigma (800-635-4567) samplers are an excellent example of a highly flexible automatic sampler (Figure 5.14). They have an integral flowmeter option and can directly connect to a liquid level actuator or a depth sensor. The depth sensor is placed in the storm drainage upstream of a flow monitoring device (such as a weir or flume, or any calibrated stage-discharge relationship can be used). The flow indicators can control sample initiation and/or sampling frequency. A rain gauge is also available that can be connected directly to the sampler. Rainfall data can therefore be logged by the sampler, along with flow information and sampling history. Rainfall can also be

Table 5.14 The Advantages and Disadvantages of Manual and Automatic Sampling

Type	Advantages	Disadvantages
Manual	Low capital cost Not a composite Point-in-time characterization Compensate for various situations Note unusual conditions No maintenance Can collect extra samples in short time when necessary	Probability of increased variability due to sample handling Inconsistency in collection High cost of labor[a] Repetitious and monotonous task for personnel
Automatic	Consistent samples Probability of decreased variability caused by sample handling Minimal labor requirement for sampling Has capability to collect multiple bottle samples for visual estimate of variability and analysis of individual bottles	Considerable maintenance for batteries and cleaning; susceptible to plugging by solids Restricted in size to the general specifications Inflexibility Sample contamination potential Subject to damage by vandals

[a] High cost of labor assumes that several samples are taken daily, large distances between sampling sites, and labor is used solely for sampling.

From EPA. *Handbook for Sampling and Sample Preservation of Water and Wastewater*, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH, EPA 600/4-82/029. 1982.

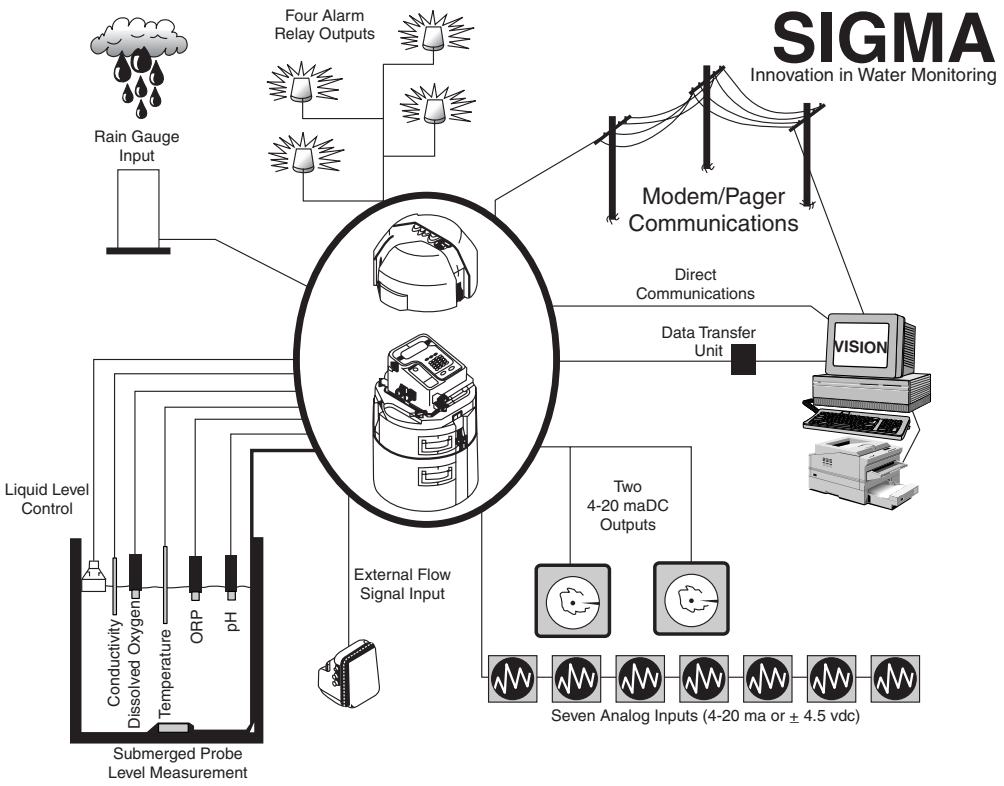

Figure 5.14 American Sigma connection options to ancillary equipment. (Used with permission.)

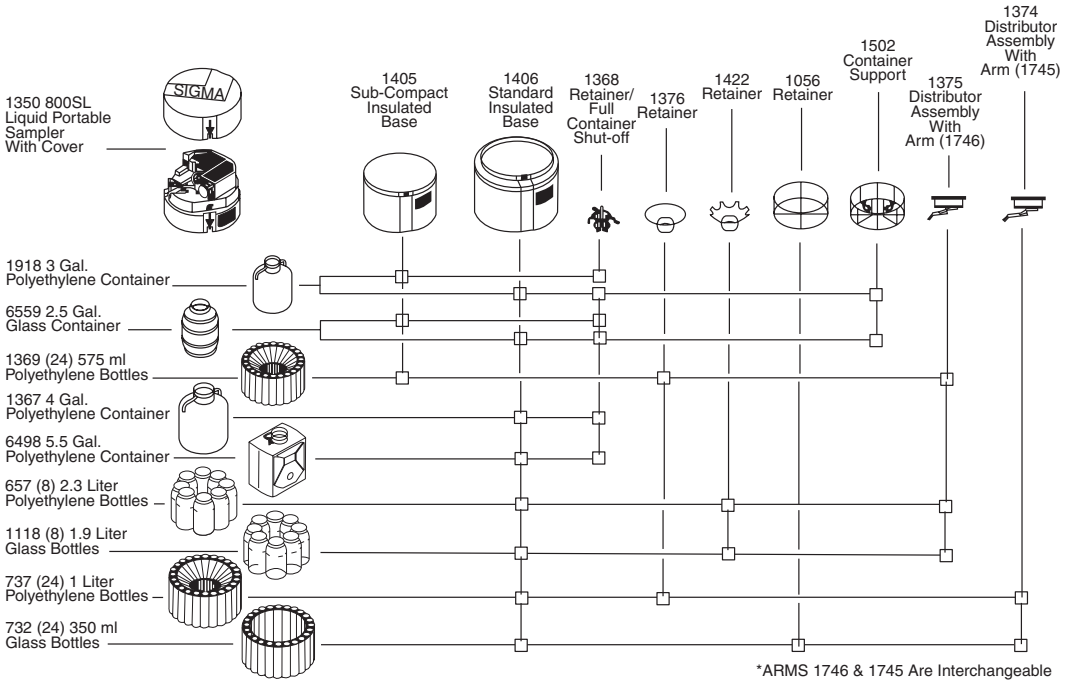

Figure 5.15 American Sigma sample bottle options. (Used with permission.)

Figure 5.16 Automatic ISCO sampler used to monitor snowmelt in Toronto, Ontario, manhole.

Figure 5.17 ISCO sampler used in instrument shelter with flow monitoring and telemetry equipment in Madison, WI.

Figure 5.18 Intermittent stream monitoring in Austin, TX.

Figure 5.19 Refrigerated automatic sampler located at detention pond outfall in Madison, WI.

used to trigger sample initiation. A solar panel is also available to keep the sampler's battery charged. Several sample bases and sample bottle options are also available (Figure 5.15). Single bottle composite sample bases are available having glass or polyethylene bottles from 2.5 to 5.5 gallons in volume. Up to four 1 gallon glass or polyethylene bottles can also be used to obtain composite samples over segments of the runoff event. In addition, several 24 bottle options are also available, with 575 mL or 1 L polyethylene bottles, or 350 mL glass bottles. American Sigma also has several AC-powered samplers that are refrigerated.

ISCO (800-228-4373) also offers a complete line of automatic water samplers that have been used for stormwater sampling for many years. Flowmeter and rain gauge options are available, along with numerous sample base and sample bottle options. ISCO also has several AC-powered refrigerated samplers. The ISCO 6100 sampler (about $8000, with bladder pump and special bottle rack for 40 mL VOC bottles) is especially designed to obtain samples for volatile analyses. Samples are collected directly in capped 40 mL VOC vials in the sampler, with minimal loss of volatile compounds. Very few volatile hydrocarbons have ever been detected in stormwater, so this sampler

Figure 5.20 Refrigerated automatic sampler in Madison, WI, instrument shelter.

Figure 5.21 Discrete sample bottle base for ISCO automatic sampler.

(and VOC analyses) would probably be used only for specialized studies where VOCs are expected (such as in commercial areas with older dry cleaners or near gasoline stations).

Sigma and ISCO also have new automatic samplers that interface with continuously recording water quality probes that can be used to control sampling during critical periods, irrespective of time or flow. McCrone (1996) describes American Sigma's options for using numerous probes (such as conductivity, DO, temperature, ORP, and pH). The sampler can be programmed to collect a special sample when any of these monitored parameters meets a preset criterion. ISCO has a new sampler series that interfaces with the YSI 6000 water quality probes, allowing specific water quality conditions to also trigger sampling (similar to Sigma's list, plus turbidity).

If a refrigerated sampler cannot be used (due to lack of AC power), ice may be used if sample chilling is needed. Ice is placed in the central cavity surrounded by the sample bottles in the sampler base. The ice must be placed soon before an expected storm event, as it will generally melt within a day. The placement of any sampler in a cool location (such as a manhole) is much preferred over placement in a small shelter that may heat excessively in the summer. In most cases, chilling stormwater during sample collection is not done due to lack of AC power and the inconvenience of using ice. If the sampler is located in a cool location and the samples retrieved soon after the storm has ended, few problems are expected. Bacteria sampling, for example, requires manual sampling to ensure sterile equipment and to minimize storage problems. VOC analyses have previously required manual sampling, but the VOC sampler from ISCO can be used for automatic sample collection. The use of probes to measure pH, ORP, and temperature *in situ* also reduces the need for manual samples for these parameters. Therefore, it is possible to conduct a stormwater sampling program using automatic samplers that do not require AC-powered refrigerated

Figure 5.22 Composite sample bottle from Toronto snowmelt sampler.

samplers, if supplemented with manual sampling for microorganism determinations, and if the samples are retrieved soon after the event has ended. Some analyses may not be available using automatically collected samples, and other options may need to be used to supplement the automatic sampling. In all cases, special storage tests can be used to determine the likely errors associated with long storage in the samplers, with and without chilling.

Required Sample Line Velocities to Minimize Particle Sampling Errors

Typical sample lines are Teflon-lined polyethylene and are 10 mm in diameter. Table 5.15 shows the particle sizes that would be lost in vertical sampling lines at a pumping rate of 30 and 100 cm/s. The water velocity in sample lines is about 100 cm/s, enabling practically all sediment to be transported to the sample containers. A water velocity of 100 cm/s (about 3 ft/s) would result in very little loss of stormwater particles. Particles of 8 to 25 mm would not be lifted in the sample line at all at this velocity, but these particles would not fit through the openings of the intake or even fit in most sample lines. They are also not present in stormwater, but may be a component of bedload in a stream, or gravel in the bottom of a storm drain pipe, requiring special sampling. Very few particles larger than several hundred micrometers occur in stormwater and these should only have a loss rate of 10% at the most. Most particles in stormwater are between 1 and 100 μm in diameter and have a density of between 1.5 and 2.65 g/cm^3. Even at 30 cm/s, these particles should experience insignificant losses. A pumping rate of about 100 cm/s would add extra confidence in minimizing particle losses. ASTM (1995) in method D 4411 recommends that the sample velocity in the sampler line be at least 17 times the fall rate of the largest particle of interest. As an example, for the 100 cm/s example above, the ASTM recommended critical fall rate would be about 6 cm/s, enabling a particle of several hundred micrometers in diameter to be sampled with a loss rate of less than 10%. This is certainly adequate for most stormwater sampling needs.

Automatic Sampler Line Flushing

Automatic samplers generally go through three phases when activated to collect a sample. First, the sample line is back-flushed to minimize sample cross-over and to clear debris from the sample intake. Next, the sample is collected. Finally, the sample is back-flushed again before going into a sleep mode to await the next sampling instruction. It can require several minutes to cycle through this process. A volume of 1850 mL of water fills a 10 mm (3/8 in) diameter sample line that is 7.5 m (25 ft) long. If a sample volume of 350 mL is to be collected for each sample interval, the following total volume of water is pumped by the sampler for each sample instruction:

Back-flush line	1850 mL
Fill tube	1850 mL
Collect sample	350 mL
Back-flush line	1850 mL

Table 5.15 Losses of Particles in Sampling Lines

	30 cm/s Flow Rate		100 cm/s Flow Rate	
% Loss	Critical Settling Rate (cm/s)	Size range (μm, for ρ = 1.5 to 2.65 g/cm^3)	Critical Settling Rate (cm/s)	Size Range (μm, for ρ = 1.5 to 2.65 g/cm^3)
100	30	2000–5000	100	8000–25,000
50	15	800–1500	50	3000–10,000
25	7.5	300–800	25	1500–3000
10	3.7	200–300	10	350–900
1	0.37	50–150	1	100–200

This totals about 6000 mL of water to be pumped. Typical automatic samplers have a pumping rate of about 3500 mL/min for low head conditions (about 1 m). It would therefore require about 1.7 min to pump this water. With pump reversing and slower pumping speeds at typical pumping heads, this could easily extend to 2 min, or more. If the sampler collects 3 L of sample instead of 350 mL, then another minute can be added to this sampling time for one cycle.

This sampler cycle time necessitates various decisions when setting up and programming a sampler, especially for flow-weighted composite sampling. The most important decisions relate to selecting the sampling interval that can accommodate expected peak flows and the sample volume needed for the smallest events to be sampled. Sample storage in the samplers is limited, further complicating the issue. The samplers are generally programmed to sample every 15 min to 1 hour for time-compositing sampling, or for an appropriate sample volume increment for flow-weighted sampling. If each sample increment is 0.25 L, a total of 40 subsamples can accumulate in a 10 L composite sample container.

Time or Flow-Weighted Composite Sampling

Automatic samplers can operate in two sampling modes, based on either time or flow increments. The sample bases can generally hold up to 24 bottles, each 1 L in volume. A single sample bottle of up to about 20 L is generally available for compositing the sample into one container. These bottle choices and the cycle time requirements of automatic samplers restrict the range of rain conditions that can be represented in a single sampler program for flow-weighted sampling. It is important to include samples from small rains (at least as small as 0.1 to 0.2 in) in a stormwater sampling program because they are very frequent and commonly exceed numeric water quality criteria, especially for fecal coliform bacteria and heavy metals. Moderate-sized rains (from about 0.5 to 2 in) are very important because they represent the majority of flow (and pollutant mass) discharges. The largest rains (greater than about 3 in) are important from a drainage design perspective to minimize flooding problems. It is very difficult to collect a wide range of rain depths in an automatic sampler using flow-weighted sampling. Conflicts occur between needing to have enough subsamples during the smallest event desired (including obtaining enough sample volume for the chemical analyses) and the resulting sampling frequency during peak flows for the largest sampling event desired. As an example, consider the following problem:

- Desired minimum rain to be sampled: 0.15 in in depth, 4-hour runoff duration, having a 0.20 Rv (volumetric runoff coefficient)
- Largest rain desired to be sampled: 2.5 in in depth, 12-hour runoff duration, having a 0.50 Rv
- The watershed is 250 acres in size and 3 samples, at least, are needed during the smallest rain

The calculated total runoff is therefore:

- Minimum rain: 0.10 (0.15 in) (250 ac) (ft/12 in) (43,560 ft^2/ac) = 13,600 ft^3
- Maximum rain: 0.50 (2.5 in) (250 ac) (ft/12 in) (43,560 ft^2/ac) = 1,130,000 ft^3

The average runoff flow rates expected are roughly estimated to be:

- Minimum rain: (13,600 ft^3/4 hr) (hr/3600 s) = 0.95 ft^3/s
- Maximum rain: (1,130,000 ft^3/12 hr) (hr/3600 s) = 26 ft^3/s

Using a simple triangular hydrograph, the peak flows are estimated to be about twice these average flow rates:

- Minimum rain: 1.9 ft^3/s
- Maximum rain: 53 ft^3/s

Actual peak flow rates are obviously related to the watershed time of concentration and other factors of the watershed and drainage system, but this triangular hydrograph has been found to roughly estimate high flows during small and moderate rains. It is certainly not an adequate procedure for drainage design, however. As the smallest storm is to be sampled three times during the runoff period, the volume of flow per subsample is simply:

$$13{,}600 \text{ ft}^3/3 \cong 4500 \text{ ft}^3$$

Therefore, the total number of samples collected during the maximum rain would be:

$$1{,}130{,}000 \text{ ft}^3/4500 \text{ ft}^3 \cong 250 \text{ samples}$$

If the minimum sample volume required was 1 L, then each subsample could be as small as 350 mL. This would result in about 1 L of sample during the minimum storm, but result in about 90 L during the maximum storm (obviously much larger than the typical 10 to 20 L container). During the estimated high flow conditions of the largest storm, a subsample would be collected every:

$$4500 \text{ ft}^3 \text{ per sample}/53 \text{ ft}^3/\text{s} \cong 85 \text{ s}$$

If the sampler required 2 min to collect 350 mL, the sampler would not complete its cycle before it was signaled to collect another subsample. This would result in the sampler pump running continuously during this peak time. Since the peak flow period is not expected to have a long duration, this continuous pumping may not be a serious problem, especially considering that about 250 samples are being collected. The biggest problem with this setup is the large volume of sample collected during the large event.

This problem was solved during numerous stormwater monitoring projects (including Pitt and Shawley 1982 during the Castro Valley, CA, NURP project, and Pitt 1985 during the Bellevue, WA, NURP project) by substituting a large container for the standard sample base and installing the sampler in a small shelter. The large container can be a large steel drum (Teflon-lined), a stainless steel drum, or a large Nalgene™ container, depending on the sample bottle requirements. In order to minimize handling the large container during most of the events, a 10 L glass jar can be suspended inside to collect all of the subsamples for the majority of the events. The jar would overflow into the large container for the largest events. Glass bottles are used in the sampler when organics are to be analyzed, with the assumption that the short period of storage in the glass would not adversely affect the metal concentrations. The small shelter should be well vented to minimize extreme temperatures, as it is difficult to ice the large container. Obviously, the sampling stations need to be visited soon after a potential runoff event to verify sample collection, to collect and preserve the collected sample, and to clean the sampler to prepare it for the next event.

Alternatives to using a large sample base (Figure 5.23) in order to accommodate a wide range of runoff events include:

- Use time-compositing instead of flow-weighted sampling
- Use two samplers located at the same location, one optimized for small events, the other optimized for larger events (Figures 5.24 through 5.26)
- Visit the sampling station during the storm and reprogram the sampler, switch out the bottles, or manual sample

The most common option is the last one, which is expensive, uncertain, and somewhat dangerous. Few monitoring stations have ever used multiple samplers, but that may be the best all-around solution, but at an increased cost. The first option above, using time-compositing instead of flow-weighted sampling, should be considered.

Figure 5.23 . Automatic sampler with large base for monitoring wide range of flows, with large chest freezer USGS discrete sampler in background, at Bellevue, WA.

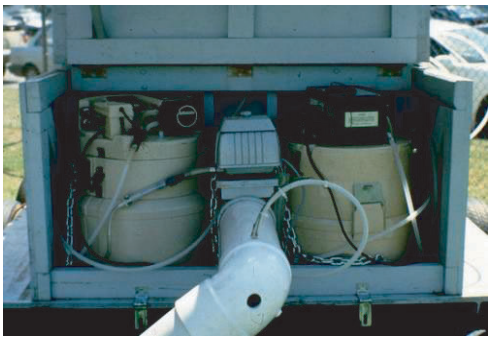

Figure 5.24 . Double monitor setup for simultaneously monitoring influent and effluent at small treatment device in Birmingham, AL.

Figure 5.25 . Double monitor setup for sampling over a wide range of flow conditions.

Figure 5.26 . Multiple flow monitor and sampler setup for simultaneously monitoring influent and effluent over wide range of flow conditions at a small treatment device in Madison, WI.

The Wisconsin Department of Natural Resources conducted a through evaluation of alternative sampling modes for stormwater sampling to determine the average pollutant concentrations for individual events (Roa-Espinosa and Bannerman 1994). Four sampling modes were compared at outfalls at five industrial sites, including flow-weighted composite sampling, time-discrete sampling, time-composite sampling, and "first-flush" sampling during the first 30 min of runoff. Based on many attributes, they concluded that time-composite sampling at outfalls is the best method due to simplicity, low cost, and good comparisons to flow-weighted composite sampling. The time-composite sampling cost was about $^1/_4$ of the cost of the time discrete and flow-weighted sampling schemes, for example (but was about three times the cost of the first-flush sampling only). The accuracy and reproducibility of the composite samples were all good, while these attributes for the first-flush samples were poor.

It is important to ensure that the time-weighted composite sampling include many subsamples. It would not be unusual to have the automatic samplers take samples every 10 min for the duration

of an event. If the minimum sample volume needed is 1 L and the shortest rain to be sampled is 30 min, then each subsample would need to be about 350 mL. The total volume collected would be about 50 L (144 samples) if a storm lasted 24 hours. The sampler would have to have an enlarged container (as in the above flow-weighted example), or the sampler would have to be visited about every 5 hours if a 10 L composite sample container was used.

Another important attribute of time-compositing sampling is that intermittent discharges and other short-term high concentration flows would be more readily detected. Flow-weighted composite sampling may allow very long periods to be unrepresented in the sample, while time-composite sampling can be adjusted to include relatively short sampling periods. Long periods between samplings could allow short-period episodes to be missed. However, sampling periods that are too short may result in almost continuous pumping activity that may exceed the continuous duty cycle of the sampler, resulting in frequent maintenance. Pump tubing should be carefully inspected and frequently replaced in any case, especially considering the gritty nature of stormwater. A new option is the use of *in situ* probes attached to the sampler that can be used to trigger sampling during unusual water quality shifts.

Automatic Sampler Initiation and the Use of Telemetry to Signal or Query Sampler Conditions

Automatic sampling equipment is typically located semipermanently in the field and is set to automatically begin sampling for a predetermined set of conditions. The most common method to start samplers is to use a stage indicator. This simple device, available from most sampler manufactures, may be a float switch (as from American Sigma) or an electronic sensor that shorts out when wet (ISCO). These devices plug into the sampler at the flow sensor connection. If flow monitoring is simultaneously being monitored, a Y connection is available to allow both connections. The stage sensor is typically placed slightly above the baseflow water elevation (in a pipe, open channel, or creek). It is difficult to sample small events that may not cause a large-enough stage elevation increase to trip the indicator. False alarms are also common when the sensor is placed too close to the baseflow water elevation or in areas of high humidity (for the moisture sensor). In addition, the baseflow water stage changes seasonally, requiring constant modifications in the sensor location. If the channel or pipe is normally dry, these problems are significantly reduced, as the sensor can be placed on the bottom of the drainage way or pipe. Flow-weighted sampling schemes can eliminate the use of sensors all together. In this case, some water may collect in the sample container during baseflow conditions, however. Frequent visits to the sampler are needed to empty and clean the sample container.

Another method used to initiate sampling is to trip the sampler using a rain gauge. Pitt and McLean (1986) used a rain gauge to initiate sampling at an industrial site in Toronto, while simultaneously monitoring flow. A tipping bucket rain gauge was used and three trips (about 0.03 in of rain) of the rain gauge within a few hours were usually used to initiate sampling.

In all cases, the use of telemetry (radio, telephone, or cellular phone) is extremely useful in minimizing false trips to a remote sampler by automatically signaling that samples have been collected (Figure 5.27). Campbell Scientific of

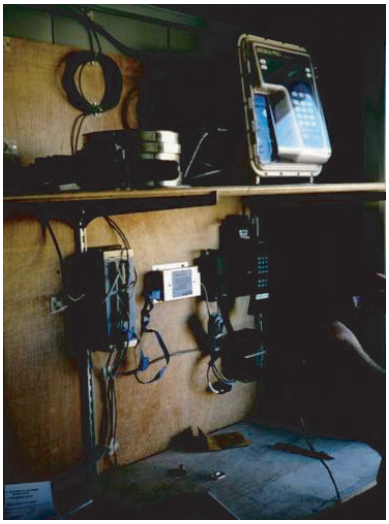

Figure 5.27 Telemetry equipment at USGS monitoring site in Madison, WI.

Figure 5.28 In-stream continuous probes at Dortmund, Germany, CSO monitoring site.

Figure 5.29 Automatic sampler connected to continuous probes and telemetry at Dortmund, Germany.

Logan, UT (801-753-2342), supplies many options allowing remote inquiring or automatic signaling to indicate sampler status. It is also possible to phone a monitoring station and immediately determine if a sampler is operating, and to download or observe instantaneous or compiled rain, flow, or continuous *in situ* water quality monitoring information. The use of telemetry is extremely important when many remote systems are being operated by a small group. It should be considered an integral part of all sampling and monitoring programs where high reliability and good quality data are needed. There are potential problems with RF interference between cellular phones and some monitoring equipment, so care must be taken to use an external antenna, to electronically shield the monitoring equipment, and to thoroughly test the setup.

An early example of an automatic stormwater monitoring program using telemetry to excellent advantage was the Champaign/Urbana NURP study conducted in the early 1980s (EPA 1983a). The Universität Gesamthochschule in Essen, Germany, has also used standard telemetry equipment components and specialized software in CSO monitoring in Dortmund, Germany, to inquire about monitoring station and flow status (Wolfgang Geiger, personal communication) (Figures 5.28 and 5.29). Numerous municipalities and state agencies in the United States have also installed telemetry-coupled monitoring stations using relatively inexpensive components, including cellular telephone service and solar-powered battery chargers. This has eliminated most of the concern about the availability of remote utility installations. Cooling collected samples still requires AC-powered chillers, or ice. For remote installations with a small sampling crew, it is impractical to ice the sampler in anticipation of a rain, but that is possible when the samplers are more accessible. It would be more important to recover the samples from the samplers as soon as possible after the event. This is made much more practical, especially with remote samplers, when telemetry is used to inquire about the sampler status.

Siphon Samplers

The USGS recently published a review of siphon samplers, compared to flow-weighted composite samplers for use along small streams (Graczyk et al. 2000). These are inexpensive units that can be utilized in many locations (Figure 5.30). They operate semiautomatically by starting to fill when the water level reaches level B (the top of the loop connected to the intake) in Figure 5.30. The sample

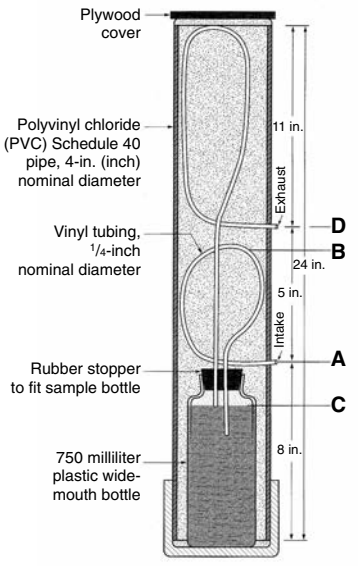

Figure 5.30 Siphon sampler. (From Graczyk, D.J. et al. *Comparison of Water Quality Samples Collected by Siphon Samplers and Automatic Samplers in Wisconsin*. USGS Fact Sheet FS-067-00. U.S. Geological Survey, Middleton, WI. July 2000.)

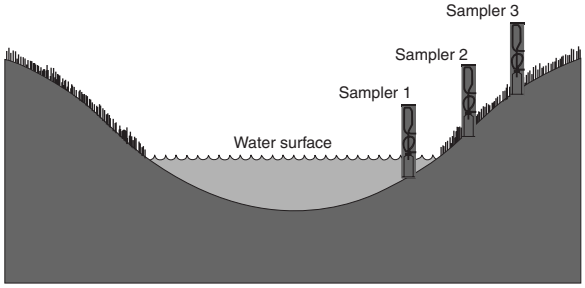

Figure 5.31 .Placement of siphon samplers along stream bank. (From Graczyk, D.J. et al. *Comparison of Water Quality Samples Collected by Siphon Samplers and Automatic Samplers in Wisconsin*. USGS Fact Sheet FS-067-00. U.S. Geological Survey, Middleton, WI. July 2000.)

bottle fills rapidly due to the hydraulic head (the elevation of the stream surface above the discharge end of the intake tube, level C, in the bottle). After the stream level reaches level D, an airlock is created in the top loop, stopping the filling. Therefore, the siphon collects a sample near the water surface when the stream stage is between levels B and D, which can be adjusted. Since they collect samples over narrow ranges of stream stages, several can be placed at different heights along a receiving water, as illustrated in Figure 5.31. Graczyk et al. (2000) compared sets of three siphon samplers, set at different elevations, along three streams that also had flow-weighted automatic samplers (ISCO) for comparison. They collected 40 to 50 pairs of samples and analyzed them for suspended solids, ammonia, and total phosphorus. Figure 5.32 illustrates the comparison for suspended solids. There was substantial scatter in the data, but the differences in the results averaged about 10% for suspended solids and ammonia,

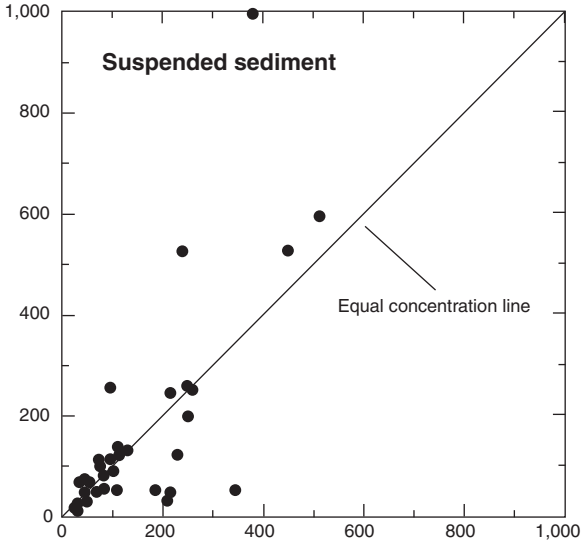

Figure 5.32 .Comparison of siphon sampler (y axis) and ISCO sampler (x axis) suspended solids observations. (From Graczyk, D.J. et al. *Comparison of Water Quality Samples Collected by Siphon Samplers and Automatic Samplers in Wisconsin*. USGS Fact Sheet FS-067-00. U.S. Geological Survey, Middleton, WI. July 2000.)

and about 25% for phosphate. However, the differences between individual pairs of samples were much greater. Some of the larger differences may reflect the siphon samplers only collecting samples at specific stage increments, while the automatic samplers collected samples at a single depth over longer periods of time. The siphon samplers may be useful when many samples can be collected and overall conditions are desired, in contrast to more accurate individual results. Their low cost and ability to sample for specific stage conditions makes them an interesting alternative to more expensive automatic samplers, or difficult manual sampling.

Retrieving Samples

Each sampler site will need to be visited soon after the runoff event to retrieve the sample for delivery to the laboratory. The storage time allowed in the sampler before collection should be determined from a special holding-time study conducted in conjunction with the analytical laboratory. Stormwater samples can usually withstand longer holding times than those implied from standard laboratory method descriptions without significant degradation. However, this will need to be verified by local tests. In all cases, the allowable holding times noted in Table 5.10 should be followed except in unusual situations and then only with specific tests. This is especially important when organizing sample deliveries to the laboratory after hours (which can happen frequently).

Manual Sampling Procedures

The following paragraphs summarize the procedures needed for manually collecting water and sediment samples from a creek or small stream.

1. Fill out the sample sheet and take photographs of the surrounding area and the sampling location. Conduct any *in situ* analyses (such as stream flow measurements, along with dissolved oxygen, pH, temperature, conductivity, and turbidity measurements in the water).
2. Use a dipper sampler to reach out into the flow of the stream to collect the sample. Slowly lower the sampler onto the water, gently rolling the top opening into the flow. Be careful not to disturb the bottom sediments. Submerge the sampler lip several inches into the water so floating debris are not collected. Lift out the sampler and pour the water into a compositing container (such as a churn sample splitter). Several samples should be collected in the area of concern and composited. In some cases, it may be useful to sample the water–air interface. This surficial layer is known to trap many types of organic chemicals (e.g., oils and surfactants) and have elevated microbial populations (e.g., pathogens).
3. Each water subsample can be poured into a large clean container during this sampling period. At the end of the sampling period, this composite sample is mixed and poured into the appropriate sample bottles (with preservatives) for delivery to the analytical laboratory.

Microbiological sampling requires special sampling techniques. ASTM (1995) in standard D 3370 describes the grab sampling procedures that must be used for collecting samples that will be analyzed for bacteria. The samples need to be glass and sterile. If the sample contains chlorine, then the sample bottle must contain sodium thiosulfate so any residual disinfection action will be destroyed. The bottle lid is removed and the bottle is placed under flowing water and filled to about $^3/_4$ of its capacity. Care must be taken when handling the bottle and lid (including not setting them down on any surface and not touching any part of the upper bottle portion) to minimize contamination. Do not rinse the bottle with the sample or submerge it under water.

Sampling sediment can be difficult (see also later discussion). The simplest method is to use a lake bottom sampler. Specifically, a small Ekman dredge sediment sampler, which is typically used for sand, silt, and mud sediments, is usually most useful. Corer samplers are generally not as successful for stream sediments. An exception is the freezing core sampler, where liquid CO_2 is pumped inside a stainless steel tube (with the bottom end sealed with a point) to freeze sediment

to the outside of the tube. Again, the sediment would have to be at least several inches deep. In all cases, multiple sediment samples would have to be obtained and composited. Any water samples should be obtained first, as the sediment sampling will create substantial disturbance and resuspension of sediment in the water column. All sampling equipment must also be constructed of noncontaminating materials. Stainless steel, polypropylene, or Teflon are the obvious choices.

Dipper Samplers

The simplest manual sampler is a dipper sampler (Figure 5.33). Markson (telephone: 800-858-2243) sells a dipper sampler that has a 1 L polyethylene beaker on the end of a two-piece, 4-m pole (catalog # MK34438 for about $60). They also sell units on 1- and 2-m poles and with 500 mL capacities. These samplers can only obtain samples from the surface of the water. If subsurface samples are needed, samplers with closure mechanisms need to be used, as described below. A dipper allows sampling of surface waters away from the immediate shoreline and from outfalls or sewerage pipes more conveniently than other types of samplers. Dippers are commonly used to sample small discharges from outfalls, where the flow is allowed to pour directly into the sampler. ASTM (1995) in standard D 5358 describes the correct stream water sampling procedure using a dipper sampler. The dipper needs to be slowly lowered into the water on its side to allow the water to flow into the sampler. The dipper is then rotated to capture the sample and is lifted from the water. Care must be taken to prevent splashing or disturbing the water. The sample is then poured directly into the sample bottles or into a larger container (preferably a churn sampler splitter, as previously described) for compositing several dipped samples.

Figure 5.33 Manual dipper sampler.

Submerged Water Samplers with Remotely Operated Closures

There are numerous historical and modern designs of samplers that can take water samples at specific depths. These all have a way to remotely operate closures in a sample container. The sampler capacities usually range from 0.5 to 3 L. Older designs include the Kemmerer and Van Dorn samplers, shown on Figure 5.34 (*Standard Methods* 1995). These samplers have a tube made of metal or plastic and end closures made of plastic or rubber. All Teflon units are available to minimize sample contamination. Newer designs commonly used for small lakes or streams are

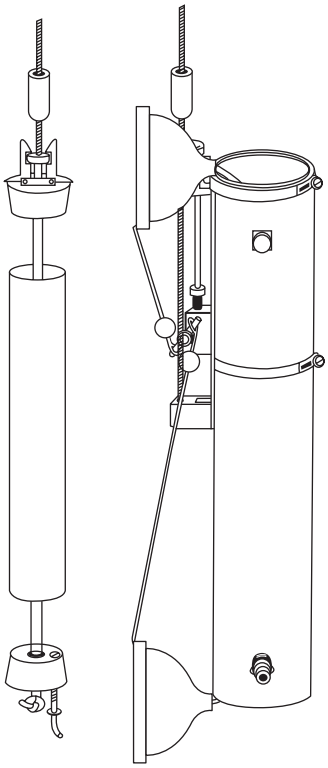

Figure 5.34 .Kemmerer and Van Dorn samplers. (From *Standard Methods for the Examination of Water and Wastewater.* 19th edition. Water Environment Federation. Washington, D.C. Copyright 1995 APHA. With permission.)

Figure 5.35 . Horizontal water sampler in open position before use.

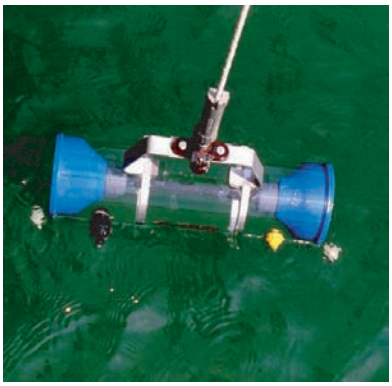

Figure 5.36 . Tripped horizontal water sampler being withdrawn from water with messenger resting on trigger mechanism.

Figure 5.37 . Open vertical water sampler being lowered into water, above a horizontal sampler on the same line.

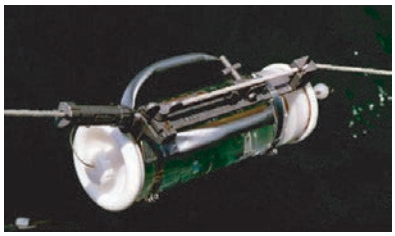

Figure 5.38 . Tripped vertical water sampler being withdrawn from water with messenger resting on trigger mechanism.

similar to the Van Dorn design (Figures 5.35 through 5.38). This design allows unhindered flow through the sample container before closure, enabling faster equilibrium with surrounding waters. These samplers are also available in horizontal models (for shallow water) or vertical models. Several of the vertical units can be used on a single line to obtain water samples from various depths simultaneously. A weighted messenger slides down the line that the samplers are attached to, striking a trigger mechanism that closes the end seals. If multiple samplers are used, the trigger releases another messenger that slides down to the next sampler to close that sampler and to release another messenger. A vertical alpha end-closure 2.2-L sampler (polyurethane end seals and transparent acrylic cylinder) is available from Forestry Suppliers, Inc. (800-647-5368) as catalog #77244, with messenger #77285, for a total cost of about $450. Several of these samplers can be installed on a line for simultaneous sampling at various depths. Forestry Suppliers, Inc., also sells a 1.2-L Teflon Kemmerer vertical bottle sampler (catalog #77190) for about $800. A water sample collected with this sampler only contacts Teflon.

Another surface operated design is a sampler that contains a 1-L glass bottle on the end of a long pole (such as catalog #53879 from Forestry Suppliers, Inc. at about $400). A stopper is spring loaded and is attached to a wire extending to the other end of the pole. The bottle end is lowered to the desired sampling depth and the wire is then pulled to fill the bottle. After a short period to allow the bottle to fill, the wire is released, resealing the bottle. This sampler was designed specifically for collecting water samples for Winkler titrations for DO analyses at sewage treatment plants. The bottle is initially full of air before the water enters and aeration may elevate the DO reading. If the bottle is prefilled with clean water, it is difficult to assume that the desired water sample will replace the water in the bottle. However, this sampler type might be useful for collecting subsurface samples for bacteriological analyses that should be collected in glass bottles with minimal handling.

Figure 5.39 Tube sampler.

Figure 5.40 .Grundfos Redi-Flo2 pump sampler with controller.

A newer alternative is a Teflon tube sampler that contains a wire-activated sealant mechanism and flow-through design (Figure 5.39). This overcomes the above limitations of the bottle sampler and still allows direct sampling at a specific depth. The AMS Cable Control Liquid Sampler is available from Forestry Suppliers, Inc. (catalog #77623), and costs about $550.

Manual Pump Samplers

A Grundfos Redi-Flo2 (Figure 5.40) pump and converter (designed and commonly used for well sampling) is available with a 300-foot polyurethane hose on a reel that can be used to deliver a water sample to a convenient location, especially useful when sampling wide and swift streams from a bridge. These pumps are available from Forestry Suppliers, Inc. (800-543-4203, catalog #76328 for pump, hose, and reel, and #76333 for voltage converter, for a total cost of about $4500). Hazco (800-332-0435) also sells (and rents) the Redi-Flo2 pump and converter for about $2100 without a hose (catalog #B-L020001 for converter and #B-L020005 for 150 motor lead and pump). A Teflon-lined polyethylene hose is available from Hazco for about $3.25 per foot, with support cable (catalog #A-N010041 and #C-L020009). This pump has an adjustable pumping rate of between 100 mL/min and 9 gal/min and can pump against a head of about 250 ft. However, this pump should be operated at least at 4.5 gal/min to meet the 100 cm/s criterion to minimize particulate settling in the 1 in ID hose. Low pumping rates from a submerged pump can also lead to "sand jamming," in addition to preventing an adequate sample from being obtained.

A less expensive alternative is the XP-100 pump, also available from Forestry Suppliers (#76216 for XP-060 pump and #76230 for control box, for a total cost of about $525). This is an adjustable rate pump and can deliver the needed 100 cm/s pump rate through a $^{3}/_{8}$-in tubing against a head of about 30 ft or less. This pump operates from a 12V DC power supply and has a limited service life, compared to the Grundfos pump. It may be useful for temporary installations having limited head, but needing several pumping locations across a stream. It is also useful for continuous sampling at different lake depths.

Depth-Integrated Samplers for Suspended Sediment

Suspended sediment is usually poorly distributed in both flowing and quiescent water bodies. The sediment is usually in greater concentrations near the bottom, as shown in Figure 5.41 (ASTM 1995). Larger and denser particles are also located predominantly in lower depths. Flowing water

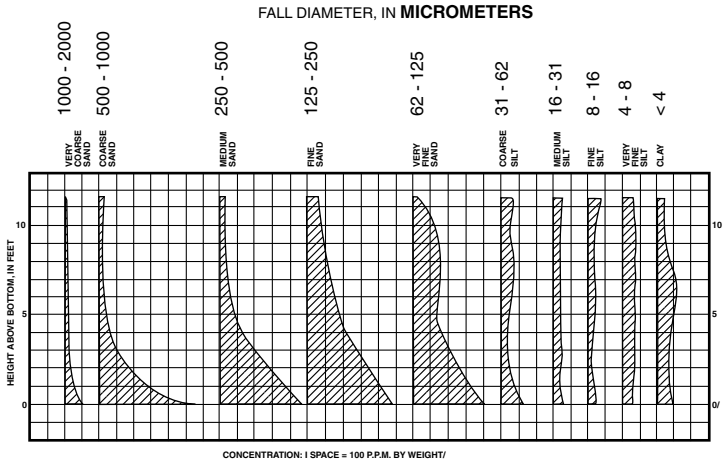

Figure 5.41 Sediment concentrations by depth and particle size, Missouri River, Kansas City, MO. (From American Society for Testing and Materials. *ASTM Standards on Environmental Sampling*. ASTM Pub Code No. 03-418095-38. ASTM, Philadelphia. 1995. Copyright ASTM. Reprinted with permission.)

in a sinuous stream also distributes the suspended sediment horizontally, as shown in Figure 5.42 (ASTM 1995), differently for large and small particles. Collecting representative samples in these situations for sediment analyses is therefore difficult. Because most of the pollutants in stormwater are associated with the particulates, this unequal distribution of sediment also affects the ability to collect representative samples of many pollutants. Depth-integrating sampling is commonly done in small upland streams. Sampling in smaller and more turbulent flows (such as in sewerage or at outfalls during moderate to large storms) is not as severely affected by sediment stratification.

Clay and silt-sized particles are generally well mixed with depth, depending mostly on water mixing conditions near discharges, etc., and not on gravity. ASTM (1995) states that the concentrations of particles smaller than about 60 μm in diameter will be uniform throughout the stream depth (Figure 5.41). However, larger particles will be more affected by gravitational forces and may not be represented well with typical sampling procedures. Conventional water samplers may be used to represent all of the sediment in flowing water (floating material, suspended sediment, and bedload), if the water is very turbulent and capable of mixing the sediment of interest. ASTM refers to these locations as "total-load" stations, allowing the collection of all sediment greater than about 2 mm in diameter. These are generally located at outfalls or other free-falling locations.

Automatic samplers (or any pumped sampler) may disproportionately collect particulates if the intake velocities vary significantly from the water velocity. Isokinetic sampling requires that

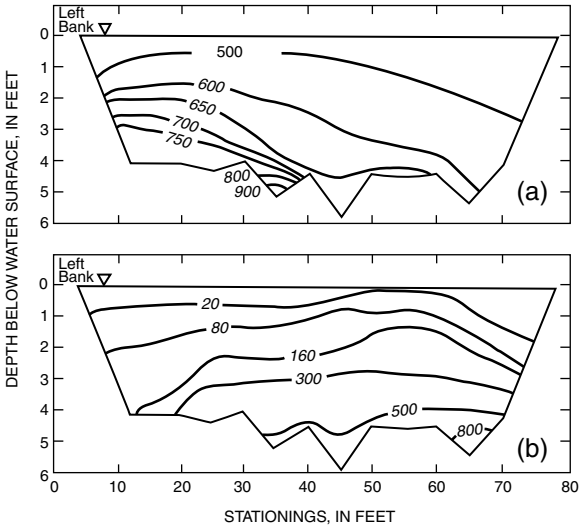

Figure 5.42 Suspended solids concentrations in the Rio Grande River, near Bernardo, NM, for different sediment sizes: (a) material between 62.5 and 125 mm; (b) material between 250 and 500 mm). (From American Society for Testing and Materials). *ASTM Standards on Environmental Sampling*. ASTM Pub Code No. 03-418095-38. ASTM, Philadelphia. 1995.Copyright ASTM. Reprinted with permission.)

Figure 5.43 . Depth-integrated sediment sampler parts.

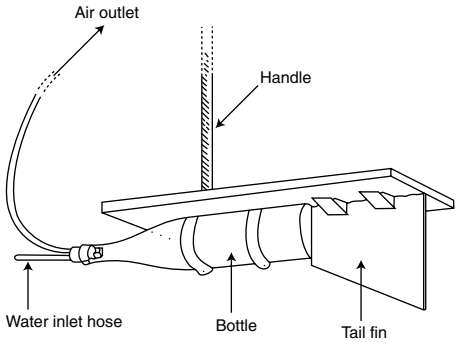

Figure 5.44 . Plan for a home-made depth integrated sampler. (Modified from Finlayson 1981.)

Figure 5.45 . Depth-integrated sediment sampler being readied for use.

the sampler intake be pointed directly into the flowing water and that the velocity in the intake be the same as the flowing water. The water and sediment streamlines will therefore be parallel in this situation and a sample representative of the flowing water will be obtained. If the sample intake velocity is greater than the water velocity, water will be drawn into the sampler, while heavier particles will tend to flow past. This effect is most evident for heavier particles (larger and denser) than for lighter particles. Berg (1982) reports that particles approaching 100 µm in diameter with densities of 2.65 g/cm^3 have less than a 20% sampling error when the velocities are not matched. Almost all stormwater and stream-suspended particulates are smaller and have a lighter density than this and would therefore generally follow the flow streamlines. These particles would therefore not be significantly affected by this possible problem.

Large-sized (larger than several hundred micrometers in diameter) suspended sediment measurements may be important for receiving water studies, especially in areas having flash flood flows in sandy soil regions (such as the southwest United States). The depth integrated sampler is designed to obtain a sample continuously as the sampler is lowered vertically through the water column at a constant velocity (Figures 5.43 through 5.45). These units vary significantly from commercial grab samplers that have remotely operated valves in that they have air vents to allow the air in the sample bottle to uniformly escape as the sample bottle fills with water. The home-made unit has a narrow-mouthed bottle mounted on a rod with stabilizing fins. The mouth of the bottle is fitted with a two-holed stopper. The top hole has a long flexible tube (which could extend above the water surface for most streams) to act as an air outlet, while the bottom hole has a rigid tube extending at least an inch to act as an intake. The intake nozzle should have a sharp front edge, with a narrow tubing thickness (less than $^1/_{16}$ in) and an inner

diameter of 5 to 6 mm ($^3/_{16}$ or $^1/_4$ in) (ASTM 1995, standard D 4411). These are available commercially from Forestry Suppliers, Inc. (800-543-4203) and in Canada from Halltech Environmental, Inc. (519-766-4568), or they can be constructed (Figure 5.44).

When collecting a depth-integrated sample, the sampler needs to stand to the side and downstream of the sampling area to minimize disturbance. The rod is lowered vertically through the water column at a constant rate of about 0.4 times the stream velocity. Detailed vertical sampling rates are presented by ASTM (1995) in standard D 4411 for the series of older depth-integrated samplers. The sampler is lowered at this constant rate from the surface of the stream to the stream bottom, and then reversed and brought back to the surface at the same rate. The sampler does not collect samples within several inches of the stream bottom. Moving sediment near the bottom is usually included in the bedload sample, which requires other sampling methods. The sample bottle should be between $^2/_3$ and $^3/_4$ full after sample collection. If it is full, then the sampler did not represent the complete stream depth and the sample should be discarded and collected again, at a faster vertical rate. If the sampler is less than $^2/_3$ full, another vertical sample pass can be collected. After the sample is collected, the sample is poured from the sampler into a sample bottle. It is possible to mount an appropriate sample bottle directly to the sampler, and sample transfer would therefore not be needed.

Several vertical samples will normally need to be collected across the stream, as the coarser suspended sediment is likely highly variable in both time and space (ASTM 1995). The location and number of sampling verticals required at a sampling site is dependent primarily on the degree of mixing at the cross section.

Settleable Solids Samplers

Sediment traps suspended in the water column can be used to capture settleable solids. Zeng and Vista (1997) describe the use of these samplers off San Diego to capture marine settleable solids for organic compound analyses in the water column at several off-shore locations. The sediment traps were located 1 and 5 m from the seafloor and were retrieved after 30 days. The traps were made of two parts, a glass centrifuge bottle at the bottom and a glass funnel positioned on the bottle through a Teflon-lined silicone rubber seal. When retrieved, the two parts of the traps were separated and water covering the particulates was carefully removed. The centrifuge bottles were then capped with Teflon-lined caps and brought to the laboratory for analysis.

Similar sediment traps were used in the Seattle area to investigate the amount and fate of CSO settleable solids in the receiving waters. These traps were generally similar to those described above but were located much closer to shore and in shallower water. Several were placed vertically on an anchored line in a grid pattern near and surrounding CSO discharge locations being investigated.

Sediment traps were also placed in Fresh Creek, New York City, at the Equi-Flow demonstration facility. These traps were placed within and outside the facility to quantify the amount of settleable material that was captured during the CSO storage operations before being pumped back to the treatment plant. This use of sediment traps was not very successful due to very dynamic flow conditions and the short exposure periods used in an attempt to obtain data during frequently occurring CSO events. Longer exposure periods would have enabled the capture of more measurable material, but would have blended together material from adjacent events.

Sediment traps can be useful sampling devices to capture and measure slowly settling solids *in situ* in the water column. This information is especially important when quantifying the effects of sediment-laden discharges into relatively large water bodies having slow to moderate currents. They may not be suitable for small streams, unless they can be miniaturized. Several traps should be suspended at one location at different depths, and redundant devices should be used to compensate for traps lost during the exposure period. Like the bedload samplers described next, the exposure periods should probably be long (several weeks). The sampler materials also need to be compatible with the constituents intended to be analyzed. A simple framework (made of

inert materials) should also be constructed to brace the assembled sediment trap and to allow easy attachment to the anchored line, but it should not extend above the funnel to minimize interference with settling materials.

Bedload Samplers

Bedload is the material that travels in almost continuous contact with the stream bed (ASTM 1995). The bedload material moves when hit by another moving particle, or when water forces overcome its resisting forces. Bedload is sampled by using a trapping sampler located on the stream bottom. The simplest bedload samplers are box or basket samplers which are containers having open ends facing upstream. Bedload material bounces and rolls into the sampler and is trapped. Other types of bedload samplers consist of containers set into the sediment with slot openings about flush with the sediment surface. The bedload material falls through a slot and is trapped. Slot widths and lengths can be varied to represent various fractions of the bedload actually moving in the stream. The errors associated with sampling bedload are greater than with sampling suspended sediment because the larger particles move more irregularly under the influence of gravitational forces and are not well mixed in the water.

Bedload may be important when characterizing stormwater sediment discharges. In northern areas where sands are used for ice control, relatively large amounts of sand can be transported along the drainage system as bedload. At the Monroe St. detention pond site in Madison, WI, the bedload accounted for about 10% of the total annual sediment loading. This fraction was much greater during the spring when most of the sand was flushed from the drainage area.

Conventional water samplers may not adequately collect bedload material. A slot sampler placed in a drilled hole in the bottom of a discharge pipe can effectively collect this material. However, the slot dimensions and placement exposure times must usually be determined by trial and error. In addition, several bedload samplers should be used in close proximity because of the varied nature of bedload transport. Bedload samplers that are full upon retrieval may not represent actual conditions. If full, then the slot widths should be reduced and/or the exposure time should be shortened. The slot length should be as long as possible for the container lid, as bouncing bedload particles may jump over openings that are too short. In addition, the slot widths should be at least $^1/_4$ in wide, as narrower slots will filter out large materials. Basket samplers are probably most applicable in streams, where the opening width is a small fraction of the stream width. Again, several samplers need to be used in close proximity, and the best exposure period needs to be determined by trial. For grab samples, both hand-held and cable suspended Helley Smith (Geological Survey) bedload samplers are available from Halltech Environmental, Inc. (519-766-4568).

Floatable Litter Sampling

One example of quantifying litter discharges during wet weather was described by Grey and Oliveri (1998). New York City has been involved in a comprehensive litter analysis and capture effectiveness program since the mid-1980s. As part of this investigation, it studied litter discharges from stormwater inlets using baskets that were inserted in manholes below catchbasins (Figure 5.46). The baskets were made of galvanized mesh and were 13 in square and 36 in high. The lower half of the baskets was made of $^1/_4$-in mesh, while the upper half was of $^1/_2$-in mesh. The baskets were positioned on a wooden platform just beneath the catchbasin outlet pipe and were held in place with ropes, allowing removal without requiring entry into the manholes. These baskets were installed at 38 locations throughout the city and were in place for 3 to 4 months. Most baskets were removed, emptied, and replaced every 2 weeks, although some were in place for only a week before emptying. The captured material was placed in sample bags, brought to the laboratory, sorted into 13 categories, counted, and weighed. The surface areas of the collected material were also measured.

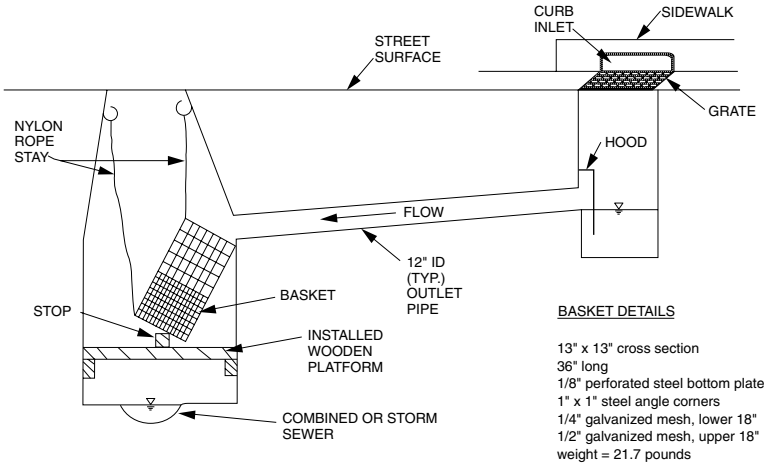

Figure 5.46 . New York City catchbasin litter sampling setup. (From HydroQual, Inc. *Floatables Pilot Program Final Report: Evaluation of Non-Structural Methods to Control Combined and Storm Sewer Floatable Materials*. City-Wide Floatables Study, Contract II. Prepared for New York City, Department of Environmental Protection, Bureau of Environmental Engineering, Division of Water Quality Improvement. NYDP2000. December 1995.)

In addition to characterizing the litter discharges, New York City also examined the effectiveness of the catchbasins in capturing this material. Grey and Oliveri (1998) also described these tests. They placed a known amount of litter (10 pieces each of 12 different floatable items, totaling about 1 ft^3 in volume of each material), including plastic bags, candy wrappers, straws, bottle caps, juice bottles, hard plastic pieces, glass vials, aluminum cans, polystyrene cups and pieces, cigarette butts, and medical syringes. They then opened a fire hydrant to produce a basic flow rate of about 75 gal/min (corresponding to a rain intensity of about 0.28 in/hour over a 40,000 ft^2 drainage area). They also ran tests at $^1/_3$ and 2× this flow. The flow was continued until no more items were transported to the sampling basket (usually about 5 to 10 min). The items remaining in the catchbasin were then retrieved and counted. This test was repeated five times for each test, and 10 tests in all were conducted (some with and some without catchbasin hoods).

Source Area Sampling

Much information can be obtained by collecting stormwater samples at source areas. Source areas are where the runoff originates before it is collected in the storm drainage system. Source area sampling also includes rainfall sampling for water quality analyses, conventionally done using a wet/dry-fall sampler. This sampler also collects dust fall during dry periods. This atmospheric contribution can have a significant affect on stormwater quality. However, very little of the dry-fall pollutants occurring over a watershed actually are washed off during rains.

This information can help identify the critical areas in the watershed where most of the problem pollutants may be originating and where control measures should be implemented (Pitt et al. 1995). These areas may include paved industrial storage areas, convenience store parking areas, vehicle maintenance areas, landscaped areas, roof runoff, etc. Conventional automatic samplers may not be efficiently used in these areas because of the small scale of the sampling areas and limited places where the samplers can be located that would only receive runoff from the area of concern. Three sampling methods have been used:

- Manual sheetflow samplers
- Semiautomatic samplers
- Special designs for automatic sample collection

Figure 5.47 .Sheetflow sampler operated by hand vacuum pump.

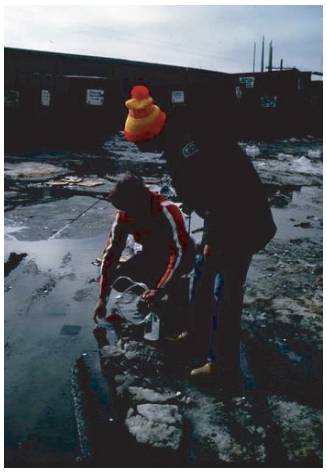

Figure 5.48 Sheetflow sampler being used to sample snowmelt.

Manual Sheetflow Samplers

Manual sheetflow samplers are usually used when collecting grab samples from many different sampling locations. A small team can visit many sampling sites during a single rain to obtain multiple grab samples for statistical comparisons (Figures 5.47 and 5.48). The main drawback is that the samples are not composited during the rain and only represent the conditions during the short sampling period. It is therefore very important to carefully document rain and flow conditions during the sampling period, and for the short time before the sample was obtained. Rain conditions up to the time of sampling can also have a significant effect on measured pollutant concentrations. In many cases, the ability to obtain many samples in a relatively short time is more important than obtaining flow-weighted composite samples. Roa-Espinosa and Bannerman (1994) found that many discrete samples (which could be composited before analysis) are just as useful in obtaining an event-mean concentration (EMC) as are more difficult to obtain flow-weighted composite samples.

Sheetflow samples should be obtained in areas where the sheetflow is originating from a homogeneous area, such as from a parking area, roof runoff, runoff from a landscaped area, etc. Sheetflow samples can be collected by collecting the flow directly into the sample containers, if the flow is deep enough. The flow may be "scooped" using a small container and by pouring the collected samples into the sample container. For shallow sheetflows, a hand-operated vacuum pump can be used to draw the sample into the sample container, as shown in Figure 5.47. A Teflon-lined lid that fits the sample containers can be fitted with two Teflon bulk-head connectors. One of the connectors has a Teflon tubing (about 18 in long and 1/4 in ID) attached that is used to draw the sample into the container. The other connector has a Tygon™ tube leading to a water trap (another bottle) that is in turn attached to a hand-operated vacuum pump (such as a Nalgene #6132-0020, at about $100). To collect a sample, the Teflon tubing is immersed in the sheetflow and the hand pump draws the water into the sample bottle. The pump should be operated slowly to prevent cavitation at the tubing inlet. The short lengths of Teflon tubing are inexpensive and can be replaced after each sample to prevent cross-contamination. Since the sample is drawn directly into the sample bottle, sample transfer is unnecessary.

An alternative to the hand-operated vacuum pump and water trap arrangement is to use a battery-operated peristaltic pump (such as a Masterflex L/S portable sampling pump, catalog #FE-07570-10, at about $850, with a Teflon tubing pump head, catalog #FE-77390-00, at about $400, available from Cole-Parmer, 800-323-4340). This battery-operated pump can be used to pump directly into the sample containers. The Teflon tubing used in this pump (catalog #FE-77390-60) costs about

$15 each and would therefore not likely be replaced after each sample. The tubing would therefore require field cleaning between each sample. Since the battery is built into this pump, and no water trap is needed, this sampling arrangement is relatively compact.

Semiautomatic Sheetflow Samplers

Source area samplers have been developed to semiautomatically collect composite stormwater samples from small drainages. Samplers (at $250 to $650) from the Vortox Company (909-621-3843) are an attractive option for some studies (Figure 5.49). These 0.8- to 5.5-gallon units (available Teflon lined) are completely passive and operate with a double ball closure system. They are installed in the bottom of intermittent flow paths, requiring a sump for installation. They have a screw closure to adjust the rate of filling. A top ball seals the inlet during dry conditions. When a flow occurs, this ball floats, opening the inlet. An inner ball on the underside of the inlet then seals the inlet when the sampler is full.

Figure 5.49 Vortox sampler.

Potential problems may occur with sediment clogging the very small inlet and fouling the ball seals. However, this sampler also collects bedload from the flowing stormwater (if the ball valve is opened sufficiently) that is not collected using conventional stormwater samplers. The sampler is somewhat awkward to clean. Another problem is the rapid time (less than 20 minutes for the 0.8-gal unit and less than 2 hours for the 5.5-gal unit) to completely fill the sampler. Sheetflows from homogeneous areas (especially small paved areas where these samplers are likely to be used) usually demonstrate strong "first-flush" conditions. The initial flows have much greater concentrations than the EMC, especially for relatively constant rain intensities. This would result in biased concentrations if only the first 20 min of the flow is represented in the sample.

Because of its low cost and passive operation, this sampler may be attractive in situations where many source areas are to be sampled with a small sampling crew. Again, caution must be expressed in interpreting the results, as the concentrations may be greater than the EMC values for source area flows. At outfalls, in complex drainage ways, or with highly variable rain intensities, the initial samples are not likely to be consistently different from the EMC. Frequent site visits will be necessary when runoff has been expected in order to retrieve samples. It may be desirable to have additional samplers so clean units can be substituted in the field for full samplers. The full samplers can then be brought to the laboratory to be emptied and cleaned.

Automatic Source Area Samplers

Problems associated with the above two sampling methods for source area sheetflows can be largely overcome using automatic samplers. Conventional automatic water samplers discussed earlier are probably the most flexible. However, they are expensive and large. Their size limits where they can be located and the size of flow they can sample. Their cost limits the number of units that can be simultaneously deployed. It is possible to rotate a relatively few samplers randomly between semipermanent sampling locations after every few storms. The samplers would be programmed for time-composite sampling (or time-discrete sampling) and automatically activated with

Figure 5.50 Prototype WI DNR/USGS automatic sheetflow sampler.

flow level sensors, or by rain gauge activity. As noted earlier, telemetry can be used to call the project personnel automatically when the sampler has been activated.

Roa-Espinosa and Bannerman (1994) describe a new automatic source area sheetflow sampler that the Wisconsin Department of Natural Resources and the Madison USGS office have jointly developed (Figure 5.50). Their initial source area sampler was similar to a slot bedload sampler and located in the flow path to be sampled. Like the Vortox unit, it usually filled quickly and did not represent the complete runoff event. This initial sampler consisted of a 10-in ID PVC pipe 12-in long. A 10-in PVC pipe coupling was cut in half and glued to the top of the pipe as a reinforcing collar. This pipe was then cemented in a drilled hole in the pavement (for pavement runoff sampling). A 1-in-thick PVC cap, having a $^5/_8$-in center hole, was fitted snugly in the coupling sleeve of the pipe section cemented in the pavement. The upper surface of this cap was flush with the pavement surface. A sample bottle lid was bolted to the underside of the removable cap, which also had a $^5/_8$-in hole matching the hole in the cap. A 2.5-L glass sample bottle was screwed into this lid and placed in the pipe cemented into the pavement when rain was expected. After the runoff ended, the bottles were retrieved and brought to the laboratory. As noted above, sample bottles commonly were full after the runoff ended, indicating that the samples did not represent the complete event. The sampling holes were reduced to reduce the inflow rate, but clogging was a concern and they still were frequently full. Investigators then developed a new sheetflow sampler that was electronically activated (Figure 5.50). A relatively large sample inlet was used to minimize clogging, but an electronically operated ball valve was added. It is possible to program the sampler to schedule the duration of the open and closed times. This enabled the complete runoff events to be represented in the sample. When commercially available, these samplers are likely to cost about $1000.

Source Area Soil Sampling

Soil sampling in urban areas usually involves collecting material from both paved and unpaved areas. Collecting particulates from paved areas (“street dirt”) is described in the following subsection and can be applied to many paved source areas, in addition to streets, the original area of most interest. Soil sampling from nonpaved areas involves more traditional soil sampling procedures and is discussed in any agricultural soils textbook. Generally, small trowels are used to collect surface soil samples for analyses, while small hand coring tools are used to collect subsurface samples down to about 1 ft in depth. Deeper soil samples can be best obtained from the walls of trenches that have been excavated using small backhoes.

If soil characteristics associated with particulates most likely to erode during rain events are of most interest, then care should be taken to emphasize the surface soils during sample collection. In this case, careful “scrapings” of surface dirt by a trowel or stiff brush into a sample container may be most efficient, as only very thin layers of most surface soils are typically eroded. If subsurface soil characteristics are needed, such as observing signs of seasonal high groundwater, then small trenches may be needed. Small soil cores should be used when measuring soil texture when soil infiltration studies are being conducted. Cores (or trenches) are also needed if soil chemical quality is needed for different soil depths.

Street Surface Particulate Sampling Procedures

The street dirt sampling procedures described in this section were developed by Pitt (1979) and were used extensively in many of the EPA's Nationwide Urban Runoff Program (NURP) projects (EPA 1983a) and other street cleaning performance studies and washoff studies (Pitt 1987). These procedures are flexible and more accurate indicators of street dirt loading conditions than previous sampling methods used during earlier studies (such as Sartor and Boyd 1972, for example). The procedures are described here in detail so that they can be used by those wishing to determine loading conditions, accumulation rates, washoff rates, and street cleaning effectiveness for their own locations.

Powerful dry vacuum sampling, as used in this sampling procedure, is capable of removing practically all of the particulates (>99%) from the street surface, compared to wet sampling. It can also remove most of the other major pollutants from the street surface (>80% for COD, phosphates, and metals, for example). Wet sampling, which would better remove some of these other constituents, is restricted to single area sampling, requires long periods of time, requires water (and usually fire hydrants, further restricting sample collection locations to areas that have no parked cars), and basically is poorly representative of the variable conditions present. Dry sampling can be used in many locations throughout an area; it is fast, and it can also be used to isolate specific sampling areas (such as driving lanes, areas with intensive parking, and even airport runways and freeways, if special safety precautions are used). It is especially useful when coupled with appropriate experimental design tools to enable suitable numbers of subsamples to be collected representing subareas, and finally, the collected dry samples can be readily separated into different particle sizes for discrete analyses.

Equipment Description

A small half-ton trailer can be used to carry the generator, two stainless steel industrial vacuum units, vacuum hose and wand, miscellaneous tools, and a fire extinguisher. This equipment can also be fitted in a pickup truck, but much time is then lost with frequent loading and unloading of equipment, especially considering the frequent sampling that is typically used for a study of this nature (sampling at least once a week, and sometimes twice a day before and after street cleaning or rains). A truck with a suitable hitch and signal light connections is needed to pull the trailer. The truck also requires warning lights, including a rooftop flasher unit. The truck is operated with its headlights and warning lights on during the entire period of sample collection. The sampler and hose tender both need to wear orange, high-visibility vests. The trailer also needs to be equipped with a caution sign on its tailgate. In addition, both the truck and the street cleaner used to clean the test area can be equipped with radios (CB radios are adequate), so that the sampling team can contact the street cleaner operator when necessary to verify location and schedule for specific test areas.

Experiments were conducted by Pitt (1979) to determine the most appropriate vacuum and filter bag combination. Two-horsepower (hp) industrial vacuum cleaners with one secondary filter and a primary dacron filter bag are recommended as the best combination. The vacuum units are heavy duty and made of stainless steel to reduce contamination of the samples. Two separate 2-hp vacuums are used together by joining their intakes with a Y connector. This combination extends the useful length of the 1.5-in vacuum hose to 35 ft and increases the suction so that it is adequate to remove all particles of interest from the street surface. Unfortunately, two vacuums need to be cleaned to recover the samples after the subsample collections. A wand and a "gobbler" attachment are also needed. The aluminum gobbler attaches to the end of the wand and is triangular in shape and about 6 in across. Since it was scraped across the street during sample collection, it wears out frequently and must be replaced. The generator needed to power the vacuum units must be of sufficient power

to handle the electrical current load drawn by the vacuum units, about 5000 watts for two 2-hp vacuums. Honda water-cooled generators are extremely quiet and reliable for this purpose. Finally, a secure, protected garage is needed to store the trailer and equipment near the study areas when they are not in use.

Sampling Procedure

Because the street surfaces are more likely to be dry during daylight hours (necessary for good sample collection), collection should not begin before sunrise nor continue after sunset. During extremely dry periods, sampling can be conducted during dark hours, but that requires additional personnel for traffic control. Two people are needed for sampling at all times, one acting as the sampler, the other acting as the vacuum hose tender and traffic controller. This lessens individual responsibility and enables both persons to be more aware of traffic conditions.

Before each day of sampling, the equipment is checked to make sure that the generator's oil and gasoline levels are adequate, and that vacuum hose, wand, and gobbler are in good condition. Dragging the vacuum hose across asphalt streets requires periodic hose repairs (usually made using gray duct tape). A check is also made to ensure that the vacuum units are clean, the electrical cords are securely attached to the generator, and the trailer lights and warning lights are operable. The generator requires about 3 to 5 min to warm up before the vacuum units are turned on one at a time (about 5 to 10 s apart to prevent excessive current loading on the generator). The amperage and voltage meters of the generator are also periodically checked. The generator and vacuums are left on during the complete subsampling period to lessen strain associated with multiple shutoffs and startups. Obviously, the sampling end of the vacuum hose needs to be carefully secured between subsamples to prevent contamination.

Figure 5.51 illustrates the general sampling procedure. Each subsample includes all of the street surface material that would be removed during a severe rain (including loose materials and caked-on mud in the gutter and street areas). The location of the subsample strip is carefully selected to ensure that it has no unusual loading conditions (e.g., a subsample should not be collected through the middle of a pile of leaves; rather, it is collected where the leaves are lying on the street in their normal distribution pattern). When possible, wet areas are avoided. If a sample is wet and the particles are caked around the intake nozzle, the caked mud from the gobbler is carefully scraped into the vacuum hose while the vacuum units are running. In addition, the hose needs to be struck against the ground at the end of the sampling period to knock loose any material stuck on the inside of the hose.

Subsamples are collected in a narrow strip about 6 in wide (the width of the gobbler) from one side of the street to the other (curb to curb). In heavily traveled streets where traffic is a problem, some subsamples consist of two separate one-half street strips (curb to crown). Traffic is not stopped for subsample collection; the operators wait for a suitable traffic break. On wide or busy roadways, a subsample is often collected from two strips several feet apart, halfway into the street. On busy roadways with no parking and good street surfaces, most particulates are found within a few feet of the curb, and a good subsample could be collected by vacuuming two strips adjacent to the curb

Figure 5.51 Street dirt subsample collection.

and as far into the traffic lanes as possible. Only a sufficient (and safe) break in traffic allows a subsample to be collected halfway across the street.

Subsamples taken in areas of heavy parking are collected between vehicles along the curb, as necessary. The sampling line across the street does not have to be a continuous line if a parked car blocks the most obvious and easiest subsample strip. A subsample can be collected in shorter (but very close) strips, provided the combined length of the strip is representative of different distances from the curb. Again, in all instances, each subsample must be representative of the overall curb-to-curb loading condition.

When sampling, the leading edge of the gobbler is slightly elevated above the street surface (0.125 in) to permit an adequate air flow and to collect pebbles and large particles. The gobbler is lifted further to accept larger material as necessary. If necessary, leaves in the subsample strip are manually removed and placed in the sample storage container to prevent the hose from clogging. If a noticeable decrease in sampling efficiency is observed, the vacuum hoses are cleaned immediately by disconnecting the hose lengths, cleaning out the connectors (placing the debris into the sample storage container), and reversing the air flows in the hoses (blowing them out by connecting the hose to the vacuum exhaust and directing the dislodged debris into the vacuum inlet). If any mud is caked on the street surface in the subsample strip, the sampler loosens it by scraping a shoe along the subsample path (being certain that street construction material is not removed from the subsample path unless it was very loose). Scraping caked-on mud is done after an initial vacuum pass. After scraping is completed, the strip is revacuumed. A rough street surface is sampled most easily by pulling (not pushing) the wand and gobbler toward the curb. Smooth and busy streets are usually sampled with a pushing action, away from the curb.

An important aspect of the sample collection is the speed at which the gobbler is moved across the street. A very rapid movement significantly decreases the amount of material collected; too slow a movement requires more time than is necessary. The correct movement rate depends on the roughness of the street and the amount of material on it. When sampling a street that has a heavy loading of particulates, or a rough surface, the wand needs to be pulled at a velocity of less than 1 ft/s. In areas of lower loading and smoother streets, the wand can be pushed at a velocity of 2 to 3 ft/s. The best indicators of the correct collection speed are achieved by visually examining how well the street is being cleaned in the sampling strip and by listening to the collected material rattle up the wand and through the vacuum hose. It is quite common to leave a visually cleaner strip on the street where the subsample was collected, even on streets that appeared to be clean before sampling.

In all cases, the hose tender must continuously watch traffic and alert the sampler of potentially hazardous conditions. In addition, the hose tender plays out the hose to the sampler as needed and keeps the hose as straight as possible to prevent kinking. If a kink develops, sampling is stopped until the hose tender straightens the hose. While working near the curb out of the traffic lane (typically an area of high loadings), the sampler visually monitors the performance of the vacuum sampler and periodically checks for vehicles. In the street, the sampler constantly watches traffic and monitors the collection process by listening to particles moving up the wand. A large break in traffic is required to collect dust and dirt from street cracks in the traffic lanes because the sampler has to watch the gobbler to make sure that all of the loose material in the cracks is removed.

When moving from one subsample location to another, the hose, wand, and gobbler need to be securely placed in the trailer. All subsamples are composited in the vacuums for each study area, and the hose must be placed away from the generator's hot muffler to prevent damage. The generator and vacuum units are left on and in the trailer during the entire subsample collection period. This helps dry damp samples and reduces the strain on the vacuum and generator motors.

The length of time it takes to collect all of the subsamples in an area varies with the number of subsamples and the test area road texture and traffic conditions. The number of subsamples required in each area can be determined using the experimental design sample effort equations described earlier in this chapter, with seasonal special sampling efforts to measure the variability

of street dirt loadings in each area. The variabilities can be measured using a single, small 1.5-hp industrial vacuum, with a short hose to make sample collection simpler. The vacuum needs to be emptied, the sample collected and placed in individual Ziploc™ baggies, and weighed (later in the lab) for each individual sample to enable the variability in loadings to be measured. As an example, during the first phase of the San Jose, CA, study (Pitt 1979), the test areas required the following sampling effort:

Test Area	No. of Subsamples	Sampling Duration, h
Downtown — poor (rough) asphalt street surface	14	0.5
Downtown — good (smooth) asphalt street surface	35	1
Keyes Street — oil and screens street surface	10	0.5–1
Keyes Street — good asphalt street surface	36	1
Tropicana — good asphalt street surface	16	0.5–1

In the oil and screens test area, the sampling procedure was slightly different because of the relatively large amount of pea gravel (screens) that was removed from the street surface. The gobbler attachment was drawn across the street more slowly (at a rate of about 3 s/ft). Each subsample was collected by a half pass (from the crown to the curb of the street) and therefore contained one half of the normal sample. Two curb-to-curb passes were made for each Tropicana subsample because of the relatively low particulate loadings in this area, as several hundred grams of sample material are needed for the laboratory tests. In addition, an "after" street cleaning subsample is not collected from exactly the same location as the "before" street cleaning subsample (they need to be taken from the same general area, but at least a few feet apart).

A field data record sheet kept for each sample contains:

- Subsample numbers
- Dates and time of the collection period
- Any unusual conditions or sampling techniques

Subsample numbers are crossed off as each subsample is collected. After cleaning, subsample numbers are marked if the street cleaner operated next to the curb at that location. This differentiation enables the effect of parked cars on street cleaning performance to be analyzed. In addition, photographs (and movies) are periodically made to document the methods and street loading conditions.

Sample Transfer

After all subsamples for a test area are collected, the hose and Y connections are cleaned by disconnecting the hose lengths, reversing them, and holding them in front of the vacuum intake. Leaves and rocks that may have become caught are carefully removed and placed in the vacuum can; the generator is then turned off. The vacuums are either emptied at the last station or at a more convenient location (especially in a sheltered location out of the wind and sun).

To empty the vacuums, the top motor units are removed and placed out of the way of traffic. The vacuum units are then disconnected from the trailer and lifted out. The secondary, coarse vacuum filters are removed from the vacuum can and are carefully brushed with a small stiff brush into a large funnel placed in the storage can. The primary dacron filter bags are kept in the vacuum can and shaken carefully to knock off most of the filtered material. The dust inside the can is allowed to settle for a few minutes, then the primary filter is removed and brushed carefully into the sample can with the brush. Any dirt from the top part of the bag where it is bent over the top of the vacuum is also carefully removed and placed into the sample can. Respirators and eye protection are necessary to minimize exposure to the fine dust.

After the filters are removed and cleaned, one person picks up the vacuum can and pours it into the large funnel on top of the sample can, while the other person carefully brushes the inside of the vacuum can with a soft 3- to 4-in paintbrush to remove the collected sample. In order to prevent excessive dust losses, the emptying and brushing is done in areas protected from the wind. To prevent inhaling the sample dust, both the sampler and the hose tender wear mouth and nose dust filters while removing the samples from the vacuums.

To reassemble the vacuum cans, the primary dacron filter bag is inserted into the top of the vacuum can with the filter's elastic edge bent over the top of the can. The secondary, coarse filter is placed into the can and assembled on the trailer. The motor heads are then carefully replaced on the vacuum cans, making sure that the filters are on correctly and the excess electrical cord is wrapped around the handles of the vacuum units. The vacuum hoses and wand are attached so that the unit is ready for the next sample collection.

The sample storage cans are labeled with the date, the test area's name, and an indication of whether the sample was taken before or after the street cleaning test, or if it was an accumulation (or other type) of sample. Finally, the lids of the sample cans are taped shut and transported to the laboratory for logging-in, storage, and analysis.

Measurements of Street Dirt Accumulation

The washoff of street dirt and the effectiveness of street cleaning as a stormwater control practice are highly dependent on the street dirt loading. Street dirt loadings are the result of deposition and removal rates, plus "permanent storage." The permanent storage component is a function of street texture and condition and is the quantity of street dust and dirt that cannot be removed naturally or by street cleaning equipment. It is literally trapped in the texture, or cracks, of the street. The street dirt loading at any time is this initial permanent loading plus the accumulation amount corresponding to the exposure period, minus the resuspended material removed by wind and traffic-induced turbulence. Removal of street dirt can occur naturally by winds and rain, or by human activity (by the turbulence of traffic or by street cleaning equipment). Very little removal occurs by any process when the street dirt loadings are small, but wind removal may be very large with larger loadings, especially for smooth streets (Pitt 1979).

It takes many and frequent samples to ascertain the accumulation characteristics of street dirt. The studies briefly described in the following paragraphs typically involved collecting many hundreds of composite street dirt samples during the course of the 1- to 3-year projects from each study area. With each composite sample made up of about 10 to 35 subsamples, a great number of subsamples were used to obtain the data. Without high resolution (and effective) sampling, it is not possible to identify the variations in loadings and effects of rains and street cleaning.

The most important factors affecting the initial loading and maximum loading values are street pavement texture and street pavement condition. When data from many locations are studied, it is apparent that smooth streets have substantially smaller street dirt loadings at any accumulation period compared to rough streets for the same land use. Very long accumulation periods relative to the rain frequency result in high street dirt loadings. During these conditions, the losses of street dirt to wind (as fugitive dust) may approximate the deposition rate, resulting in relatively constant street dirt loadings. At Bellevue, WA, typical inter-event rain periods average about 3 days. Relatively constant street dirt loadings were observed in Bellevue because the frequent rains kept the loadings low and very close to the initial storage value, with little observed increase in dirt accumulation over time (Pitt 1985). In Castro Valley, CA, the rain inter-event periods were much longer (ranging from about 20 to 100 days) and steady street dirt loadings were only observed after about 30 days when the loadings became very high and fugitive dust losses caused by the winds and traffic turbulence moderated the loadings (Pitt and Shawley 1982).

An example of the type of sampling needed to obtain accumulation rate values was conducted by Pitt and McLean (1986) in Toronto. They measured street dirt accumulation rates and the effects

of street cleaning as part of a comprehensive stormwater research project. An industrial street with heavy traffic and a residential street with light traffic were monitored about twice a week for 3 months. At the beginning of this period, intensive street cleaning (one pass per day for each of 3 consecutive days) was conducted to obtain reasonably clean streets. Street dirt loadings were then monitored every few days to measure the accumulation rates of street dirt. The street dirt sampling procedures previously described were used to clean many separate subsample strips across the roads, which were then combined for physical and chemical analyses.

In Toronto, the street dirt particulate loadings were quite high before the initial intensive street cleaning period and were reduced to their lowest observed levels immediately after the last street cleaning. After street cleaning, the loadings on the industrial street increased much faster than on the residential street. Right after intensive cleaning, the street dirt particle sizes were also similar for the two land uses. However, the loadings of larger particles on the industrial street increased at a much faster rate than on the residential street, indicating more erosion or tracking materials were deposited on the industrial street. The residential street dirt measurements did not indicate that any material was lost to the atmosphere as fugitive dust, likely due to the low street dirt accumulation rate and the short periods of time between rains. The street dirt loadings never had the opportunity to reach the high loading values needed before they could be blown from the streets by winds or by traffic-induced turbulence. The industrial street, in contrast, had a much greater street dirt accumulation rate and was able to reach the critical loading values needed for fugitive losses in the relatively short periods between the rains.

A street dirt sampling program must be conducted over a long enough period of time to obtain accumulation information. Infrequent observations hinder the analyses. It requires a continuous period of sampling, possibly with samples collected at least once a week, plus additional sampling close to the beginning and end of rains. Infrequent sampling, especially when interrupted by rains, does not allow changes in loadings to be determined. In addition, seasonal measurement periods are also likely needed because street dirt accumulation rates may change for different periods of the year. Infrequent and few samples may be useful to statistically describe the street dirt loading and to measure pollutant strengths associated with the samples, but they are not suitable for trend analyses. Chapter 7 presents statistical test procedures for identifying trends and should be consulted for different alternative methods to measure street dirt accumulation rates.

Small-Scale Washoff Tests

Washoff tests may be necessary to directly measure the energy available to dislodge and transport street dirt from paved areas to the drainage system. These tests are not usually conducted, as many rely on the process descriptions contained in commonly used stormwater models. Unfortunately, many of the process descriptions are in error due to improper interpretations of the test data. The following discussion therefore briefly describes these tests to encourage watershed researchers to obtain local data for accurate model calibration.

Observations of particulate washoff during controlled tests using actual streets and natural street dirt and debris are affected by street dirt distributions and armoring. The earliest controlled street dirt washoff experiments were conducted by Sartor and Boyd (1972) during the summer of 1970 in Bakersfield, CA. Their data were used in many stormwater models (including SWMM, Huber and Heaney 1981; STORM, COE 1975; and HSPF, Donigian and Crawford 1976) to estimate the percentage of the available particulates on the streets that would wash off during rains of different magnitudes. Sartor and Boyd used a rain simulator having many nozzles and a drop height of $1^1/_2$ to 2 m in street test areas of about 5 by 10 m. Tests were conducted on concrete, new asphalt, and old asphalt, using simulated rain intensities of about 5 and 20 mm/hour. They collected and analyzed runoff samples every 15 min for about 2 hours for each test. Sartor and Boyd fitted their data to an exponential curve, assuming that the rate of particle removal of a given size is proportional to the street dirt loading and the constant rain intensity:

$$dN/dt = krN$$

where: dN/dt = the change in street dirt loading per unit time
k = proportionality constant
r = rain intensity (in/hour)
N = street dirt loading (lb/curb-mile)

This equation, upon integration, becomes:

$$N = N_o e^{-krt}$$

where: N = residual street dirt load (after the rain)
N_o = initial street dirt load
t = rain duration

Street dirt washoff is therefore equal to N_o minus N. The variable combination rt, or rain intensity (in/h) times rain duration (h), is equal to total rain depth (R), in inches. This equation then further reduces to:

$$N = N_o e^{-kR}$$

Therefore, this equation is only sensitive to the total depth of the rain that has fallen since the beginning of the rain, and not rain intensity. Because of decreasing particulate supplies, the exponential washoff curve also predicts decreasing concentrations of particulates with time since the start of a constant rain (Alley 1980, 1981).

The proportionality constant, k, was found by Sartor and Boyd to be slightly dependent on street texture and condition, but was independent of rain intensity and particle size. The value of this constant is usually taken as 0.18/mm, assuming that 90% of the particulates will be washed from a paved surface in 1 hour during a 13 mm/hour rain. However, Alley (1981) fitted this model to watershed outfall runoff data and found that the constant varied for different storms and pollutants for a single study area. Novotny (as part of Bannerman et al. 1983) also examined "before" and "after" rain event street particulate loading data from the Milwaukee Nationwide Urban Runoff Program (NURP) project and found almost a threefold difference between the constant value of k for fine (<45 μm) and medium-sized particles (100 to 250 μm). The calculated values were 0.026/mm for the fine particles and 0.01/mm for the medium-sized particles, both much less than the "accepted" value of 0.18/mm. Jewell et al. (1980) also found large variations in outfall "fitted" constant values for different rains compared to the typical default value. Either the assumption of the high removal of particulates during the 13 mm/hour storm was incorrect or the equation cannot be fitted to outfall data (most likely, as this would require that all the particulates originate from homogeneous paved surfaces during all storm conditions).

This washoff equation has been used in many stormwater models, along with an expression for an availability factor. An availability factor is needed, as N_o is only the portion of the total street load available for washoff. This availability factor (the fraction of the total street dirt loading available for washoff) is generally used as 1.0 for all rain intensities greater than about 18 mm/hour and reduces to about 0.10 for rains of 1 mm/hour.

The Bellevue, WA, urban runoff project (Pitt 1985) included about 50 pairs of street dirt loading observations close to the beginnings and ends of rains. Very large reductions in street dirt loadings during rains were observed in Bellevue for the smallest particles, but the largest particles actually increased in loadings (due to deposited erosion materials originating from off-street areas). The particles were not source limited, but armor shielding may have been important. Most of the

particulates in the runoff were in the fine particle sizes (<63 µm). Very few particles greater than 1000 µm were found in the washoff water. Care must be taken to not confuse street dirt particle size distributions with stormwater runoff particle size distributions. The stormwater particle size distributions are much more biased toward the smaller sizes, as described later.

Washoff tests can be designed to investigate several important factors and interactions that may affect washoff of different sized particulates from impervious areas (Pitt 1987):

- Street texture
- Street dirt loading
- Rain intensity
- Rain duration
- Rain volume

Multiple parameters that may affect a process can be effectively evaluated using factorial tests as described by Box et al. (1978) and earlier in this chapter. As an example, the tests conducted by Pitt (1987) were arranged as an overlapping series of 2^3 factorial tests, one for each particle size and rain total, and were analyzed using factorial test procedures. Nonlinear analyses were also used to identify a set of equations to describe the resulting curve shapes. The differences between available and total loads were also related to the experimental factors. This experimental setup can be effectively repeated elsewhere, with possible adjustments in the levels used in the experiments to reflect local conditions.

All tests were conducted for about 2 hours, with total rain volumes ranging from about 5 to 25 mm. The test code explanations follow:

Test Code	Rain Intensity	Street Dirt Loading	Street Texture
HCR	High	Clean	Rough
HDR	High	Dirty	Rough
LCR	Light	Clean	Rough
LDR	Light	Dirty	Rough
HCS	High	Clean	Smooth
HDS	High	Dirty	Smooth
LCS	Light	Clean	Smooth
LDS	Light	Dirty	Smooth

Unfortunately, the streets during the LDS (light rain intensity; dirty street; smooth texture) test were not as dirty as anticipated and actually replicated the LCS tests. The experimental analyses were modified to indicate these unanticipated duplicate observations.

A simple artificial rain simulator was constructed using 12 lengths of "soaker" hose, suspended on a wooden framework about 1 m above the road surface (Figures 5.52 and 5.53). "Rain" was applied by connecting the hoses to a manifold having individual valves to adjust constant

Figure 5.52 Washoff test site in Toronto.

Figure 5.53. Runoff collection area for Toronto washoff tests.

Figure 5.54. Sprinklers at freeway washoff test site in Austin, TX.

Figure 5.55 Sampler and rain gauge location at Austin freeway washoff test site.

rain intensities for the different areas. The manifold was in turn connected to a fire hydrant. The flow rate needed for each test was calculated based on the desired rain intensity and the area covered. The flow rates were carefully monitored by using a series of ball flow gauges before the manifold. The distributions of the test rains over the study areas were also monitored by placing about 20 small graduated cylinders over the area during the rains. In order to keep the drop sizes representative of sizes found during natural rains, the surface tension of the water drops hanging on the plastic soaker hoses was reduced by applying a light coating of Teflon spray to the hoses.

A different washoff test site is shown in Figures 5.54 through 5.56, where large sprinklers were located along the side of a freeway in Austin, TX. The sprinklers rained water directly onto the freeway during traffic conditions to better represent the combined effect of rain and auto-induced turbulence. Unfortunately, in order to get "rain" over a substantial area of the freeway, the "rain intensity" was extremely high, supplying much more energy than was typical, even for extreme events. In addition, this setup, while useful in obtaining hard-to-get data, may also have imposed an unusually high accident risk to freeway users (although large amounts of publicity, signage, and available alternate routes were all used to reduce this risk). This semipermanent installation was also used to monitor runoff from natural rains for comparison.

It was difficult to obtain even distributions of rain during the light rain tests in Toronto using the manifold, so a single hose was used that was manually moved back and forth over the test area during the smaller rain tests (three people took 30-min shifts). To keep evaporation reasonable for the rain conditions, the test sites were also shaded during sunny days. Blank water samples were also obtained from the manifold for background residue analyses. The filterable residue of the "rain" water (about 185 mg/L) could cause substantial errors when calculating washoff.

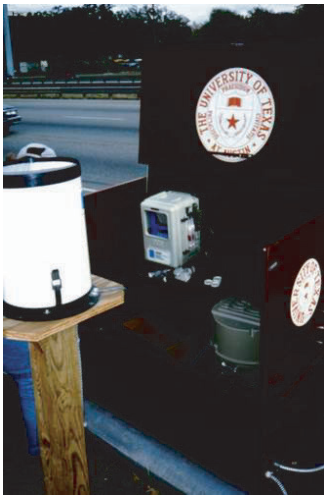

Figure 5.56. Sampler and flow monitoring equipment at Austin freeway washoff test site.

The areas studied were about 3 by 7 m each. The street side edges of the test areas were edged with plywood, about 30 cm in height and embedded in thick caulking, to direct the runoff toward the curbs with minimal leakage. All runoff was pumped continuously from downstream sumps (made of caulking and plastic sand bags) to graduated 1000-L Nalgene containers. The washoff samples were obtained from the pumped water going to the containers every 5 to 10 min at the beginning of the tests, and every 30 min near the end of the test. Final complete rinses of the test areas were also conducted (and sampled) at the tests' conclusions to determine total loadings of the monitored constituents.

The samples were analyzed for total residue, filtrate residue, and particulate residue. Runoff samples were also filtered through 0.4-μm filters and microscopically analyzed (using low power polarized light microscopes to differentiate between inorganic and organic debris) to determine particulate residue size distributions from about 1 to 500 μm. The runoff flow quantities were also carefully monitored to determine the magnitude of initial and total rainwater losses on impervious surfaces.

These tests are different from the important early Sartor and Boyd (1972) washoff experiments in the following ways:

- They were organized in overlapping factorial experimental designs to identify the most important main factors and interactions.
- Particle sizes were measured down to about 1 μm (in addition to particulate residue and filterable residue measurements).
- The precipitation intensities were lower in order to better represent actual rain conditions of the upper Midwest.
- Observations were made with more resolution at the beginning of the tests.
- Washoff flow rates were frequently measured.
- Emphasis was placed on total street loading, not just total available loading.
- Bacteria population measurements were also periodically obtained.

Sampling of Atmospheric Contributions

Atmospheric processes affecting urban runoff pollutants include dry dustfall and precipitation quality. These have been monitored in many urban and rural areas. In many instances, however, the samples were combined as a bulk precipitation sample before processing. Automatic precipitation sampling equipment can distinguish between dry periods of fallout and precipitation. These devices cover and uncover appropriate collection jars exposed to the atmosphere. Much of this information has been collected as part of the Nationwide Urban Runoff Program (NURP) and the Atmospheric Deposition Program, both sponsored by the U.S. Environmental Protection Agency (EPA 1983a).

One must be very careful in interpreting this information, however, because of the ability of many polluted dust and dirt particles to be resuspended and then redeposited within the urban area. In many cases, the atmospheric deposition measurements include material that previously resided and was measured in other urban runoff pollutant source areas. Also, only small amounts of the atmospheric deposition material would directly contribute to runoff. Rain is subjected to infiltration and the dry-fall particulates are most likely incorporated with surface soils and only small fractions are then eroded during rains. Therefore, mass balances and determinations of urban runoff deposition and accumulation from different source areas can be highly misleading, unless transfer of material between source areas and the effective yield of this material to the receiving water is considered. Depending on the land use, relatively little of the dustfall in urban areas likely contributes to stormwater discharges. The major exception would be dustfall directly on receiving waters.

Dustfall and precipitation affect all of the major urban runoff source areas in an urban area. Dustfall, is typically not a major pollutant source, but fugitive dust is mostly a mechanism for

pollutant transport. Most of the dustfall monitored in an urban area is resuspended particulate matter from street surfaces or wind erosion products from vacant areas (Pitt 1979). Point source pollutant emissions can also significantly contribute to dustfall pollution, especially in industrial areas. Transported dust from regional agricultural activities can also significantly affect urban stormwater.

Wind-transported materials are commonly called "dustfall." Dustfall is normally measured by collecting dry samples, excluding rainfall and snowfall. If rainout and washout are included, one has a measure of total atmospheric fallout. This total atmospheric fallout is sometimes called "bulk precipitation." Rainout removes contaminants from the atmosphere by condensation processes in clouds, while washout is the removal of contaminants by the falling rain. Therefore, precipitation can include natural contamination associated with condensation nuclei in addition to collecting atmospheric pollutants as the rain- or snowfalls. In some areas, the contaminant contribution by dry deposition is small, compared to the contribution by precipitation (Malmquist 1978). However, in heavily urbanized areas, dustfall can contribute more of an annual load than the wet precipitation, especially when dustfall includes resuspended materials.

Much of the monitored atmospheric dustfall and precipitation would not reach the urban runoff receiving waters. The percentage of dry atmospheric deposition retained in a rural watershed was extensively monitored and modeled in Oakridge, TN (Barkdoll et al. 1977). They found that about 98% of the lead in dry atmospheric deposits was retained in the watershed, along with about 95% of the cadmium, 85% of the copper, 60% of the chromium and magnesium, and 75% of the zinc and mercury. Therefore, if the dry deposition rates were added directly to the yields from other urban runoff pollutant sources, the resultant urban runoff loads would be very much overestimated.

Rubin (1976) stated that resuspended urban particulates are returned to the earth's surface and waters in four main ways: gravitational settling, impaction, precipitation, and washout. Gravitational settling, as dry deposition, returns most of the particles. This not only involves the settling of relatively large fly ash and soil particles, but also the settling of smaller particles that collide and coagulate. Rubin stated that particles that are less than 0.1 μm in diameter move randomly in the air and collide often with other particles. These small particles can grow rapidly by this coagulation process. They would soon be totally depleted in the air if they were not constantly replenished. Particles in the 0.1 to 1.0 μm range are also removed primarily by coagulation. These larger particles grow more slowly than the smaller particles because they move less rapidly in the air, are somewhat less numerous, and, therefore, collide less often with other particles. Particles with diameters larger than 1 μm have appreciable settling velocities. Those particles about 10 μm in diameter can settle rapidly, although they can be kept airborne for extended periods and for long distances by atmospheric turbulence.

The second important particulate removal process is impaction. Impaction of particles near the earth's surface can occur on vegetation, rocks, and building surfaces. The third form of particulate removal from the atmosphere is precipitation, in the form of rain and snow. This is caused by the rainout process in which the particulates are removed in the cloud-forming process. The fourth important removal process is washout of the particulates below the clouds during the precipitation event. Therefore, it is easy to see that reentrained particles (especially from street surfaces, other paved surfaces, rooftops, and from soil erosion) in urban areas can be readily redeposited through these various processes, either close to the points of origin, or some distance away.

Pitt (1979) monitored airborne concentrations of particulates near typical urban roads using Climat Particle Counters (Figure 5.57). He found that on a particle count basis, the downwind roadside particulate concentrations were about 10% greater than upwind conditions. About 80% of the concentration increases, by particle count, were associated with particles in the 0.5 to 1.0 μm range. However, about 90% of the particle concentration increases by weight were associated with particles greater than 10 μm. He found that the rate of particulate resuspension from street surfaces increases when the streets are dirty (cleaned infrequently) and varied widely for different street and traffic conditions. The resuspension rates were calculated based upon observed long-term accumulation conditions on street surfaces for many different study area conditions, and varied from about 0.30 to 3.6 kg/curb-km (1 to 12 lb/curb-mile) of street per day.

Figure 5.57 Hi-vol suspended particulate sampler, along with particle counters and wind velocity meters used to measure fugitive dust losses caused by traffic-induced turbulence and dirty roads in San Jose, CA, tests.

Murphy (1975) described a Chicago study in which airborne particulate material within the city was microscopically examined, along with street surface particulates. The particulates from both of these areas were found to be similar (mostly limestone and quartz) indicating that the airborne particulates were most likely resuspended street surface particulates, or were from the same source. PEDCo (1977) found that the reentrained portion of the traffic-related particulate emissions (by weight) is an order of magnitude greater than the direct emissions accounted for by vehicle exhaust and tire wear. They also found that particulate resuspensions from a street are directly proportional to the traffic volume and that the suspended particulate concentrations near the streets are associated with relatively large particle sizes. The medium particle size found, by weight, was about 15 µm, with about 22% of the particulates occurring at sizes greater than 30 µm. These relatively large particle sizes resulted in substantial particulate fallout near the road. They found that about 15% of the resuspended particulates fall out within 10 m, 25% within 20 m, and 35% within 30 m from the street (by weight). In a similar study Cowherd et al. (1977) reported a wind erosion threshold value of about 5.8 m/s (13 mph). At this wind speed, or greater, significant dust and dirt losses from the road surface could result, even in the absence of traffic-induced turbulence. Rolfe and Reinbold (1977) also found that most of the particulate lead from automobile emissions settled out within 100 m of roads. However, the automobile lead does widely disperse over a large area. They found, through multielemental analyses, that the settled outdoor dust collected at or near the curb was contaminated by automobile activity and originated from the streets.

The experimental design and interpretation of atmospheric contributions must therefore be done carefully. Measurements can be obtained using numerous procedures, as summarized below:

- Conventional air pollution monitoring equipment, especially hi-vol samplers for particulates. The captured particulates can be chemically analyzed for pollutants, especially heavy metals.
- Real-time air pollution monitoring equipment, such as nephelometers and particle counters (Figure 5.57). These are especially useful for short-term measurements of resuspended particulates from nearby pavements to indicate turbulence effects from vehicles or natural winds. They are also useful for fugitive dust measurements from construction sites and can also be used to indicate the effects of vehicular traffic and wind losses from construction roads, etc.
- Sticky paper fugitive dust samplers. These are simple upright cylinders about 10 cm in diameter and 20 cm in height that are carefully oriented to enable moderate- or long-term measurements of fugitive dust losses from specific directions. Simple measurements are made by comparing the color and tone of the exposed paper for different exposed directions to standards. The exposed

Figure 5.58 Wet-dry atmospheric deposition sampler in Bellevue, WA.

Figure 5.59 Large surface area used to capture sufficient rain for chemical analyses in early San Jose, CA, tests.

paper can also be examined under a microscope for more specific measurements and identification of particle characteristics.

- Wet- and dry-fall automatic samplers (Figure 5.58). These were commonly used during the EPA's NURP and Atmospheric Deposition Program and allow long-term sampling of dustfall during dry weather and rainwater during wet weather. A lid, connected to a moisture sensor, automatically moves to cover the appropriate sampling bucket. The collected samples are rinsed from the appropriate buckets after the desired exposure periods and chemically analyzed. If a single bucket sampler is used (without the automatic lid), then the dry dustfall and the rainwater samples are combined in one sample for a bulk precipitation analysis. Evaporation of the rainwater sample and obvious chemical transformations occur in these samplers during the typically long-term exposures. These samplers are therefore most useful for evaluations for stable compounds (such as suspended solids and most heavy metals) and are not very suitable for nutrient, bacteria, or organic analyses.
- Precipitation sampler. Because rainwater has little buffer capacity, short-term collections of rainwater are needed for many constituents (especially major ions, pH, and nutrients). However, in order to collect sufficient sample volume in a short period, a large collection area is needed. One simple solution is to construct a large collection area using a plastic tarp supported around its edges (Figure 5.59). The tarp is allowed to sag toward the center, where a weight surrounds a central hole that is located over an appropriate sample bottle. A tarp having about a 10 m^2 surface area can collect several liters of rainwater in a few minutes during a relatively light rainfall. Of course, potential contamination of the sample is possible through the use of the tarp. For a semipermanent installation, it would be possible to construct a relatively large collection area using a piece of glass (being careful of joint materials), or a Teflon-coated surface could be used with fewer interferences than a plastic surface. See the earlier discussion on sample contamination potential from various materials. Many laboratory suppliers sell Teflon-coated sticky paper that is used for covering laboratory benches. It may be possible to use this material to cover a simple seamless rigid platform, having a central trough for rainwater collection.

SEDIMENT AND PORE WATER SAMPLING

Sediment Sampling Procedures

As discussed previously, sediments act as sinks and sources of contaminants and have been implicated as the cause of beneficial use impairments, such as fish consumption advisories, at

numerous sites throughout North America. Sediments that should be targeted as potential problem sources during any receiving water assessment are the small-grained, depositional-type sediments in urban, industrial, and agricultural drainages. Stormwater discharges can cause metal and organic chemicals, nutrients, and pathogens to accumulate in depositional sediments. These contaminants then may enter groundwater or reenter surface waters for further transport, or contaminate resident organisms and the overlying food web (see also Chapter 6). Once stormwater flows subside, the influence of contaminated sediments on overlying water persists and even increases during low flow conditions. Even though the short-term BOD of stormwater is not very high (BOD_5 of about 25 mg/L), the long-term BOD (BOD_{90} of about 250 mg/L) is high and resulting accumulations of organic debris in urban streams create anaerobic sediment conditions (Pitt 1979). These depositional sediments will continue to degrade in quality as long as organic and contaminant loadings continue, resulting in replacement with pollution-tolerant benthic macroinvertebrates, such as midges and worms, and also degrade the fish community (Burton and Scott 1992). Assessing the role of sediments in beneficial use attainment and ecosystem health is a necessary aspect of a receiving water investigation. As noted previously, heavy metals and nutrient and organic toxicants are of most interest in urban stream sediments while nutrients and pesticides are of primary concern in agricultural waterways. Pathogens may be a problem in either urban or agricultural watersheds. Contaminated stream sediments likely impart the most important impairments to aquatic life in urban areas (after direct habitat destruction and frequent high flows) and may also in agricultural areas. Collecting and analyzing these sediments and their biota are therefore necessary to establish water quality and the sources of any degradation.

In many ways, sampling and evaluating the quality of sediments is more difficult than water quality sampling. Though sediments vary less than waters on a temporal basis, they exhibit greater variation spatially, in a complex, semisolid, three-dimensional structure. Understanding and preserving this structure has tremendous ramifications in the assessment process. The surficial sediment layers that interface with overlying waters are the most dynamic and recent sediments, subject to resuspension and downstream deposition, oxidation, and rapid changes in quality based on overlying water conditions. As sediment depth increases, the biological communities and chemical conditions may change orders of magnitude over a millimeter to centimeter scale. This has been observed in oxygen-redox vertical gradients (Carlton and Klug 1990) and toxicity (horizontally and vertically) (Stemmer et al. 1990b). In addition to the high degree of heterogeneity often observed, maintaining sediment structure integrity is crucial when attempting to characterize the sample based on physical (e.g., redox potential, percent fines), chemical (e.g., metal speciation, nutrient concentration and speciation, volatile components), biological (e.g., biotransformations, microbial-meiofaunal communities), and toxicity (e.g., contaminant bioavailability) characteristics (ASTM 1991b; Burton 1992b). Maintaining complete sediment integrity is nearly impossible since the very process of sample collection is disruptive (Figure 5.60). There are effective methods, however, by which to reduce this disruption (see also Chapter 6). The importance of maintaining sample integrity depends on the type of problem and the data quality objectives (DQOs) of the study. Several guidance documents exist that address sediment sampling in detail. The most comprehensive and current guidance documents to date include ASTM 1994 and EPA 2001.

Figure 5.60 The fine-grained and muddy nature of most urban sediments requires specific sediment sampling procedures.

Disrupting the sensitive sediment environment is a major concern when collecting samples for toxicity studies, since the bioavailability and resulting toxicity can change significantly when in-place sediments are disturbed. An additional major concern is that the sediment depth sampled and chemically analyzed matches that being assessed for organism exposure (indigenous organisms and/or toxicity and bioaccumulation using surrogate species). Too often sediment grab samples are collected at unknown sediment depths (0 to 30 cm). The sediments are homogenized and then subsampled for chemical and physical analyses. Contaminant peaks occurring near the surface or deeper in the sediments may be diluted via the mixing process and then compared to biological effects. Resident benthic organisms are likely not being exposed to the same chemicals or concentrations that result from this process. In addition, laboratory toxicity testing will yield results that may bear little resemblance to field conditions. Therefore, it is best to establish whether recent or historical contamination is a concern, sample the appropriate sediment depth, and match the chemical analyses with realistic organism exposures.

A number of sampling-related factors can contribute to loss of the sediment sample's original characteristics, including sampler-induced pressure waves, washout of fine-grained sediments during retrieval, compaction due to sampler wall friction, sampling vessel or person-induced disturbance of surficial layers, disruption during subsampling or transport, oxidation, and temperature alterations. While it is impossible to remove all of these factors from routine assessments, reducing their influence increases the certainty that the data generated and resulting weight-of-evidence conclusions will be reliable.

Choosing the most appropriate sediment sampler for a study will depend on the sediment's characteristics, the volume and efficiency required, and the study's objective (Tables 5.16 through 5.18; Figures 5.61 through 5.63). Numerous sediment samplers are available. Two general categories include core samplers (which can obtain samples that can be analyzed by depth) and surface grab samplers (which only collect surface sediment). ASTM (1995) standard 4823 contains much information concerning core sampling in unconsolidated sediments that is applicable to urban streams. ASTM standard E 1391 also presents additional useful information concerning the sampling of sediment for toxicological testing. The preferred sampling method is to use core samplers whenever possible. However, they collect relatively little sediment and represent only a very small area. In addition, it may be difficult to retain samples in the samplers for retrieval in some types of bottom conditions (especially sandy sediment).

Grab samplers only collect samples from the surface layers of the sediment (10 to 50 cm in depth, at maximum). They also greatly disturb the sediment that is being sampled. Common problems include shallow depth of penetration and presence of a shock wave that results in loss of the fine surface sediments. However, they are much easier to use than corers under a wide variety of conditions. A common grab sampler is the Ponar sampler (Figures 5.64 through 5.67). It comes in a standard size and a "petite" size that weighs substantially less and is more practical for urban streams. The Ponar sampler is useful for sand, silt, and clay sediments and can be used in relatively deep water or shallow waters. It has a flexible cover over a top screen that helps to minimize the loss of fines during sampling. Forestry Suppliers, Inc. (800-543-4203) sells a petite 6" × 6" Wildco Ponar bottom dredge (catalog #77250 for about $450) and a larger 9" × 9" Wildco Ponar bottom dredge (catalog #77249 for about $800). The Peterson grab sampler is similar to the Ponar, but doesn't have a screened top plate. It is heavy and is more suitable for deeper water and harder clay bottoms than the Ponar sampler. Because of its weight, it requires the use of a winch. Cole Parmer (800-323-4340) sells a Peterson dredge sampler (catalog #H-05472-00 for about $1000). An Ekman sampler is also commonly used in small urban streams and ponds, but is limited to sampling soft bottoms. Forestry Suppliers, Inc. sells a light 6" × 6" Wildco–Ekman bottom dredge (catalog #77251 for about $350, including line, messenger, and case). Cole Parmer also sells a larger 9" × 9" Ekman dredge (catalog #H-05470-10 for about $600).

Dredge samplers that quantitatively sample surface sediments have been described (Grizzle and Stegner 1985). The depth profile of the sample may be lost in the removal of the sample from the

Table 5.16 Popular Sediment Samplers: Strengths and Weaknesses

Sampler	Strengths	Weaknesses
	Core Samplers	
Hand and gravity corers 0–30 cm depth 0.1–1.5 L volume	Maintains sediment layering of inner core. Fine surficial sediments retained. Replicate samples efficiently obtained. Removable liners. Inert liners may be used. Quantitative sampling allowed.	Small sample volume. Liner removal required for repetitive sampling. Not suitable in large-grain or consolidated sediments. Spillage possible.
Freeze core sampler 0–1 m depth 1 L volume	Maintains sediment layering of core. Fine sediments retained. Replicates samples efficiently obtained. Can be made of inert materials.	Small sample volume. Freezing may disturb sediment. Uses liquid CO_2 or dry ice for collecting sample. Requires several minutes to obtain each sample. May not collect large material. Not suitable for consolidated sediments.
Box corer 0–50 cm depth 1–30 L volume	Maintains sediment layering of large volume of sediment. Surficial fines retained relatively well. Quantitative sampling allowed.	Size and weight require power winch, difficult to handle and transport. Not suitable in consolidated sediments.
Vibratory corers 3–6 m depth 6–13 L volume	Samples deep sediments for historical analyses. Samples consolidated sediments. Minimal disturbance. May be used on small vessels.	Expensive and requires winch. Outer core integrity slightly disrupted.
	Grab Samplers	
Ekman or box dredge 0–10 cm depth Up to 3.5 L volume	Relatively large volume may be obtained. May be subsampled through lid. Lid design reduces loss of surficial sediments as compared to many dredges. Usable in moderately compacted sediments of varying grain sizes.	Loss of fines may occur during sampling. Incomplete jaw closure occurs in large-grain sediments or with large debris. Sediment integrity disrupted. Not an inert surface.
Ponar 0–10 cm depth Up to 1 L volume (petite) Up to 7.5 volume (standard)	Commonly used. Large volume obtained. Adequate on most substrates. Weight allows use in deep waters.	Loss of fines and sediment integrity occurs. Incomplete jaw closure occurs occasionally. Not an inert surface.
Van Veen or Young Grab 0–30 cm depth Up to 75 L volume	Useful in deep waters and on most substrates. Young grab coated with inert polymer. Large volume obtained.	Loss of fines and sediment integrity occurs. Incomplete jaw closure possible. Van Veen has metal surface. Young is expensive. Both may require winch.
Peterson 0–30 cm depth Up to 9.5 L volume	Large volume obtained from most substrates in deep waters.	Loss of fines and sediment integrity. Not an inert surface. Incomplete jaw closure may occur. May require winch.
Orange-Peel 0–30 cm depth 10–20 L volume	Large volume obtained from most substrates. Efficient closure.	Loss of fines and sediment integrity. Not an inert surface. Requires winch.
Shipek 0–10 cm depth Up to 3 L volume	Adequate on most substrates.	Small volume. Loss of fines and sediment integrity. Not an inert surface.

Modified from ASTM (American Society for Testing and Materials). *Standard Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing*. American Society for Testing and Materials, Philadelphia, Standard E 1391. 1991.

Table 5.17 Sediment and Interstitial Water Sampler Selection Guidelines

1. Sediment grain size effects on sampler selection
   - Silt-clay = core, grab, or peeper*
   - Sand = grab or peeper
   - Cobble = peeper
2. Sediment compacted: powered core
3. Sediment vertical gradient must be maintained: core or peepers
4. Sediment volumes
   Large volumes over small vertical gradients: dredge
   Small to moderate volumes: dredge, core, or peeper
5. Optimal samplers, in order of maintaining original sediment characteristics:
   1. *In situ* peeper*
   2. *In situ* suction*
   3. Core
   4. Grab
   5. Dredge
6. Optimal methods of collecting interstitial water (in order of preference, see Table 5.18)
   1. *In situ* peepers
   2. *In situ* suction (airstone or core-port)
   3. Centrifugation @ 10,000 × *g* (4°C) (without subsequent filtration)
   4. Centrifugation @ lower speeds
   5. Basal cup
   6. Squeezing or pressurization
   7. Suction or filtration

* For interstitial water collection only.

sampler. Dredge sampling promotes loss of not only fine sediments, but also water-soluble compounds and volatile organic compounds present in the sediment (ASTM 1991a). A comparison of sampler precision for macrobenthic purposes showed the Van Veen sampler to be the least precise; the most precise were the corers and Ekman dredge (Figures 5.68 and 5.69). The Smith–McIntyre and Van Veen samplers are more commonly used in marine studies, due to their weight. Shipek samplers are also used in marine investigations but may lose the top 2 to 3 cm of sediment fines from washout (Mudroch and MacKnight 1991).

Many of the problems associated with dredge samplers are largely overcome with the corers. The best corers for most sediment studies are hand-held polytetrafluoroethylene plastic, high-density polyethylene, or glass corers (liners), or large box corers. Corer samplers can penetrate the sediment by several meters, but that is rarely necessary (or possible) in urban receiving water studies. Their most important advantage is that samples collected by corers can be separated by depth for analyses. However, conventional corer samplers are difficult to use in the highly variable bottom sediment conditions commonly found in urban streams. The freezing core samplers, described later, overcome many of the sample loss and disturbance problems associated with conventional corers.

If used correctly, box corers can maintain the integrity of the sediment surface while collecting a sufficient depth for most toxicity studies. Conventional gravity corers may compress the sediment as evidenced by altered pore water alkalinity gradients, and box coring was superior for studies of *in situ* gradients (Lebel et al. 1982). The box core can be subcored or sectioned at specific depth intervals, as required by the study. Unfortunately, the box corer is large and cumbersome; thus, it is difficult to use and usually requires a lift capacity of 2000 to 3000 kg. Box cores typically require fine-grained sediments of at least a 30 cm depth. Other coring devices that have been used successfully include the percussion corer (Gilbert and Glew 1985), vibratory corers (Imperato 1987; Figure 5.70), and freeze corers (Pitt 1979; Spliethoff and Hemond 1996; Figures 5.71 and 5.72).

When only chemical testing is to be conducted (that is, not toxicity testing), a useful type of corer sampler is the freezing core sampler. Sediments to be used for SOD, BOD, or toxicity

Table 5.18 Optimal Interstitial Water Collection Methods

Device	Sediment Depth (cm)	Volume (cm^3)	Advantages	Disadvantages
Peeper	0.2–10	1–500	Most accurate method, reduced artifacts, no lab processing; relatively free of temperature, oxidation, and pressure effects; inexpensive and easy to construct; some selectivity possible on nature of sample via specific membranes, wide range of membrane/mesh pore sizes, and/or internal solutes or substrates.	Deployment easiest by hand. in >0.6-m depth waters; allow hours to days for equilibration, which will vary with site and chamber; methods not standardized and used infrequently; some membranes such as dialysis/cellulose are subject to biofouling; must deoxygenate chamber and materials to prevent oxidation effects; some chambers only allow small sample volumes; care must be used on collection to prevent sample oxidation.
In situ suction	0.2–30	1–250	Reduced artifacts, gradient definition; shallow water (<60 m) air stone method ease; core method deployment may not require diving in deep water, rapid collection, no lab processing; closed system possible which prevents contamination; methods include air stone, syringes, probes, and cores.	Requires custom, nonstandard collection devices; small volumes; limited to softer sediments; core method may require diving for waters; methods used infrequently and by limited numbers of laboratories.
Centrifugation	— Sampler dependent		Most accurate of lab processing methods; allows anoxic/cold processing; large volumes; commonly used.	Some chemical loss/alteration; results depend on centrifugation conditions; requires high-speed centrifuge; difficult with sandy sediments.
Suction	— Sampler dependent		Use with all sediment types; may process in field; large volumes possible with some sediments; closed system possible.	Alteration of chemical characteristics may occur; increased loss of metals and organics; loss of vertical gradient resolution.
Squeezing	— Sampler dependent		Use with all sediment types; may process in field; large volumes possible with some.	Alteration of chemical characteristics may occur; increased loss of metals and organics; loss of vertical gradient resolution sediments.

Note: Incorporation of filtration into any of the collection methods may result in loss of metal and organic compounds.

testing should not be frozen, as the bioavailability of nutrients and toxicants is altered. All of the freezing core samplers rely on CO_2 (either as a liquid or a solid — dry ice). The use of CO_2 must be carefully evaluated and minimized in consideration of its role as a greenhouse gas. Pitt (1979) devised a freezing core sampler to collect profiles in sandy deposits of catchbasins that would also work well in shallow streams. This sampler was a 19-mm-diameter stainless steel tube, with a stainless steel point attached to one end. This was pushed into the sediment. A length of flexible 6 mm copper tubing was then inserted into the free end of the stainless probe (which is above the water depth), extending to the bottom of the stainless probe. The other end of the copper tubing was attached to a high-pressure hose and to a valve on a CO_2 fire extinguisher. The fire extinguisher was modified with a valve in place of the standard squeeze release, and

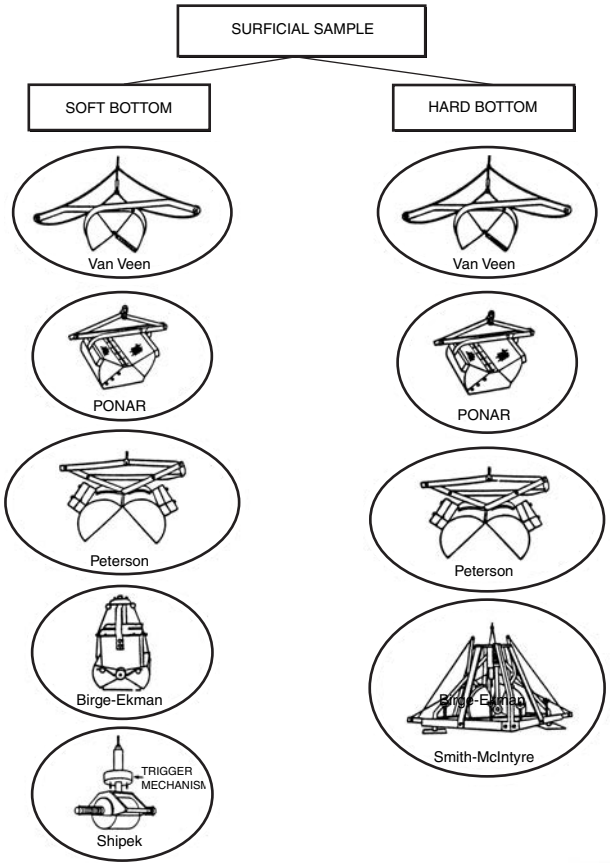

Figure 5.61 Some recommended devices for collecting surficial sediments. (From EPA. *Methods for Collection, Storage and Manipulation of Sediments for Chemical and Toxicological Analyses.* Office of Water. U.S. Environmental Protection Agency. Washington, D.C. In press.)

with an internal "delivery" tube that extended to the bottom of the fire extinguisher. This enabled liquid CO_2 to be delivered to the probe sampler, instead of gaseous CO_2 from the top of the fire extinguisher tank (the fire extinguisher is kept upright during operation). The valve was opened slightly and a continuous flow of CO_2 was delivered to the stainless steel probe (Figure 5.71). Care must be taken to turn off the flow of CO_2 at the fire extinguisher if it appears that a jam has occurred inside the probe (such as from ice forming due to water inside the probe sampler). The vaporization of the liquid CO_2 quickly chills the probe and freezes the sediment sample to the outside of the tube. In operation, the CO_2 is allowed to flow for about 1 min, but this can be changed depending on specific conditions and desired sample thickness. The probe is then removed from the sediment (with the sediment frozen to the outside) after the CO_2 flow is terminated and the copper tube is withdrawn. The probe with frozen sample is then laid on a stainless steel tray and the sample is removed by section and bottled separately, according to desired depth. A flame torch can be used to gently heat the probe uncovered by sample to allow the easier removal of the sample. It may be difficult to separate the sample into precise segments unless the sample is allowed to warm slightly first.

Another version of a freezing core sampler suitable for deeper water use was described by Spliethoff and Hemond (1996). They developed two versions of core samplers using dry ice within a probe that was used to measure the history of heavy metal contamination in an urban lake. One sampler (Figure 5.72) was made of a 96-cm length of 7.6-cm-diameter aluminum tubing. The bottom half of the tube was cut away lengthwise, and a flat aluminum plate was welded to act as a freezing surface. Stabilizing fins were also attached, along with weights to control penetration. PVC was also used to insulate the sampler where sample was not wanted. The sampler nose piece

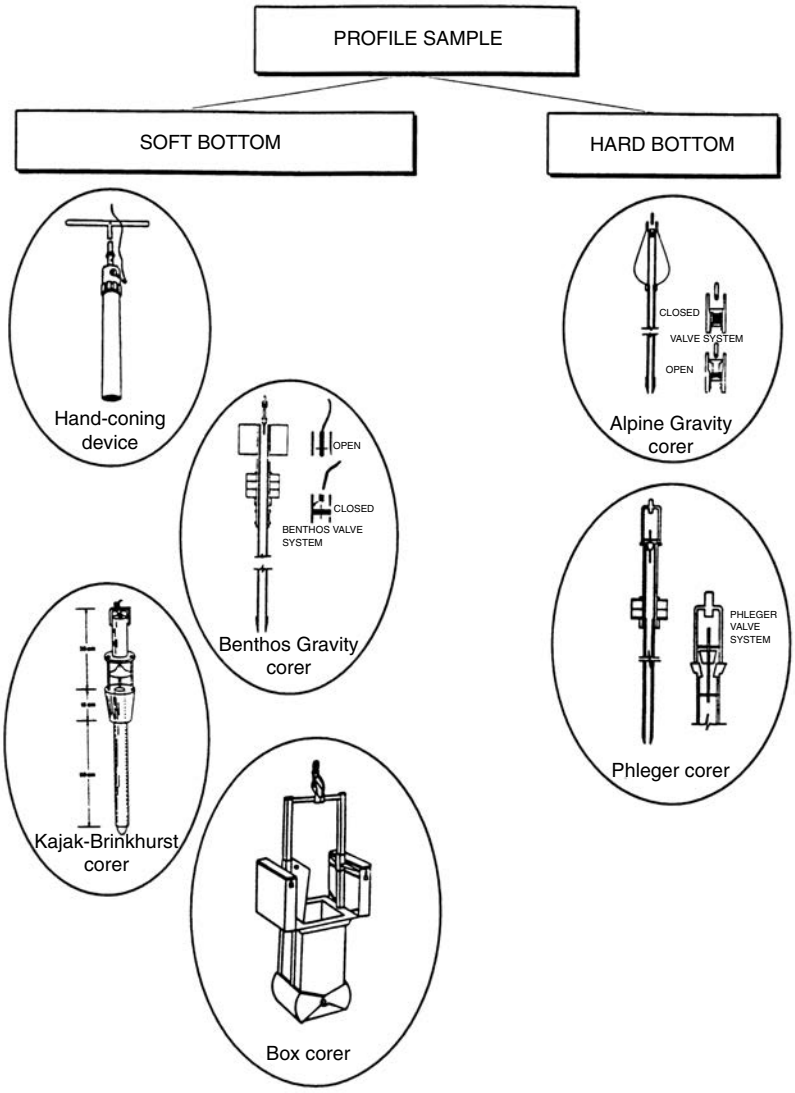

Figure 5.62- Some recommended devices for obtaining sediment profiles. (From EPA. *Methods for Collection, Storage and Manipulation of Sediments for Chemical and Toxicological Analyses*. Office of Water. U.S. Environmental Protection Agency. Washington, D.C. In press.)

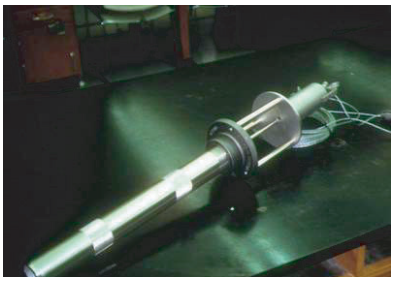

Figure 5.63 Gravity and hand corers.

Figure 5.64 Petite Ponar dredge.

Figure 5.65- Petite Ponar sediment dredge being lifted from water after sampling.

Figure 5.66- Emptying Ponar sample into stainless steel sample pan.

Figure 5.67 Winch with Ponar dredge.

Figure 5.68 Hand-held corer and Ekman dredge.

Figure 5.69 Collecting sediment with an Ekman dredge.

Figure 5.70 Shallow water vibratory core collection.

Figure 5.71- Freezing core sampler venting CO_2 used to sample catchbasin sediment in San Jose, CA.

was of solid aluminum. A screw cap was fitted to the other end which had a vent hole drilled in it. Another sampler was also constructed by Spliethoff and Hemond that allowed longer samples to be obtained (also in Figure 5.72). This sampler was made using a 125-cm length of 7.6-cm-square Extren tubing (a fiberglass reinforced resin). One side of the square tubing was machined off and an aluminum plate was attached to act as a freezing surface. A point-shaped lead weight was attached to one end and a cap with gas relief valve was attached to the other end. They used a slurry of dry ice and denatured ethanol to act as a coolant in both samplers. The samplers were dropped from the lake surface to test the penetration depth. The samplers were then retrieved, filled with the coolant mixture, and dropped again. After about 15 min, the CO_2 bubbles reaching the lake surface subsided, and the corers were retrieved. The samplers were then cleaned of unfrozen sediment and filled with warm lake water to help in releasing the frozen sample from the sampler. The frozen samples were sealed in plastic wrap and transported to the lab in dry ice filled coolers where they were separated into segments for analysis.

The above described freezing core samplers result in relatively undisturbed cores for analyses; plus they enable effective sampling in conditions where sample retention using conventional core samplers is difficult (unconsolidated coarse-textured sediment).

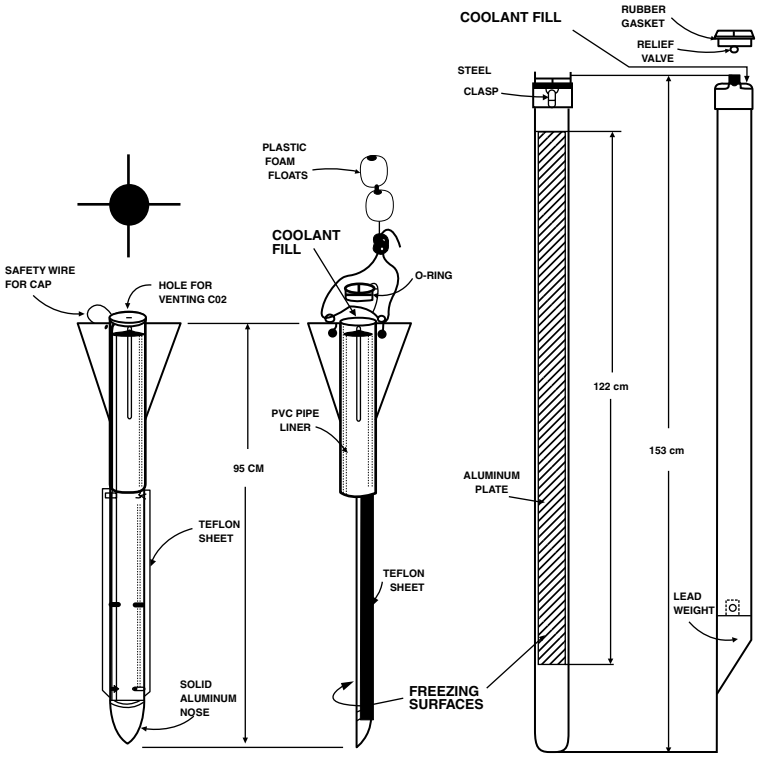

Figure 5.72 Freezing core samplers. (From Spliethoff, H.M. and H.F. Hemond. History of toxic metal discharge to surface waters of the Aberjona watershed. *Environ. Sci. Tech.*, 30(1): 121. January 1996. Copyright 1995 American Chemical Society. Reprinted with permission.)

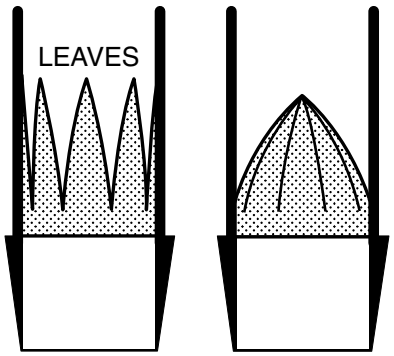

Figure 5.73 Leaf core catcher. (From American Society for Testing and Materials). *ASTM Standards on Environmental Sampling*. ASTM Pub Code No. 03-418095-38. ASTM, Philadelphia. 1995. Copyright ASTM. Reprinted with permission.)

ASTM (1995) in standard D 4823 describes many other types of core samplers. The most common sampler is the open tube sampler with a core catcher. This sampler is commonly used in shallow waters where it is manually pushed into the sediment. When the desired penetration depth is reached, the sampler is carefully withdrawn. A leaf core catcher is commonly used to help retain the sample in the corer (Figure 5.73). The leaves separate and fold against the inside walls of the sampler when the corer penetrates the sediment. The leaves fold closed when the sampler is withdrawn, holding the sample in the corer. Plastic liners are also commonly used inside the sampler, simplifying core removal from the corer. The liners usually have plastic end caps that can be placed on the liner ends, holding the cores inside until analyses. These conventional core samplers are most effective with clayey sediments. Sandy sediments tend to easily wash out of most corers upon retrieval, irrespective of the core catcher used. ASTM (1995) mentions excavating around a core sampler and sliding a flat plate under the bottom of the corer before retrieval in shallow water to capture most of the sample. Forestry Suppliers, Inc. sells the Wildco hand core sediment sampler that is 2" in diameter and 20" long, made of stainless steel with a plastic core liner tube and eggshell catcher (catalog #77258 for about $340). Extra plastic liners are also available (catalog # 77260) for about $12 each. They also sell stainless steel liners and core catchers (catalog #77303 for the stainless steel liner for about $70 each and catalog #77304 for the stainless steel eggshell sample catcher for about $40 each).

Corer samplers also have limitations in some situations (ASTM 1991a). Most corers do not work well in sandy sediments or in extremely soft (high water content) sediments; dredge samplers or diver-collected material remain the only current alternatives. In general, corers collect less sediment than dredge samplers that may provide inadequate quantities for some toxicity studies. Small cores tend to increase bow (pressure) waves (disturbance of surface sediments) and compaction, thus altering the vertical profile. However, these corers provide better confidence limits and spatial information when multiple cores are obtained (EPA 1983b; Elliott and Drake 1981). As shown by Rutledge and Fleeger (1988) and others, care must be taken in subsampling from core samples, since surface sediments might be disrupted even in hand-held core collection. They recommend subsampling *in situ* or homogenizing core sections before subsampling. Slowing the velocity of entry of coring equipment also reduces vertical disturbance. Samples are frequently of a mixed depth, but a 2-cm sample is recommended and the most common depth obtained, although depths up to 40 ft have been used in some dredging studies.

For dredging, remediation, and/or historical pollution studies, it is sometimes necessary to obtain cores of depths up to several meters. This often requires the use of vibracores that are somewhat destructive to sediment integrity but are often the only feasible alternative for deep or hard sediment sampling (Figures 5.74 through 5.76). In most studies of sediment toxicity, it is advantageous to subsample the inner core area (not contacting the sampler) since this area is most likely to have maintained its integrity and depth profile and not be contaminated by the sampler. Subsamples from the depositional layer of concern, for example, the top 1 or 2 cm, should be collected with a nonreactive sampling tool, such as a polytetrafluoroethylene-lined calibration scoop

Figure 5.74 Vibratory core collection.

Figure 5.75 Lowering vibratory corer.

(Long and Buchman 1989). Subsamples are placed in a nonreactive container and mixed until texture and color appear uniform. Due to the large volume of sediment that is often needed for toxicity or bioaccumulation tests and chemical analyses, it might not be possible to use subsampled cores because of sample size limitations. In those situations, the investigator should be aware of the above considerations and their possible effect on test results as they relate to *in situ* conditions.

Once sediment samples are collected, it is important, in most situations, to reduce the possibility of sediment oxidation. The majority of fine-grained sediments that are of concern in toxicity assessments are anaerobic below the top few millimeters (Carlton and Klug 1990), and any introduction of oxygen will likely alter the valence state of many ionic chemicals. This alteration may significantly change the bioavailability and toxicity of the sample. To protect sediments from oxygenation, the use of a glove box or bags with an inert gas supply for subsampling and processing, e.g., preparation of sediments for centrifugation, might be necessary.

While coring is preferred for maintaining a sediment's vertical integrity, care must be taken to reduce the possibility of spillage. Sediment cores should be stoppered immediately upon retrieval to prevent accidental loss of sediment. During all handling procedures, cores should be kept in an upright position as a general precaution against disturbance of the sediment. This is particularly important to prevent mixing of the uppermost part of the sediment column, which usually consists of very fine, soft, and unconsolidated material. The intact core samples (liners) should also be capped or stoppered and taped closed, secured in an upright position (e.g., rack), and labeled with appropriate information regarding sampling site, location, sample number and/or identification, time and date of collection, method of collection, and name or initials of the collector. When using clear plastic liners, the appearance of each sediment core should be recorded prior to any subsampling, along with other descriptive features such as the length of the core, thickness of various sediment units, occurrence of fauna, presence of

Figure 5.76 Emptying vibratory corer.

oil or noticeable odor, and sediment color, texture, and structure (Environment Canada 1994; Mudroch and Azcue 1995; Figure 5.77).

Figure 5.77 Vertical layers of a sliced core.

Once samples are collected, some form of subsampling and/or compositing is often performed. Removal of a portion of the collected sediment from the grab sampler (i.e., subsampling) can be performed using a spoon or scoop made of inert, noncontaminating material (e.g., Teflon, titanium, or high-quality stainless steel). It is recommended when subsampling to exclude sediment that is in direct contact with the sides of the grab sampler as a general precaution against any potential contamination from the device. Each subsample may be placed into a separate clean, prelabeled container. As a general rule, each labeled sample container must be tightly sealed and the air excluded. However, if the sample is to be frozen, it is advisable to leave a small amount of headspace in the container to accommodate expansion and avoid breakage.

Compositing of core samples or subsamples, if necessary, can be done in the field or laboratory, such as by using a drill auger mixer shown in Figure 5.78. The quality of the core sample must be acceptable and only sediment depths with similar stratigraphy should be combined. Although there might be occasions when it is desirable to composite incremental core depths, it is recommended that only horizons of similar stratigraphy be composited. Depending on the study objectives and desired sampling resolution, individual horizons within a single core can be homogenized to create one or more depth composites for that core, or corresponding horizons from two or more cores might be composited. Thorough homogenization of the composite sample, by hand or using a mechanical mixer, is recommended prior to analysis or testing.

The type of sediment characterization needed will depend on the study objectives and the contaminants of concern; however, a minimum set of parameters should be included which are known to influence toxicity and will aid data interpretation. At a minimum, the following physical and chemical characterization of sediment is recommended: total solids (dry weight), total organic carbon (TOC), acid volatile sulfides (AVS) (when metals are of concern), ammonia, and grain size fractionation. The following parameters are also frequently useful in characterization and data interpretation of contaminant effects: pH, ORP (oxidation–reduction potential), temperature, salinity-conductivity, hardness, total volatile solids (ash free weight), nitrogen and phosphorus species, cation exchange activity (CEC), sediment or suspended solids biochemical oxygen demand (BOD), and/or chemical oxygen demand (COD). Many of the characterization methods have been based on analytical techniques for soils, wastewaters, and waters, and the literature should be consulted for further information (EPA 1977; Black 1965; USGS 1969; ASTM 1989; Page et al. 1982).

Figure 5.78 Mixing sediment with a drill auger.

Interstitial Water and Hyporheic Zone Sampling

Interstitial water (pore water) is defined as the water occupying space between sediment or soil particles and is often isolated to provide either a matrix for toxicity testing or provide an indication of the concentration and partitioning of contaminants within the sediment matrix. U.S. EPA sediment quality criteria are based on the assumption that the primary route of exposure to benthic organisms is via the interstitial water (Di Toro et al. 1991). However, this route of exposure does not include uptake from ingestion of contaminated sediment particles. In addition, contaminants in interstitial waters can be transported into overlying waters through diffusion, bioturbation, and resuspension processes (Van Rees et al. 1991). The usefulness of interstitial water sampling for determining chemical contamination and/or toxicity will depend on the study objectives and nature of the sediments at the study site. Sediments that are either very large grain-sized (such as gravel or cobble) or hard, compacted clays will likely not have interstitial waters that are significantly contaminated. Therefore, sampling of interstitial waters should be restricted to sediments ranging from sandy to noncompacted clays. Interstitial waters from depositional zones containing smaller-sized sediments (clays) are usually the most contaminated.

Frequently, surface waters and groundwaters intermix via upwelling or downwelling transition zones (TZ). The ecosystem associated with this transition zone is sometimes referred to as the hyporheic zone or hyporheous. It can be a very important zone for many reasons: provides essential habitat and refugia for micro-, meio-, and macrofauna or flora; affects contaminant attenuation, removal, or transport; cycles nutrients and carbon; and provides trophic links between the microbes and invertebrates and their macrofaunal predators (Duncan 1999). To date these zones have largely been ignored in environmental contaminant assessments and conceptual models, even though they are quite common. They provide a challenge in that their assessment requires collaboration of hydrogeologists, hydrologists, ecologists, chemists, and toxicologists.

The biological and physicochemical conditions within the groundwater and surface water are different, and hence may affect the partitioning (e.g., bound or freely dissolved), mobility, and bioavailability of sediment-associated contaminants. For example, changes in pH may affect the binding of metals, whereas the rate and extent of microbial processing of sediment organic matter may affect the partitioning of persistent organic contaminants. Upwelling zones (where groundwater and interstitial water move up toward surface water) are generally anoxic, with low pH. Anaerobic microbial processes dominate and may include reductions, denitrification, ammonification, and methanogenesis. Dissolved organic carbon (DOC) is of low quality and species diversity is often quite low in upwelling zones. However, benthic consumers are attracted to this habitat. Downwelling zones (the downward movement of surface water into the stream bed) are generally higher in oxygen content and pH. Aerobic microbial processes such as oxidation and nitrification are dominant. DOC quality, species diversity, and productivity are high in downwelling habitats. The hydrological interface between upwelling groundwater and downwelling surface water within the stream bed contains large gradients for a variety of physicochemical parameters (e.g., temperature, dissolved oxygen, pH, and pE). Previous studies have shown that organic contaminant and metals concentrations can vary over several orders of magnitude (Benner 1995).

There are several scenarios in which data on groundwater–surface water interactions would be useful in evaluations of the fate and dynamics of sediment contaminants and the *in situ* exposure of biota. Upwelling groundwater can affect benthos and surface water biota if either or both the groundwater and sediments are contaminated. Aqueous phase chemicals (e.g., freely dissolved, colloid-bound) in the upward flowing groundwater and/or the mobilization of sediment-bound contaminants by upwelling groundwater are the potential inputs to the surficial environs under these conditions. Downwelling surface water can affect benthic, hyporheic, and phreatic (groundwater-associated) biota if either or both the surface water and sediments are contaminated. Under such conditions, the potential exists for the transport of sediment contaminants to deep layers within the stream bed and the

contamination of groundwater by the downward-flowing contaminant load. We have observed this at sites contaminated by PCBs and chlorinated benzenes (Greenberg and Burton 1999).

Selection of Measurement Methods for Interstitial Water

Isolation of sediment interstitial water can be accomplished by a wide variety of methods, which can be grouped as laboratory or field (*in situ*) based. The common laboratory-based methods can be categorized as (1) centrifugation, (2) pressurization, or (3) suction. Field-based methods include suction and "peepers" (for reviews, see Adams et al. 1991; ASTM 1994; Burton et al. 2001; Environment Canada 1994). Peepers are small chambers with membrane or mesh walls, which are buried in sediments, and surrounding interstitial water then equilibrates within the chamber. Chambers are typically retrieved from 2 to 20 days after deployment.

It is important to work with the analytical and toxicity testing laboratories to determine the least amount of sample needed, because of the difficulty of obtaining large amounts of interstitial water for analyses. As an example, the use of an anodic stripping voltammeter is suitable for direct analyses (undigested) of heavy metals in interstitial water using only about 5 mL of water for several metals (at least copper and lead) simultaneously, instead of about 50 mL typically required. Organic analyses may be conducted with about 250 mL of water, using the modified methods described in Chapter 6, instead of the typically required 1-L sample sizes, but with loss of sensitivity. The use of an automated water analyzer (such as the TrAAcs 2000 analyzer from Bran+Luebbe) can dramatically reduce the water volume needed for conventional nutrient analyses. Ion chromatography also requires only a very small amount of sample for complete cation and anion analyses. Microtox, from Azur Environmental, is also a very useful indicator of toxicity and requires only a very small amount of sample (about 1 mL). Bacteria tests can also be conducted using small sample volumes (using methods from IDEXX, Inc., for example), especially if the bacteria densities are high, as is likely in contaminated urban streams, allowing dilution of the samples.

When relatively large volumes of water are required (such as 20 mL or greater), only grab and core sampling with subsequent centrifugation and sediment squeezing methods are typically used. Other methods such as suction and *in situ* samplers do not easily produce sufficient volumes for most required analyses. However, larger-sized peepers (500 mL volume) have been used for collecting samples for chemical analyses and for exposing test organisms *in situ* (Burton 1992a,b; Sarda and Burton 1995; see also Chapter 6).

Most sediment collection and processing methods have been shown to alter interstitial water chemistry (e.g., Schults et al. 1992; Bufflap and Allen 1995a,b; Sarda and Burton 1995) and, therefore, can potentially alter contaminant bioavailability and toxicity. Some important interstitial water constituents, e.g., dissolved organic carbon, dimethylsulfide, ammonia, major cations, and trace metals can be significantly altered by the collection method (e.g., Martin and McCorkle 1993; Carignan et al. 1994; Bufflap and Allen 1995a,b; Sarda and Burton 1995). Increased sample handling associated with methods such as grab or core sampling and centrifugation, squeezing, or suction may cause significant increases in key constituents, such as ammonia, sulfide, and DOC concentrations, as compared to those collected via *in situ* "peepers" or core-port suction. Other constituents, such as salinity, dissolved inorganic carbon, sulfide, and sulfate, might not be affected by collection, providing oxidation is prevented. If sediments are anoxic, as most depositional sediments are, all steps involved in sample processing should be conducted in inert atmospheres or by limited contact with the atmosphere to prevent oxidation (and subsequent sorption/precipitation) of reduced species. When anoxic sediments are exposed to air, volatile sulfides will be lost which may increase the availability of sulfide-bound metals. In addition, iron and manganese oxyhydroxides are quickly formed which readily complex with trace metals, thus altering metals-related toxicity (e.g., Bray et al. 1973; Troup et al. 1974; Burton 1991). There is no need for maintaining anoxic processing conditions when the study objectives are concerned only with exposures to oxic sediments, or if target contaminants are unaffected by oxidation in short-term

toxicity or bioaccumulation testing. For example, often studies of dredged material toxicity do not consider ammonia-related toxicity, and oxidation is actually promoted to remove ammonia from overlying waters of the toxicity test beakers.

Immediate collection and analysis of interstitial water is recommended since chemical changes might occur even when sediments are stored for short periods (e.g., 24 h) at *in situ* temperatures (Sarda and Burton 1995). Coagulation and precipitation of the humic material was noted when interstitial water was stored at 4°C for more than 1 week. Oxidation of reduced arsenic species in interstitial water of stored sediments was unaffected for up to 6 weeks when samples were acidified and kept near 0°C, without deoxygenation. When samples were not acidified, deoxygenation was necessary. Others have recommended interstitial waters be frozen after extraction, prior to toxicity testing, to prevent changes, but others have recommended against freezing samples that will undergo toxicity testing. The optimal collection method will depend upon the purpose of the sample (e.g., acidification for metal analysis and not toxicity testing), characteristics of the sediment, and the contaminants of concern. Sediments that are highly contaminated with strongly nonpolar organics (such as PCBs) are not likely to change in toxicity during storage.

The conditions for isolation of interstitial waters by centrifugation have varied considerably. For toxicity testing, interstitial waters have been isolated over a range of centrifugal forces and temperature ranges (Ankley and Schubauer-Berigan 1994; Schults et al. 1992) with centrifuge bottles of various compositions. When centrifugation followed by filtration has been compared with *in situ* dialysis, higher speed centrifugation followed by filtration with 0.2 membrane filters has produced results that were more similar for metals and organic carbon. Centrifugation at low speeds or use of a larger pore size filtration membrane (e.g., 45 μm mesh) will result in retention of dissolved contaminants, colloidal materials, and aquatic bacteria in the pore water sample. High-speed centrifugation (e.g., $10{,}000 \times g$) is necessary to remove colloids and dispersible clays (Ankley and Schubauer-Berigan 1994). Typically, toxicity is reduced with high-speed centrifugation or filtration due to the removal of particle-associated contaminants (Ankley and Schubauer-Berigan 1994; Schults et al. 1992; Bufflap and Allen 1995a). While the duration of the centrifugation has been variable, 30 min is relatively common. The temperature for the centrifugation should reflect the ambient temperature of collection to ensure that the equilibrium between particles and interstitial water is not shifted.

Filtration through glass fiber or polycarbonate membranes may cause the loss of some dissolved metals and organics (Schults et al. 1992). If filtration is employed, a nonfiltered sample should also be tested for toxicity and contaminant concentrations. The effects of centrifugation speed, filtration, and oxic conditions on some chemical concentrations in interstitial waters have been well documented (e.g., Ankley and Schubauer-Berigan 1994; Schults et al. 1992; Bufflap and Allen 1995b; Bray et al. 1973). It is recommended that, for routine toxicity testing of interstitial waters, sediments should be centrifuged at $10{,}000 \times g$ for a 30-min period at 4°C. It is difficult to collect interstitial water from sediments that are predominantly coarse sand. A modified centrifuge bottle has been developed with an internal filter which can recover 75% of the interstitial water as compared to 25 to 30% from squeezing.

Polytetrafluoroethylene (PTF) bottles will collapse at 3000 *g* but have been used successfully up to 2500 *g* when filled to 80% of capacity. Isolation of interstitial water in this case should be at the temperature of collection, at a slower speed of $2500 \times g$ for 30 min. This material will contain colloidal material as well as dissolved compounds. At low centrifugation speeds, without filtration, removal of the colloids may not be possible. The influence of dissolved and colloidal organic carbon may be estimated by measuring the organic carbon content. If small volumes of water are required for testing, higher speed centrifugation can be performed with glass tubes (up to $10{,}000 \times g$). If metal analysis of toxicity is not a concern, then high-speed centrifugation in stainless steel centrifuge tubes is an option. When working with samples contaminated with organics, efforts should be made to reduce sample exposure to light to reduce photo-related degradation or alteration of any potentially toxic compounds. This can be accomplished by using amber bottles and yellow lights.

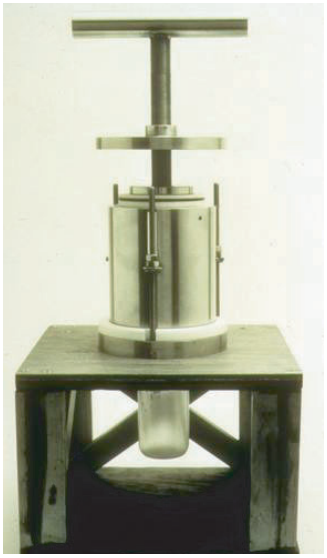

Figure 5.79 Pore water squeezer — stainless steel with Teflon liner.

Isolation of interstitial water by squeezing has been performed with a variety of procedures. In all cases, the interstitial water is passed through a filter that is a part of the apparatus. Filters have different sorptive capacities for different compounds. Numerous studies have shown filters reduce toxicity and contaminant concentrations by retaining contaminant-associated particles and also by contaminant sorption onto the filter matrix (Schults et al. 1992; Bray et al. 1973; Troup et al. 1974; Sasson-Brickson and Burton 1991). The characteristics of filters and the filtering apparatus should be carefully considered. Squeezing has been shown to produce a number of artifacts due to shifts in equilibrium from pressure, temperature, and gradient changes (e.g., Schults et al. 1992; Troup et al. 1974; Mangelsdorf et al. 1969; Fanning and Pilson 1971; Figure 5.79). Squeezing can affect the electrolyte concentration in the interstitial water with a decrease near the end of the squeezing process. It is therefore recommended that moderate pressures be used with electrolyte (conductivity) monitoring during extraction. Significant alterations to interstitial water composition occurred when squeezing was conducted at temperatures different from ambient (e.g., Mangelsdorf et al. 1969). Other sources of alteration of interstitial water when using the squeezing method are contamination from overlying water, internal mixing of interstitial water during extrusion, and solid-solution reactions as interstitial water is expressed through the overlying sediment. As interstitial waters are displaced into upper sediment zones, they come in contact with solids with which they are not in equilibrium. This intermixing causes solid-solution reactions to occur. The chemistry of the sample may be altered due to the fast kinetics (minutes to hours) of these reactions. Most interstitial water species are out of metastable equilibrium with overlying sediments and are rapidly transformed, as observed with ammonia and trace metals. Bollinger et al. found elevated levels of several ions and dissolved organic carbon in squeezed samples as compared to samples collected by peepers. The magnitude of the artifact will depend on the element, sediment characteristics, and redox potential. It is unlikely that reactive species gradients can be established via squeezing of sediment cores.

Many studies have demonstrated the usefulness of *in situ* collection methods (e.g., Barnes 1973; Belzile et al. 1989; Bottomley and Bayly 1984; Buddensiek et al. 1990; Howes et al. 1985; Jahnke 1988; Mayer 1976; Murray and Grundmanis 1980; Sayles et al. 1973; and Whiticar 1982). These methods of interstitial water collection are superior to more traditional methods in that they are less likely to alter the chemistry of the sample. The principal methods of interstitial water collection are through the use of peepers (e.g., Bufflap and Allen 1995a,b; Carignan 1984; Bottomley and Bayly 1984) or *in situ* suction techniques. These methods have the greatest likelihood of maintaining *in situ* conditions and have been used to sample dissolved gases (Sarda and Burton 1995) and volatile organic compounds.

Suction using an aquarium air stone recovered up to 1500 mL from 4 L of sediment suctioned in an anoxic environment (Galli 1997). Hand vacuuming using an aquarium stone has shown to be an effective method of collecting interstitial water (Sarda and Burton 1995). The air stone is attached to a 50-mL syringe via plastic tubing. The stone is inserted in the sediment to the desired depth and then suction applied. Clogging of the air stone is a problem in some sediments; however, it is effective in most tested. The collection system can be purged of oxygen prior to leaving the laboratory. Ammonia concentrations in water obtained by this system were similar to those collected

Figure 5.80 Disassembled small-volume, high-resolution peepers.

Figure 5.81 Small-volume peeper assembly showing 75-mm nylon screening.

with *in situ* peepers (Sarda and Burton 1995). Problems common to suction methods are loss of equilibration between the interstitial water and the solids, filter clogging, and oxidation. However, *in situ* suction or suction via core ports has been shown to accurately define small gradients of some sediment-associated compounds, including ammonia, the concentrations of which can change by an order of magnitude over a 1-cm depth interval. However, these small-scale suction methods may not provide an adequate volume for conducting most standard toxicity test procedures.

Small-volume, high-resolution peepers, made by the University of Alabama at Birmingham, were designed for chemical and bacteriological analyses of interstitial water (Lalor and Pitt 1998). These peepers were made from Delrin and are about 10 to 15 cm wide and 45 to 60 cm long, with one end tapered to a point (Figures 5.80 through 5.83). The main body is made of 20-mm-thick stock and has numerous deep and wide slots (not cut through), spaced 1 cm apart, that hold about 5 to 10 mL of water each. This common peeper design enables vertical stratification of pore water quality to be determined. However, because the water volume for each separate chamber is very small, special laboratory analysis procedures are needed that minimize water volume requirements. In order to collect larger volumes of water, these peepers are frequently placed in a cluster arrangement allowing compositing from similar depth slots from adjacent peepers.

The slots should not extend any closer than about 20 mm from the edge, to prevent cracking of the thinner cover piece (common in peepers made from Plexiglas, for example). A nylon screen having 75-µm apertures is placed over this thick piece and is then covered with a thinner sheet of

Figure 5.82 Peeper placement near shore in urban lake.

Figure 5.83 Ten replicate high-resolution peepers (to obtain larger water composite samples).

Delrin that is 6 mm thick. This cover piece has identically located slots cut through the material and has countersunk holes matching tapped holes in the main body. For use, the cavities in the main body are filled with distilled or deionized water, covered with the nylon screen, and the two Delrin pieces are screwed together using plastic screws, sandwiching the nylon screen (Figures 5.80 and 5.81). The unit is then pushed into the stream or lake sediment, gently pushing down on the unit until resistance prevents further penetration, leaving about five slots above the sediment/water interface (Figure 5.82). The unit is left in place until equilibrium is established, and is then removed (several hours using the large aperture screening). The unit may require up to 2 weeks for equilibrium to become established when using small aperture screenings (such as 0.45 or 2 µm membrane filter material). A recent modification has added a thin stainless steel cover to the peeper that slides over the front slots to protect them while inserting or withdrawing the peepers in sediment. The cover is slid off after the peepers are pushed into the sediment to the appropriate depth. In addition, the water is extracted from the peeper wells after disassembling the units and carefully rolling back the nylon screening, instead of puncturing the screening and inserting a syringe for sample withdrawal. These modifications have significantly reduced the disturbance to the sediments when using the peepers and have reduced contamination of the sample water.

The optimal equilibration time for *in situ* peepers is a function of membrane aperture, sediment type, contaminants of concern, and temperature. There are several artifact problems associated with peepers which use dialysis membranes. Total organic carbon may be elevated in peepers (4 to 8 µm pore size) due to biogenic production; however, colloidal concentrations are lower than centrifuged samples. Cellulose membranes are unsuitable because they decompose too quickly. A variety of polymer materials have been used, some of which may be inappropriate for studies of certain nonpolar compounds.

More recently, larger pore sized mesh has been used (Figures 5.84 through 5.87) which dramatically shortens equilibration time (Fisher 1992; Sarda and Burton 1995), as illustrated in Figure 5.88 during tests at UAB. In this test, 75-µm nylon screening was used on a peeper placed in a bucket of saline water (about 5.5 mS/cm). Every few minutes, the peeper was removed, and a syringe was used to remove water from an individual cell. This was then measured for conductivity.

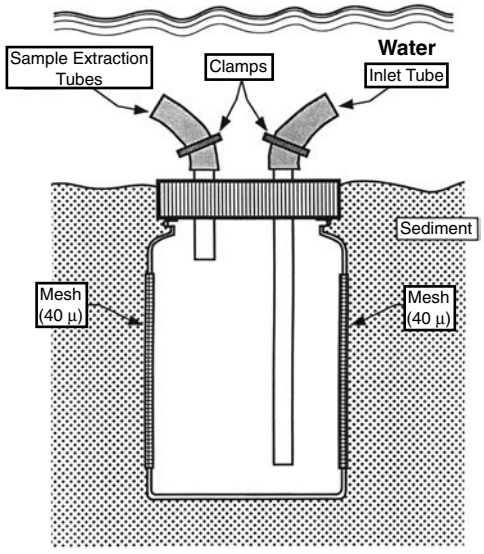

Figure 5.84 Large-volume peeper with large aperture mesh. (From Burton, G. A., Jr., Ed. *Sediment Toxicity Assessment.* Lewis Publishers. Boca Raton, FL. 1992b. With permission.)

Figure 5.85 Withdrawing interstitial water sample from large-volume peeper.

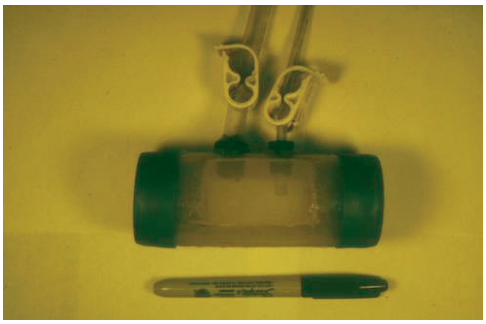

Figure 5.86 Medium-volume peeper with large aperture mesh for water sampling.

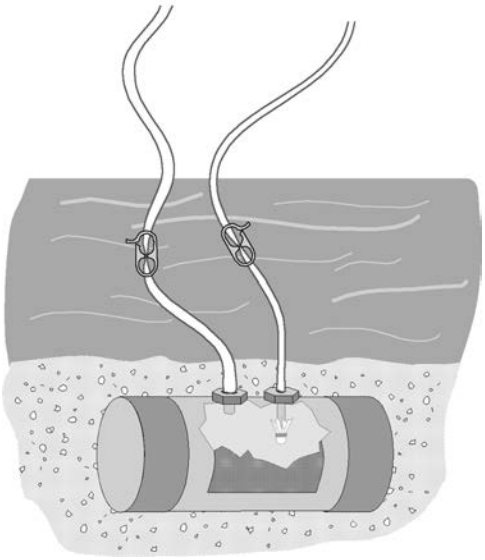

Figure 5.87 Medium-volume peeper buried in sediment.

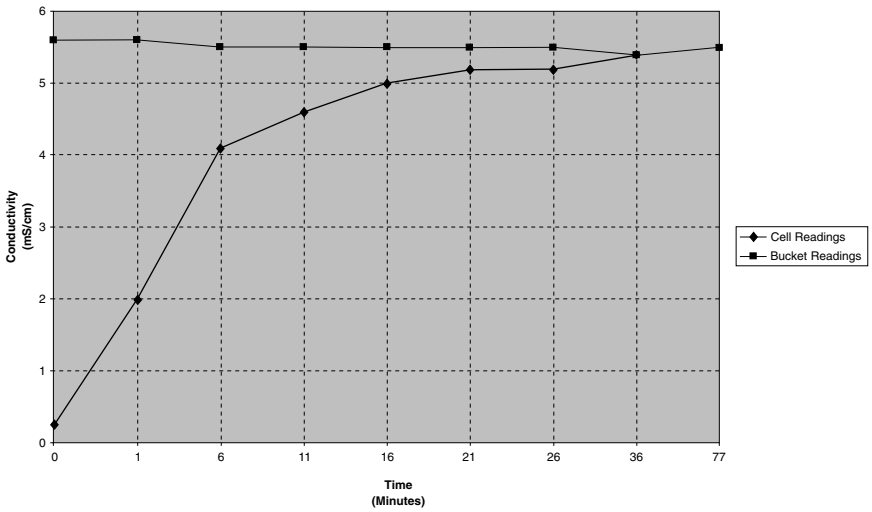

Figure 5.88 Equilibrium plots for 75-µm nylon screening in small-volume peeper.

Effective equilibrium was reached after about 20 min. In comparison, Figure 5.89 is an equilibrium plot for a 0.22-µm polyethersulfone membrane filter used in a diffusion peeper (Easton 2000). This test was conducted in a small laboratory flume with water flowing about 1 ft/s. Saline water was placed in the peeper (about 18 mS/cm), and the flume water was regular tap water (about 200 µS/cm). Samples were withdrawn from the peeper frequently at the beginning of the test, and at longer intervals later, and analyzed for conductivity. In this case, it required about 20 hours to reach equilibrium, although about 90% of the equilibrium was established at 10 hours.

When using sampler peepers and 75-µm membrane material, we commonly leave the peepers in place for about 2 to 24 hours to ensure equilibrium. Solids that pass through the mesh tend to settle to the bottom of the peeper chamber. Long exposure times may be impractical due to security problems and high flows in streams. The samplers need to be taken to the laboratory where the water

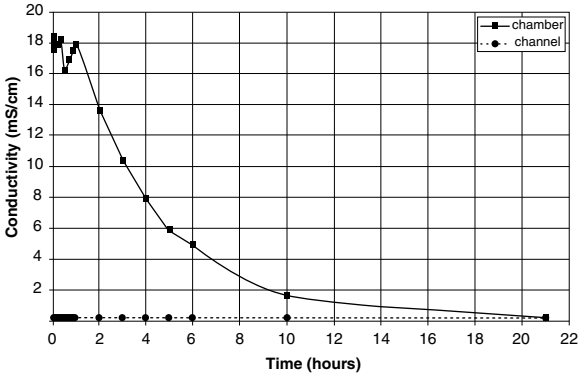

Figure 5.89 Equilibrium plot for 0.22-mm polyethersulfone membrane filter in diffusion peeper (From Easton, J. *The Development of Pathogen Fate and Transport Parameters for Use in Assessing Health Risks Associated with Sewage Contamination*. Ph.D. dissertation, Department of Civil and Environmental Engineering, University of Alabama at Birmingham. 2000. With permission)

is immediately analyzed. It is also possible to remove the samples from the slots in the field (using a syringe and needle), transferring the water into sealed and full bottles (such as small VOC vials). Four or five high-resolution peepers located close together can provide a 20 to 50 mL composite sample of pore water in 1-cm depth increments for chemical analyses (as shown on Figure 5.83).

When ionizable compounds, e.g., metals, are to be collected, it is important to preequilibrate the samplers with an inert atmosphere to avoid introducing oxygen into the sediments, thereby changing the equilibrium. Plastic samplers can contaminate anoxic sediments with diffusable oxygen and should be stored before testing in inert atmospheres (Carignan et al. 1994). In addition, when samples are collected and processed, they should also be kept under an inert atmosphere and processed quickly. Metals sampling of interstitial waters can be accomplished using a polyacrylamide gel probe (Krom et al. 1994) More recently, semipermeable membrane devices (SPMDs) filled with a nonpolar sorbant have been used effectively to show potential for bioaccumulation of nonpolar organic compounds.

Recently, test organisms have been exposed within peeper chambers where larger mesh sizes of 149 μm were used successfully in oxic sediments. Chambers can be buried several centimeters or in surficial sediment depending on the study objectives (Figures 5.90 and 5.91). Equilibration of conductivity was observed within hours of peeper insertion into the sediment (Fisher 1992). Replicate peepers revealed extreme heterogeneity in sediment interstitial water concentrations of ammonia and dissolved oxygen (Frazier et al. 1996; Sarda and Burton 1995; Sherman et al. 1994). Sediments that were high in clay and silt fractions usually were anoxic and did not allow for organism exposure *in situ* (Fisher 1992).

The Birmingham SSO (sanitary sewer overflow) evaluation project is a recent example of the use of peepers with large apertures. *Enterococcus, Escherichia coli*, total coliform bacteria, Micro-

Figure 5.90 Medium-volume peepers *in situ* with sampling tubes exposed.

Figure 5.91 Surficial sediment chambers.

tox toxicity screening, heavy metals (copper and lead), major ions, and nutrients are being analyzed on most of the pore water samples by combining water from three adjacent 10-cm chambers, and by using five replicate peepers located close together. This allows a total of about 150 mL of water for analysis. The careful selection of test methods (and dilution of water for the bacteria analyses) allows a relatively comprehensive evaluation of pore water chemical and bacteriological conditions. Changes in pore water chemical and bacteriological quality for different depths can be used to calculate diffusion coefficients and kinetic rate coefficients.

In situ and real-time chemical measurements of interstitial water are also possible using continuously recording *in situ* water quality sondes. The University of Alabama at Birmingham is currently using YSI 6000 monitoring probes to continuously monitor interstitial water pH, ORP, conductivity, DO, and temperature in urban streams as part of an EPA-sponsored research project investigating SSO impacts. These instruments are capable of unattended operation for several weeks. The probe end of the instrument is wrapped with a nylon screen having 150-µm apertures. Equilibrium should be obtained within a few hours using this large aperture. The instrument can be placed vertically with the probe end buried several hundred mm in the sediment in slow-moving streams for short periods. The instrument is completely buried horizontally for longer periods or for higher flows. The use of a direct readout (hand-held readout from YSI, or a portable computer) is useful in determining equilibrium times during preliminary trials. The available turbidity probe is also used to indicate the effects of placement of the probe by measuring the exchange of water in the probe chamber. A similar unit placed simultaneously in the water column can be used to measure the lag time of any chemical changes (such as conductivity) in response to storm events and to directly determine diffusion coefficients. Of course, this method does not provide accurate vertical placement of the analytical results, but it is expected to be generally representative of near-surface conditions where most of the benthic organism activity occurs. These probes are extremely useful to illustrate the variation of these parameters with time, especially during wet weather events, and to measure the recovery of conditions after events.

Mini-Piezometer Measurements of Pore Water Conditions

Mini-piezometers (Lee and Cherry 1978) are useful tools because they allow for the detection of upwelling groundwater and downwelling surface water on a local scale (i.e., cm to m). Additionally, these simple, inexpensive devices allow for samples of pore water to be withdrawn from desired depths within the stream bed for chemical analysis. Mini-piezometers are comprised of lengths of 1/8" ID plastic tubing that is perforated and screened with 300-µm mesh along the bottom 5 cm (Figures 5.92 through 5.94). A nest is a group of mini-piezometers of different lengths attached to a 1-m dowel rod that will sample at desired levels beneath the sediment surface (e.g., 10, 25, 50, 75, and 100 cm). Once piezometers are installed, they can be left in place indefinitely for repeated sampling and measurements. To detect areas of upwelling and downwelling, transects of nested mini-piezometers are installed in the riffle and pool areas of *in situ* test sites. Hydraulic heads (in cm) are determined by measuring the heights of water columns drawn simultaneously from the inserted mini-piezometer and overlying surface water into a manometer (Winter et al. 1988; Figure 5.94). Relative to surface water, a positive or negative hydraulic head indicates an upwelling or downwelling zone, respectively.

The hydrologic data from mini-piezometer pore water samples and hydraulic head measurements have improved our ability to interpret often complex exposure–effects relationships that result from *in situ* toxicity tests. We have found that contaminant concentrations in samples of sediments and pore water are not always predictive of *in situ* chamber (actual) exposure levels and observed effects in the test species. For example, in an *in situ* study of three sites in a stream system with similar levels of sediment contamination by chlorinated benzenes, one site was downwelling at all mini-piezometer nest locations and two sites had no net hydraulic pressure differences. Total chlorinated benzenes in water samples taken from the piezometer nests ranged

Figure 5.92 Placement of mini-piezometers into support tubing.

Figure 5.93 Placement of mini-piezometer array into sediments via temporary support pipe.

from 100 to 1300 µg/L at all sites. The highest concentrations generally occurred in piezometers installed 30 cm or deeper into the stream bed. Concentrations of total chlorinated benzenes in water samples taken from the chambers used during 4-day *in situ* exposures of *Ceriodaphnia dubia, Hyalella azteca,* and *Chironomus tentans* to surficial sediments were near 100 µg/L at the two no-exchange sites, whereas the level was only 3 µg/L at the downwelling site. Survival of all three test species was significantly higher at the downwelling site (>80%) than at the no-exchange sites (<20%). For *C. dubi*a and *H. azteca,* survival between the downwelling and reference sites was not significantly different. It appears the downward flow of surface water through the sediments might have removed bioavailable contaminants in the surficial sediments to deeper zones within the stream bed (Greenberg and Burton 1999). However, this condition places transition zone species and groundwater resources at risk.

Sediment chemists, toxicologists, and risk managers have primarily focused their research efforts and the development of sediment quality guidelines on the effects of contaminants on benthic and water column organisms associated with the surficial sediments (0 to 10 cm depth). Implicit in this approach is that the historical contamination buried beneath the top sediment bed layer is biologically unavailable and hence poses little to no ecological risk. However, deeper sediments (ca. 10 to 100 cm depth), and more specifically sediments within the transition zone, serve important ecosystem functions and therefore may be sensitive to chemical perturbation. Vertical transport of dissolved or colloid-bound contaminants within the sediment interstices can potentially exert deleterious effects in the surficial sediments, surface water, or groundwater, or it can exacerbate preexisting degraded conditions. Therefore, ecosystem integrity can be more effectively evaluated if the scientific and regulatory community adopts a holistic approach to stream health that includes focusing on the transition zone. At the present time, we have begun to incorporate this added hydrologic perspective in our *in situ* sediment toxicity research program through the use of mini-piezometers. Continuing this line of research by developing assessment tools capable of measuring biological effects within the transition zone is the next step.

Figure 5.94 Field manometer connected to mini-piezometer to measure vertical flow through sediments.

Case Example 1. Sediment Sampling for Interstitial Pore Water in an Ice-Covered Lake

A site was sampled in northern Minnesota in January which had depositional sediments (non-consolidated silts and clays) and was ice-covered with water depths of 50 to 60 ft. Site conditions prevented use of peeper sampling and no *in situ* core-port sampling equipment was available. The study design required collection of 30 L of sediment. Based on these restrictions, a Ponar grab sampler was most appropriate for sediment collection.

Replicate Ponar grabs were collected through holes drilled in the ice and were deposited into a 20-L high-density polyethylene bucket and gently stirred to homogenize. Nitrogen gas was bubbled into any overlying water and added to the headspace prior to lid closure. Sediments were placed in ice chests at approximately 4°C and returned to the laboratory for processing.

Interstitial waters were collected using centrifugation. Sediments were distributed to the appropriate type of centrifuge bottles under a nitrogen atmosphere and centrifuged at 10,000 × *g* at 4°C for 30 min. The supernatant was gently decanted under nitrogen atmosphere. Note: if solids are resuspended with the supernatant, a second centrifugation of the interstitial water should be conducted. The interstitial water from all bottles was combined under nitrogen and then split for chemical analyses and toxicity testing. Chemical samples were preserved and stored as appropriate. Toxicity testing was initiated within 48 hours, at which time the sample temperature was raised from 4°C to the required test temperature and dissolved oxygen checked to ensure adequate levels.

Case Example 2. Shallow Stream with Contaminated Sediments

A shallow stream in Ohio with sediment contamination was studied to develop site-specific sediment quality criteria. Site conditions allowed the placement of peeper samplers. The sediment depth of concern was from 0 to 5 cm. Peepers were constructed from high-density polyethylene bottles with 70- to 140-µm PTF mesh windows on the chamber walls, 1 to 5 cm from the top of the chamber (similar to Figure 5.84). Chambers were filled with sterile deionized water and placed in a nitrogen atmosphere for 24 to 48 hours prior to site placement. Five replicate (total volume approximately 2.5 L) chambers were placed at the site by removing a plug of sediment the size of the chamber, inserting the chamber and gently packing the sediment around the chamber so that only the lid was exposed. Equilibration time can be reduced and time series sampling of the interstitial water is possible by constructing an outlet tube into the chamber lid (Sarda and Burton 1995). Degassed syringe samplers can then be attached to the outlet port and interstitial water removed without disturbing the peeper unit. Equilibration time with 140-µm mesh windows occurs within several hours. However, it may take days for the sediment gradients to reestablish adjacent to the chamber. Sampling of interstitial waters at the sediment surface (0 to 1 cm depth) is not readily feasible when large samples are required. However, toxicity may be determined on surficial sediment using *in situ* toxicity test chambers which expose organisms either directly to the sediments or via mesh barriers (Burton 1992a,b; see also Chapter 6). Microanalytical sampling of near-surface sediments is possible using narrow plate chamber designs (see reviews in Adams 1991, Burton 1991, and above citations). Samples are returned to the laboratory on ice and then processed by the appropriate chemical and toxicity test methods.

SUMMARY: BASIC SAMPLE COLLECTION METHODS

This chapter presented methods to determine the needed sampling effort, including the number of samples and the number of sampling locations. These procedures can be utilized for many different conditions and situations, but some prior knowledge of the conditions to be monitored is

Table 5.19 General Sampling Guidelines[a]

	Location
1.	Locate stations at sites representative of least and greatest impact from each pollutant source and for the total system, considering each ecosystem component (e.g., substrate, flow, biota).
2.	Sample depositional areas and critical habitats such as riffles and spawning areas.
3.	Collect replicate samples at each station which characterize the site spatially.
4.	Sample during baseflow and various stormflow conditions.
5.	Sample during different seasons.
6.	Sample during recovery periods (following storm events) noting different periods of disturbance (i.e., storm recurrence period).
7.	Note diurnal, weekly, monthly, and seasonal cycles of various ecosystem components-endpoints (e.g., DO, redox, tissue residues, toxicity, life stage).
	Type
8.	In areas where effects are uncertain, use a "weight-of-evidence" integrated approach (see Chapter 8). Characterize the inputs and receiving water system both physically (e.g., flow, solids, temperature, habitat) and chemically (e.g., oxygen, hardness, organics, metals). Measure key indigenous biological communities (indices), indicators (e.g., trout), and endpoints (e.g., fish abnormalities). Measure toxicity of effluents, waters, and sediments using sensitive and relevant species representing multiple levels of biological organization (e.g., fish, zooplankton, algae, benthic macroinvertebrates). *In situ* toxicity testing is the preferred approach.
	Method
9.	Process samples quickly (refrigerate and/or preserve immediately upon collection).
10.	Reduce sample manipulation whenever possible (e.g., mixing, sieving, aeration, filtration).
11.	Maintain sample integrity when possible (e.g., using core rather than grab [dredge] collection).
12.	Characterize key components of all sample replicates.
13.	Follow proper QA/QC practices.

[a] All sampling issue decisions must be based on the study objectives and their associated data quality objectives.

needed. A phased sampling approach is therefore recommended, allowing some information to be initially collected and used to make preliminary estimates of the sampling effort. Later sampling phases are then utilized to obtain the total amount of data expected to be needed.

Descriptions of data quality objectives and associated QA/QC requirements are also given. The use of different sample blanks and other quality control samples are described, along with dealing with typical problems associated with detection limits.

The main component of this chapter covers sampling methods, including water, source area, sediment, and pore water sampling options. Numerous examples are given illustrating the use of the many sampling methods and approaches. There are few universal methods that can be used for all sampling activities, and much discretion and professional judgment is needed to select the most appropriate methods for any specific project. However, there are some general guidelines for sampling streams and lakes which should apply to most studies, as listed in Table 5.19. Each of the points listed in this table are also discussed in greater detail elsewhere in this handbook, especially in Chapter 6 and the appendices.

There are a number of factors to consider when selecting a sampling site after preliminary surveys and design elements are completed. The selection factors and their relative importance are often study specific, but some general considerations do exist, as shown in Table 5.20. The factors that influence the representativeness of a sample are numerous and cross many disciplines, as do all ecosystem evaluations. Therefore, it is important to select sampling stations based on professional judgment(s) from an individual(s) with expertise in aquatic ecosystem assessments (hydrology, environmental chemistry, biosurveys, and ecotoxicology), taking into account spatial and temporal variation and the characteristics of base- and stormwater flow; habitat; pollutant loadings, fate and effects; aquatic communities; and sensitive indicator species.

These same selection criteria should then be used to establish reference area sampling, if preexisting reference data are not available. The reference station (upstream), stream or lake, and

Table 5.20 Sampling Size Selection, Sampling Media, and Sampling Frequency Considerations

Consideration	Sample	Influencing Factors
Heterogeneity	Ambient water, sediment effluent, runoff, biotic communities	Flow, mixing, depth, particle size distribution, land use patterns, runoff coefficients, season, life-cycle, behavior, patch dynamics, pollutant partitioning (fate)
Pollutant sources	Upstream-downstream, tributary mouths, sensitive habitats, dilution gradient, beneficial uses, "typical" habitats	Pollutant partitioning (fate), mixing, loading characteristics, toxicity target species and endpoints, habitat complexity
Beneficial uses	"Beneficial" component (e.g., water supply, fishery, swimming) at critical areas	Above factors

watershed should, ideally, have baseline characteristics identical to those of the test system when the pollutant problem (e.g., stormwater) being assessed is removed. However, since no two ecosystems are identical, this reference should be considered as a *general* benchmark from which to determine relative effect.

The next chapter presents much detail and information on evaluating samples and conditions (flow, rainfall, soil, aesthetics, habitat, water, sediment, microorganisms, benthos, zooplankton, fish, and toxicity), heavily supported with case study examples. Chapter 7 discusses statistical evaluations of the data, and Chapter 8 discusses data interpretation.

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CHAPTER 6

Ecosystem Component Characterization

"*Things don't turn up in this world until somebody turns them up.*"

James A. Garfield

CONTENTS

OVERVIEW

Ecosystem Structure and Integrity, Chaos and Disturbance

It is impossible to produce meaningful, representative, and reliable data to be used in decisions regarding the status of, or possible impacts to, the environment without first defining the environment, critical receptors, influencing factors, and natural dynamics. This requires the measurement of many aspects of the watershed, as previously described in this book. Simplistic and rapid approaches are fine for initial assessments, but may fall short in providing understanding of the causes of the observed problems. Therefore, later phases of watershed assessment projects generally need to examine more detailed aspects of a study area in order to obtain a better understanding of possible interactions. As an example, the majority of studies dealing with aquatic toxicity have used surrogate species (or a small number of species) and have not attempted to investigate ecosystem interactions *a priori*, such as ecosystem energetics or stress–productivity–predation relationships. For example, surrogate responses have simply been quantified based on sample toxicity, and then effects have been extrapolated to *in situ* conditions. While these exercises might satisfy the study objectives of defining sample toxicity to the test species, they do little to document or define ecosystem disturbance. Ecological processes can be ignored, to a degree, when acute toxicity scenarios are studied, such as in sediments that are severely degraded. However, "significant cases of acute toxic effects have been encountered infrequently" (Chapman 1986), and the more common situations in which effects and zones of contamination are "gray" (Chapman 1986) dictate that natural and anthropogenic effects be separated. This cannot be done accurately without an understanding of ecosystem dynamics such as spatial and temporal variance of chemical, physical, and biological systems and their interactive processes.

Community ecology in lotic and lentic systems has progressed substantially in recent years. "Biotic dynamics and interactions are intimately and inextricably linked to variation in abiotic factors" (Power et al. 1988), and lotic systems are not in equilibrium due to natural disturbances

which may occur frequently or infrequently (Resh et al. 1988). Disturbance can be defined as a discrete event that alters community structure and changes the physical environment and resource availability. These disturbances vary in type, frequency, and severity, both among and within ecoregions. The frequency and intensity of disturbances cannot be predicted (Resh et al. 1988). Intermediate levels of disturbance maximizes species richness (Resh et al. 1988). Equilibrium or steady-state conditions will tend to occur if disturbances are infrequent, thus excluding opportunistic species (Minshall 1988). In stream ecology, disturbance is the dominating organizing factor, having a "major impact on productivity, nutrient cycling and spiraling, and decomposition" (Resh et al. 1988). Disturbances such as storm events or the presence of toxicants can eliminate biota (Power et al. 1988). Recovery and succession of these systems between disturbances is typified by recurrent or divergent patterns (Pringle et al. 1988; Resh et al. 1988). Despite this inherent variability, benthic communities have been used effectively to classify community structure and functioning in aquatic ecosystems.

Ecotones are defined as zones of transition between adjacent ecosystems. Disturbance plays a major role in determining the structure and dynamics of ecotones, such as stream bank riparian zones. High relief areas are less stable due to more frequent and diverse disturbances combined with complex topographic effects. Both fluvial and geomorphic processes influence vegetation development along stream and lake embankments (Decamps et al. 1990).

The major role that natural and anthropogenic disturbances have on aquatic ecosystems increases the level of spatial and temporal variance. Spatial and temporal dimensions span 16 orders of magnitude in stream ecology (Minshall 1988; Pringle et al. 1988). Some suggest that spatial heterogeneity enhances the ability of an ecosystem to resist and recover after a disturbance (Fisher 1990). Significant spatial variance in sediments is common (Stemmer et al. 1990). Each level of the system has different dimensions, has different variances associated with it, and is interacting simultaneously with other ecosystem levels and their respective dimensions and variances. This complex reality is difficult, if not impossible, to define accurately but must be considered in all assessments of water quality or ecosystem health.

Orians (1980) stated that one of the greatest challenges in ecology (and ecotoxicology) is bridging the conceptual gap between micro- and macroecology. Aquatic systems can be considered as a mosaic of patches (Pringle et al. 1988). "A patch is a spatial unit that is determined by the organism and problems in question" (Pringle et al. 1988). The heterogeneous environment has highly clumped distributions (patches) of organisms whose spatial and temporal patterns and relationships change seasonally due to factors such as food (resource) patterns (Findlay 1981). These clumped distributions, therefore, pose severe sampling problems. The appropriate sampling scale will depend on the organism size, density, distribution, life cycle, and question being asked (Pringle et al. 1988), which, unfortunately, are often not considered. Aquatic ecosystems are open nonequilibrium systems (Carpenter et al. 1985; Pringle et al. 1988) where patches are in transitory steady state with other patches (Sheldon 1984). Many "ecosystems" are not independent units, and some processes (e.g., nutrient cycling) show no spatial threshold. That is, no one area bounds all processes, showing that ecosystems have both an open nature and are connected in many complex ways. Most aquatic organisms are aggregated at certain spatial scales and are random on other scales. In order to accurately determine total organism numbers and distribution patterns (patches) within and among sites, presampling should be conducted whereby the site is divided into quadrants, sampled, and coefficients of variation (standard deviation divided by the mean) determined. This will detect heterogeneity in density measurements (Westman 1985). Unfortunately, this level of accuracy is often beyond the resource capabilities of typical studies. Different life histories and variable interactions between species may prevent equilibrium (Carpenter et al. 1985).

Ecosystems tend to restore balance (homeostasis or resilience). Diversity does not equate to integrity. Biological integrity may be defined as the ability of species to interact and maintain their structure and function in some self-regulating, homeostatic fashion (Westman 1985). The rate, manner, and extent of recovery following a disturbance is a measure of resilience.

The influence of storm events and watershed characteristics on chemical element dynamics is poorly understood, particularly because some are lumped into operationally defined units such as dissolved or total organic carbon. Significant heterogeneity (62 to 100%) has been observed between adjacent sediment cores in concentrations of organic matter, water, and total phosphorus (Downing and Roth 1988). Some heterogeneity is likely due to small-scale variations in bottom profiles.

In stream benthic communities, hydraulics appear to be more important than substrate in determining distribution (Statzner et al. 1988). As in fish communities, populations will vary in type and number between pool and riffle areas. Most benthic macroinvertebrate testing occurs in riffle areas where continual flow exists and more types of organisms are present. Small-scale sampling is more likely to define benthic invertebrate patches than large-scale sampling, which homogenizes patchiness differences. The replicate number needed to obtain a given precision decreases with increased density and sample size, and the optimal sample size (considering cost and precision) depends on mean density (Morin 1985).

Other important considerations in valid hazard assessments are contaminant interactions and subsequent distribution in the aquatic system via solids. Sediment contaminant data should be evaluated based on grain size correction, which reduces the inert fractions (e.g., hydrates, sulfides, amorphous, and fine-grained organics). The most useful size fraction for contaminant assessments appears to be <63 μm (Håkanson 1984). This size fraction will tend to predominate in deposition areas and will play a major role in the transport, deposition, and resuspension of the fine-grained sediments. Particle diameters of suspended solids vary over two orders of magnitude and settling speeds in waters vary over four orders of magnitude (Gailani et al. 1991). Predicting transport is complicated by the lack of understanding of sizes and settling speeds, floc disaggregation due to shear, processes governing entrainment and deposition, and turbulence description (Gailani et al. 1991).

When resuspension events occur, predicting metal remobilization may be possible in site-specific studies; however, remobilization is dependent on particle residence time in the water column, which varies between sites, storms, and systems. In most systems, however, remobilization of metals from resuspended sediments is likely to be insignificant due to the slow reaction rates (Kersten and Forstner 1987).

Though resuspension effects appear limited if one considers the scavenging effects of solids, laboratory studies of bioturbation effects on contaminant movement and toxicity to planktonic species have shown otherwise. Bioturbation by benthic and epibenthic invertebrates occurs in many ways: by pumping pore water constituents out of the sediment into overlying waters; by injecting water into the sediment; by pumping particulates to the sediment-water interface; by depositing fecal pellets on the sediment surface; and by disrupting horizontal and vertical layering (Petr 1977).

Given the above discussion on the complexities of aquatic ecosystems, it is evident that it is no longer adequate to simply study separate components of the ecosystem, such as planktonic species in water-only systems or chemical dynamics in a water-only or sediment slurry system. This "reductionist" approach is essential for defining processes, but does not provide an accurate picture of the component–ecosystem interactions and, in fact, may produce misleading results. Examples of this disparity are becoming increasingly obvious, particularly in the field of aquatic toxicology, as more "holistic" types of studies are published (Chapman et al. 1992).

Sediments play a major role in ecosystem processes and ecosystem health (Chapman et al. 1992). Generally speaking, the surficial layer (upper few centimeters) is the active portion of the ecosystem, while deeper sediments are passive and more permanently "in-place." These deeper layers are of interest as a historical record of ecosystem activity, but may also be reintroduced into the active portion of the ecosystem via dredging activities and severe storm or hydrogeological events. The usefulness of a sediment monitoring station as an indicator of contaminant presence is a function of the interactions between the change in contaminant net deposition rate, sediment accumulation rate, mixing zone depth and dynamics, sampling method and frequency, the type of laboratory method, and its precision and accuracy (Larsen and Jensen 1989). Sediments and soils typically exhibit more spatial variability from overlying waters but less temporal variation. This reality affects sampling design and statistical analyses.

This chapter describes a wide variety of tools that can be used for assessing the ecosystem and watershed physical characteristics because of the likely need to consider a broad range of assessment procedures. This chapter starts with discussions of rainfall and flow monitoring, as it is difficult to understand pollutant transport, fate, and effects without appreciating the physical movement of the water. The main sections of this chapter pertain to examinations of specific receiving water uses and associated ecosystem components: aesthetics and safety, habitat, water and sediment, microorganisms, benthos, zooplankton, fish, and toxicity and bioaccumulation.

FLOW AND RAINFALL MONITORING

It is essential that there be an accurate description of the system's hydrodynamics when assessing the effects of stormwater runoff on receiving waters. Flow represents the pollutant loading mechanism, and its power and frequency of occurrence can degrade the physical habitat. One of the principal reasons there is a relatively poor understanding of stormwater runoff effects is because of the difficult logistics involved in measuring short-term, high-flow events quickly and accurately. Flow and rainfall monitoring are considered separately from other physical characteristics, which are discussed in the following section on habitat. The hydrology of the stream, reservoir, or lake which receives stormwater runoff is interrelated, directly and indirectly, with many other characteristics, such as substrate composition, temperature, suspended solids, channel morphology, and biological communities. Hydrology, as discussed here, is composed of flow, velocity, power, turbulence, mixing, sedimentation, and resuspension subcomponents. Each of these subcomponents is important to varying degrees depending on the site and study objectives, and each is relatively difficult to quantify accurately during storm events.

As with other major ecosystem components, the storm event hydrodynamics of the receiving water must be evaluated based on references for comparison. References may include an upstream station, present day baseflow conditions, predevelopment conditions, and/or a least disturbed watershed of similar natural characteristics (e.g., soil, topography, drainage area, stream order, stream substrate, biological communities). The assessment should attempt to characterize the hydrology of the system by defining the loading dynamics (i.e., magnitude, duration, frequency) and the receiving system response (e.g., flow, spatial-temporal patterns). The physical characterization of the loading and system response will dictate the chemical sampling from which to determine pollutant (stressor) loading dynamics and optimal stormwater control programs and associated remediation measures.

The rate of stream discharge (flow) (Q) is a function of the channel cross-sectional area (A) and the mean velocity (V), which is usually expressed as cubic feet per second (cfs). So, $Q = AV$. Velocity is a function of runoff quantity, stream width, depth, and gradient, and channel roughness. Roughness is affected by channel sinuosity, substrate size, bottom topography, stream vegetation, debris, and other obstructions. Channelization increases velocity and also tends to make velocity more uniform (EPA 1983). Channelization practices, such as straightening, vegetation and debris removal, berming and leveeing, all increase drainage efficiency. These practices also produce sharper flow hydrographs, with much greater peak flow rates. The resulting higher flow rate and power increases the impact of storm events, including increased scour, erosion, bank cutting, sediment transport, flooding below the channelized section, reduced groundwater levels and stream dewatering, degraded habitat and water quality, promotion of land development, and lowered recreational values. Assessing channelization effects on habitat quality is discussed more fully in the following section on habitat.

Stream staff gauges, which measure stream depth, may be used to indirectly measure flow through the use of a rating curve which shows the relationship between stream depth and flow rate. The rating curve is developed by making velocity measurements in a cross-sectional area of the stream channel where the channel morphology and flow patterns are simple. This is done over a range of flows so that the curve can be constructed. This is discussed in a following subsection.

Stream power is the rate of potential energy expenditure per unit weight of water in a channel and is calculated as:

$$SP = \frac{\Delta Y}{\Delta t} = \frac{\Delta X}{\Delta t}\frac{\Delta Y}{\Delta X} = VS_f$$

where SP = stream power (ft-lbs/lb H_2O/s)
t = time (s)
V = velocity (ft/s)
S_f = stream friction slope (ft/ft) (energy gradient)
Y = energy grade line elevation above a point, equivalent to potential energy (ft-lb)/lb/H_2O) = water surface elevation and velocity head ($V^2/2g$)
X = longitudinal distance
g = gravitational constant

Stream power can be used to estimate the energy available for sediment transport. This energy can be reduced by other habitat factors (e.g., bank and substrate stability, vegetation, or surface erosion).

"Time of passage" has been recommended as a parameter of pollutant movement through a stream more useful than the kinematic wave velocity that is typically used in hydrograph routing calculations (Velz 1970). The distinction is that the kinematic wave (hydrograph crest) moves faster than the waste in the body of water, particularly in large, deep water systems. Time of passage (as seconds or days) is based on the average flow rates that are measured when using current meters. It is determined by dividing the occupied channel volume (from cross-sectional area) (as cubic feet) by the runoff (from drainage area and yield) (as cfs).

Flow Requirements for Aquatic Biota

A popular evaluation tool for evaluating flow effects on aquatic communities was published by Tennant (1976). He found the following in 11 streams of three western states:

- Changes in habitat were similar among streams with similar flow regimes.
- A depth of at least 0.3 m and velocity of at least 0.75 ft/s were required for most fish.
- Thirty percent of the annual flow provided good habitat.
- Sixty percent of the annual flow provided outstanding habitat.

Stream velocity plays a major role in determining the composition of benthic communities (Cummins 1975): invertebrate drift increases as the velocity increases (Minshall and Winger 1968; Walton 1977; Zimmer 1977).

The U.S. Fish and Wildlife Service developed the Instream Flow Incremental Methodology (IFIM) computer program to evaluate changes in aquatic life from alteration of channel morphology, water quality, and hydraulic components. Each species has a range of habitat (including flow) conditions it can tolerate, which can be defined (or is defined) for the species, as can stream conditions. IFIM simulates hydraulic conditions — habitat availability for a species and size class, or usable waters for a particular recreational activity. This is done through use of the Physical Habitat Simulation Model (PHABSIM), which relates changes in flow and channel structure to changes in physical habitat availability.

The basic steps in the IFIM can be summarized by the following:

- Project scoping — Define objectives for the delineation of study area boundaries, determine the species, and define their life history, food types, water quality tolerances, and microhabitat.
- Study reach and site selection — Identify and delineate critical reaches to be sampled, delineate major changes and transition zones and the distribution of the evaluation species.

- Data collection — Transects are selected to adequately characterize the hydraulic and in-stream habitat conditions. Data gathering must be compatible with IFIH computer models.
- Computer simulation — Reduce field data and input into programs described above.
- Interpretation — The output is expressed as the Weighted Usable Area (WUA), a discrete value for each representative and critical study reach, for each life stage and species, and for each flow regime.

For further information on IFIM and PHABSIM, consult *A Guidance to Stream Habitat Analysis Using the Instream Flow Incremental Methodology.* U.S. FWS/OBS-82/26, June 1982.

Urban Hydrology

Basic watershed characteristics need to be known in order to understand stream flow conditions. These include topography (watershed divide plus stream and land slopes), drainage efficiency (stream orders and types of artificial drainage systems), and, to a lesser extent in urban areas, soil characteristics (disturbed or compacted, age since development, type of ground cover, soil texture, etc.). It is important that characteristics throughout the watershed be evaluated when studying streams. Looking only at characteristics adjacent to the stream is very misleading, as urban drainage systems are very efficient transporting systems, capable of carrying water and pollutants to the stream from locations far away. These topics are beyond the scope of this book, but several good books are available that describe urban hydrology and associated drainage design (including McCuen 1989; WEF and ASCE 1992; Debo and Reese 1995; and Wanielista et al. 1997).

Urban hydrology can be used to divide rain into different major categories, each reflecting distinct portions of the long-term rainfall record (Pitt et al. 1999). When monitoring runoff, it is therefore important to include a sampling effort that represents each of these categories. All too often, the small rains are not sampled because of misunderstandings of their significance. It is easy to ignore these small events, considering the problems that occur when trying to program automatic sampling equipment. However, small events are extremely important when conducting a receiving water investigation. As an example, consider the following rainfall and runoff data for Milwaukee, WI, what were obtained during the Nationwide Urban Runoff Program (EPA 1983). Figure 6.1 shows measured rain and runoff distributions for Milwaukee during the 1981 NURP monitored rain year. Rains between 0.05 and 5 in were monitored during this period. Two very large events

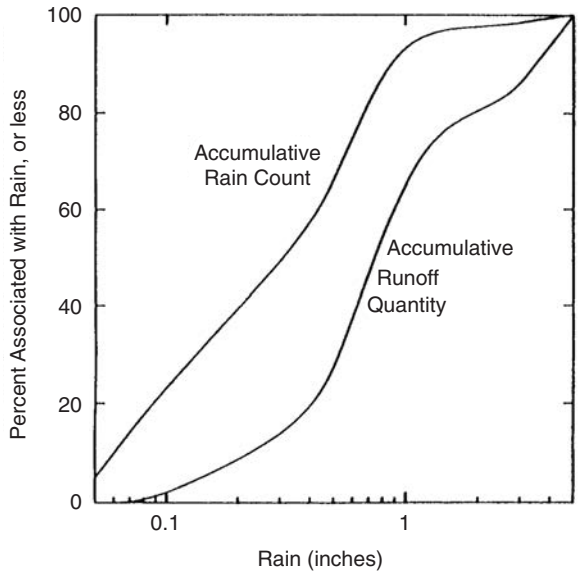


Figure 6.1 Milwaukee rain and runoff distributions.

(greater than 3 in) occurred during this monitoring period, which greatly bias this distribution, compared to typical rain years. The following observations are evident:

- The median rain depth was about 0.3 in.
- 66% of all Milwaukee rains are less than 0.5 in in depth.
- For the medium-density residential area, 50% of the runoff was associated with rains less than 0.75 in for Milwaukee.
- Observable runoff occurred with rain as small as 0.05 in in depth.

In addition, a 100-year, 24-hour rain of 5.6 in for Milwaukee could produce about 15% of the typical annual runoff volume, but it only contributes about 0.15% of the average annual runoff volume, when amortized over 100 years. Typical 25-year drainage design storms (4.4 in in Milwaukee) produce about 12.5% of typical annual runoff volume but only about 0.5% of the average runoff volume.

Figure 6.2 shows measured Milwaukee pollutant discharges associated with different rain depths for a monitored medium-density residential area. Suspended solids, COD, lead, and phosphate discharges are seen to closely follow the runoff distribution shown in Figure 6.1. Therefore, the concentrations of most runoff pollutants do not vary significantly for runoff events associated with different rain depths.

The monitored rains at this Milwaukee medium-density residential location can be divided into four categories:

- <0.5 inch. These rains account for most of the events, but little of the runoff volume. They produce much less pollutant mass discharge and probably have fewer receiving water effects than other rains. However, the runoff pollutant concentrations likely exceed regulatory standards for several categories of critical pollutants, especially bacteria and some total recoverable metals. They also cause large numbers of overflow events in uncontrolled combined sewers. These rains are very common, occurring once or twice a week (accounting for about 60% of the total rainfall events and about 45% of the total runoff events that occurred), but they only account for about 20% of the annual runoff and pollutant discharges. Rains less than about 0.05 in did not produce noticeable runoff.
- 0.5 to 1.5 inches. These rains account for the majority of the runoff volume (about 50% of the annual volume for this Milwaukee example) and produce moderate to high flows. They account for about 35% of the annual rain events, and about 20% of the annual runoff events. These rains

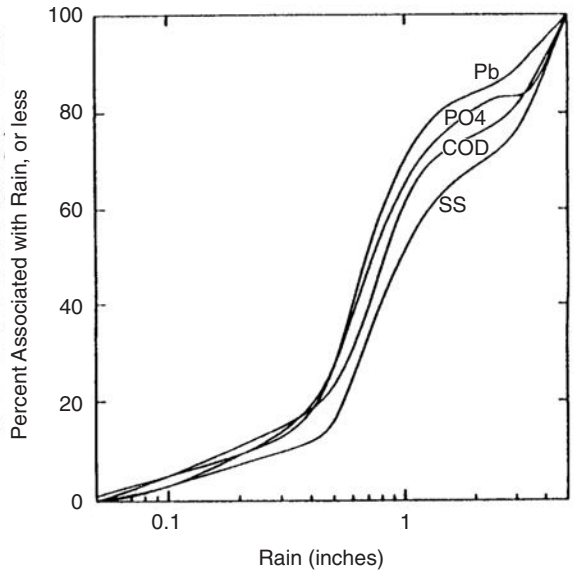


Figure 6.2 Milwaukee pollutant discharge distributions.

occur on the average about every 2 weeks from spring to fall and subject the receiving waters to frequent high pollutant loads and moderate to high flows.

- 1.5 to 3 inches. These rains produce the most damaging flows, from a habitat destruction standpoint, and occur every several months (at least once or twice a year). These recurring high flows, which were historically associated with much less frequent rains, establish the energy gradient of the stream and cause unstable stream banks. Only about 2% of the rains are in this category, and they are responsible for about 10% of the annual runoff and pollutant discharges. Typical storm drainage design events fall in the upper portion of this category.
- >3 inches. The smallest rains in this category are included in design storms used for drainage systems in Milwaukee. These rains occur only rarely (once every several years to once every several decades, or even less frequently) and produce extremely large flows. The monitoring period during the Milwaukee NURP program was unusual in that two of these events occurred. Less than 2% of the rains were in this category (typically <<1%), and they produced about 15% of the annual runoff quantity and pollutant discharges. During a "normal" period, these rains would produce only a very small fraction of the annual average discharges. However, when they do occur, great property and receiving water damage results. Receiving waters can conceivably recover naturally from this damage (mostly associated with habitat destruction, sediment scouring, and the flushing of organisms great distances downstream and out of the system) and return to before-storm conditions within a few years, depending on riparian vegetation growth rates and nearby "reservoir or refugia" areas for aquatic organisms.

The above specific rain values are given for Milwaukee, WI, selected because of the occurrence of two very rare rains during an actual monitoring period. Obviously, the critical values defining the design storm regions would be highly dependent on local rain and development conditions. Computer modeling analyses from 24 urban locations from throughout the United States were conducted by Pitt et al. (1999) to examine these patterns nationwide. These locations represent most of the major river basins and much of the rainfall variations in the country. These simulations were based on 5 to 10 years of rainfall records, usually containing about 500 individual rains each. The rainfall records were from certified NOAA weather stations and were obtained from CD-ROMs distributed by EarthInfo of Boulder, CO. Hourly rainfall depths for the indicated periods were downloaded from the CD-ROMs into an Excel spreadsheet. This file was then read by a utility program included in the Source Loading and Management Model (SLAMM) package (Pitt and Voorhees 1995). This rainfall file utility combined adjacent hourly rainfall values into individual rains, based on user selections (at least 6 hours of no rain was used as the criterion to separate adjacent rain events and all rain depths were used, with the exception of the "trace" values that were <0.01 in). These rain files for each city were then used in SLAMM for typical medium-density and strip commercial developments. SLAMM utilizes unique prediction methods that were especially developed by Pitt (1987) to accurately predict runoff during these small rains. Conventional runoff prediction methods are based on drainage design storms (of several inches in depth) and are not accurate when predicting runoff during small rains.

Table 6.1 summarizes these rain and runoff distributions for these different U.S. locations. Lower and upper runoff distribution breakpoints were identified on all of the individual distributions. The breakpoints separate the distributions into the following three general categories (similar to the regions identified for the Milwaukee rains):

- Less than lower breakpoint: small, but frequent rains. These generally account for 50 to 70% of all rain events (by number), but only produce about 10 to 20% of the runoff volume. The rain depth for this breakpoint ranges from about 0.10 in in the arid Southwest, to about 0.5 in in the wet Southeast. These events are most important because of their frequencies, not because of their mass discharges. They are therefore of great interest where water quality violations associated with urban stormwater occur. This would be most common for bacteria (especially fecal coliforms) and for total recoverable heavy metals, which typically exceed receiving water numeric criteria during practically every rain event in heavily urbanized drainages having separate stormwater drainage systems.

Table 6.1 Rainfall and Runoff Distribution Characteristics for Different Locations from Throughout the U.S. (Pitt, et al. 1999)

	Median Rain Depth, by Count (in)	Corresponding Percentage of Runoff for the Median Rain Depth	Rain Depth Associated with Median Runoff Depth (in)	Lower Breakpoint Rain Depth (in)	Percentage of Rain Events Less Than Lower Breakpoint	Percentage of Runoff Volume Less Than Lower Breakpoint	Upper Breakpoint Rain Depth (in)	Percentage of Rain Events Less Than Upper Breakpoint	Percentage of Runoff Volume Less Than Upper Breakpoint	Percentage of Runoff Volume Between Breakpoints	Percentage of Rain Events Between Breakpoints
Columbia North Pacific											
Boise, ID	0.07	3–5	0.30–0.35	0.10	52	9–11	0.91	99	89–93	80–82	47
Seattle, WA	0.12	4–6	0.62–0.80	0.18	60	8–11	3.4	99	92–96	84–85	39
California											
Los Angeles, CA	0.18	3–5	1.2–1.5	0.29	64	7–10	3.5	99	92–98	85–88	35
Great Basin											
Reno, NV	0.07	3–5	0.35–0.41	0.10	61	8–10	1.7	99	93–95	85	38
Lower Colorado											
Phoenix, AZ	0.10	4–6	0.55–0.68	0.19	64	9–12	2.3	99	94–98	85–87	35
Missouri											
Billings, MT	0.06	2–4	0.55–0.60	0.12	64	8–10	1.6	99	89–93	81–83	35
Denver, CO	0.08	2–4	0.50–0.60	0.19	71	13–17	1.8	99	91–95	78	28
Rapid City, SD	0.06	2–4	0.50–0.55	0.15	69	10–13	1.9	99	92–96	82–83	30
Arkansas-White-Red											
Wichita, KS	0.13	2–5	1.1–1.4	0.31	65	10–13	3.0	99	88–93	78–80	34
Texas Gulf											
Austin, TX	0.14	2–3	1.4–1.8	0.50	72	8–12	6.0	99	88–94	80–82	27
Upper Mississippi											
Minneapolis, MN	0.11	3–5	0.73–1.0	0.22	65	9–13	2.8	99	94–96	83–85	34
Madison, WI	0.12	3–5	0.78–0.98	0.23	65	9–13	3.5	99	97–99	86–88	34
Milwaukee, WI	0.12	2–4	0.9–1.1	0.25	65	9–12	2.5	99	89–95	80–83	34
St. Louis, MO	0.14	4–6	1.0–1.2	0.31	65	10–13	2.8	99	90–95	80–82	34

Great Lakes											
Detroit, MI	0.20	7–11	0.72–0.81	0.20	50	7–11	2.4	99	92–95	85–84	49
Buffalo, NY	0.11	2–4	0.61–0.72	0.12	64	8–12	2.1	99	88–93	80–81	35
Ohio											
Columbus, OH	0.12	3–5	0.80–1.0	0.22	63	8–12	2.2	99	85–91	77–79	36
North Atlantic											
Portland, ME	0.15	2–4	1.1–1.5	0.30	64	8–12	4.5	99	90–96	82–84	35
Newark, NJ	0.28	6–12	1.2–1.5	0.33	54	8–12	3.3	99	89–94	81–82	45
Lower Mississippi											
New Orleans, LA	0.25	3–5	1.7–2.2	0.45	62	7–11	4.0	99	88–93	81–82	37
South Atlantic Gulf											
Atlanta, GA	0.22	3–5	1.2–1.7	0.32	58	5–9	4.0	99	91–95	86	41
Birmingham, AL	0.20	3–5	1.2–1.5	0.40	64	8–13	5.0	99	90–96	82–83	35
Raleigh, NC	0.18	4–6	1.0–1.2	0.26	60	7–11	2.5	99	87–93	80–82	39
Miami, FL	0.13	3–5	1.2–1.6	0.30	67	9–13	4.0	99	87–93	78–80	32

From Pitt, R. et al. *Guidance Manual for Integrated Wet Weather Flow (WWF) Collection and Treatment Systems for Newly Urbanized Areas (New WWF Systems). Second year project report: model integration and use.* Wet Weather Research Program, U.S. Environmental Protection Agency. Cooperative agreement #CX 824933-01-0. February 1999.

- Between the lower and upper breakpoint: moderate rains. These rains generally account for 30 to 50% of all rain events (by number), but produce 75 to 90% of all the runoff volume. The rain depths associated with the upper breakpoint range from about 1 to 2 in in the arid parts of the United States and up to 5 or 6 in in wetter areas. These runoff volume distributions are approximately the same as the pollutant distributions. Therefore, these intermediate rains also account for most of the pollutant mass discharges and many of the actual receiving water problems associated with stormwater discharges.
- Above the upper breakpoint: large but rare rains. These rains include the typical drainage design events and are therefore quite rare. During the period analyzed, many of the sites only had one or two, if any, events above this breakpoint. These rare events do account for about 5 to 10% of the runoff on an annual basis. Obviously, these events must be evaluated to ensure adequate drainage.

The fourth category, evident in the Milwaukee monitoring results and shown in Figures 6.1 and 6.2, was not obvious during these computer analyses. These extremely rare events, which exceed the drainage capacity of most areas, do not significantly affect these long-term probability distributions. During the isolated years when they occur, such as during the monitoring period in Milwaukee, they have significant effects, but when averaged over long periods, their contributions diminish rapidly.

The small rains, generally less than about 0.5 in, are very important in a wet-weather monitoring program. They represent the vast majority of rains that occur in an area, and may represent the majority of runoff events. Water quality violations associated with wet-weather flows are typically common for these events. Similarly, the medium-sized events (from the 0.5-in rains to rains of several inches in depth) contribute the majority of runoff volume and mass pollutant discharges and are therefore likely responsible for most of the biological effects (especially habitat destruction and sediment contamination) in receiving waters. The largest rains (greater than several inches) are the primary focus of drainage design. Therefore, efforts must be made to characterize runoff and receiving water conditions in each of these different categories in order to understand the varying receiving water problems that may be occurring.

Pollutant Transport

The routing of pollutants through a watershed is a complex issue and beyond the scope of this book. One of the most important goals of a monitoring effort is collecting representative samples. In many cases, pollutant routing can affect pollutant concentration distributions. At outfalls, or in receiving waters, stormwater pollutant concentrations are random, with little of the observed variations being explainable by normal parameters (such as time since the event started or rain depth). As noted by Roa-Espinosa and Bannerman (1994), obtaining many discrete subsamples over the event duration likely results in a composite sample that has pollutant concentrations very similar to a flow-weighted composite sample. However, if collecting samples from a relatively small homogeneous area (such as a paved parking area), high concentrations of practically all pollutants are commonly observed near the beginning of the rain.

This “first-flush” phenomenon is most prevalent for rains having relatively constant intensities and for small areas. As a drainage area size increases (or as the surfaces become more complex, such as in a residential area), multiple first-flush waves travel through the drainage system, arriving at a single downstream location at different times. This moderates obvious concentration trends with time during the event. Also, as the rain intensity varies throughout an event, the washoff of pollutants at the sources also varies. Peak washoff occurs during periods of peak rain energy (high rain intensity). Therefore, periods of high concentrations may also occur later in a rain, as high intensities occur. Generally, lighter (more soluble) hydrocarbons and the smallest particles will “always” show a first-flush of high concentrations from small paved areas, while larger particulates and heavier hydrocarbons will wash off more effectively with high rain energies, which may occur randomly during a rain.

Sampling strategies must therefore consider these possible scenarios. The most effective sampling (but most expensive) is flow-weighted composite sampling throughout the entire storm event.

However, compositing many discrete subsamples collected throughout the event is likely to result in similar concentration values. If sampling a small critical source area (such as a gas station or convenience store), it may be useful to obtain an initial sample during the first few minutes of the event, and a composite over the complete event. In all cases, it would be difficult to justify analyzing many individual discrete samples collected throughout an event.

Flow Monitoring Methods

There are a wide variety of methods (Table 6.2) to determine flow in open and closed (e.g., conduit) channels. For additional information, see EPA (1982 and 1987a). Most flow measurements to assess receiving water effects from stormwater are conducted in relatively small streams. Often, channel cross-sectional area is determined and the velocity measured at intervals across the channel using a current meter. In some situations, discharge from a pipe, notched weir, or small dam can be caught in a container of known volume and mean fill-up time used to calculate flow (e.g., liters per second). A variety of flumes and weirs have been used successfully in assessing flow and runoff.

Mechanical current meters are commonly used because they are simple, rugged, accurate at low velocities (0.03 m/s, 0.1 ft/s) and operate at shallow depth (0.1 m). A manufacturer's calibration table converts the meter rotation number into meters or feet per second. Many modern meters are direct reading. The mean velocity at each cross-sectional interval is multiplied by the area of the subsection to calculate volumetric flow for each subsection. These are then summed to obtain the total stream flow.

Salt or fluorescent dyes have been used effectively to estimate velocities and time of passage when other methods are not practical, especially for highly irregular stream shapes or highly turbulent low flows. They depend on determining the amount of dilution that a known concentration of tracer receives as it mixes in the stream. The velocity between two stations is determined by knowing the travel time of the dye, or by comparing the dilute dye concentration to the injected dye concentration. The tracer may be added continuously or as a slug. A common tracer is Rhodamine WT dye which is measured with a fluorometer.

Flow monitoring in streams and other open channels is usually a necessary component of receiving water investigations. Flow estimates need to be made whenever any in-stream measurements are made, or samples collected, for example. In addition, equipment for continuous flow monitoring must be periodically calibrated using manual procedures. The following paragraphs briefly describe several common manual flow monitoring procedures.

Drift Method

The drift method is simply watching and timing debris floating down the stream. This velocity is then multiplied by the estimated or measured stream cross-sectional area to obtain the stream discharge rate. Of course, this method is usually the least accurate flow estimation method. The accuracy can be improved by choosing drift material that floats barely under the stream surface (such as an orange). Do not use material that floats high in the water (such as Styrofoam debris, for example), as it will be strongly influenced by winds. Drift measurements made in the center of a stream will tend to be the highest stream velocities, so the values should be reduced (by roughly 0.6, but highly variable) to better represent average stream flow rates.

Current Meter Method

The most traditional method of measuring flow is using a mechanical current meter. This method requires at least two people (one person should never be working alone near a stream anyway), a current meter, and simple surveying equipment. The stream discharge is measured at a cross section, usually selected along a relatively straight stretch (about 10 stream widths downstream from any major bends). If the stream discharge is being used to calibrate a stage recorder for continuous

Table 6.2 Methods for Flow Measurement and Their Application to Various Types of Problems

Device or Method	Flow Range Measurement	Applicable to Type of Water and Wastewater	Cost	Ease of Installation	Accuracy of Data[a]	Pressure Loss thru the Device	Volumetric Flow Detector	Flow Rate Sensor	Transmitter Available
Formula	Small to large	All	Low	NA	Fair	NA	NA	NA	NA
Bucket and stopwatch	Small	All	Low	Fair	Good	NA	NA	NA	NA
Floating objects	Small to medium	All	Low	NA	Good	NA	NA	NA	NA
Rotating elements current meters	Small to medium	All	Low	NA	Good	NA	Yes	NA	Yes
Dyes	Small to medium	All	Low	NA	Fairly good	NA	NA	NA	NA
Salt dilution	Small to medium	All	Low	NA	Fair	NA	NA	NA	NA
Magnetic flowmeters	Small to large	All	High	Fair	Excellent ½–1%	None	Yes	Yes	Yes
Weirs	Small to large	All	Medium	Difficult	Good to excellent 2–5%	Minimal	Yes	Yes	Yes
Flumes	Small to large	All	High	Difficult	Good to excellent 2–5 %	Minimal	Yes	Yes	Yes
Acoustic flowmeters	Small to large	All	High	Fair	Excellent 1%	None	Yes	Yes	Yes

[a] Assume proper installation and maintenance.

Data from Blasso, L. Flow measurement under and conditions, *Instruments Control Syst.,* 48: 45–50. 1975; Thorsen, T. and R. Owen. How to measure industrial wastewater flow. *Chem. Eng.* 82: 95–100. 1975.

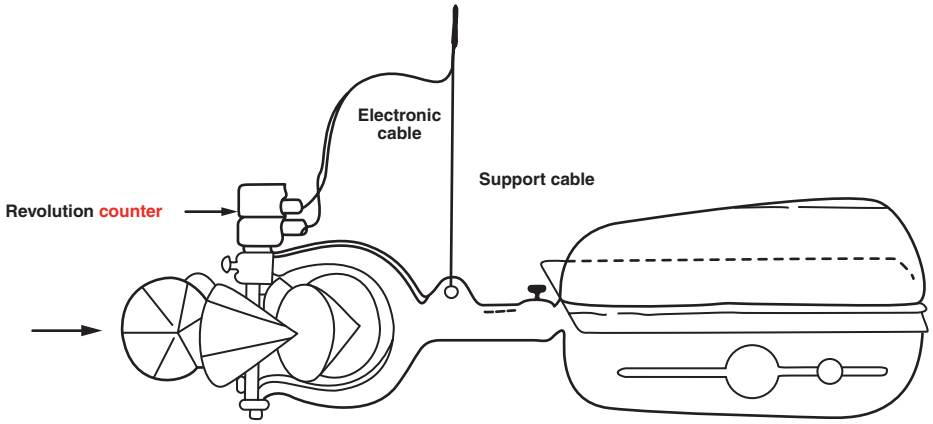


Figure 6.3 Price meter.

Figure 6.4 Student current meter.

flow monitoring, the cross section being measured must not be affected by backwater conditions. If the selected cross section is in the vicinity of sampling and will not be used to calibrate a flow equation but will be used to determine the instantaneous current conditions at the time of sampling, then backwater influences and affects from meanders need to be included in the measurements. Instantaneous flows are determined using current meters to document flows occurring in a sampling period. However, this procedure can also be used to calibrate a state–discharge curve that can be used in conjunction with a conventional continuous stage recording device for long-term studies. Figures 6.3 and 6.4 illustrate common current meters used for stream studies.

In order to calibrate a flow or discharge model (especially the Manning's equation), the stream is assumed to have normal flow where the water surface is parallel with the stream bottom. This is unusual under real stream conditions, where actual water surface profiles exist. In this case, Manning's equation can still be used, but by substituting the friction slope for the water surface (or stream bed) slope. The friction slope is elevated above the water surface by the velocity head ($v^2/2g$). It is therefore easy to adjust the surveyed water surface slope to the friction slope by adding the velocity heads at the upstream and downstream locations. The calibration procedure usually involves calculating the Manning's roughness factor (n) in the stream stretch. Manning's equation is (in U.S. customary units):

$$V = 1.49(R^{2/3})S_f^{0.5}/n$$

where V = velocity of the open channel flow (ft/s)
R = hydraulic radius (area/wetter perimeter, ft)
S_f = friction slope
n = Manning's roughness coefficient

Biological monitoring is normally conducted during relatively low flow periods, whereas Manning's equation was developed for channel design for large, rare events. Manning's equation is a

Table 6.3 Example Calculation for Flow and Current Measurements

Section Interval (ft)	Midpoint, Distance from Shore (ft)	Depth at Midpoint (ft)	Section Area (ft^2)	Velocity at 0.2 Depth (ft/s)	Velocity at 0.8 Depth (ft/s)	Average Velocity (ft/s)	Discharge (ft^3/s)
0–1	0.5	0.21	0.2	0	0	0	0
1–3	2	0.74	1.5	0.3	0.4	0.4	0.6
3–5	4	1.42	2.8	1.1	1.6	1.4	3.9
5–7	6	1.70	3.4	1.8	2.0	1.9	6.5
7–9	8	1.93	3.9	1.5	2.5	2.0	7.8
9–11	10	1.94	3.9	1.4	2.5	2.0	7.8
11–13	12	1.79	3.6	2.0	3.0	2.5	9.0
13–15	14	1.54	3.1	1.5	2.2	1.9	5.9
15–17	16	1.19	2.4	0	0	0	0
17–18.5	17.75	0.46	0.7	0	0	0	0
Total			26			1.6	42

conservative design formula (when using the published roughness coefficients). It is not an analysis method and it must be used with care during low flow conditions. During low flows, the roughness coefficient is usually much greater than during high flows, for example, requiring equation calibration at different stream stages.

Current meter flow monitoring requires that the stream be divided into several sections. About 10 sections from 1 to several feet wide are usually adequate, depending on overall stream width. The depth of the stream is measured at each section edge, and the current velocity is measured in a vertical profile in the center of each section. The average velocities in each section are multiplied by the section areas to obtain the discharge rates for each section. These are then summed to obtain the total stream discharge. Table 6.3 is an example calculation for a section on Cahaba Valley Creek, in Shelby County, AL, that is generally used as a field demonstration site for UAB hydrology classes. Figure 6.5 is a cross-sectional diagram of this site, also showing the flow profile distributions. It is interesting to note that the peak water velocity for this stream section is seen to be near the bottom of the stream, close to the middle, but off-set, likely due to the slight meandering of the stream at this location. This is in contrast to the typically assumed velocity profile where the peak velocity is very near the top of the stream (and near the center). Figures 6.6 and 6.7 are photographs of a UAB hydrology class obtaining current measurements at this location.

Stream discharge monitoring is obviously a multiperson job, both from a safety standpoint and in order to take the actual measurements. A safety throw rope should always be ready, and great care should be exercised when working in a fast-moving or deep stream. If a stream has too great a velocity (especially greater than about 2.5 ft/s), or if it is too deep, then current measurements

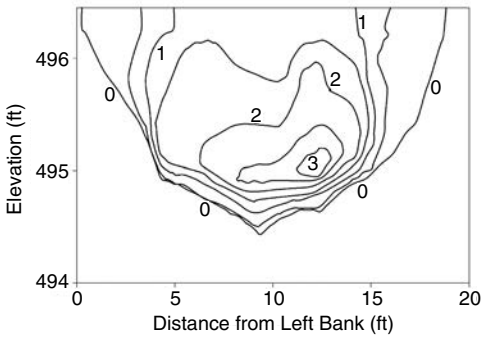


Figure 6.5 Cross section of stream velocities (ft/s) at Cahaba Valley Creek, Shelby County, AL.

Figure 6.6 Obtaining elevation contours at Cahaba Valley Creek, Shelby County, AL.

Figure 6.7 Obtaining current readings across Cahaba Valley Creek, Shelby County, AL.

should be conducted from a bridge, or cable system, and personnel should not be allowed to enter the water. Urban streams are also known for hidden debris and very soft bottoms. As in all work in urban streams, waders are necessary to minimize water contact and to prevent injuries from sharp objects. Riparian plants (such as poison oak and poison ivy) and slippery banks can also present additional hazards near streams. And do not step on any water moccasins.

A suitable current meter is obviously needed for a stream discharge study. Direct-reading digital meters (instead of older audible counter meters, where the operator must count clicks that are related to the water velocity) are now most commonly used. The current meter should be able to measure to 0.1 ft/s, have a threshold velocity of at least 0.2 ft/s, and preferably have an averaging mode in addition to an instantaneous mode. The meter should also be capable of measuring in very shallow water and next to the stream bottom (within a few inches of the stream bottom). The readout should also be selectable between metric and U.S. customary units. The meter must be recalibrated at least every year, preferably in the manufacture's tow tank or in an open channel test facility. Numerous hand-held current meters are available. Forestry Suppliers, Inc. (800-543-4203) has several different mechanical models, as listed below:

Swoffer Model 2100-1514 (#94161)	0.1 to 25 ft/s 1% accuracy	$2300
Handheld Flowmeter (#94303)	0.5 to 25 ft/s ± 0.5 ft/s accuracy	$700
Gurley Model 625 Pigmy (#94993)	0.05 to 3 ft/s audible counter	$1320
Gurley Model 625 Pigmy (#94983)	0.05 to 3 ft/s digital indicator	$2600
Gurley Model D622F Meter (#94982)	0.2 to 32 ft/s digital indicator	$2940

All of these current meters meet the desirable performance criteria, except for the much less expensive flowmeters. Newer portable meters are available that have no moving parts, typically using sonic pulses and Doppler measurements of reflected sound waves from moving particles in the water. These meters are costly (>$3000) and may have a more limited life span than the traditional current-driven meters.

An engineering level, rod, stakes, and tape are also needed to measure the water surface slope between adjacent cross sections when calibrating Manning's equation. Fiberglass tapes are suitable for measuring the stream widths, and rigid (but thin) rules are useful for measuring water depth at the stream sections. When measuring water velocities with a current meter, operators must stand to the side and behind the meter and ensure that no turbulence from their legs (or from others) affects the measurements.

Flow Monitoring Using Tracers

The most precise method of stream current measurements is through the use of tracers. This method is especially important when measuring flows in areas having karst conditions where surface waters frequently lose and/or gain substantial flows to and from underground flows. A single upstream dye injection location and multiple downstream sampling stations through the study area are used in this situation. Tracers are also needed if there is an obviously large fraction

of inter-bed flow or if the stream flow is very turbulent. The flow in very shallow streams, especially when the stream is cobble lined, is also very difficult to monitor with current meters, requiring the use of tracers. Another common use of tracers is when measuring the transport and diffusion of a discharge into a receiving water. Hydraulic detention times in small ponds and lakes can also be determined using tracers. Orand and Colon (1993) state that the use of tracers for water discharge measurements is not a new concept. They admit that the use of current meters is usually much simpler and therefore more common. However, current meters are not applicable in many situations, as noted above. As an example, they routinely use dye tracers and a field fluorometer with continuously recording output to measure the discharge of very turbulent mountain streams, which would not be possible with current meters.

Unfortunately, tracers are rarely useful for continuously monitoring flows, but they can be used for instantaneous flow determinations or for calibrating conventional continuous flow monitoring equipment in actual installations.

Brassington (1990) lists the desired traits for a tracer:

- An ideal tracer should be detectable in very small concentrations.
- It should not be naturally occurring.
- If an artificial tracer is being used, it should exhibit conservative behavior.
- It should be safe to use and produce no harmful environmental effects.
- It should be relatively inexpensive and readily available.

Three main classifications of tracers are generally used. Dyes give a specific and distinctive color to the water that can be detected easily. Chemicals, especially naturally occurring salts, can be used effectively if a discharge into a receiving water has a unique water chemistry and the tracer study objective is to determine the behavior of the discharge. Mechanical tracers can also be used to tag the water, much like the drift method described previously.

The most common mechanical tracer is a spore of *Lycopodium*, a club moss (Brassington 1990). The spores can be dyed and used to measure the surface and groundwater interactions in complex systems. Another approach in monitoring complex surface–groundwater interaction is to use bacteriophages to trace groundwater movement, including the role of septic tank discharges on local receiving waters. Paul et al. (1995) injected prepared bacteriophage cultures (ϕHSIC-1 and Salmonella phage PRD1) as viral tracers, along with 1-μm fluorescent spheres and fluorescein dye, into septic tanks and injection wells and identified their presence in local surface waters in Key Largo, FL. They found relatively rapid movement of the viral tracers (from 0.5 to 25 m/h) in the subsurface limestone environment into the surrounding marine waters. These rates were more than 500 times faster than had been previously measured. They concluded that the subsurface flows may not have reflected uniform diffusion through a homogeneous matrix, but were rather "channeling" through the limestone. Another possibility they suggested was that viruses travel like colloids through the subsurface, moving faster than the bulk water flow. They concluded that the bacteriophages were much more efficient than the fluorescent tracers due to their much better detection limits.

The most efficient tracer is a naturally occurring one. Johnson (1984) concluded that using naturally occurring materials (such as salinity, turbidity, temperature, or other suspended or dissolved materials) allows much more data to be collected and is usually relatively inexpensive (compared to using artificial tracers). In order to use a natural tracer, the material must be:

- Conservative
- Highly soluble under a variety of conditions
- Not amenable to sorption or precipitation or degradation
- Linear with mixing
- Present in greatly contrasting concentrations in the two water bodies that are mixing

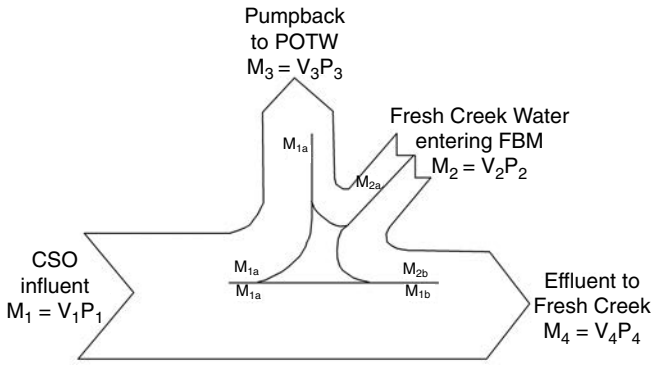


Figure 6.8 Schematic depicting mass balance at Fresh Creek, NY. (From Field, R. R. Pitt, D. Jaeger, and M. Brown. Combined sewer overflow control through in-receiving water storage: an efficiency evaluation. *Water Resources Bulletin, Journal of the American Water Resources Association,* Vol. 30, No. 5. pp. 921–928. October 1994. With permission.)

The tracer must also be easily and cheaply analyzed. In many cases, specific conductivity can be used. Specific conductivity is especially useful when examining freshwater inflows into saline receiving waters. Field et al. (1994) described the use of specific conductivity to measure the effectiveness of a combined sewer overflow (CSO) capture and control device in Brooklyn, NY. In this example, the CSO (which had a specific conductivity of about 1000 µS/cm and a standard deviation of about 250 µS/cm) was contrasted with Fresh Creek water (which had a specific conductivity of about 20,600 µS/cm and a standard deviation of about 2600 µS/cm). Standard conductivity meters were used to trace the CSO water as it displaced the Fresh Creek water in the treatment facility during rains, and to measure the leakage of Fresh Creek water into the treatment facility between rains, as shown in Figure 6.8. The mass (M) of the tracer is equal to the water volume (V) times the concentration (P). It does not matter that there is no adequate conversion for specific conductivity to be expressed as a mass, as specific conductivity concentrations were shown to be linearly related to dilution with the receiving water. A Monte Carlo mixing model was used to calculate the unknowns in this diagram, considering the variabilities of the concentrations in the two water bodies. Stable isotopes have been used successfully as tracers by some researchers with access to sensitive mass spectrophotometers, if the waters being distinguished have a sufficiently different source (Sangal et al. 1996). Ratios of major ions have also been used successfully to identify different waters, especially in groundwater studies.

In most cases, naturally occurring tracers cannot be effectively used because of their nonconservative behavior, insufficient concentration contrasts, or expense. A later section in this chapter discusses the use of natural tracers to identify sources of discharges. Commercially produced fluorescent dyes have been available for many years and have been extensively used for water tracer analyses. Fluorescein (a green fluorescent dye) has been used since the late 1800s, for example, but is not very stable in sunlight. However, it is still commonly used in visual leak detection tests and to visually trace discharge connections (such as determining if floor drains are connected to the sanitary wastewater lines or the storm drain system). Color Figures 6.1 and 6.2* show fluorescein being used to trace sanitary sewage connections to a storm drainage system in Boston.

Rhodamine B was used in the 1950s for water tracing in Chesapeake Bay because it was more stable in sunlight than fluorescein, but it readily adsorbed to sediments, making quantitative measurements difficult (Johnson 1984). Forestry Suppliers, Inc. (800-543-4203) sells liquid and compressed tablets and cakes of Rhodamine B and fluorescein for visual tracer work (but not for use

* Color Plates follow p. 370.

near water intakes). Bottles of 200 tablets of either dye, having a total weight of about 10 oz., or a 3" donut, also weighing 10 oz., of either dye costs about $35.

The most common artificial tracer currently used is Intracid Rhodamine WT dye, a 20% (by weight) stock of dye in water and other solvents having a specific gravity of 1.2. It is available from Crompton and Knowles (Reading, PA, 215-582-8765), at about $400 per 10 L. It is greatly diluted before use in the working stock solution for continuous dye injection studies. Chemical and laboratory suppliers also sell much more dilute mixtures (but at a much greater cost per unit of dye). Forestry Suppliers, Inc., sells a 1-gallon bottle of Rhodamine WT, unspecified dilution, (catalog #92969) for about $100, and bottles of 200 compressed Rhodamine WT tablets (catalog #92991) (weighing 11 oz.) for about $36.

Rhodamine WT was specifically developed in the 1960s for water tracing applications and is much superior for quantitative work compared to the earlier dyes. It is generally easier to detect in much lower concentrations, less toxic, has lower sorption to particles, and exhibits slower decay. Even though it is very expensive by volume, its very low detection limit (about 0.01 ppb of the 20% stock solution) and conservative behavior make it cost-effective.

Rhodamine WT is generally thought to have low toxicity; however, the USGS limits its concentrations at water supply intakes to 10 ppb (Johnson 1984). The biggest toxicity problem associated with Rhodamine WT is apparently associated with reactions with very high concentrations of nitrates. In all cases, it is important to contact local drinking water and state water regulatory agencies when planning a dye tracer study. The largest concern is probably associated with complaints of red water (which should not occur if proper dye concentrations are used).

The Corps of Engineers (Johnson 1984) has published a comprehensive description of the use of water tracing using fluorescent dyes. This report stresses monitoring inflows into reservoirs, with information applicable for a wide range of surface water conditions, including small streams, large rivers, and lakes. Johnson (1984) reports that no significant decay of Rhodamine WT is likely to occur due to chemical or photochemical decay for conditions found in natural waters. However, high chlorine levels (several mg/L, such as are found in many drinking waters) can cause significant decay during long exposure tests (tens of hours). As an example, Johnson reports that chlorine concentrations of 5 mg/L in tests run over 20 hours caused about a 5% decay of fluorescent activity. If operating in urban areas, where the chlorine concentrations may be periodically high or the turbidity variable, it is important to test decay and sorption of the dye. This is best done by using actual receiving water collected at the time of the tracer study as the dilution water when preparing the dye standards. These standards should be compared to standards using proper laboratory dilution water (preferably prepared using ion exchange, and/or reverse osmosis, as laboratory distilled water can contain very high chlorine levels).

Johnson (1984) states that total fluorescent decay of Rhodamine WT is probably about 0.04/day, from both sorption and photochemical decay. Almost all of this loss is likely associated with sorption. The sorption of Rhodamine WT onto particles, according to Orland and Colon (1993), had less than a 7% effect on the measured stream discharges (overestimated) in water having suspended solids concentrations ranging from 200 to 2000 mg/L (particle diameter <200 µm).

Johnson (1984) also reports the effects of pH, temperature, and salinity on the fluorescence of Rhodamine WT. The most serious problem with precise measurements is that the fluorescent intensity decreases with increasing temperature, requiring temperature corrections. This change is a decrease in fluorescence by about 5% for every 2°C increase in temperature. If collecting discrete samples that are brought back to the laboratory for analysis, the samples and the standards can be kept at the same temperature for analysis, eliminating this problem. *In situ* fluorescent measurements require temperature corrections (available as an option in the Turner Designs 10-AU, for example). It is recommended that discrete samples also be periodically collected, along with the continuous field measurements, for temperature-controlled laboratory analysis to confirm the automatic corrections.

The pH of the receiving water affects the sorption of the Rhodamine WT to organic material. Below a pH value of 5.5, the carboxyl acid group of the dye becomes protonated, increasing

adsorption. Johnson (1984) reviewed studies that showed that humic sediment solutions of 2.0 and 20 g/L and 100 ppb Rhodamine WT caused 18 and 89% decreases in fluorescence, respectively. The high humic concentrations lowered the pH values of the water and increased the organic content of the water. In similar solutions using a kaolinite clay, the fluorescent losses were only 11 and 23%. These clay concentrations are very high (2000 and 20,000 mg/L) and would be likely to occur only in construction site runoff in urban areas. The very high associated turbidity of these samples would also greatly complicate fluorescent measurements. The samples would likely have to be clarified (by centrifuge or filtering) before measurement (see also below).

The most commonly used fluorescent measurement instrumentation for fluorescent dye studies has been the older and obsolete Turner model 111 fluorometer that is still available in many laboratories, and the newer Turner Designs (408-749-0994) model 10-AU fluorometer (Figures 6.9 and 6.10). Both of these instruments are filter fluorometers and are very sensitive. The Turner Designs 10-AU is a much superior unit for field measurements, as it is designed to operate on 12-volt batteries, has newer and more stable electronics, a wider dynamic range, and has a water-resistant case. It is also suitable for laboratory measurements. The Turner Designs unit also has a flow-though cell, plus built-in temperature correction and data logging options, which are convenient for field use.

The downstream dye concentrations should be measured over a long period and at many locations across the stream to obtain the best flow estimate. In practice, an automatic water sampler is used to obtain samples, or manual grab samples are obtained, at the downstream location for laboratory analyses, or less commonly, a flow-through fluorometer is used to measure the dye concentrations on a real-time basis. If manual sampling is used, subsamples can be obtained from several locations across the stream for compositing. If a flow-through instrument is used, the intake can be moved to various locations across the stream to investigate mixing conditions. In all cases, the downstream location should be well beyond the predicted fully mixed area. Variations in dye concentrations observed are therefore assumed to be associated with flow variations in the stream.

Background fluorescence in the water must be determined before and during the test. During some tests, we have detected residual background fluorescence. In receiving waters affected by sanitary sewage (such as from raw overflows, inappropriate connections, leaks, septic tank influences, and treated effluent), background fluorescent can be very high due to detergents in the water. Almost all of this interference is eliminated by using specific Rhodamine WT filter sets in the fluorometer. The use of the actual water being tested as the injection water diluent during a continuous test reduces background problems, as do the highly selective optics available for Rhodamine WT analyses. However, background water samples need to be collected for analyses before any dye is added to the water. In addition, it is a good idea to collect upstream water

Figure 6.9 Older Turner model 111 fluorometers used in the laboratory.

Figure 6.10 Current model Turner 10-AU fluorometer being calibrated in the laboratory before field deployment.

periodically during the test to check for changing background conditions (especially important when conducting a tracer test in a sanitary sewer where background water quality can change dramatically over a relatively short period of time). If turbidity levels vary greatly during the test, Johnson (1984) recommends that the samples be filtered or centrifuged prior to analysis. Continuous dye analysis in the field does not allow a correction for turbidity (like the built-in temperature correction option available from Turner Designs), but periodic grab samples analyzed in the laboratory after turbidity reduction enables these effects to be determined.

An example of continuous background corrections was described by Dekker et al. (1998) using Rhodamine WT in Detroit to accurately calibrate flow-metering equipment. They found that abrupt changes in suspended solids in the sewage were very common and that this could radically change the fluorescent response. They therefore collected background (upstream) sewage samples every 15 min during the dye tests and prepared calibration curves with this water, changing the response factors for the measurements accordingly. They also monitored the light absorbance at the Rhodamine WT excitation wavelength (550 nm) simultaneously with the dye concentrations to screen out periods of abrupt changes in suspended solids that would affect the calibration curves.

The careful calibration of fluorometers is critical because of their great sensitivity. Calibration solutions from about 0.1 to 500 ppb should be used (these concentrations are in relation to the 20% stock solution). Two sets of calibration solutions need to be prepared. The initial laboratory series is prepared using laboratory-grade clean water, and another set must be prepared using the receiving water. As noted previously, if using distilled water, ensure that the chlorine concentrations are very low. Never use tap water. Deionized water (at 18 meg-ohm resistance) is probably the best. Preparing such low concentration standards requires a great deal of care, especially when withdrawing the stock and making the initial dilution. Needless to say, the largest hazard associated with working with Rhodamine WT is the mess that it can make if splashed or spilled. The stock solution is stratified in the shipping container, requiring stirring, but trying to stir or shake the stock container is a challenge, as it is heavy and minor spills or leaks are a great nuisance.

It is recommended that the amount of dye needed for the test be withdrawn from the stock shipping container, including the minor amount needed for preparing the standards. This will be only a very small amount, usually only a few hundred mLs for a slug dose test, or a few liters if conducting a continuous injection experiment in an urban stream. This aliquot doesn't have to be perfectly representative of the stock solution. The goal is to withdraw the amount needed without spilling any, with minimal mixing. The initial dilution is usually made using 10 mL of the stock diluted in a liter of dilution water, using a volumetric flask. The 10 mL of stock is very dark and viscous, making it almost impossible to measure with a standard pipette. Many people weigh the initial amount, correcting for the 1.2 specific gravity, but unless the aliquot was from a well-mixed stock container, the specific gravity can be quite different. An automatic pipette (capable of handling viscous fluids) is probably better, as volume dilutions are being measured during the test. Serial dilutions are then usually made, making weaker and weaker standards. The strong concentrations foam if violently mixed, making it difficult to fill the volumetric flasks accurately to the calibration marks.

Analytical chemists do not approve of serial dilutions, as errors are easily compounded, but the nature of Rhodamine WT and the great dilutions needed would otherwise require measuring very small quantities of stock. Using a 1-µL pipette and a 1-L volumetric flask would only produce a 1 ppm (1000 ppb) solution, by volume. At least a second (serial) dilution would still have to be made to obtain a 1 ppb concentration, and a third dilution to obtain a fraction of a ppb standard. Inaccuracies associated with serial dilutions are probably less of a problem than trying to pipette such small amounts.

Fluorescent analyses can be conducted in the field or in the laboratory. *In situ* (flow-through) dye analyses (for which the Turner Designs 10-AU is specifically designed) can be much more efficient than collecting water samples and bringing them back to the laboratory for analyses. However, a combination approach is usually best, where periodic samples are collected and brought to the laboratory for temperature controlled analyses for comparison to the *in situ* values. The *in*

situ analyses allow immediate evaluation of the sampling program, especially when the dye is being used at proper concentrations, making it nearly invisible to the eye, or if complex hydraulics (such as in an estuary with strong currents) prohibit easy prediction of the flow path. However, using a fluorometer in flow-through mode presents special problems. Johnson (1984) stresses the need to ensure that all water connections are air tight to prevent bubbles from entering the flow path. In addition, the pump should be located above the light cell to decrease bubbles from leaky pump seals. The intake of the water delivery system should also be screened to decrease the chance of sand and other debris from scratching the instrument optics.

The two main types of dye injection include instantaneous or continuous releases. Instantaneous dye releases are much more efficient in the use of dye. The amount of dye quickly added to the water usually results in a visible dye cloud that is easy to follow manually. In addition, no special dye injection equipment is required, as the dye is simply poured quickly into the water body. However, continuous releases of dye, especially in conjunction with *in situ* analyses, is necessary when simply tracking the dye is challenging. Continuous dye releases require substantially more dye and usually more field personnel, but changing conditions can be easily measured (Color Figures 6.3 and 6.4).

Thomann and Mueller (1987) present a USGS method used to estimate the amount of Rhodamine WT dye needed for an instantaneous release experiment. The amount is usually much less than needed for a continuous release experiment. They also present several methods to evaluate the observations and obtain estimates of flow, diffusion coefficients, and recovery of dye.

Continuous release rates of dye are dependent on the desired downstream concentration of dye, the concentration of the dye being released, the injection rate, and the estimated stream discharge. Figure 6.11 shows a basic mass balance for a discharge into a river or stream. This can be easily applied to a dye injection experiment, with the dye being considered as the effluent being discharged into the receiving water.

The mass balance for this situation is:

$$\text{upstream mass} + \text{effluent mass} = \text{downstream mass, or}$$
$$Q_u s_u + Q_e s_e = Qs$$

where Q_u = upstream flow rate
s_u = upstream concentration
Q_e = effluent discharge (or dye injection) rate
s_e = effluent (or dye injection solution) concentration
Q = resulting downstream discharge rate (equal to $Q_u + Q_e$)
s = resulting downstream concentration

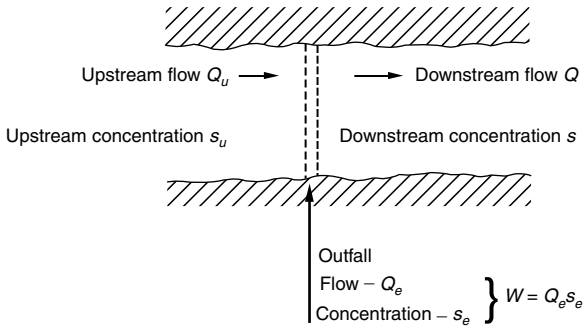


Figure 6.11 Notation for mass balance calculations for dye injection current measurements. (From Thomann, R.V. and J.A. Mueller. *Principles of Surface Water Quality Modeling and Control.* Harper & Row. New York. 1987. With permission.)

Solving for Q, the downstream discharge rate:

$$Q = (Q_u s_u + Q_e s_e)/s$$

If the background concentration (s_u) is zero (as desired in a tracer experiment), this further reduces to:

$$Q = Q_e(s_e/s)$$

where (s_e/s) is the dilution ratio of the dye

Therefore, the stream discharge (Q) is the ratio of the concentration of the dye injection solution (s_e) to the measured downstream dye concentration (s), multiplied by the dye injection rate. As an example, assume the following conditions:

Q_e = 10 cm^3/s
s_e = 1.0 (injection dye solution concentration, a given arbitrary concentration of 1.0)
s = 12 ppm_{vol} compared to injection concentration (average dye concentration from numerous samples collected).

The average value for s was determined to be 12 ppm (relative to the injection dye concentrations); therefore, the calculated stream discharge rate is:

$$Q = Q_e(s_e/s) = 10\ cm^3/s\ (1.0/12 \times 10^{-6}) = 830{,}000\ cm^3/s$$

This is equal to 830 L/s, or about 29 ft^3/s (cfs). As noted in this example, the absolute concentration of the injection solution does not need to be known, as long as calibration solutions are made using the injection solution and the receiving water.

The injection solution needs to be discharged at a constant rate. This is made much easier by using a special metering pump (as supplied by Turner Designs, for example, or a battery-operated peristaltic pump available from Cole-Parmer). In all cases, someone must be at the injection site for the duration of the experiment to ensure that the discharged dye is well mixed and that constant pumping of the injection solution is occurring. This is achieved by periodically measuring the time needed to fill an appropriate graduated cylinder (retain some of the solution from the filled cylinder for use in later calibration solutions, and dump the remainder of the material from the cylinder into the stream when finished timing). The injection solution samples should be analyzed to detect variations in injection dye concentration during the study period.

Fortunately, as is evident from the above equation, everything is relative to the injection concentration, or the mass of dye used, with tracer work. The stock solution concentrate is never directly used in dye studies because the intense color would make the injection plume visible for a large downstream distance; also, the high 1.2 specific gravity affects the plume buoyancy, and precisely pumping very small dye injection rates is difficult. The stock is therefore greatly diluted (by about 10 to 100 times) to create an injection solution to minimize these problems. When conducting a continuous injection experiment, one measures the ratio in concentrations between the injection dye stream and the resulting receiving water concentration. This initial dilution causes a loss of sensitivity, so more dye is required in a continuous injection experiment. In small urban streams, this loss of efficiency is not too serious. When conducting a large-scale injection experiment, specific gravity adjustments are usually made and close to full-strength dye is injected to minimize costs. In a slug discharge test, much less dye is usually needed, and the full amount of tracer dye is introduced into the water as rapidly as possible (within a few seconds). During

instantaneous tests, the strength of the dye solution is not important. It is only necessary to know the mass of the dye used. Therefore, the small amount of dye needed can be effectively diluted in a several-gallon container that can be rapidly poured into the stream to initiate the test.

Experimental conditions needed for various estimated stream discharges can be predetermined by knowing the injection pump rates available and the sensitivity of the fluorometer. A Cole-Parmer Masterflex peristaltic pump can supply a wide range of dye injection rates, depending on the pump rotational speed and the size of tubing. With #13 tubing, the pump can be set to deliver between 0.2 and 0.5 mL/s. Number 16 tubing has a useful range of between 2.0 and 8.0 mL/s, while #18 tubing can be used between 10 and 40 mL/s. A Turner model 111 fluorometer has a range of sensitivity from less than 1 to more than 150 ppb Rhodamine WT, depending on the sensitivity setting. The newer Turner Designs model 10-AU has a much wider dynamic range. The combination of these settings allows measurement of a wide range of flow rates. Table 6.4 illustrates some of the flow rates that can be measured using some of these combinations. The downstream concentrations shown on this table are in relation to the injection concentration, which should be diluted by at least 10 times compared to the 20% stock solution. Therefore, the downstream concentration of 10 ppb shown may actually be closer to 1 ppb of the 20% stock. Intermediate downstream concentrations should be targeted to ensure that variations in stream flow can be accommodated. If a needed injection rate is too low, it may be unstable. The concentration of the dye being injected should then be decreased so a higher pumping rate can be used.

As an example, consider a stream having an estimated discharge rate of 25 cfs and the target downstream concentration is 25 ppb (compared to the injection dye strength which is diluted 10 times from the 20% stock solution; the actual downstream dye concentration is therefore about 2.5 ppb, which would be about mid-scale on the most sensitive setting for a Turner model 111 fluorometer). An injection rate of about 20 mL/s will therefore be required. Therefore, 2 mL of 20% stock will be used per second, or 120 mL of stock per minute of the test, or 7.2 L of stock per hour of the test — a large amount of dye. The injection duration depends on the duration of the steady flow period to be monitored. This should be long in comparison to the flow duration from the injection location to the monitoring location to minimize sampling problems. The sampling location must be located far enough downstream to ensure complete mixing. This length (in feet) can be estimated using the equation presented by Thomann and Mueller (1987):

$$L_m = (2.6\ UB^2)/H$$

where U = the stream velocity in ft/s
B = the average stream width in feet
H = average stream depth in feet

As an example, the discharge rate is estimated to be 25 cfs, the stream velocity is estimated to be about 1 ft/s, the stream width about 25 ft, and the depth about 1 ft. The “complete mixing” length is therefore about 1600 ft. About half of this distance would be needed if the dye injection

Table 6.4 Stream Discharge Rates (cfs) That Can Be Measured for Different Experimental Conditions

Injection Rate (mL/s)	Downstream Conc. = 50 ppb	Downstream Conc. = 25 ppb	Downstream Conc. = 10 ppb	Downstream Conc. = 1 ppb
0.3	0.21	0.42	1.1	11
0.5	0.35	0.71	1.8	18
2	1.4	2.8	7.1	71
10	7.1	14	35	350
20	14	28	71	710
30	21	42	110	1100

point is located at the centerline of the stream. The travel time needed (if injected at midstream) is about 13 min, at least. Therefore, an hour-long injection period would not be unusually long, requiring about 7 L of 20% Rhodamine WT dye, for this example.

The Use of a Multiparameter Probe to Indicate the Presence, Duration, Severity, and Frequency of Wet-Weather Flows

Most receiving water problems are highly dependent on the duration, severity, and frequency of wet-weather events. Habitat effects, for example, are greatly dependent on the frequency of erosive flows that cause bank instability. Sediment scour and deposition is also dependent on the flow energy. Bacteria, turbidity, and other water quality standard violations are much more serious if they occur commonly. Toxicity effects on receiving water organisms are also greatly dependent on the frequency and duration of exposure to excessive concentrations. Knowing when an event occurred, plus knowing the duration and severity of the event, is critical when conducting a long-term exposure experiment using many of the techniques described in this book. Therefore, knowing these basic wet weather event parameters is very important and enables a more complete evaluation of wet-weather problems in receiving waters. The following discussion presents a simple way to automatically monitor these important hydraulic characteristics in a stream without installing a permanent flow monitoring station.

Continuous sondes for water quality monitoring have been available for some time, but current models are vastly improved compared to earlier ones. It is now possible to deploy a water quality sonde for up to several weeks, with little drift and other degradation in performance. This allows the units to be left unattended for extended periods to obtain diurnal variations of constituents (such as DO, temperature, conductivity, turbidity, and water depth) for varying environmental conditions. One application is to examine the duration of degraded receiving water quality conditions following rains.

The following example is based on work by Easton et al. (1998) as part of an investigation studying the effects of SSOs (sanitary sewer overflows) on small urban streams. This study used YSI 6000 UPG water quality sondes to indicate the duration, frequency, and magnitude of wet-weather events in both surface waters and surficial sediments. Short-term, or runoff-induced, pollution effects can be studied in detail using these instruments. Long deployment time and the continuous monitoring capability of the YSI 6000 enables acquisition of data for multiple events, i.e., as many as occur during the time of deployment. The YSI 6000UPG sonde is a multiparameter water quality monitor manufactured by YSI Incorporated, Yellow Springs, OH. The 6000 UPG is capable of performing a subset of the following measurement parameters: dissolved oxygen, conductivity, specific conductance, salinity, total dissolved solids, resistivity, temperature, pH, ORP (oxidation reduction potential), depth, ammonium/ammonia, nitrate, and turbidity. The 6000 UPG can be left unattended in the field for approximately 45 days, depending on the frequency of data logging and parameters being recorded. The instrument is constructed of PVC and stainless steel and is 3.5 in in diameter and 19.5 in in length. It weighs approximately 6.5 lb, with batteries. The sonde is capable of interfacing with an IBM PC-compatible computer for downloading data, or a hand-held unit can be used for direct observations. In addition, a software package, Ecowatch for Windows, is available for sonde setup, data acquisition, and data presentation/analysis. The sondes used in these experiments were configured to acquire the following parameters: dissolved oxygen, conductivity, specific conductance, temperature, pH, ORP, turbidity, and depth.

Five-Mile Creek (which is actually about 50 miles long) is a typical medium-sized Alabama stream, originating in a rural area, then flowing through a suburban, and then a heavily urbanized area. The flow in the creek ranged from approximately 2 to 10 m^3/s, depending on recent rainfall conditions. At each test site, one sonde was located on the creek bottom and the second was buried under approximately 6 in of sediment. The buried sondes were protected by placing them inside 75-mm-aperture nylon mesh bags and were used to measure interstitial water characteristics *in situ* and continuously. The sondes were anchored to the bottom by a chain attached to cinder

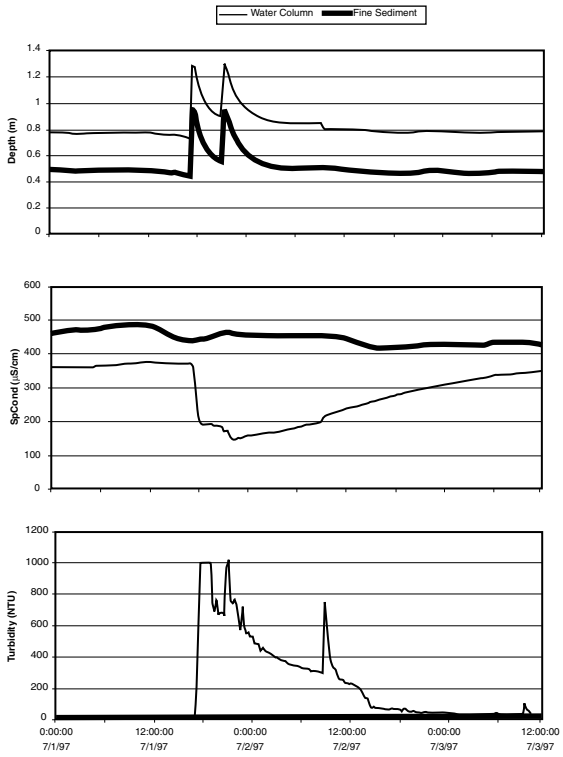


Figure 6.12 Event plots of depth, specific conductance, and turbidity at fine sediment site. (Note: the maximum range of the turbidity probe is 1000 NTU). (From Easton, J.H. et al. The use of a multiparameter water quality monitoring instrument to continuously monitor and evaluate runoff events. Presented at *Annual Water Resources Conference of the AWRA,* Point Clear, AL. 1998. With permission.)

blocks. The cinder blocks were then attached to a tree to prevent the sondes from being washed downstream during major events. One set of sondes was located in an area having coarse sediments (stones of about 1 in in diameter), while the other set was located in an area having finer sediments (sandy grained).

The duration, frequency, and magnitude of runoff events is apparent from an examination of plots constructed from the sonde data (Figures 6.12 and 6.13). These sonde data show a large fluctuation in depth, specific conductance, and turbidity in the water column at both sites on July 1 at 5:00 pm, roughly corresponding to the 0.6 in of rain observed at the Birmingham International Airport several miles away. No site-specific rain information was available, as may be typical for many small-scale studies.

The rise period for all of the parameters was very rapid, and the peaks occurred very early in the runoff event. They then returned to previous levels within 1 to 2 days, depending upon the parameter. The data acquired for water depth are obviously the parameters that best correlate to tracking runoff hydrographs as they pass. There is an obvious change in flood stage (approximately 0.5 m increase in depth), as indicated on these figures. There were two slightly separated, but very similar, runoff hydrographs that passed through the creek; the depth data show two obvious peaks spaced about 3 hours apart. The other two parameters do not distinguish between these two separate, but close events, as is evident in the time taken to return to baseline (Tables 6.5 and 6.6). The turbidity and specific conductance data also substantiate the presence of a runoff event, but with an additional perspective on the duration of the potential effects from elevated turbidity levels and possibly other pollutants. Notice the almost immediate increase in depth and turbidity, and corresponding decrease in specific conductance. These changes are easily explained by a sudden increase in runoff water within the creek. Furthermore, the depth sensors indicate the timing and severity of the runoff event from a hydrologic perspective, while the specific conductance and turbidity sensors indicate the extended duration of probable adverse water quality conditions due to contaminated baseflows entering the stream.

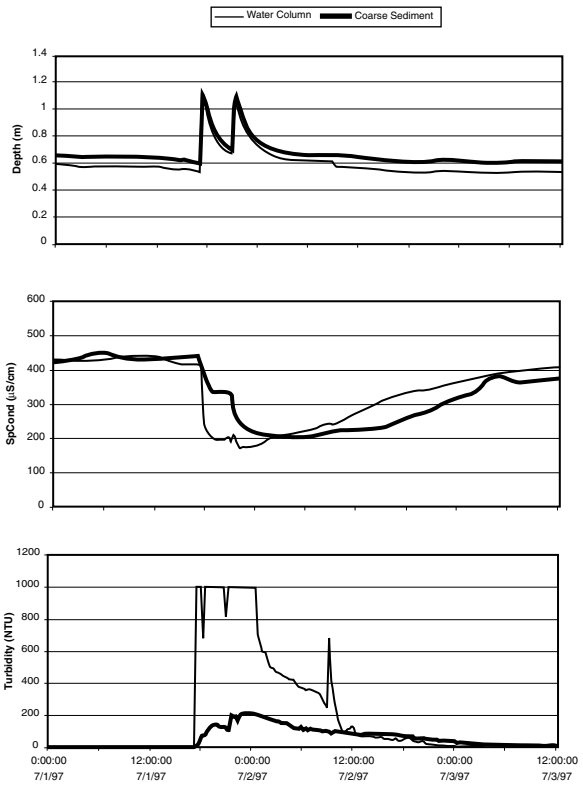


Figure 6.13 Event plots of depth, specific conductance, and turbidity at coarse sediment site. (From Easton, J.H., et al., The use of a multiparameter water quality monitoring instrument to continuously monitor and evaluate runoff events. Presented at *Annual Water Resources Conference of the AWRA,* Point Clear, AL. 1998. With permission.)

The data in Tables 6.5 and 6.6 show the differences in water exchange between the water column and the interstitial water occurring in the two different sediment types (coarse and fine). These experiments show that the interstitial water at the coarse sediment site changes with the water column, although at a slightly reduced magnitude, while the interstitial water at the fine sediment site shows no change. Most urban streams have sediments represented by the fine sediment site (sand sized) or finer. Therefore, very little direct water exchange occurs between the water column and the interstitial water. The interstitial water quality is much more affected by the quality of the deposited sediments (especially decomposable material and toxicants) than by the water column quality directly. This rapid fluctuation of interstitial water in coarse-grained sediments has important implications on evaluations of sediment quality. The benthic micro-, meio-, and macrofaunal exposures in these environments will be more dynamic than typically assumed. Interstitial water sampling and sediment sampling were discussed in Chapter 5.

Table 6.5 Values for Magnitude of Change and Time to Return to Baseline for Specific Conductance, Due to Period of High Flow

Sonde Location	Magnitude of Change (µS/cm)	Time to Return to Baseline (hr)
Water column	210	42
Fine sediment	Not obvious	Not obvious
Water column	260	44
Coarse sediment	230	46

From Easton, J.H., Lalor, M., Pitt, R., and Newman, D.E., The use of a multiparameter water quality monitoring instrument to continuously monitor and evaluate runoff events. Presented at *Annual Water Resources Conference of the AWRA,* Point Clear, AL. 1998. With permission.

Table 6.6 Values for Magnitude of Change and Time to Return to Baseline for Turbidity, Due to Period of High Flow

Sonde Location	Magnitude of Change (NTU)	Time to Return to Baseline (hr)
Water column	>1000	30
Fine sediment	0	0
Water column	>1000	30
Coarse sediment	210	30

From Easton, J.H., Lalor, M., Pitt, R., and Newman, D.E., The use of a multi-parameter water quality monitoring instrument to continuously monitor and evaluate runoff events. Presented at *Annual Water Resources Conference of the AWRA,* Point Clear, AL. 1998. With permission.

The duration of the water column effects from the wet-weather events is seen to be much greater than the duration indicated by the high flows alone (30 to 45 hours vs. 12 hours). This has a major impact on evaluating biological effects of the receiving waters. As an example, rains only occur for about 4.5% of all hours in Birmingham. Periods of extended high flows in Five-Mile Creek may occur for about 15% of the time. However, periods of elevated turbidity (and likely other constituents of concern) may occur for about 40% of the time. This extended time has a significant effect on in-stream beneficial uses and risk assessments from wet-weather toxicants and pathogens.

In-Stream and Outfall Flow Monitoring

Monitoring of flows in storm drainage systems is typically done to supplement stormwater sampling activities. In most cases, flow monitoring equipment available from the same vendor that supplied the automatic water samplers is selected. The flow sensors typically measure depth of flow in the sewerage and apply Manning's equation to calculate the flow rate and discharge. Unfortunately, Manning's equation was developed as a design equation and not as an analysis equation. It was not intended for accurate measurements for shallow flows and does not consider debris that accumulates in sewerage. A better approach is to use a control section in the sewerage and calibrate a stage-discharge relationship. The ultimate solution is to use a special prefabricated manhole that contains a flume. Plasti-Fab (503-692-5460) offers many options of manhole and flume sizes and types for a broad range of sites and conditions. A less expensive alternative (and more suitable for temporary installations) is a manhole flume insert. These are available from Plasti-Fab and from Badger Meter (918-836-8411). These are installed in the discharge sewer line from a manhole, causing a backwater in the manhole that provides an accurate stage-discharge relationship that can be measured. Acoustical flowmeters (measuring water surface distances from a reference location above the water using reflected sound) or bubbler flowmeters (measuring the depth of water above the sensor based on hydrostatic pressure) are usually used to measure the water depth. If the storm sewer line is debris and obstruction free, Manning's equation can be used, but a site-specific stage–discharge relationship must be developed and calibrated over a wide range of depths. Flow calibration is most effectively conducted using Rhodamine WT dye as a tracer, as described previously.

It is critical that the flow monitoring sites be selected to provide accurate flow measurements, along with providing safe and easy access. Sites for flow monitoring must meet numerous criteria in order to obtain accurate results. The most critical criteria require the absence of backwater conditions at the monitoring location and a reasonably straight and homogeneous stream character upstream of the monitoring location for a length of at least 10 times the stream width. Since the stream depth measurements will need to be translated into flow values using a depth–discharge curve, the stream banks and stream bottom need to be reasonably stable at the monitoring locations. The best way to provide the stability and constant stage–discharge relationship at a flow monitoring

station is to construct a control section, usually a flume or a weir. If the stream to be monitored is moderate in size and in a natural setting, especially with important in-stream biological resources, constructing a flume or weir is usually not practical.

The electronic components of typical in-stream flowmeters need to be secured near the stream edge, but outside the zone of common flooding. It would be best to secure them within a heavy steel contractor's box permanently mounted onto an oversized concrete slab. A heavy padlock normally provides adequate security. This enclosure can also contain the necessary deep-cycle batteries recommended for power. If an external data logger is needed, it can also be secured within the box. In many instances, a solar panel can be installed to provide a trickle charge to the battery (but the solar panel would be exposed to vandalism, and riparian locations might be heavily shaded). The bubble tube can be easily run inside a steel pipe (2 to 3 in in diameter) buried in the stream bank. The upper end can come through the concrete pad directly into the steel instrument shelter. The lower end must terminate below the lowest expected stream depth, coming up through a moderate-sized concrete pad to protect the pipe and bubbler tube. The bubbler tube end must lie on top of the in-stream concrete pad and needs a heavy, but shallow, wire cage covering. This covering needs to be relatively easy to remove (while submerged) in order to provide intermittent service to the end of the bubbler tube. This installation can be easily upgraded to include an automatic water sampler, with the sampler (and its deep cycle battery) also enclosed in the steel shelter and the sampler tube also running down the pipe. If a water sampler is also to be used, a galvanized steel pipe must not be used because of zinc contamination. A very heavy-duty plastic pipe, sufficiently buried and protected may be suitable, or a much more expensive stainless steel pipe could be used to encase the bubbler and sampler tubes.

Another option for a shelter is to use concrete pipe rings stacked to a sufficient height and a steel plate padlocked to the top. This is a more temporary (and cheaper) alternative that usually works well. The bubbler tube should also be protected, if possible, within a large-diameter heavy plastic pipe. Another alternative is to mount the flowmeter and ancillary components on a road crossing where a stilling well can be run down into the water, usually on the downstream side of a bridge pier. The equipment can be mounted inside a heavy plywood box on top of the stilling well and accessed from the bridge. In this case, the pier may cause water level interferences.

Many flow measurement equipment vendors now offer simultaneous stage and velocity sensors. The velocity sensors directly measure the flow rate of the water, reducing the need for a stage–discharge relationship. The two major types of velocity sensors are the time-of-transit flowmeter and the Doppler flowmeter. Time-of-transit flowmeters use acoustical signals directed diagonally across the water flow path to a receiver. The acoustical signal travel time can be very accurately predicted. Any difference between the predicted and measured travel time is associated with the water motion. Accusonic (508-548-5800) is one vendor of these devices, which have been reliably used in large conduits. A series of three Accusonic sensors is placed in each of three parallel 10 ft × 15 ft CSO outfalls in Brooklyn, NY, as part of the Fresh Creek CSO treatment study (Field et al. 1995). The three sensor and receiver pairs in each outfall are placed in three vertical zones in each outfall, representing three layers of flow that can measure the severe backwater conditions due to daily tides. As an example, the individual sensors can measure tidal flows entering the bottom of the outfall and any floating CSO discharging on top of the saline receiving water.

Rob Washbusch and Dave Owens of the USGS in Madison, WI, recently (1998) tested several different flow monitoring devices simultaneously in a single storm drain pipe for comparison (Figures 6.14 to 6.19). A unique aspect of these tests was the use of continuous dye injection and downstream water sampling that was automatically activated when rainfall started. The samples were then brought to the laboratory for fluorometric determinations and actual flow values. These actual flows were then compared with the flows indicated by the different flow monitoring equipment. The box plots show the observations from 60 events examined over a 6-month period of time. Flow measurement errors of ±25% were not uncommon. They emphasize that these results

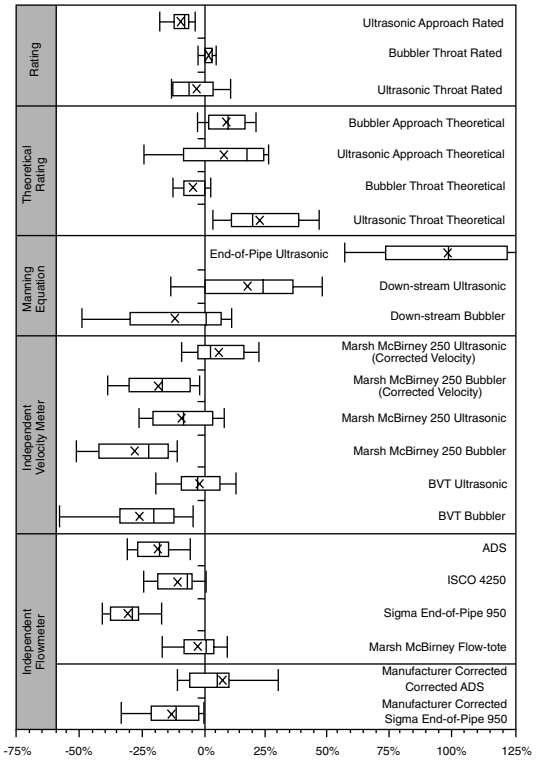


Figure 6.14 Box plots of differences observed when using different flow monitoring methods. (From Waschbusch, R. and D. Owens. *Comparison of Flow Estimation Methodologies in Storm Sewers.* Report prepared by the USGS for the FHWA. Madison, WI. January 1998.)

Figure 6.15 Sigma bubbler flowmeter at USGS test site.

Figure 6.16 ADS acoustic flowmeter at USGS test site.

are for only one site (an industrial area in Madison, WI) and are not likely directly indicative of conditions that might be found elsewhere. They recommend that all runoff flow monitoring equipment be carefully calibrated at the time of installation and periodically rechecked.

Doppler velocity sensors are more commonly used in small storm and sanitary sewer lines. These reflect acoustic signals from particles flowing toward the sensors. The signals reflect off the fastest moving particles, and signal processing then determines the average water velocity. Several vendors sell Doppler units that are constantly improving in accuracy and ease of use. ADS Environmental

Figure 6.17 Acoustic flowmeter at USGS test site.

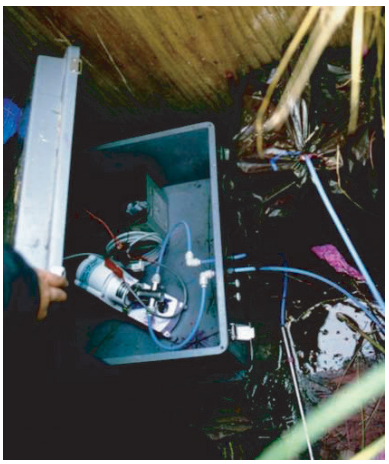


Figure 6.18 Automatic dye injection installation at USGS flowmeter test site.

Figure 6.19 Automatic sampler at outfall at USGS flowmeter test site for collecting real-time dye samples for calibration of flowmeters.

Services, Inc. (800-633-7246) maintains many large-scale flow monitoring networks around the world using its Doppler velocity and ultrasonic level sensors. ISCO (800-228-4373) also sells a Doppler unit that can be used in conjunction with its automatic water samplers. Unidata America (503-697-3570) sells the Starflow ultrasonic/Doppler flowmeter that is very compact and can be used in small open channels and sewer and drainage lines.

Summary of Flow Monitoring Methods

Table 6.7 is a list of some of the advantages and disadvantages of the different flow monitoring/measurement techniques that are most commonly used in urban receiving water studies. The previous discussion presented both manual flow monitoring procedures and methods for flow monitoring that can be used in conjunction with automatic water samplers. In most cases, standard bubble depth sensors supplied by the sampler manufacturer are probably the best choice for an automated station. However, these should be placed in a control section where the stage–discharge curve is specifically known and has been calibrated. Time-of-travel (sonic) current meters can be extremely valuable in situations where stratified flow may occur, but custom interfaces with the sampling equipment may be needed. Basic velocity meters are best used for more casual flow measurements, especially when flow measurements are being taken simultaneously with biological sampling. Dye testing is usually reserved for absolute calibration of flow monitoring setups and to measure in difficult situations, especially during low flow conditions in rocky streams where much of the flow may be actually occurring within gravel deposits, and in streams in karst areas where the interactions between surface and subsurface flows can be dramatic.

Table 6.7 Comparisons of Available Flow Measurement Instruments

Flow Monitoring Instrument Type	Advantages	Disadvantages
Manual Instruments	Simple and rapid results	Instantaneous results, not long-term
Velocity meters	Direct readout of current velocity	Requires multiple measurements across stream to obtain average condition. Can be dangerous during high flows.
Tracers (fluorescent dye)	Considered the standard flow calibration procedure	May be subject to interferences from changing water quality (solids and temperature) or pipe materials. May be difficult to design and to conduct measurements for large systems. Required fluorometer is expensive.
Tracers (naturally occurring salts)	Used for mixing and dilution studies. Inexpensive if using naturally occurring salts in major flow components.	Requires unique and conservative tracer material in mixing components, such as mixing studies for outfalls in marine environment, or industrial discharges.
Automated Instruments	Long-term placement	More expensive and needed for each monitoring location
Bubble sensor depth indicators	Simple and easy to interface with automatic samplers. Most choice and experience from many vendors.	Only measures depth; requires stage-discharge relationship. Should be used in conjunction with a control section (weir or flume) and be verified with frequent velocity meter studies (not commonly done).
Propeller velocity meters	Direct measurement of current velocity.	Foul easily and only indicate velocity at location of propeller.
Time-of-travel (sonic) velocity meters	Direct measurement of velocity. Can be used to measure velocity of specific layer of the water to indicate shear; especially useful in tidal conditions with stratified water moving in different directions.	Relatively expensive and several may be needed to accurately measure flow in different flow strata.
Acoustic velocity meters	Direct measurement of current velocity. Usually measures the peak velocity, and the average velocity for the relatively large sensing zone is calculated as a fraction of the peak velocity.	Current models with supporting software enable relatively easy interpretation of the monitoring results. However, these units generally suffer from a lack of precision and seem to be more subject to error than traditional flow monitoring units.

Rainfall Monitoring

Rainfall data are very important when monitoring receiving water quality and quantity. As an example, the rainfall history in a watershed is needed before interpretation of biological monitoring data can be used to identify possible sources of degraded conditions. The hydrology texts listed previously all contain excellent summaries of rainfall aspects of importance in runoff studies. An especially important reference on rainfall depth measurements and interpretation is the *National Engineering Handbook Series* (Part 630, Chapter 4, Storm Rainfall Depth) published by the USDA (Soil Conservation Service, SCS, now the Natural Resources Conservation Service, NRCS), commonly referred to as NEH-4. This is available from the Consolidated Forms and Distribution Center, 3222 Hubbard Road, Landover, MD 20785. This handbook is supplied in a three-ring binder and sections are periodically updated.

Figure 6.20 Tipping bucket rain gauge with data logger.

Figure 6.21 Close-up of tipping bucket rain gauge mechanism.

Placement and selection of rain gauges are described in these references, along with calculating and interpreting watershed-wide rainfall. This section briefly summarizes several important aspects of rainfall monitoring not usually discussed in available reference texts, especially selecting the proper rain gauge network density and the need for calibration.

Rain gauges suitable for stormwater monitoring are available from many sources. A new small and self-contained weather station is available from Hazco (800-332-0435) that contains sensors for wind speed, wind direction, temperature, relative humidity, dew point, barometric pressure, and rainfall. It has a built-in data logger for up to 6 months of recording and is even available with a modem for connecting to a cellular telephone for telemetry. The cost is about $8500 (catalog #B-W010010M) with a modem and $6600 (catalog #B-W010010) without a modem. Tipping bucket recording rain gauges and data loggers, standard 8" rain gauges, and wind screens are available without the other sensors from several sources, including Qualimetrics, Inc. (800-824-5873) and Global Water (916-638-3429) (Figures 6.20 and 6.21).

The other extreme in rainfall monitoring is the “Clear View” rain gauge from Cole-Parmer (800-323-4340) that is only about $35 (catalog #H-03319-10). This is a nonrecording rain gauge (having a 4" funnel diameter) requiring manual readings of the rain depth. Many other types of “garden store” accumulative rainfall gauges (Figure 6.22) are also available for as little as $5 each, including simple ones that can be made using 3-L plastic soft drink bottles (requiring the collected rain to be poured out and measured). As noted below, relatively few recording rain gauges (for accurate rainfall intensity measurements and start and end rain times) are needed for most urban catchment

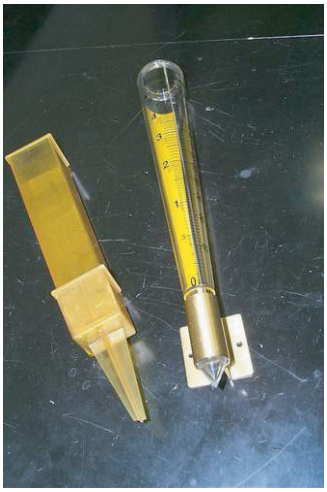


Figure 6.22 Inexpensive “garden/household” rain gauges.

studies. However, numerous nonrecording gauges should be placed throughout the study area to indicate rainfall variations, especially for small rains.

Determining Watershed Averaged Rainfall Depths

Three methods are most commonly used to determine representative watershed-wide rainfall amounts from several point observations. These include the station-average method, the Thiessen polygon method, and the isohyetal method. These methods are briefly described in the following paragraphs.

Station-Average Method

The simplest and easiest method of estimating watershed-wide rainfall amounts is simply to compute the numerical average of all observed values in the watershed. Only those rain gauges physically located in the watershed of interest are usually considered. This method yields good estimates if most of the following conditions are present: the watershed has little topographical relief, a sufficient number of rain gauges are present, the rain gauges are reasonably uniformly distributed throughout the area, and the individual rain depths observed for the different rain gauges do not vary widely from the overall mean. The most important criterion is the need for a large number of rain gauges uniformly distributed throughout the area.

Thiessen Polygon Method

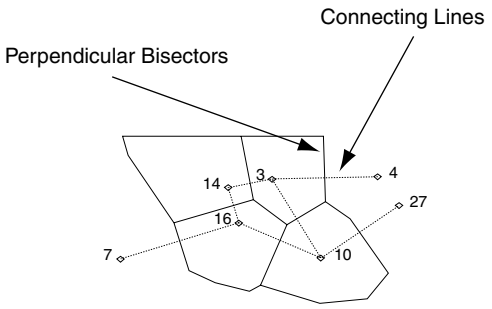


Figure 6.23 Thiessen polygon construction.

The Thiessen method uses a weighted average for the rain gauge network, based on the area assumed to be represented by each rain gauge. Closely spaced rain gauges have smaller weightings than do rain gauges spaced farther apart. The area weightings generally do not consider topography, or other watershed characteristics, although the polygons can be manually adjusted to account for these potential effects, with experience. The area represented by each station is assumed to be the area that is closer to it than to any other station. These areas are determined by drawing connecting lines between all adjacent rain gauges. These connecting lines are then bisected. The perpendicular bisectors then describe a polygon surrounding each rain gauge. Figure 6.23 is a simple illustration of the construction of the polygons surrounding each rain gauge. Figure 6.24 is an example of a Thiessen polygon system for the Toronto, Ontario, metropolitan area which has 35 rain gauges over an area of about 4000 km^2. These polygons were prepared using the SYSTAT computer program.

Results from the Thiessen polygon method are usually assumed to be more accurate than those obtained by the simple station-average method because the Thiessen method accounts for nonuniform distributions of stations. Rain gauge measurements from surrounding areas are also used in the analysis. The polygons also do not change for different rains, unless data are missing from one or more rain gauges. The weightings therefore are relatively constant, making the calculations reasonably simple for multiple rains, after the polygons are initially determined and measured.

Isohyetal Method

This is the most complex method for determining rainfall depths over a watershed and is usually considered the most accurate. It was rarely used before the common availability of computers that

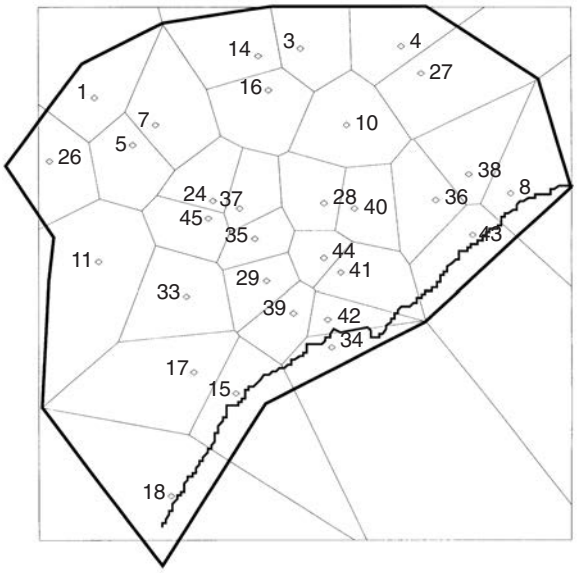


Figure 6.24 Thiessen polygons for Toronto rain gauges.

simplified the necessary calculations. In contrast to the Thiessen polygon method, the isohyetal method requires extensive calculations for each individual rain event. In this method, contours of equal precipitation depth are constructed over the watershed. The construction of the contours can consider the presence of topographic or lake effects. The precipitation averaged over the entire area is computed by multiplying the area enclosed between adjacent isohyetal lines by the average rain depth values of the two adjacent isohyetal lines. Figure 6.25 is an isohyetal map (rain depths in mm) for a single rainfall over the Toronto area, using data from many individual rain gauges. This map was also prepared using SYSTAT.

The Toronto rain gauge network density resulted in small differences between the three averaging methods because of the large number of rain gauges available. The use of the 35 rain gauges was a lot compared to available rain gauge networks in most urban areas. The resulting errors in using the simple averaging method or the Thiessen polygon method, compared to the

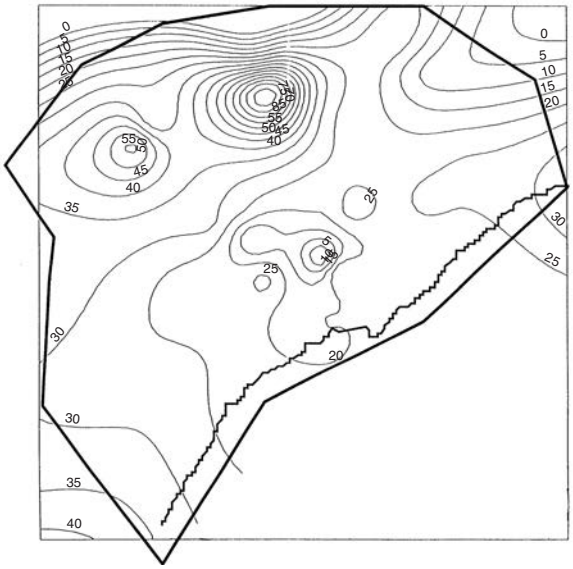


Figure 6.25 Isohyetals prepared for a single Toronto rainfall (mm).

isohyetal method, were all less than 1 mm in rain depth for rains of just a few mm in depth to over 25 mm in depth.

Rain Monitoring Errors

There are several common aspects of rainfall monitoring that can cause measurement errors. Most of these errors result in decreased rainfall values compared to true conditions. These include too few rain gauges for the area, poor placement of the rain gauges, wind effects, splashing of rain out of the gauge during high-intensity rains, tipping rate of tipping bucket rain gauge not keeping up with high-intensity rains, and calibration errors. These problems can usually be identified when reviewing the data. The errors can be corrected during the monitoring period, one hopes; otherwise the rain data might not be usable.

The easiest way to identify questionable rainfall data is to compare the site data with data collected from nearby and independent rain gauge locations. Residual analyses (differences between the site data and surrounding data) may indicate a consistent bias. This may be expected if there is a good reason for the bias (such as topographic differences or nearby large water bodies). The residuals also need to be examined for changes with time. This pattern should also be random, with no obvious trends or abrupt changes. In all cases, a recording rain gauge (especially a tipping bucket rain gauge) must have a standard rain gauge located in close proximity. The total rainfall recorded between observation times of the tipping bucket rain gauge is adjusted based on the standard gauge readings. These adjustment factors should be reasonably consistent. Another way to check rain gauge data is by comparing the watershed rainfall quantity with the stream flow quantity. This relationship should follow a reasonable rainfall–runoff pattern, with no abrupt deviations. Finally, recording rain gauges need to be periodically calibrated against different artificial rain intensities. The measured rainfall causing a tip of the bucket in a tipping bucket rain gauge should remain constant for a wide range of rain intensities. This quantity should also not change abruptly with time.

Needed Rain Gauge Density

One of the most common problems with rainfall monitoring is simply not having enough rain gauges in the watershed. Typical guidance for appropriate rain gauge densities does not consider the likely errors associated with too few gauges located in relatively small urban watersheds. The absolute number of rain gauges is probably more important than the simple rain gauge density. In all cases, multiple rain gauges are needed, even in the smallest study area. The number of rain gauges required depends on local conditions (Curtis 1993). Areas of higher rainfall variability require a greater number of rain gauges to adequately estimate rainfall over a watershed. As an example, mountainous areas will require more gauges than flat lands, and areas subject to convective storms will require more gauges than areas subject to frontal-type storms.

The spatial variability and intended use of the data should be used in determining the needed number of rain gauges. Typical guidance for flat terrain indicates rain gauge spacing of about 25 to 30 km, while this spacing is reduced to 10 to 15 km for mountainous areas. Most monitored urban watershed areas are quite small: almost all are less than 100 km^2, and typically less than 10 ha in area. These small areas seem to justify only a single rain gauge. Wullshleger et al. (1976) made one of the earliest recommendations for the number of rain gauges needed in small urban runoff catchments. They found about one gauge was needed in 0.5- to 1-km^2 watersheds, and about 12 gauges for larger (25-km^2) watersheds. However, multiple rain gauges are needed in all monitored watersheds. This should include a tipping bucket rain gauge and a single standard rain gauge, at least, for the smallest area, if rain intensities are to be monitored. When the study area increases, and if smaller rains are of interest, the number of rain gauges must be increased to compensate for the increased variation in the rain depth throughout the area. These additional rain gauges can be

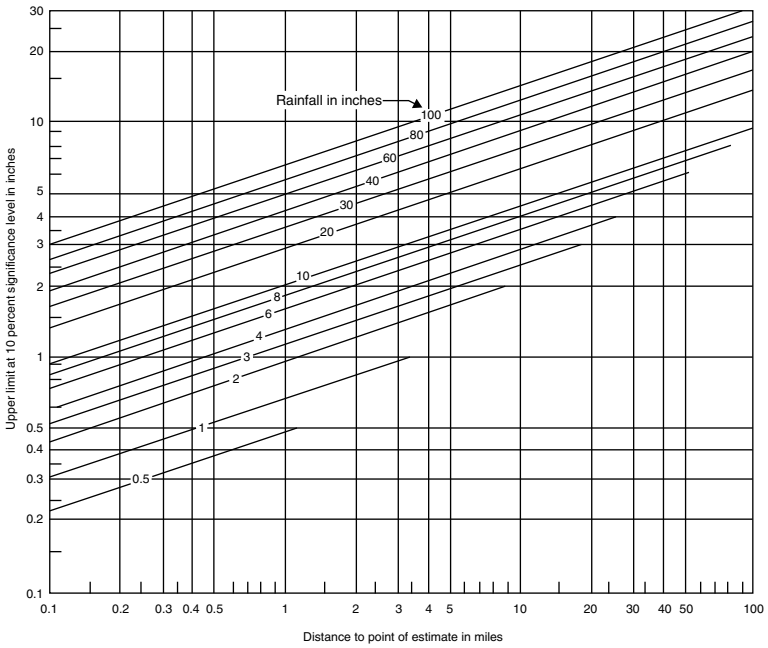


Figure 6.26- Confidence limits based on rain gauge spacing. (From NEH (National Engineering Handbook). Part 630, Chapter 4, *Storm Rainfall Depth* (NEH-4). USDA (Natural Resources Conservation Service), Consolidated Forms and Distribution Center, 3222 Hubbard Road, Landover, MD 20785. Periodically updated.)

additional pairs of tipping bucket and standard rain gauges, or simple accumulative (garden-store type) rain gauges, if intensities are not needed.

The *National Engineering Handbook Series* contains a simple chart, shown here as Figure 6.26, that can be used to estimate the 90% confidence limits of a rainfall located a specific distance from a rain gauge (NEH undated). As an example, if the measured rainfall at a rain gauge is 2 in, the 90% confidence limit in rain depth for a location 0.5 miles away can be estimated as:

- The "plus error" is about 0.8 in, or 2.8 in for the upper limit.
- The "minus error" is assumed to be about one half this amount, or 0.4 in, with a lower limit of 1.6 in.

The NEH also contains a nomograph (Figure 6.27) that can be used to estimate the error in measurement of watershed average rainfall depth, based on the size of the watershed, the number of rain gauges, the annual average precipitation depth, and the storm rainfall depth of concern. The example shown in this figure is for a watershed of 200 acres, having two rain gauges. In the example shown, the annual rainfall is about 33 in, and the rain of interest is 5 in. The average error is estimated to be about ±12%, or ±0.6 in.

Lei and Schilling (1993) studied the rainfall distribution in two urban watersheds located in Essen, Germany. The catchment had an area of 34 km^2 and was represented by 17 rain gauges. Rainfall data for five summers (1980–1984) were analyzed. They only examined rains that had all stations represented and that had at least 0.5 mm of rain. They compared catchment-wide averaged rain depth using subsets of the complete rain gauge network against the data from all 17 rain gauges as a reference. Figure 6.28 shows the basin-wide runoff volume errors that would result if only one rain gauge was used in rainfall–runoff modeling. It shows that relative errors of computed runoff volume decreased with increasing rain depth. Rains greater than about 8 mm had about ±20% errors in modeled runoff volume with a single rain gauge over the 34 km^2

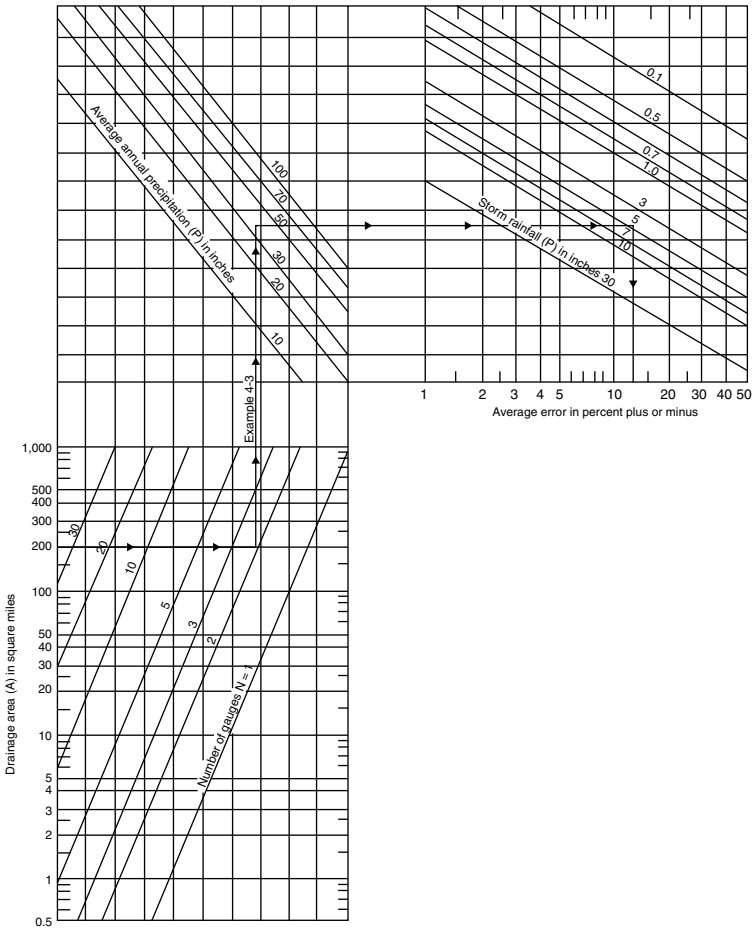


Figure 6.27- Errors in watershed rain depth. (From NEH (National Engineering Handbook). Part 630, Chapter 4, *Storm Rainfall Depth* (NEH-4). USDA (Natural Resources Conservation Service), Consolidated Forms and Distribution Center, 3222 Hubbard Road, Landover, MD 20785. Periodically updated.)

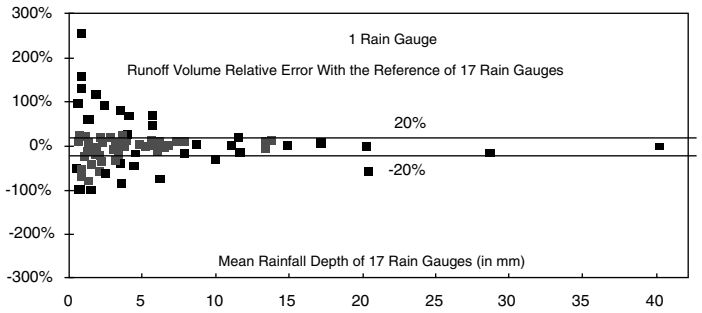


Figure 6.28- Relative runoff volume errors while using one rain gauge in Essen, Germany. (From Lei, J.L. and W. Schilling. Requirements of spatial rain data resolution in urban rainfall runoff simulation, in *Proceedings of the 6th International Conference on Urban Storm Drainage,* Niagara Falls, Ontario, Sept. 12–17, 1993. pp. 447–452. IAHR/IAWQ. Seaport Publishing, Victoria, B.C. 1993. With permission.)

drainage area. However, smaller rains could have rain depth errors of up to 250% with only a single rain gauge.

Ciaponi et al. (1993) studied rainfall variability in the 11.4-ha Cascina Scala experimental urban catchment watershed in Pavia, Italy, for a 3-year period. Two rain gauges separated by 310 m were used in this study. During this period, 233 storm events were selected for analysis, all greater than 1 mm in depth. The following list shows the percentage differences between the rain depths measured at the two monitoring locations for three rain depth categories:

- For 1 mm < h < 5 mm (135 storms), the average error was 31%.
- For 5 mm < h < 20 mm (75 storms), the average error was 10%.
- For h > 20 mm (23 storms), the average error was 8%.

These results show that the rainfall monitoring variations over even a very small watershed and with two closely spaced rain gauges can be quite large for small rain depths (<5 mm), with the differences decreasing for larger rains.

The National Weather Service guideline (Curtis 1993) used to determine the minimum number of gauges required in a local flood warning system is:

$$N = A^{0.33}$$

where A is the basin area in square miles. As an example, a 10-mi^2 watershed would require at least two rain gauges, while a 100-mi^2 watershed would require at least five.

Figure 6.29 shows the expected coefficients of variation for different rain gauge numbers and watershed sizes (Curtis 1993). For a fast-responding watershed, a coefficient of variation (the standard deviation divided by the mean) goal of 0.10 would require about six rain gauges for a 50-mi^2 watershed, while a 500 mi^2 watershed would require about 13 rain gauges for the same COV of observed rain depths in the watershed. Average and slow-responding watersheds would require slightly fewer rain gauges for the same watershed areas.

Rodda (1976) presented recommendations (Tables 6.8 and 6.9) for the minimum number of rain gauges required for small and moderate-sized watersheds and for larger watersheds. Table 6.8 shows the number of rain gauges needed for observations of daily rain depth totals and for monthly rain depth totals.

According to Chow (1964), one rain gauge per 625 mi^2 is the minimum for general climato< logical purposes, while for hydrologic purposes, each study basin should have at least one rain gauge per 100 mi^2. However, one rain gauge per 1 mi^2 was recommended for the analysis of thunderstorms.

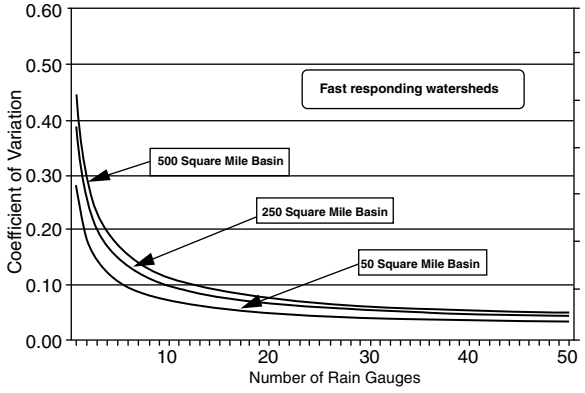


Figure 6.29- Areal rainfall accuracies for fast-responding watersheds.

Table 6.8- Recommended Minimum Numbers of Rain Gauges Needed in Small and Medium-Sized Watersheds

Area (mi^2)	Daily	Monthly	Total
1	1	2	3
2	2	4	6
8	3	7	10
16	4	11	15
31	5	15	20
47	6	19	25
63	8	22	30

Data from Rodda, D.W.C. Water data collection and use. *Water Pollution Control,* Maidstone, England. Vol. 75, No. 1, pp. 115–123. 1976. With permission.

Table 6.9- Recommended Minimum Number of Rain Gauges Needed for Large Watersheds

Area (mi^2)	Number of Rain Gauges
10	1
100	2
500	3
1000	4
2000	5
3000	6

Data from Rodda, D.W.C. Water data collection and use. *Water Pollution Control,* Maidstone, England. Vol. 75, No. 1, pp. 115–123. 1976. With permission.

Pitt and McLean (1986) investigated rainfall distributions in the Toronto area as part of the Humber River pilot watershed study. Rainfall data were available for 35 rain gauges over an area of about 4000 km^2. This high number of gauges allowed sensitivity calculations to be made to determine the appropriate number of rain gauges that may be needed. Numerous random subsets of these rain gauge data were used to analyze potential errors associated with using fewer gauges for 46 different rains greater than 1 mm in depth. Figure 6.30 shows the likely errors for different numbers of rain gauges over this area. The largest rains (>20 mm) had the smallest rainfall variations over the area and therefore had the smallest errors for a specific number of rain gauges. The smallest rains (<5 mm in depth) had much greater errors because their variations were much larger throughout the area. This plot shows that the errors would be very large (several hundred percent in error) for all rains with only one rain gauge for the complete area. The errors somewhat leveled off after about 12 rain gauges were used. However, the rain depth errors for the largest rain category would remain greater than 10% even for 25 rain gauges, and the smallest rains may still have about 50% errors associated with this large number of gauges.

The small catchment monitoring effort by Pitt and McLean (1986) in Toronto illustrated the need to include multiple rain gauges even in very small areas. The two urban watersheds monitored were 39 and 154 ha in area and were located about 3 km apart. Rainfall was monitored at one of the areas only, and the rainfall at the airport several kilometers away was used for comparison. Part way through the monitoring program, a large deviation was noted between the local and airport monitored rain depths. The local rain gauge was then recalibrated, with a 40% increase in the

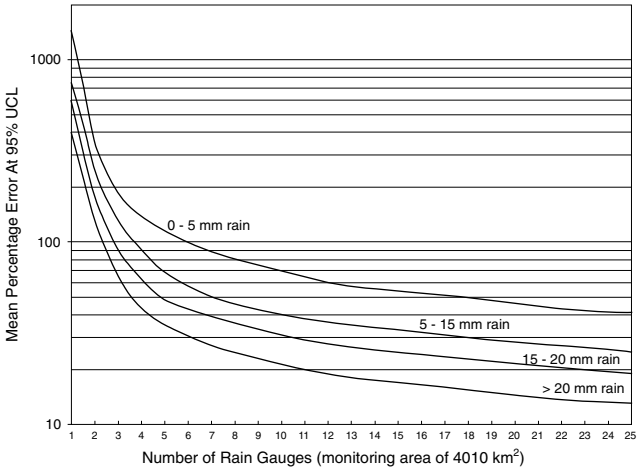


Figure 6.30 Calculated errors for using various rain gauge densities for different sized storms in Toronto.

volume needed for a single bucket tip compared to the initial calibration value. This of course had a significant effect on the rainfall quantity monitored, and much time was spent in identifying why and when the rain gauge had changed so much since its initial calibration. After much analysis using surrounding rainfall data and investigating the history of the specific rain gauge, it was determined that the rain gauge used had a historical problem with its bearings and several repairs had been made in an attempt to correct it. Unfortunately, the gauge calibration was found to be highly variable, and all the locally monitored data were therefore questionable and not used. Thankfully, the Toronto rain gauge network had six other rain gauges surrounding the two study areas within a few km. These data were extensively evaluated, including examining the storm tracks across the city during all monitored rains, to derive suitable rain depth and intensity values for the storms of interest. This analysis required much time, but was possible because of the additional rain gauge data. This problem could have been prevented with the use of a standard rain gauge located next to the tipping bucket rain gauge (as required in professional rain monitoring installa< tions) for more frequent checks on the calibration factor. Nonrecording rain gauges could also have been located in several locations in the small test watersheds to indicate variations throughout the drainage. Both of these options would have cost a fraction of the amount associated with the additional detailed rainfall analysis required during this project and would have alerted the field personnel to the rainfall monitoring problem much sooner.

Proper Placement of Rain Gauges

Precipitation measurements are greatly influenced by wind. Careful placement and shielding of rain gauges are both necessary to reduce wind-induced errors. The upward movement of air over a rain gauge reduces the amount of precipitation captured in a rain gauge. Proper placement is needed to minimize wind-induced turbulence (and to minimize rain shadow effects) from nearby obstructions.

Linsley et al. (1982) concluded that reliable measurements of wind-induced errors are difficult because of problems involved in determining the actual amounts of precipitation reaching the ground. They reported that wind-induced errors during rainfall monitoring exceed about 10% for winds greater than about 8 mph, for both shielded and unshielded rain gauges. This error increases to about 20% during 20 mph winds. Shielded rain gauges perform slightly better, with a wind< induced error about 3% less than for an unshielded rain gauge during 10 mph winds, and about 5% less during 20 mph winds. The effects of winds on snowfall is much greater, with shielded gauges having about half the magnitude of errors as unshielded gauges when monitoring snowfalls. Snowfall errors (all underreported) for unshielded gauges may be about 50% for 10 mph winds and increase to about 70% for 20 mph winds. Various types of wind shields have been used, but the Alter shield (loose-hanging vanes in a circle around the rain gauge) has been adopted as a standard in the United States. Its open and flexible construction provides less opportunity than solid shields for snow buildup, and the flexible design allows wind movement to help keep the shield free of accumulated snow and ice.

Rain gauge exposure and placement are very important to reduce rainfall measurement errors. The higher the rain gauge is located above the ground, the greater the wind error. It is therefore best to locate the rain gauge on level ground, definitely avoiding roof installations and steep hillsides. Linsley et al. (1982) and Shaw (1983) both recommend a partially sheltered site. Brassington (1990) stated that the rain gauge should be located at a distance that is at least twice the height of surrounding obstructions: the vertical angle from the rain gauge to the top of the surrounding trees and buildings should be no greater than 30°. Also, Shaw (1983) recommended that a turf wall be used in overexposed locations where natural shelter is rare. A surrounding small grassed embankment decreases wind turbulence around the rain gauge which can inhibit raindrops from falling into an unprotected gauge. The turf wall should form a circle having an inside diameter of about 3 m, and be built up to the top of the rain gauge. The inside wall should have vertical walls, while the outside should have a slope of about 1 to 4. The inner area must be drained to the outside to prevent flooding.

Rain gauges must also be placed level. If a rain gauge is inclined 10° from the vertical, it will catch 1.5% less than it should due to a decreased open area exposed to the rain. In addition, if a rain gauge is inclined slightly toward the wind, it will catch more rain than the true amount.

Proper Calibration of Rain Gauges

The standard U.S. Weather Bureau rain gauge is a nonrecording, but accumulating rain gauge that has an 8-in-diameter funnel opening. The opening directs the water into a measuring tube that has 1/10 the cross-sectional area of the gauge opening. The depth of accumulated rain in the measuring tube is therefore 10 times the depth of rain that fell since the gauge was last checked. This gauge is usually used to measure the 24-hour total rain depths, usually read at 8:00 am each day. This standard gauge should be located adjacent to any recording rain gauge to check the total amount of rain that has fallen during the observation period.

A tipping bucket rain gauge is the most common type that measures rainfall intensity. This gauge has an internal tipping mechanism that fills with water from the funnel connected to the standard 8-in-diameter opening (see Figure 6.21). The tipping mechanism is balanced to dump its contents after a specific amount of water has accumulated (usually 0.01 in). Upon dumping, another small bucket rises to collect the next increment of rainfall. Each tipping motion is recorded on an event recorder, along with its time. Rainfall intensity is therefore related to the number of tips per time period.

Tipping bucket rain gauges must be periodically calibrated by measuring the number of tips associated with a specific amount of water slowly introduced into the gauge. The calibration water must be introduced at a rate comparable to that of the rainfall of interest. Several rainfall rates should be checked over the range of interest. This calibration should be conducted in the field, with the gauge installed, at least every 6 months. As noted previously, tipping bucket rain gauges are most accurate for small to moderate rain intensities. Significant rain can be missed during the time that the tipping action is moving and before the other bucket is in place. Heavy rains also tend to hold the buckets in intermediate positions for long periods, preventing the rain from accumulating in the buckets. The use of a standard accumulating rain gauge adjacent to any recording rain gauge is therefore highly recommended.

Table 6.10 shows the water delivery rate to a tipping bucket rain gauge needed for calibration for different equivalent rainfall intensities, assuming a standard 8-in opening. The rates needed to calibrate a tipping bucket rain gauge for the smallest rainfall intensities shown on this table are very low and would require special low flow pumps. As an example, a Masterflex® portable pump can pump from 0.06 to 1100 mL/min, depending on pump head, tubing size, and pump speed (available from Forestry Suppliers, catalog #76899, model 7570-10 variable speed pump with rechargeable battery, and #76888 pump head with #16 tubing, for 0.80 to 320 mL/min, at a total cost of about $900). This pump can therefore be used for all the rainfall intensity calibrations listed in Table 6.10. Of course, other available peristaltic pumps can also be used for this calibration.

Table 6.10 Water Delivery Rates for Recording Rain Gauge Calibration (standard 8-in opening)

Rainfall Intensity (mm/hour)	Rainfall Intensity (in/hour)	Water Delivery Rate for Calibration (mL/min)
2	0.078	1.1
5	0.20	2.7
10	0.39	5.4
25	0.98	14
50	2.0	27
100	3.9	54
200	7.9	110

When the rainfall intensity becomes great, the tipping bucket mechanism cannot keep up, resulting in a decreased amount of rain recorded. As an example, Ciaponi et al. (1993) used a peristaltic pump to calibrate two gauges in an urban test watershed in Pavia, Italy. The calibrations showed that the rain gauges could accurately measure rainfall intensities at 44 mm/hour (the lowest rate calibrated with the pump) with errors less than 1%. However, at rain intensities of about 250 mm/hour, the errors were about 10%, and at 400 mm/hour, the errors increased to about 15%. The measured rain intensities were all less than the actual intensities due to missing rain during the tipping time of the individual buckets. Of course, very few rains would be expected to have prolonged large intensities that would cause errors greater than about 10%. However, short-duration, very high rain intensities are much more likely, and accurate rates in these high-intensity ranges may be needed. Therefore, care must be taken when calibrating rain gauges to use appropriate water delivery rates that correspond to a wide range of expected rainfall intensities.

Summary of Rainfall Monitoring Methods

Table 6.11 lists the main advantages and disadvantages of the different basic types of rainfall monitoring methods. In all cases, a tipping bucket rain gauge is needed in an urban study area, with a standard gauge located nearby for proper calibration. In addition, at least several rain gauges (need not be recording, but that would obviously be most helpful) must be placed throughout the study area. For large areas, many gauge installations are needed. In areas of snowfall, special modifications are also required. Proper placement and shielding of the rain gauges are also needed but frequently overlooked. Radar rainfall information can be valuable, but only as a supplement to standard rain gauges in a study area. Proper use of radar rainfall data generally requires an expert and specialized software, and it is only useful relatively close to the radar installation.

SOIL EVALUATIONS

Knowing local soil properties is critical for many aspects of watershed evaluations. Soil prop< erties are extremely important for less-developed areas, because they control many of the hydrologic and sediment aspects of the stormwater. As a watershed becomes developed, however, soil char< acteristics may become less important than other aspects (especially the nature and extent of the paved and roofed surfaces). Nonetheless, it is important to acknowledge that soils become dramat-

Table 6.11 Advantages and Disadvantages of Different Rainfall Monitoring Methods

Rainfall Monitoring Method	Advantages	Disadvantages
Tipping bucket rain gauges	Most commonly used and available gauge. Obtains high resolution rainfall intensity data. Relatively inexpensive for current versions of recording models.	Must be frequently calibrated and located adjacent to a standard rain gauge (not usually done). Usually insufficient numbers of recording gauges in most local networks.
Standard rain gauges	Standard rain gauge and most accurate. Can be heated and used for monitoring snowfall.	Does not obtain rain intensity information. Must be manually read at least once a day.
“Garden store” rain gauges	Inexpensive and can be placed throughout a study area. Best use to supplement standard and tipping bucket rain gauges.	Does not obtain rain intensity information. Must be manually read.
Radar rainfall measurements (such as NEXRAD)	High resolution data over a large area. Real-time measurements.	Most indicative of severe weather conditions. Can be very inaccurate and requires substantial calibration from standard rain gauges. Only suitable for areas relatively close to a radar installation.

Figure 6.31 Double-ring infiltrometer measurements in disturbed urban soils in Oconomowac, WI.

Figure 6.32 Infiltration test apparatus at University of Essen, Germany.

ically altered with typical urban development and to understand how these changes affect local stormwater. The following paragraphs describe the unusual soil conditions found during some studies of urban soils and the methodologies that were used.

Local USDA Soil Conservation Service (SCS) (now NRCS, Natural Resources Conservation Service) offices have a wealth of information pertaining to soils in all areas of the nation. The county soil surveys should be carefully reviewed for important information. However, urbanization typically alters many "natural," or mapped, soil characteristics beyond recognition through removing vegetation and topsoil, large-scale cut-and-fill operations, compaction, and artificial landscaping. Unfortunately, these changes usually all adversely affect the soils' abilities to infiltrate runoff and to retain soil during storms. It may therefore be important to directly measure some of these critical soil characteristics in watersheds undergoing study. This section briefly describes the experimental design and numerous test procedures and some results for a recent EPA-sponsored research project (Pitt et al. 1999a) that investigated adverse soil changes with urbanization (mostly compaction) and possible mitigation methods (amending soil with compost).

Numerous methods have been used to measure infiltration in urban areas. Figure 6.31 is a double-ring infiltration apparatus used to measure infiltration through disturbed urban soils in Oconomowac, WI. Figures 6.32 and 6.33 are photographs of an infiltration test apparatus developed by Dr. Wolfgang Geiger at the University of Essen, Germany, and Figures 6.34 through 6.35 are photographs of the Pac Forest soil infiltration test site developed by Dr. Rob Harrison of the Ecosystem Science and Conservation Division, College of Forest Resources at the University of Washington, Seattle.

Case Study to Measure Infiltration Rates in Disturbed Urban Soils

The soil characteristics of most interest for a receiving water investigation include the soil texture, the soil erosion factors (NRCS K and T factors), and the soil infiltration rates. Because soils in urban areas are greatly disturbed during construction activities, the information contained in the county soil surveys will not be directly applicable, requiring site investigations. Soil infil< tration may be related to the time since the soil was disturbed by construction or grading operations (turf age). In most new developments, compact soils are expected to be dominant, with reduced infiltration compared to preconstruction conditions. In older areas, the soil may have recovered

Figure 6.33 Adjustments being made to rain drop tubes at Essen infiltration test apparatus.

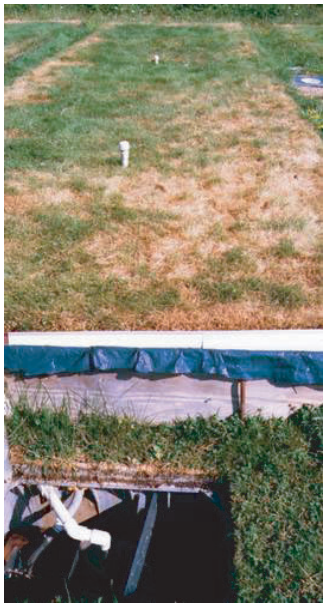


Figure 6.34 Soil infiltration test plot at University of Washington, Seattle.

some of its infiltration capacity due to root structure development and from soil insects and other digging animals.

The following discussion presents a case study that was conducted by Pitt et al. (1999) that investigated infiltration rates in disturbed urban soils. These types of data can be used to more accurately predict watershed hydrology and associated receiving water problems, compared to using published information for natural soil conditions. The results presented in the following example

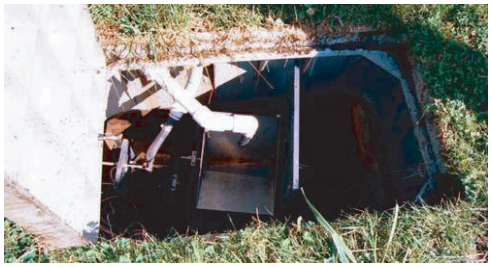


Figure 6.35 Tipping bucket flow measurement device for measuring groundwater flows at UW test plot.

Figure 6.36 Weather station at UW soil infiltration test plot.

show how site measurements can be significantly different from published and traditional data. This case study is presented as an example of how this type of study can be conducted to obtain this critical, site-specific information.

Experimental Design

A series of 153 double-ring infiltrometer tests were conducted in disturbed urban soils in the Birmingham and Mobile, AL, areas. The tests were organized in a complete 2^3 factorial design (Box et al. 1978) to examine the effects of soil moisture, soil texture, and soil compactness on water infiltration through historically disturbed urban soils. Moisture and soil texture conditions were determined by standard laboratory soil analyses. Compaction was measured in the field using a cone penetrometer (Dickey-John Corp. 1987) and confirmed by the site history. Moisture levels were increased using long-duration surface irrigation before most of the saturated soil tests. From 12 to 27 replicate tests were conducted in each of the eight experimental categories in order to measure the variations within each category for comparison to the variation between the categories.

Table 6.12 shows the analytical measurement methods used for measuring the infiltration rates, and supporting measurements, during the tests of infiltration at disturbed urban sites. Table 6.13 defines the different levels for the experimental factors used during these tests.

Infiltration Rate Measurements

The infiltration test procedure included several measurements. Before a test was performed, the compaction of the soil was measured with the DICKEY-john Soil Compaction Tester and a sample was obtained to analyze moisture content. TURF-TEC Infiltrometers (1989) were used to measure the soil infiltration rates. These small devices have an inner ring about 64 mm (2.5 in) in diameter and an outer ring about 110 mm (4.25 in) in diameter. The water depth in the inner compartment starts at 125 mm (5 in) at the beginning of the test, and the device is pushed 50 mm (2 in) into the ground. The rings are secured in a frame with a float in the inner chamber and a pointer next to a stopwatch. These units are smaller than standard double-ring infiltrometers, but their ease of use allowed many tests to be conducted under a wide variety of conditions. The use of three infiltrometers placed close together also enabled better site variability to be determined than if larger, standard-

Table 6.12 QA Objectives for Detection Limits, Precision, and Accuracy for Critical Infiltration Rate Measurements in Disturbed Urban Soils

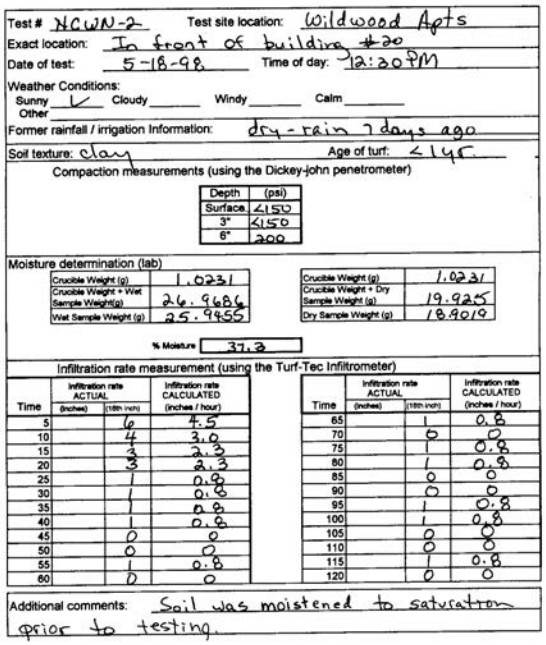
Measurement	Method[a]	Reporting Units	MDL	Precision
Double-ring infiltration rate measurements	ASTM D3385-94	in/hr	0.05	10%
Soil texture	ASTM D 422-63, D 2488-93, and 421	plots	na	10%
Soil moisture (analytical balance)	ASTM D 2974-87	Percentage of moisture in soil (mg)	5% (0.1 mg)	10% (1%)
Soil compaction	History of site activities and cone penetrometer	psi	5	10%
Soil age	Age of development	years	na	na

[a] ASTM 1994 and Dickey-John Corp. 1987.

Table 6.13 Experimental Test Levels during Infiltration Rate Tests

	Moisture	Disturbance[a]	Soil Texture[b]
Enhanced infiltration	Dry (<20% moisture)	Uncompacted (<300 psi)	Sandy (per ASTM D 2487)
Decreased infiltration	Wet (>20% moisture)	Compact (>300 psi)	Clayey (per ASTM D 2487)

[a] Dickey-John Corp. 1987.
[b] ASTM 1994.



Test # NCWN-2 Test site location: Wildwood Apts
Exact location: In front of building #20
Date of test: 5-18-98 Time of day: 12:30 PM
Weather Conditions:
Sunny ✓ Cloudy ____ Windy ____ Calm ____
Other ____
Former rainfall / irrigation Information: dry - rain 7 days ago
Soil texture: clay Age of turf: < 1 yr.

Compaction measurements (using the Dickey-john penetrometer)

Depth	(psi)
Surface	<150
3"	<150
6"	200

Moisture determination (lab)

Crucible Weight (g)	1.0231	Crucible Weight (g)	1.0231
Crucible Weight + Wet Sample Weight (g)	26.9686	Crucible Weight + Dry Sample Weight (g)	19.925
Wet Sample Weight (g)	25.9455	Dry Sample Weight (g)	18.9019

% Moisture 37.3

Infiltration rate measurement (using the Turf-Tec Infiltrometer)

Time	Infiltration rate ACTUAL (inches)	(1/8th inch)	Infiltration rate CALCULATED (inches / hour)
5		6	4.5
10		4	3.0
15		3	2.3
20		3	2.3
25		1	0.8
30		1	0.8
35		1	0.8
40		1	0.8
45		0	0
50		0	0
55		1	0.8
60		0	0
65		1	0.8
70		0	0
75		1	0.8
80		1	0.8
85		0	0
90		0	0
95		1	0.8
100		1	0.8
105		0	0
110		0	0
115		1	0.8
120		0	0

Additional comments: Soil was moistened to saturation prior to testing.

Figure 6.37 Field observation sheet. (From Pitt et al., 1999.)

sized units were used. These small units are available from Forestry Suppliers, Inc., while the standard-sized units are available from Gilson, or other soil engineering equipment suppliers.

Three infiltrometers were inserted into the turf within a meter of each other to indicate the infiltration rate variability of soils in close proximity. Both the inner and outer compartments were filled with clean water by first filling the inner compartment and allowing it to overflow into the outer compartment. As soon as the measuring pointer reached the beginning of the scale, the timer was started. Readings were taken every 5 min for 2 hours. The instantaneous infiltration rates were calculated by noting the decline in the water level in the inner compartment over the 5-min period.

Tests were recorded on a field observation sheet as shown in Figure 6.37. Each document contained information such as relative site information, testing date and time, compaction data, moisture data, and water level drops over time, with the corresponding calculated infiltration rate for the 5-min intervals.

All measurements were taken in soils in the field (leaving the surface sod in place), with no manipulation besides possibly increasing the moisture content before "wet" soil tests are conducted (if needed).

Soil Moisture Measurements

Moisture values relating to dry or wet conditions are highly dependent on soil texture and are mostly determined by the length of antecedent dry period before the test. Soil moisture was determined in the laboratory using the ASTM D 2974-87 (1994) method (basically weighing a soil before and after oven drying). For typical sandy and clayey soil conditions at the candidate test areas, the dry soils had moisture contents ranging from 5 to 20% (averaging 13%) water, while wet soils had moisture contents ranging from 20 to 40% (averaging 27%) water.

The moisture condition at each test site was an important test factor. The weather occurring during the testing enabled most site locations to produce a paired set of dry and wet tests. The dry tests were taken during periods of little rain, which typically extended for as long as 2 weeks with no rain and with sunny, hot days. The saturated tests were conducted after thorough artificial soaking of the ground, or after prolonged rain. The soil moisture was measured in the field using a portable moisture meter (for some tests) and in the laboratory using standard soil moisture methods (for all

tests). The moisture content, as defined by Das (1994), is the ratio of the weight of water to the weight of solids in a given volume of soil. This was obtained using ASTM method D 2974-87 (1994), by weighing the soil sample with its natural moisture content and recording the mass. The sample was then oven-dried and its dry weight recorded.

Soil Texture Measurements

At each site location, a soil sample was obtained for a texture classification. The texture of the samples was determined by ASTM standard sieve analyses (1994) to verify the soil conditions estimated in the field and for comparison to the NRCS soil maps. The sieve analysis used was the ASTM D 422-63 *Standard Test Method for Particle Size Analysis of Soils* for the particles larger than the No. 200 sieve, along with ASTM D 2488-93 *Standard Practice for Description and Identification of Soils (Visual — Manual Procedure).* The sample was prepared based on ASTM 421 *Practice for Dry Preparation of Soil Samples for Particle Size Analysis and Determination of Soil Constants.*

The texture analyses required a representative dry sample of the soil to be tested. After the material was dried and weighed, it was crumbled to allow a precise sieve analysis. The sample was then treated with a dispersing agent (sodium hexametaphosphate) and water at the specified quan< tities. The mixture was then washed over a No. 200 sieve to remove all soil particles smaller than the 0.075 mm openings. The sample was then dried again and a dry weight obtained. At that point, the remaining sample was placed in a sieve stack containing No. 4, No. 8, No. 16, No. 30, No. 50, No. 100, No. 200 sieves, and the pan. The sieves were then placed in a mechanical shaker and allowed to separate onto their respective sieve sizes. The cumulative weight retained on each sieve was then recorded.

The designation for the sand or clay categories follows the *Unified Soil Classification System*, ASTM D 2487. Sandy soils required that more than half of the material be larger than the No. 200 sieve, and more than half of that fraction be smaller than the No. 4 sieve. Similarly, for clayey soils, more than half of the material is required to be smaller than the No. 200 sieve. Figure 6.38 is the standard soil texture triangle defining the different soil texture categories.

Soil Compaction Measurements

The extent of compaction at each site was also measured before testing using a cone penetro< meter. The compaction of the test areas was obtained by pushing a DlCKEY-john Soil Compaction Tester (available from Forestry Suppliers, Inc.) into the ground and recording the readings from

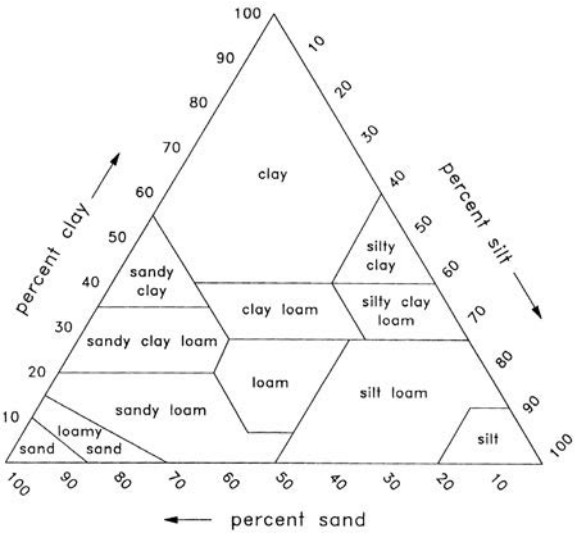


Figure 6.38 Standard soil triangle.

the gauge. For these tests, compact soils were defined as a reading of greater than 300 psi at a depth of 3 in, while uncompacted soils had readings of less than 300 psi.

Compaction was confirmed based on historical use of the test site location, as moisture levels affected the cone penetrometer readings. Soils, especially clay soils, are obviously more spongy and soft when wet compared to when they are extremely dry. Therefore, the penetrometer measurements were not made for saturated conditions, and the degree of soil compaction was also determined based on the history of the specific site (especially the presence of parked vehicles, unpaved lanes, well-used walkways, etc.). Other factors that were beyond the control of the experiments, but also affect infiltration rates, include bioturbation by ants, gophers and other small burrowing animals, worms, and plant roots.

Bulk Density

Bulk density was estimated using a coring device of known volume (bulk density soil sampler). The core was removed, oven dried, and weighed. Bulk density was calculated as the oven-dry weight divided by the core volume. Particle density was determined by using a gravimetric displacement. A known weight of soil or soil/compost mixture was placed in a volumetric flask containing water. The volume of displacement was measured and particle density was calculated by dividing the oven-dry weight by displaced volume.

Gravimetric water-holding capacity was determined using a soil column extraction method that approximates field capacity by drawing air downward through a soil column. Soil or soil/compost mixture was placed into 50 mL syringe tubes and tapped down (not compressed directly) to achieve the same bulk density as the field bulk density measured with coring devices. The column was saturated by drawing 50 mL of water through the soil column, then brought to approximate field capacity by drawing 50 mL of air through the soil or soil/compost column.

Volumetric water-holding capacity was calculated by multiplying gravimetric field capacity by the bulk density. Total porosity was calculated by using the following function:

$$\text{total porosity} = 1 - \left(\frac{\text{bulk density}}{\text{particle density}} \right) \times 100\% \qquad (6.1)$$

Particle size distribution was determined both by sieve analysis and sedimentation analysis for particles less than 0.5 mm in size. Due to the light nature of the organic matter amendment, particle size analysis was sometimes difficult, and possibly slightly inaccurate. Soil structure was determined using the feel method and comparing soil and soil/compost mixture samples to known structures.

Subsurface Flow Measurements

Subsurface flows and surface runoff during rains were measured and sampled using special tipping bucket flow monitors collecting the samples from the tubing shown in Figure 6.39 (Harrison et al. 1997). The flow amounts and rates were measured by tipping-bucket-type devices attached to an electronic recorder, as shown in Figure 6.40 (a close-up of the tipping bucket flowmeters shown previously in Figure 6.35), taken at the University of Washington installation. Each tip of the bucket was calibrated for each site and checked on a regular basis to give rates of surface and subsurface runoff from all plots.

Observations — Infiltration Rates in Disturbed Urban Soils

The initial exploratory analyses of the data showed that sand was most affected by compaction, with little change due to moisture levels. However, the clay sites were affected by a strong interaction

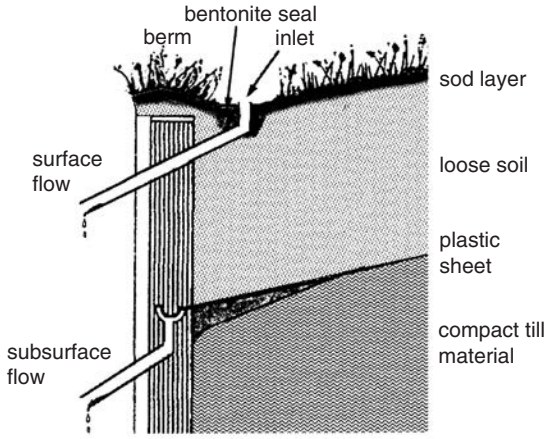


Figure 6.39 Drawing of surface and subsurface flow collectors for use in field sites. (From Harrison, R.B., M.A. Grey, C.L. Henry, and D. Xue. *Field Test of Compost Amendment to Reduce Nutrient Runoff*. Prepared for the City of Redmond. College of Forestry Resources, University of Washington, Seattle. May 1997.)

Figure 6.40 Picture of the tipping bucket installation for monitoring surface runoff and subsurface flows at the University of Washington.

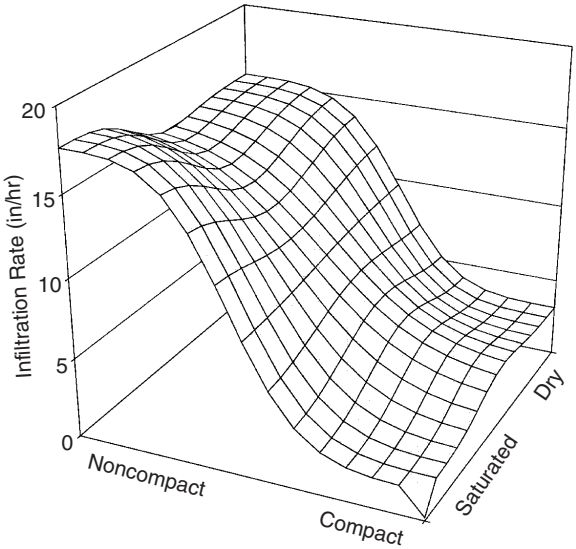


Figure 6.41 Three-dimensional plot of infiltration rates for sandy soil conditions. (From Pitt et al. 1999.)

of compaction and moisture (see Figures 6.41 and 6.42). The variations in the observed infiltration rates in each category were relatively large, but four soil conditions were found to be distinct, as shown in Table 6.14.

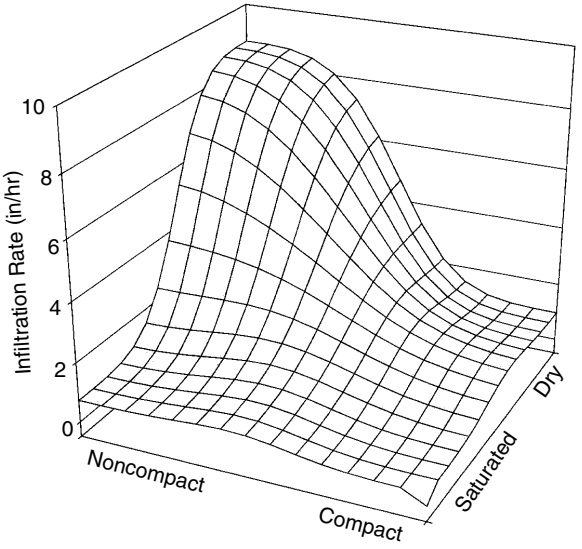


Figure 6.42 Three-dimensional plot of infiltration rates for clayey soil conditions. (From Pitt et al. 1999.)

The data from each individual test were fitted to the Horton (1939) equation (Table 6.15), but the resulting equation coefficients were relatively imprecise, and it may not matter much which infiltration model is used, as long as the uncertainty is considered in the evaluation. Therefore, when modeling runoff from urban soils, it may be best to assume relatively constant infiltration rates throughout an event, and to utilize Monte Carlo procedures to describe the observed random variations about the predicted mean value, possibly using time-averaged infiltration rates and COV values.

Importance of Field Tests of Soil Infiltration Characteristics

Very large errors in soil infiltration rates can easily be made if published soil maps and most available models are used for typically disturbed urban soils, because these tools ignore compaction.

Table 6.14 Infiltration Rates for Distinct Groupings of Soil Texture, Moisture, and Compaction Conditions

Group	Number of Tests	Average Infiltration Rate (in/hr)	COV
Noncompacted sandy soils	36	16.3	0.4
Compact sandy soils	39	2.5	0.2
Noncompacted and dry clayey soils	18	8.8	1.0
All other clayey soils (compacted and dry, plus all saturated conditions)	60	0.7	1.5

From Pitt et al. 1999.

Table 6.15 Observed Horton Equation Parameter Values for Sandy and Clayey Soils

	f_o (in/hr)		f_c (in/hr)		k (l/min)	
	Mean	Range	Mean	Range	Mean	Range
Observed noncompacted sandy soils	39	4.2 to 146	15	0.4 to 25	9.6	1.0 to 33
Observed compact sandy soils	15	0.1 to 86	1.8	0.1 to 9.5	11	1.8 to 37
Observed dry noncompacted clayey soils	18	2.5 to 58	6.6	0.1 to 24	8.8	–6.2 to 19
Observed for all other clayey soils (compacted and dry, plus all saturated conditions)	3.4	0 to 48	0.4	–0.6 to 6.7	5.6	0 to 46

From Pitt et al. 1999.

Knowledge of compaction (which can be mapped using a cone penetrometer, or estimated based on expected activity on grassed areas) can be used to more accurately predict stormwater runoff quantity.

It is therefore recommended that certain site-specific soil measurements be made in the watershed being studied. These tests should at least include actual soil texture near the surface and the shallow root zone area. Soil compaction greatly affects runoff rates and amounts and should be measured during moderately dry to moist conditions. Care should be taken when using a cone penetrometer during excessively dry or wet soil conditions. The simple double-ring infiltrometer tests, such as described for the Alabama tests, can be easily used to examine the effects of disturbing soils during development and use.

Water Quality and Quantity Effects of Amending Soils with Compost

Surface runoff decreased by five to 10 times after amending the soil with compost (4 in of compost tilled 8 in into the soil), compared to unamended sites. However, the concentrations of many pollutants increased in the surface runoff, especially associated with leaching of nutrients from the compost. The surface runoff from the compost-amended soil sites had greater concentrations of almost all constituents, compared to the surface runoff from the soil-only test sites. The only exceptions were some cations (Al, Fe, Mn, Zn, Si) and toxicity, which were all lower in the surface runoff from the compost-amended soil test sites. The concentration increases in the surface runoff and subsurface flows from the compost-amended soil test site were quite large, typically in the range of five to 10 times greater. Subsurface flow concentration increases for the compost-amended soil test sites were also common and about as large. The only exceptions were for Fe, Zn, and toxicity. Toxicity tests indicated reduced toxicity with filtration at both the soil-only and at the compost-amended test sites, likely due to the sorption or ion exchange properties of the compost.

Compost-amended soils caused increases in concentrations of many constituents in the surface runoff. However, the compost amendments also significantly decreased the amount of surface runoff leaving the test plots. Table 6.16 summarizes these expected changes in surface runoff and subsur-

Table 6.16 Changes in Pollutant Discharges from Surface Runoff and Subsurface Flows at New Compost-Amended Sites, Compared to Soil-Only Sites

Constituent	Surface Runoff Discharges, Amended-Soil Compared to Unamended Soil	Subsurface Flow Discharges, Amended-Soil Compared to Unamended Soil
Runoff volume	0.09	0.29
Phosphate	0.62	3.0
Total phosphorus	0.50	1.5
Ammonium nitrogen	0.56	4.4
Nitrate nitrogen	0.28	1.5
Total nitrogen	0.31	1.5
Chloride	0.25	0.67
Sulfate	0.20	0.73
Calcium	0.14	0.61
Potassium	0.50	2.2
Magnesium	0.13	0.58
Manganese	0.042	0.57
Sodium	0.077	0.40
Sulfur	0.21	1.0
Silica	0.014	0.37
Aluminum	0.006	0.40
Copper	0.33	1.2
Iron	0.023	0.27
Zinc	0.061	0.18

From Pitt et al. 1999.

face flow mass pollutant discharges associated with compost-amended soils. All of the surface runoff mass discharges were reduced from 2 to 50% of the unamended discharges. However, many of the subsurface flow mass discharges increased, especially for ammonia (340% increase), phosphate (200% increase), plus total phosphorus, nitrates, and total nitrogen (all with 50% increases). Most of the other constituent mass discharges in the subsurface flows decreased.

Importance of Measuring Chemical Properties of Soils When Making Soil Modifications

The use of soil amendments, or otherwise modifying soil structure and chemical characteristics, is becoming an increasingly popular stormwater control practice. However, little information is available to reasonably quantify benefits and problems associated with these changes. An examination of appropriate soil chemical characteristics, along with surface and subsurface runoff quantity and quality, was done during these Seattle tests. It is recommended that researchers considering soil modifications as a stormwater management option conduct similar local tests, including at least the detail contained in this case study, in order to understand the effects these soil changes may have on runoff quality and quantity. During the Seattle tests, the compost was found to have significant sorption and ion-exchange capacity that was responsible for pollutant reductions in the infiltrating water. However, the compost also leached large amounts of nutrients to the surface and subsurface waters. Related tests with older test plots in the Seattle area found much less pronounced degradation of surface and subsurface flows with aging of the compost amendments. In addition, it is likely that the use of a smaller fraction of compost would have resulted in fewer negative problems, while providing most of the benefits. Again, local studies using locally available compost and soils would be needed to examine this emerging stormwater management option more thoroughly.

AESTHETICS, LITTER, AND SAFETY

Safety Characteristics

Chapter 3 discussed safety-related problems associated with urban receiving waters. This is a critical beneficial use and should therefore be considered in evaluations of receiving water use impairment studies. The important safety-related information should be collected as part of the habitat survey process, as the recognized safety hazards are also indicative of poor habitat conditions for aquatic life. These include rapidly changing flows and common high flows, steep or cut banks, muddy and slippery banks, and fine-grained/mucky stream sediments. The presence of trash and other hazardous debris should also be noted as part of stream habitat surveys. Most of these problems are related to high flows from developed areas and erosion from developing areas. Watershed surveys may therefore also be important in identifying these specific sources and the necessary preventive measures to reduce safety hazards associated with urban stormwater.

Aesthetics, Litter/Floatables, and Other Debris

Aesthetics and these other elements were also described in Chapter 3 as important basic receiving water uses. Again, they should be considered in any urban receiving water evaluation investigation. Stream habitat surveys typically collect information relating to general aesthetics, including litter and other debris. An example of a beach litter survey was reported by Williams and Simmons (1997) who conducted surveys at 50 sites in South Wales and 20 sites in Devon, U.K., over a 1-year period. The surveys were conducted in both winter and summer. At each site, three transects were made, each 5 m wide, perpendicular to the beach and covering all litter strand lines. The number and types of litter were recorded in each transect. Supplemental surveys were also

carried out along 1-km lengths of beach specifically for containers (material, size, color, original contents, age, and geographical origin). Plastic fragments, bags, and plastic sheeting were the most numerous litter items found. Investigators determined that little of this material accumulates along U.K. riverbanks, leaving more for deposition along marine beaches.

HydroQual (1995) reported New York City's major efforts in characterizing litter loadings and in measuring the effectiveness of litter control devices. New York City has a Scorecard Litter Rating (SLR) Program with regular inspections of sidewalks and streets. The SLR has a numeric rating of 1.0 for streets with no litter to 3.0 for streets with a continuous line of litter. An acceptably clean rating is 1.5, with 70 to 75% of all New York City streets meeting this criterion since 1986. An extensive field monitoring program to quantify litter loadings was conducted in the summer of 1993, simultaneous with SLR inspections. This monitoring program quantified the amounts and characteristics of floatable litter. Ninety blockfaces (each 80 ft in length) were selected throughout the city for monitoring. The cleanest rating was between 1.10 and 1.19, while the dirtiest was between 2.00 and 2.09. Five to six visits were made that summer to each test area, resulting in almost 7 miles of street being directly sampled. Litter samples were collected Monday through Friday, with about half collected in the morning and half in the afternoon. At each test area, the streets and the sidewalks were individually swept with push brooms and the litter collected. The litter was then brought to a central laboratory where it was weighed and separated into 13 floatable categories (listed in Table 6.17) and nonfloatables. The material in each category was counted, weighed, and the accumulative surface areas (after laying out on a table) were measured.

The sampling procedure involved a two-person crew, one cleaning the street and the other cleaning the sidewalk. Each person used a push broom, a long-handled sweep pan, and a wheeled garbage bin lined with a plastic sample bag. The loose litter was collected and deposited into the appropriate bin labeled for the street or the sidewalk. Natural materials (sticks, leaves, etc.), gravel, sand, bricks, animal droppings and remains, and items pinned under parked vehicles were ignored. Hazardous items (syringes, glass shards, etc.) were retrieved with tongs and placed inside hard plastic containers for safe handling. Bulky items (large appliances, tires, etc.) were noted on the field sheets and not collected. Containers having liquids were drained (unless they were tightly capped or contained petroleum) before collecting. The collection bags were carefully tagged. Several bags were sometimes needed for any one sampling site. The sample bags were brought to the laboratory for analysis.

Table 6.17 Discharged Litter Material Categories Captured during New York City Tests

	Category	Examples
1	Sensitives	Syringes, crack vials, baby diapers
2	Paper-coated/waxed	Milk cartons, drink cups, candy wrappers
3	Paper-cigarette	Cigarette butts, cigarettes
4	Paper-other products	Newspaper, cardboard, napkins
5	Plastic	Spoons, straws, sandwich bags
6	Polystyrene	Cups, packing material, some soda bottle labels
7	Metal/foil	Soft drink cans, gum wrappers
8	Rubber	Pieces from automobiles, pieces from toys
9	Glass	Bottles, light bulbs
10	Wood	Popsicle sticks, coffee stirrers
11	Cloth/fabric	Clothing, seat covers, flags
12	Misc. floatables	Citrus peels. Pieces of foam
13	Non-floatables	Opened food cans, broken bottles, bolts

Data from Grey, G. and F. Oliveri. Catch basins — effective floatables control devices. Presented at the *Advances in Urban Wet Weather Pollution Reduction* conference. Cleveland, OH, June 28–July 1, 1998. Water Environment Federation. Alexandria, VA. 1998.

Three laboratory technicians would then weigh an unopened bag before pouring the contents onto a sorting table. A water-filled test bucket was also available to determine if an item was floatable or nonfloatable, if in doubt. After sorting, counting, and measuring areas (using a grid on the table), the material was placed into 20-gallon bins where the sorted material was weighed and the total volume measured. Periodically, individual categories were further subdivided into 47 subcategories to attempt to "fingerprint" the types of material found on streets and sidewalks to compare to the similarly monitored material being collected during the floatable capture activities in the receiving waters.

HABITAT

Habitat can be defined as the total physical and chemical environment where organisms live. Some of these environmental components of habitat are very dynamic, such as flow, and may change by an order of magnitude within minutes, while others change on a seasonal basis (e.g., riparian vegetation) or annual (e.g., channel morphology). As noted in the preceding discussion on flow, watershed development may dramatically alter the temporal dynamics of many of these habitat components, in addition to changing spatial relationships (e.g., patch dynamics) and general habitat character. These habitat alterations play a major role, if not *the* major role, in determining the type, size, and diversity of species, populations, and communities that will reside in the affected water system (Figure 6.43).

The other major determinants (stressors) of ecosystem quality are the pollutant types and loading dynamics that are present. Habitat and pollution stress are often interwoven, interacting components which are difficult to separate. Fortunately, it usually is not necessary to accurately determine the nature, type, and/or degree (quantity) of each individual stressor. It is often necessary, however, to determine to what degree runoff effects are due to development (anthropogenic activities) or to particular sources (e.g., construction site) as compared to natural, predevelopment, or least-impacted conditions. This necessitates use of qualitative or quantitative measures of habitat conditions in a test and reference site, as discussed in Chapter 3 and Appendix A.

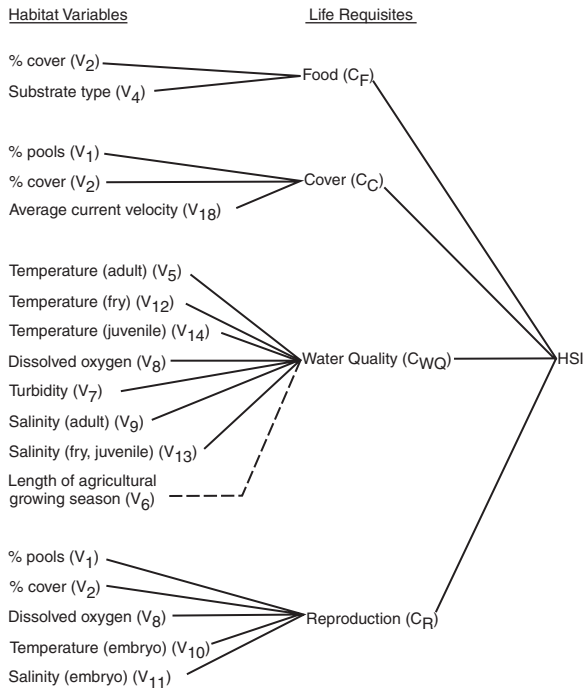


Figure 6.43 The relationship between habitat and biological condition. (From EPA. *Ecological Assessment of Hazardous Waste Sites.* Environmental Research Laboratory, U.S. Environmental Protection Agency, Corvallis, OR. EPA 600/3-89/013. 1989a; EPA. *Rapid Bioassessment Protocols for Use in Streams and Rivers: Benthic Macroinvertebrates and Fish.* Office of Water, U.S. Environmental Protection Agency, Washington, D.C., EPA 444/4-89/001. 1989c.)

Figure 6.44 Pool and riffle area in Milwaukee. (Courtesy of Wisconsin Department of Natural Resources.)

Figure 6.45 Long riffle in Milwaukee.

Figures 6.44 through 6.48 illustrate various relatively natural habitat types found in urban areas. These various types, plus the heavily modified urban streams that are also common (see Figures 3.7 through 3.11), all require investigation and specialized sampling techniques, because all are expected to be significantly different biologically. Habitat plays an important role in the natural ecosystem, and these natural differences must be evaluated when trying to understand the specific effects associated with urbanization. The following discussion will show the usefulness of characterizing physical habitat in evaluations of stormwater runoff effect, while later sections of this chapter will address specific biological sampling methods that should be used for the different habitat types.

For some studies, quantification of habitat effects is useful and necessary to meet the Data Quality Objectives (DQOs). These methods do, of course, require more resources (time, equipment, expertise, and/or expense) than qualitative assessments. Quantitative approaches include the Habitat Suitability Indices (HSI) (Figure 6.49) (Terrell 1984), Habitat Quality Index (Binns and Eiserman 1979), and the Physical Habitat Simulation Model (PHABSIM) (Hilgart 1982).

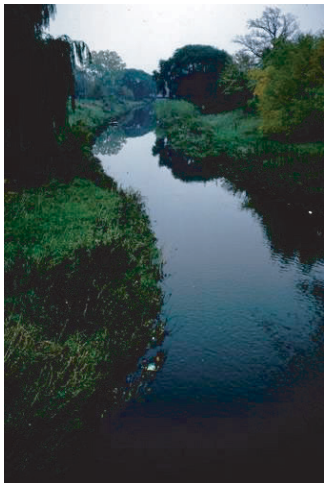


Figure 6.46 Long pool in Milwaukee.

Figure 6.47 Dry creek in Austin, TX.

Figure 6.48 Rocky substrate in Milwaukee area stream. (Courtesy of Wisconsin Department of Natural Resources.)

The HSI were developed on a species-specific basis and may be useful when particularly sensitive or economically important species are of concern. The HSI models provide information on species habitat requirements and are effective tools for beneficial use attainability analyses. These models are based on two assumptions: an HSI value has a positive relationship to potential animal numbers and a positive relationship between habitat quality and some measure of carrying capacity (EPA 1983b). HSI values range from 0 to 1, with 1 equating to optimal conditions. When comparing before and after impact data, "habitat units" may be used.

Habitat area × habitat quality (HSI) = Habitat units

Since these methods are models, they contain subjective data and such components as determining which habitat variables to include; using incomplete data sets; using data from different species of different life stages; determining independence or codependence of variables; determining when, where, and how variables should be measured; and converting assumed relationships into an aggregate suitability index (Terrell et al. 1982). The subjectivity level has been reduced, however, through extensive peer-review by the USFWS (EPA 1983b).

Most runoff effect assessments can be successful, however, without quantifying habitat effects. Rather, structured qualitative assessments exist which have been used successfully in a wide variety of ecoregions across the United States (EPA 1989c). The Qualitative Habitat Evaluation Index (QHEI) (OEPA) and the Habitat Quality Assessment Procedure (EPA 1989c) of the Ohio EPA and EPA are similar and effective at measuring six to nine interrelated metrics, including substrate, stream canopy, channel morphology, riparian and bank condition, pool and riffle quality, and gradient characteristics. All of these parameters have been shown to be related to fish and benthic macroinvertebrate community composition (OEPA 1989; EPA 1989a,c).

A key component in effectively evaluating habitat effects is the availability of baseline (predevelopment) non-(least)-impacted, reference condition information. These data are seldom available for predevelopment periods at the test site. Usually, the reference site must be in a nearby watershed that has the desired, unimpacted conditions. This approach falls within the "ecoregion" concept, which has been recommended by the EPA and successfully used by Ohio and Arkansas in their surface water quality programs (EPA 1989a).

Ecoregions are defined based on regional patterns in land-surface topology, soil and vegetation types, and land use (Omernik 1987). The biotic communities within each ecoregion are expected to be relatively similar due to habitat similarities. Studies in Ohio, Arkansas, and Oregon have suggested that fish community patterns coincide with ecoregions (Hughes et al. 1986, 1987; Larsen et al. 1986; Rohm et al. 1987; Whittier et al. 1988; Omernik 1987). Benthic macroinvertebrates show smaller habitat distribution patterns than fish (Omernik 1987) and may be influenced more by stream size, hydrologic regime, and riparian vegetation (EPA 1989a).

The QHEI, used by the State of Ohio (OEPA 1989), has shown good relationships between macrohabitat quality and fish community composition, and has been an effective tool both for implementing a biological criteria program and for assessing use impairment. Table 6.18 shows the metrics that are used in the assessment with their associated scoring ranges. When fish communities were evaluated using the Index of Biotic Integrity (IBI) (Karr 1981) and scores were less than 20, impacts were usually from a "toxicant(s)" source(s), showing greatly reduced abundance, biomass, species diversity, or other community components. However, when habitat was severely

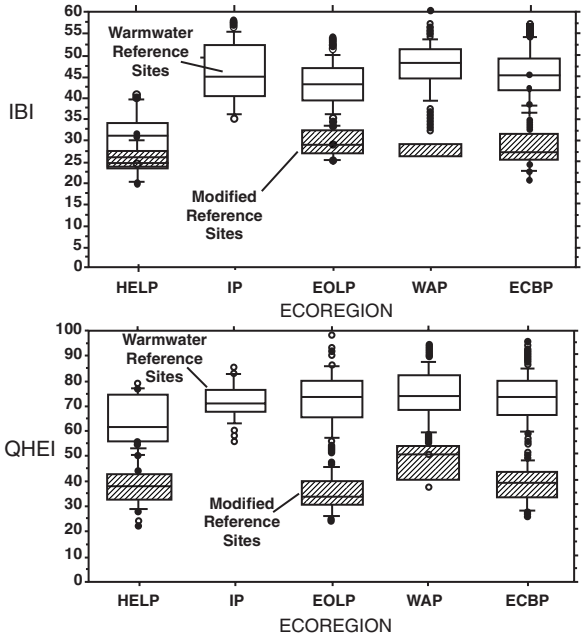


Figure 6.49 Box and whisker plots (medians, 25th and 75th percentiles, maximum value, minimum value, and outliers > two interquartile ranges from the median) for warm-water (open boxes) and modified (shaded boxes) reference sites for the IBI (top panel) and QHEI (bottom panel). (From Ohio Environmental Protection Agency. *The Qualitative Habitat Evaluation Index (QHEI): Rationale, Methods, and Application.* Ecological Assessment Section, Ohio Environmental Protection Agency, Columbus, OH. 1989.)

modified, the fish community usually responded by a shift in community function, such as from insectivore to omnivore species dominance. IBI scores rarely dropped below 20 in these situations when toxicants were absent. By utilizing individual IBI metrics and another index, the modified Index of Well-Being (mIWB), community response due to habitat or toxic impacts can be further separated (OEPA 1989).

By doing extensive surveys of habitat and aquatic communities in each ecoregion, reference site conditions can be quantified, with associated variances (for example, see Figure 6.49). Reference conditions can be tailored to meet different criteria. For example, in many states there has been extensive channel modification during the previous century.

These areas may be unable to ever recover to premodification conditions, particularly if low gradients exist (<5 ft/mi), or maintenance activities (e.g., dredging) recur. For areas where there is no evidence of or expected recovery over extended periods (i.e., decades), a channel modified reference station may be appropriate (Table 6.19, Figure 6.50) (OEPA 1989). These "irretrievable anthropogenic modifications" do not allow waters to be degraded, but rather attempt to manage historically modified streams in a realistic manner.

Factors Affecting Habitat Quality

The degree to which any habitat characteristic controls the "use" or quality of the aquatic ecosystem will vary with the site and ecoregion. There are, however, some general relationships that have been observed in a wide variety of stream systems. Small streams are more likely to be affected by riparian conditions and modifications than larger streams. Removal of riparian vegetation in headwater streams may increase water temperature 6 to 9°C and disrupt allochthonous inputs (Karr and Schlosser 1977). Another factor affecting biotic community indices is the presence of refuge areas and nearby unaffected "sources" of species (Palliam 1988; Levin 1989). If an upstream reach or tributary is unimpacted, species from this "source area or refuge" may drift or migrate into the impacted area and both assist in recovery and complicate the assessment process. Refuge areas in urban streams tend to be quite small and more limited to a protective function (e.g., debris piles) rather than a source of unaffected organisms. There are enough site-specific habitat variables to prevent the use of habitat alone as an absolute site-specific predictor of fish community quality (OEPA 1989).

Table 6.18 Metrics and Scoring Ranges for the Qualitative Habitat Evaluation Index

Metric	Score
Substrate	**20 pts**
1) Type	0–21
2) Quality	–5–3
In-stream Cover	**20 pts**
1) Type	0–10
2) Amount	1–11
Channel Quality	**20 pts**
1) Sinuosity	1–4
2) Development	1–7
3) Channelization	1–6
4) Stability	1–3
Riparian/Erosion	**10 pts**
1) Width	0–4
2) Floodplain quality	0–3
3) Bank erosion	1–3
Pool Riffle	**20 pts**
1) Max depth	0–6
2) —	—
3) Current available	–2–4
4) Pool morphology	0–2
5) Riffle/run depth	0–4
6) Riffle substrate stab.	0–2
7) Riffle embeddedness	–1–2
Drainage Area	Not included
Gradient	0–15 pts
Total score	0–100 pts.

From OEPA (Ohio Environmental Protection Agency). *The Qualitative Habitat Evaluation Index (QHEI): Rationale, Methods, and Application.* Ecological Assessment Section, Ohio Environmental Protection Agency, Columbus, OH. 1989.

Surveys of five different ecoregions in Ohio by three fish collection methods found some significant relationships between habitat components (metrics) and fish community quality (Table 6.20). Three metrics were frequently related to the IBI, namely, pool, channel, and substrate quality (OEPA 1989).

Channelization

The process of channelizing a stream alters flow (Figure 6.51), channel morphology, and stream bank and adjacent riparian zone characteristics. When these projects cover small areas, such as for road or bridge construction, adverse impacts may be limited to the short term and affect only tens to hundreds of meters. The long-reach channelization projects, however, may cause severe ecosystem quality degradation. The most significant ecosystem alterations are usually the loss of the run–riffle–pool sequence, refuge areas, substrate composition characteristics change (e.g., particle

Table 6.19 Habitat Characteristics of Modified Warm-Water Streams and Warm-Water Streams in Ohio

Modified Warm-Water Streams	Warm-Water Streams
1. Recent channelization[1] or recovering[2]	1. No channelization or recovered
2. Silt/muck substrates[1] or heavy to moderate silt covering other substrates[2]	2. Boulder, cobble, or gravel
3. Sand substrates — Boat[2], Hardpan origin[2]	3. Silt free
4. Fair–poor development[2]	4. Good–excellent development
5. Low–No sinuosity[2], Headwater[1]	5. Moderate–high sinuosity
6. Only 1–2 cover types[2], cover sparse to none[1]	6. Cover extensive to moderate
7. Intermittent or interstitial — with poor pools[2]	7. Fast current, eddies
8. Lack of fast current[2]	8. Low–normal substrate embeddedness
9. Max. depth <40- Wading[1], -Headwater[2]	9. Max. depth > 40
10. High embeddedness of substrates[2]	10. Low/no embeddedness

Note: Superscripts for MWH streams refer to the influence of a particular characteristic in determining the use (1 = high influence, 2 = moderate influence).

From OEPA (Ohio Environmental Protection Agency). *The Qualitative Habitat Evaluation Index (QHEI): Rationale, Methods, and Application.* Ecological Assessment Section, Ohio Environmental Protection Agency, Columbus, OH. 1989.

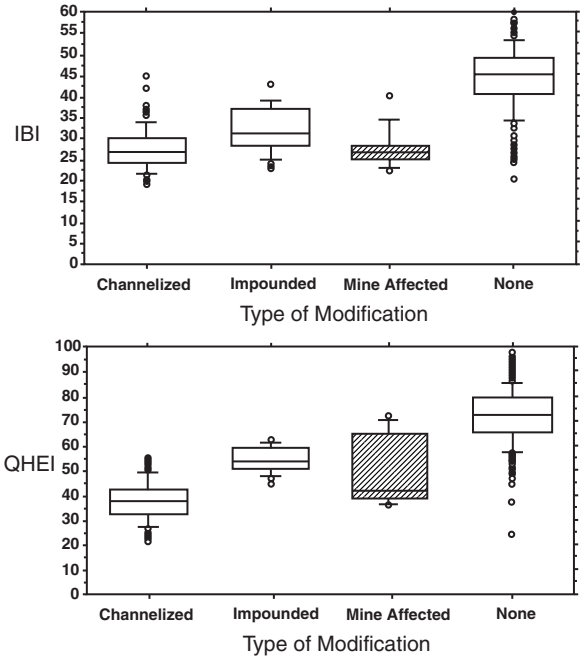


Figure 6.50 Box and whisker plots (medians, 25th and 75th percentiles, maximum value, minimum value, and outliers > two interquartile ranges from the median) from modified reference sites with channel modifications, impoundments, and mine affects (cross-hatched) and warmwater reference sites for the IBI (top panel) and QHEI (bottom panel). (From Ohio Environmental Protection Agency. *The Qualitative Habitat Evaluation Index (QHEI): Rationale, Methods, and Application.* Ecological Assessment Section, Ohio Environmental Protection Agency, Columbus, OH. 1989.)

Figure 6.51 Stream flow-altering channel conditions.

size reduction, increased embeddedness), and increased temperature, and an altered productivity and trophic level regime (EPA 1983b). By straightening a stream channel, length, habitat diversity (e.g., edge habitat), and quantity are all reduced. Since fish and benthic invertebrates are habitat selective, they are directly affected by these alterations. Numerous studies have documented stream modification effects on ecosystem, structure, function, and quality (see OEPA 1989; EPA 1983b, 1977).

Table 6.20 Relative Ranking by the Magnitude of Significant (P < 0.05) Correlation Coefficients (r) between the QHEI and IBI for Ohio Ecoregions and Fish Sampling Methods

Ecoregion[a]	N[b]	Metric Ranking
		Boat Methods
HELP	28	Substrate > Channel > Riffle
IP	7	*No significant correlations*
EOLP	22	Channel > Riffle > Substrate > Pool > Gradient > Riparian > Cover
WAP	26	Substrate > Gradient > Channel > Cover > Riparian > Riffle > Pool
ECBP	56	Pool > Channel > Gradient > Substrate > Riffle > Cover
		Wading Methods
HELP	16	*No significant correlations*
IP	20	Gradient
EOLP	28	Gradient > Riffle > Channel > Pool > Substrate
WAP	47	Substrate > Cover > Channel > Gradient
ECBP	73	Cover > Channel > Pool > Gradient > Substrate > Riffle > Riparian
		Headwater Methods
HELP	8	*No significant correlations*
IP	13	Pool
EOLP	35	Channel > Cover > Substrate > Pool
WAP	31	Substrate > Channel > Cover
ECBP	52	Channel > Cover > Pool > Substrate > Riffle > Riparian > Gradient

[a] Ecoregion classifications = HELP, Huron/Erie Lake Plain; IP, Interior Plateau; EOLP, Erie/Ontario Lake Plain; WAP, Western Allegheny Plateau; and ECBP, Eastern Corn Belt Plains

[b] Number of sample data sets.

From OEPA (Ohio Environmental Protection Agency). *The Qualitative Habitat Evaluation Index (QHEI): Rationale, Methods, and Application.* Ecological Assessment Section, Ohio Environmental Protection Agency, Columbus, OH. 1989.

Substrate

The substrate composition is a direct function of watershed and channel characteristics and to a large extent controls the composition of benthic macroinvertebrates, meio- and microfauna, periphyton, and fish communities (e.g., EPA 1983b). Algal (phytoplankton) and zooplankton communities are indirectly affected by nutrient availability, which changes as the rate of cycling changes in different sediment environments. Microbial communities are influenced structurally and functionally by sediment quality (see Benthos section).

Though substrates consist of any inorganic or organic material that is utilized as a growth surface or is solid in nature, most substrate classifications are based on inorganic particle sizes (Table 6.21). Generally, mean particle sizes decrease (get finer) farther downstream due to reduced bottom shear stress and stream power. Current velocities of >50 cm/s on steep gradients typically result in substrate that is gravel size or larger. Velocities of 20 to 50 cm/s result in substrate that is sandy, while <20 cm/s velocities result in substrate dominated by silt and clay-sized particles. Channelization impacts are often greater in headwater streams that have high gradients and where coarse substrates are necessary to provide protection from strong currents (EPA 1983b). Few to no impacts have been observed in low gradient, high order, large streams where particle sizes are smaller and food sources for sensitive species are fewer.

Large-grained (e.g., gravel) sediments typically have macrobenthic communities indicative of higher quality water. These substrates have a greater amount of living space, provide protection, trap more organic material, and are well oxygenated. High flows (storm events) tend to wash out

Table 6.21 Substrate Particle Size Classification for Sieve Analysis

Name	Particle Size (mm)	Particle Size (μm)	U.S. Standard Sieve Number
Boulder	>256 (10 in)		
Rubble	64 to 256		
Coarse gravel	32 to 64		
Medium gravel	8 to 32		
Fine gravel	2 to 8		10
Coarse sand	0.5 to 2	500–2000	35
Medium sand	0.25 to 0.5	250–500	60
Fine sand	0.125 to 0.25	125–250	120
Very fine sand	0.0625 to 0.125	62–125	230
Silt	0.0039 to 0.0625	4–62	
Clay	<0.0039	<4	

Modified from Wentworth, 1922; see Cummins, 1962 (EPA 1990c).

organic matter and thereby decrease food availability. Other important substrates include cobble, macrophytes, roots, and organic debris (sticks to leaves), which are used by numerous groups of organisms (e.g., periphyton, protozoa, filamentous algae, fungi, bacteria, and invertebrates) for attachment and as a food source.

Siltation is a significant stressor for many desirable species. Silt and clay have been shown to decrease habitat diversity by filling interstitial spaces (embeddedness), standing crop, density, taxa richness, reproductive success, and productivity, and to increase pollution-tolerant species (EPA 1983b).

In unchannelized, nonsandy streams, there is often an alternating pool–riffle structure. Riffles are stationary, comprised of gravel, cobble, and boulders, which may move. The increased flow, habitat space, and food in riffle areas support greater benthic macroinvertebrate populations than pool areas. For many fish, a 1:1 ratio of pool to riffle run areas is optimal for survival and reproduction.

The importance and heterogeneity of substrates in stormwater assessments necessitates the collection of multiple samples at each site and characterization of both organic and inorganic constituents. Useful characterization parameters are listed in Table 6.22.

Table 6.22 Substrate Characterization

Parameter	Method			Ref.
Particle size distribution	Sieve: wet sieve		Sample	Welch 1948
	Sedimentation:	Pipette		Allen 1975
		Hydrometer		APHA 1985
	Particle size:	Coulter counter		ASTM 1991
		Laser		ASTM D854-83
Dry weight	60-105MC 24 h			APHA 1985 ASTM D4318-84
Volatile solids (ash-free)	500MC 1 h			ASTM 1987
Total organic carbon				APHA 1985
Acid volatile sulfides	Spectrophotometric or gravimetric			EPA 1990
Synthetic organics	Variety			EPA SW-846 8010-8310 3510-3550
Metals	Variety			EPA SW-846 7040-7951 3010-3060
Total organic halides				
Cation exchange capacity				
Total nitrogen				APHA 1985
Ammonia				APHA 1985
Total phosphorus				APHA 1985
Extractable phosphorus				

Scour of Bottom Sediments

A critical component of habitat quality is substrate stability. Frequent scouring or sedimentation is obviously detrimental to benthic organisms. Classical methods to monitor scour have been to use standard surveying procedures and carefully measure stream cross sections and slope. This should be supplemented with scour pins and scour chains. Scour pins are long rods (with a scale) driven deeply into stream banks, with a bright end exposed. Frequent visits are needed to note the length of pin exposed at any time. With receding banks, the pins will become more and more exposed, and the bank loss rate can be calculated. They can be reset when too much of the pin is exposed. Scour pins should be used at several locations at any cross section.

Scour pins cannot be effectively used in the stream to measure scour and sedimentation separately. The use of scour chains, as described by May et al. (1999) in the following comments for work on salmon streams in the Pacific Northwest, works well in many stream locations. Nawa and Frissell (1993) monitored stream bed stability using bead-type scour monitors installed in salmonid spawning riffles in selected reaches. Figure 6.52 illustrates these devices. They found that larger scour and/or fill events normally resulted from larger storms and the resultant higher flows, as would be expected. Cooper (1996) found that increased urbanization leads to increased stream power and stream bed instability and that basin urbanization in Puget Sound lowland streams was found to have the potential to cause locally excessive scour and fill. Urban streams in the Puget Sound lowland area having gradients greater than 2% and lacking in large woody debris (LWD) were found to be more susceptible to scour than undeveloped streams.

May et al. (1999) used a stream stability classification similar to Booth (1996): stream segments with >75% of the reach classified as stable were given a score of 4; stream segments having between 50 and 75% stable banks were scored as a 3; those with 25 to 50% stable banks were scored as a 2; and those having less than 25% stable banks were scored as a 1. The presence of artificial stream bank protection (such as rip-rap) was considered a sign of bank instability and scored as a 1. Researchers found that only two undeveloped reference stream segments (watershed areas having total imperviousness area < 5%) had a stability rating less than 3. In basins that had from 5 to 10% imperviousness, the stream bank ratings were generally 3 or 4. However, in basins having between 10 and 30% impervious area, there was a fairly even mixture of stream bank conditions, from stable and natural to highly eroded or artificially "protected." For basins having total imperviousness areas of 30%, there were no segments having stream bank stability ratings of 4 and very few with ratings of 3 (only found in segments with intact and wide riparian corridors). Artificial stream bank protection was a common feature of all highly urbanized streams (those that had total imperviousness areas > 45%). May et al. (1999) also found that stream bank stability was influenced by the condition of the riparian vegetation surrounding the stream, with the stream bank stability rating being strongly related to the width of the riparian buffer and inversely related to the number of breaks in the riparian corridor.

Sediment Transport

Sediment may be composed of organic or inorganic material ranging in size from colloidal humus (<1 μm) to boulders. Total sediments are the sum of suspended, bedload, and consolidated

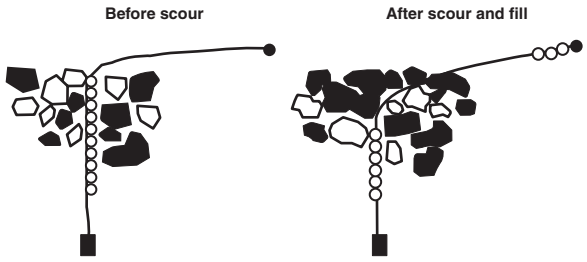


Figure 6.52 Sliding-bead type scour monitors. (From May, C., R.R. Horner, J.R. Karr, B.W. Mar, and E.B. Welch. *The Cumulative Effects of Urbanization on Small Streams in the Puget Sound Lowland Ecoregion*. University of Washington, Seattle. 1999. With permission.)

sediments, each of which may have deleterious effects on ecosystem quality. Sediment erosion, watershed yields, or loading can be estimated by various simple sampling methods. The total sediment yield includes both suspended and bedload sediment. This provides a good indicator of land-use changes. Bedload sediments are more of a concern in high flow waters, as they can scour, abrade, and smother benthic biota. Sediments may also release or adsorb nutrients and toxicants. The partitioning coefficients and controlling conditions are not well understood. Sedimentation and resuspension are affected by biological and physical processes. The physical processes include bioturbation and fluid flow (laminar or turbulent). Particle movement and settling will depend on particle size, shape, and density, cohesion-flocculation properties, temperature, solids concentration, and water velocity and turbulence. Organic settleable solids can accumulate at velocities of 0.6 ft/s or less. The sediment particle size distribution is directly related to the system's hydraulics. The most significant changes in particle size distributions occur when flow dynamics change in the stream or receiving water body, e.g., river mouth, riffle–pool boundary, or river bend. By knowing the watershed and substrate particle size characteristics and channel velocity, areas of sediment accumulation or scour may be predicted. Combining this information with time of passage data, sludge deposit areas were located (Velz 1970). As time since deposition increases, solids will tend to compact and higher velocities (e.g., 1.5 fps) will be required to induce scour.

Typical automatic water samplers are limited in their ability to sample particles in the water, as discussed in Chapter 5. If particles larger than several hundred μm need to be included in the sampling program, then manual depth-integrated (Helley–Smith) or bedload samples also need to be used, as described in Chapter 5. The Helley–Smith sampler (Helley and Smith 1971) effectively collects water and bed sediment at the same flow velocity that occurs at the stream bottom. Samples must be collected at several intervals across the channel bottom and integrated for total transport. With a depth-integrated sampler, water passes into the vented sampler at the same flow velocity as the stream, so as it is lowered it collects in proportion to the total discharge. Suspended sediment is then measured by filtering and weighing (Guy and Normal 1970; Guy 1969; Kunkle and Comer 1971).

Bedload sediment moves along the streambed by traction and saltation mechanisms (slide, roll, bounce, or hop) (Davis 1983) and may comprise approximately 10% of the total sediment load. It is more difficult to measure than suspended sediment. Bedload in streams varies greatly with stormwater discharge conditions and by season. These measurements must therefore be repeated frequently. Bedload trapping samplers can be used to measure the material moving along stream bottoms over a period of time. There are several designs for these samplers. A simple sampler is made by burying cans (bottom intact, top removed) in sediment (top flush with sediment surface). The cans are filled with large, uniformly sized marbles to provide an effective trap and prevent scour of the finer material that filters down between the much larger marbles. More exotic samplers are scoop shaped and face upstream to allow moving sediment to enter the trap and accumulate in a deeper sump.

Riparian Habitats

The importance of lake, streamside, or wetland (riparian) ecosystems in determining water quality is well known. The relationship or correlation is essentially a holistic system. However, no one riparian component or parameter can be used to predict water quality (EPA 1983b). Obviously, the effect of the riparian zone is much greater in small stream systems (i.e., high riparian area:stream area ratio). These unique ecosystems are often described as ecotones, a gradient of changing habitat between terrestrial and aquatic systems, which supports greater diversity and abundance of terrestrial species than adjacent areas.

The three principal stressors that result when riparian zones are removed are: (1) elevated temperatures from lack of shading; (2) increased siltation from the ecoregion with associated nutrients (salts, metals, pesticides, and other synthetic organics); and (3) more dynamic changes in

flow-runoff. Solids, nutrients, and toxicant loadings may increase orders of magnitude when riparian zones are removed (EPA 1991c; Lowrance et al. 1983). Another less noticeable yet important ecosystem perturbation that might occur when riparian zones are removed from small watersheds or small streams is the loss of allochthonous inputs of organic matter. The principal energy process in these systems is detritus processing and is accomplished by several biotic groups. Benthic macroinvertebrates, called "shredders," produce fine particulate organic matter which is used by "collectors." The organic matter processing is assisted by fungi and bacteria. When coarse particulate organic matter inputs are reduced, light and temperature are increased. The ecosystem changes to one of herbivorous grazers which feed on the periphytic algal populations (Cummins et al. 1973, 1974, 1975; Marzolf 1978; Vannote et al. 1980). The other interactive effects are discussed in previous sections.

Accurate assessments of riparian zone measures and their contribution to water quality are difficult and require extensive sampling and expertise. Some of the many variables factors of importance are listed in Table 6.23.

Field Habitat Assessments

When conducting qualitative assessments, the procedures outlined by the Ohio EPA or in the EPA Rapid Bioassessment Protocols (OEPA 1989; EPA 1989c, 1999) should be used. The methods are very similar and are presented with field data sheets in Appendix A.

Recommended Stream Bed/Sediment Monitoring

Unstable stream sediments may be one of the most common causes of degradation of biological uses in urban streams. It is therefore important that indicators of unstable stream beds be included in any habitat evaluation effort. As discussed above, the use of scour pins can be used to indicate unstable stream banks, while sliding bead scour monitors can be used to indicate sediment deposition and scour. These techniques can be used to supplement conventional cross section surveying at established stream stations. Pins and chain monitors can be much more rapidly examined for many intermediate locations between survey stations, enabling a better overall understanding of the magnitude and location of unstable stream bed conditions. If bedload samples are desired, or if bedload movement needs to be quantified, special bedload samplers (traps) should be used, because automatic water quality samplers cannot adequately collect the larger material that comprises bedload.

Temperature

Elevated temperatures of urban streams caused by heated stormwater has caused much concern. Much-needed research is currently being conducted by Steve Greb, of the WI Department of Natural Resources, Madison, WI. Figures 6.53 through 6.57 show some of the temperature measurement equipment he is using to investigate surface temperatures and sheetflow temperature increases from many different urban surfaces.

Fish are cold-blooded poikilothermic organisms and are sensitive to water temperature changes. Gradual changes can induce metamorphosis, migration, and spawning behavior. As with many stressors, effects are greater during the sensitive early life stages. Fish may survive in suboptimal temperatures which may favor competitors, predators, parasites, and disease, and alter food sources. Metabolic activity is increased at warmer temperatures, which increases feeding until threshold levels are reached, and it also affects toxicokinetics.

Temperature profiles in streams and rivers are generally more homogeneous than deeper, less turbulent reservoirs and lakes. As previously discussed, stormwater runoff from developed land (commercial, residential, or agricultural) is often significantly warmer than from vegetated non-developed areas. In a small receiving water system, this may quickly raise water temperatures.

Table 6.23 Riparian Zone Components That May Affect Water Quality

- Geomorphology (erosion, runoff rates and variations, sediment loads)
  - Slope
  - Topography
  - Parent material
- Soils (sediment loads, nutrient inputs, runoff rates)
  - Particle size distribution
  - Porosity
  - Field saturation
  - Organic component
  - Profile (presence or absence of mottling)
  - Cation exchange capacity
  - Redox (Eh)
  - pH
- Hydrology (water budget, flooding potential, nutrient loads)
  - Groundwater
    - a. Elevation
    - b. Chemical quality
    - c. Rate of movement
  - Climatic factors
    - a. Total annual rainfall and temporal distribution
      - – Chemical quality
    - b. Temperature
    - c. Humidity
    - d. Light
- Vegetative and Faunal Characteristics
  - Floristics (“community health,” disturbance levels)
    - a. Presence/absence
    - b. Nativity
  - Vegetation (nutrient loads, “community health,” disturbance levels)
    - a. Production
    - b. Biomass
    - c. Decomposition
    - d. Litter (Detritus) dynamics
      - – Size
      - – Transportability
      - – Quantity
    - e. Plant size classes
      - – Grasses, herbs (forbs), shrubs, trees
    - f. Canopy density and cover
      - – Light intensity
    - g. Cover values
  - Fauna (community disturbance, community health)
    - a. Production
    - b. Biomass
    - c. Mortality
  - Community Structure
    - a. Diversity
    - b. Evenness
  - Physiological Processes
    - a. Transpirational water loss (community health)
    - b. Photosynthetic rates (community health)
  - Stream bank characteristics
    - a. Stream sinuosity
    - b. Stream bank stability (sediment loads, habitat availability)

EPA. *Technical Support Manual: Waterbody Surveys and Assessments for Conducting Use Attainability Analyses.* Office of Water Regulations and Standards, U.S. Environmental Protection Agency, Washington, D.C. 1983b.

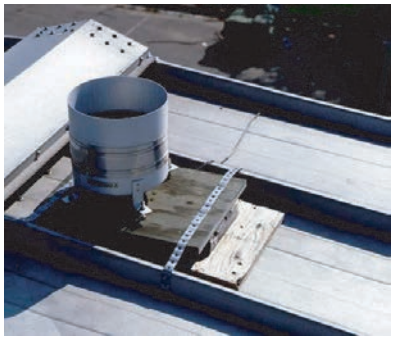


Figure 6.53 Rain temperature monitoring by WI DNR in Madison, WI.

Figure 6.54 Roof temperature data loggers being used by WI DNR.

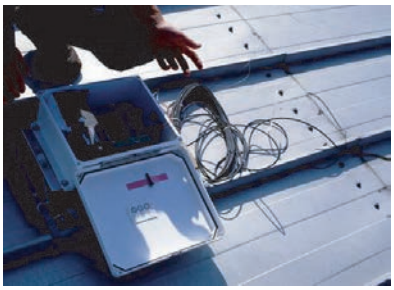


Figure 6.55 Rooftop temperature data logging used by WI DNR.

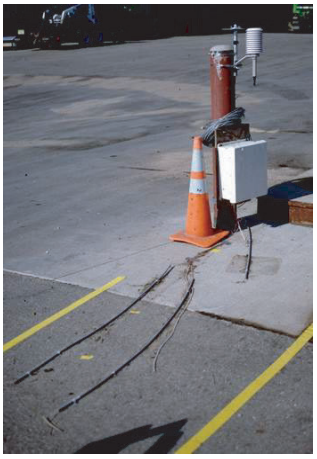


Figure 6.56 Pavement temperature monitoring by WI DNR.

This change may not exceed the temperature threshold of the species but could exceed its acclimation ability. Many urban channels also have had their natural cover removed, causing further temperature increases.

A sizable database exists on temperature effects on fish. In areas where temperature patterns change, fish populations can be expected to change. Table 6.24 shows preferred temperatures for some fish species.

Temperature also affects physical stratification (water density) in reservoirs and lakes, and thus mixing, partitioning, and the fate of feeder stream loadings. Productivity and organic matter cycling are dramatically affected by temperature both through changes in metabolic rates and changes in species (planktonic and benthic microorganisms and algae) composition. These factors combined with the physical effect of temperature on dissolved oxygen concentrations will affect macrofaunal distribution, community composition, BOD rates (waste assimilation capacity), and metal-nutrient partitioning, and thus bioavailability as soluble or insoluble species. Dissolved oxygen levels should not drop below 5 mg/L during spawning seasons (EPA 1991c) in most areas of the United States where desirable habitats exist.

Figure 6.57 Pavement temperature data loggers used by WI DNR.

Temperature is an easy parameter to define in stormwater assessments. It should accompany the collection of all samples at all sites. Background data are frequently available from nearby areas, but the land use similarity to current test conditions should be known due to its significant effect on temperature. The diurnal and seasonal patterns should be defined at reference and test sites, during baseflow, stormflow, and post-storm event conditions.

Turbidity

In many developing urban areas, urban receiving waters are typically characterized by high turbidity levels caused by high erosion rates from ongoing construction activities. Large discharges of sediment in urban runoff are mostly associated with poorly controlled construction sites, where 30 to 300 tons of sediment per acre per year of exposure may be lost. These high rates can be 20 to 2000 times the unit area sediment discharge rates associated with other land uses. Unfortunately, much of this sediment reaches urban receiving waters, where massive impacts on the aquatic environment can result. With complete development, sediment discharges from urban stormwater are significantly reduced. Unfortunately, high rates of sediment loss can also be associated with later phases of urbanization, where receiving water channel banks widen to accommodate the increased runoff volume and frequency of highly erosive flow rates. The associated increased levels of turbidity can interfere with algal productivity and with aquatic life. Increased turbidity is also typically associated with increases in settleable solids that can smother the natural bottom material and benthic organisms. These changes in the bottom characteristics of streams and lakes can produce dramatic interferences with spawning and rearing of fish.

Schueler (1997a) listed the impacts that can be associated with suspended sediment:

- "Abrades and damages fish gills, increasing risk of infection and disease
- Scouring of periphyton from streams (plants attached to rocks)
- Loss of sensitive or threatened fish species when turbidity exceeds 25 NTU
- Shifts in fish communities toward more sediment-tolerant species
- Decline in sunfish, bass, chub, and catfish when monthly turbidity exceeds 100 NTU
- Reduces sight distance for trout, with reduction in feeding efficiency
- Reduces light penetration that causes reduction in plankton and aquatic plant growth
- Reduces filtration efficiency of zooplankton in lakes and estuaries
- Adversely impacts aquatic insects, which are the base of the food chain
- Slightly increases stream temperature in summer
- Suspended sediments are major carriers of nutrients and metals
- Turbidity increases the probability of boating, swimming, and diving accidents
- Increased water treatment to meet drinking water standards
- Increased wear and tear on hydroelectric and water intake equipment
- Reduces anglers chances of catching fish
- Diminishes direct and indirect recreational experience of receiving waters"

Bolstad and Swank (1997) examined the in-stream water quality at five sampling stations in Cowetta Creek in western North Carolina over a 3-year period. The watershed is 4350 ha and is relatively undeveloped (forested) in the area above the most upstream sampling station and becomes

Table 6.24 Preferred Temperature of Some Fish Species

Common Name (Species)	Life Stage[a]	Acclimation Temperature, °C	Preferred Temperature, °C
Alewife (*Alosa pseudoharengus*)	J	18	20
	J	21	22
	A	24	23
	A	31	23
Threadfin shad (*Dorosoma petenense*)	A		>19
Sockeye salmon (*Oncorhynchus nerka*)	J		12–14
	A		10–15
Pink salmon (*O. gorbuscha*)	J		12–14
Chum salmon (*O. keta*)	J		12–14
Chinook salmon (*O. tshawytscha*)	J		12–14
Coho salmon (*O. kisutch*)	J		12–14
	A		13
Cisco (*Coregonus artedii*)	A		13
Lake whitefish (*C. clupeaformis*)	A		13
Cutthroat trout (*Salmo clarki*)	A		9–12
Rainbow trout (*S. gairdneri*)	J	—	14
	J	18	18
	J	24	22
	A		13
Atlantic salmon (*S. salar*)	A		14–16
Brown trout (*S. trutta*)	A		12–18
Brook trout (*Salvelinus fontinalis*)	J	6	12
	J	24	19
	A		14–18
Lake trout (*Salvelinus namaycush*)	J		8–15
Rainbow smelt (*Osmerus mordax*)	A		6–14
Grass pickerel (*Esox americanus vermiculatus*)	J, A		24–26
Muskellunge (*Esox masquinongy*)	J		26
Common carp (*Cyprinus carpio*)	J	10	17
	J	15	25
	J	20	27
	J	25	31
	J	35	32
	A		33–35
Emerald shiner (*Notropis atherinoides*)	J		25
White sucker (*Catostomus commersoni*)	A		19–21
Buffalo (*Ictiobus* sp.)	A		31–34
Brown bullhead (*Ictalurus nebulosus*)	J	18	21
	J	23	27
	J	26	31
	A		29–31
Channel catfish (*Ictalurus punctatus*)	J	22–29	35
	A		30–32
White perch (*Morone americana*)	J	6	10
	J	15	20
	J	20	25
	J	26–30	31–32
White bass (*M. chrysops*)	A		28–30
Striped bass (*M. saxatilis*)	J	5	12
	J	14	22
	J	21	26
	J	28	28
Rock bass (*Ambloplites rupestris*)	A	26–30	
Green sunfish (*Lepomis cyanellus*)	J	6	16
	J	12	21
	J	18	25

Table 6.24 Preferred Temperature of Some Fish Species *(Continued)*

Common Name (Species)	Life Stage[a]	Acclimation Temperature, °C	Preferred Temperature, °C
	J	24	30
	J	30	31
Pumpkinseed (*L. gibbosus*)	J	8	10
	J	19	21
	J	24	31
	J	26	33
	A		31–31
Bluegill (*L. machrochirus*)	J	6	19
	J	12	24
	J	18	29
	J	24	31
	J	30	32
Smallmouth bass (*Micropterus dolomieui*)	J	15	20
	J	18	23
	J	24	30
	J	30	31
Spotted bass (*M. punctulatus*)	J	6	17
	J	12	20
	J	18	27
	J	24	30
	J	30	32
Largemouth bass (*M. salmoides*)	J		26–32
White crappie (*Pomoxis annularis*)	J	5	10
	J	24	26
	J	27	28
	A		28–29
Black crappie (*P. nigromaculatus*)	J		27–29
	A		24–31
Yellow perch (*Perca flavescens*)	J, A		19–24
Sauger (*Stizostedion canadense*)	A		18–28
Walleye (*S. vitreum*)	J, A		20–25
Freshwater drum (*Aplodinotus grunniens*)	A		29–31

[a] J = juvenile, A = adult.

EPA. *Technical Support Manual: Waterbody Surveys and Assessments for Conducting Use Attainability Analyses.* Office of Water Regulations and Standards, U.S. Environmental Protection Agency, Washington, D.C. 1983b.

more urbanized at the downstream sampling station. Baseflow water quality was good, while most constituents increased in concentration during wet weather. Water quality was compared to building density for the different monitoring stations. Stormwater pollutant-related concentrations of turbidity increased as building densities increased. Baseflow concentrations also typically increased with density, but at a much lower rate. In addition, the highest concentrations observed during individual events corresponded to the highest flow rates.

There has been conflicting evidence on the role of elevated turbidity levels on eutrophication processes and resulting highly fluctuating DO levels. Because of the high sediment loads, urban lakes are quite different compared to most impoundments. Burkholder et al. (1998) described a series of enclosure experiments they conducted in Durant Reservoir, near Raleigh, NC. Secchi disk transparency ranged from 0.5 to 1.3 m during the summer of 1990 when these experiments were conducted. The algal communities are P-limited until late summer, when N becomes the primary limiting nutrient. The phytoplankton biomass significantly increases during the summer growing season. Several 2-m-diameter enclosures were constructed, isolating sediment to water surface columns of water. The experimental design allowed investigating the effects of different levels of

sediment and nutrients on algal productivity. They found that the effects (reduction of light and coflocculation of clay and phosphate) of low (about 5 mg/L) and moderately high clay (about 15 mg/L) loadings added every 7 to 14 days did not significantly reduce the algal productivity simulation caused by high phosphate loadings. However, higher clay loadings (about 25 mg/L added every 2 days) did produce depressed effects of phosphorus enrichment on the test lake. They concluded that dynamically turbid systems, such as is represented in southeastern urban lakes, have complex interacting mechanisms between discharged clay and nutrients that make simple predictions of the effects of eutrophication much more difficult than in the more commonly studied clear lakes. In general, increased turbidity will either have no effect, or will have a mitigating effect, on the cultural eutrophication process.

Sediment is typically listed as one of the most important pollutants causing receiving water problems in the nation's waters, and turbidity is therefore an important indicator of water quality. Turbidity, along with associated water column transparency, are two of the most commonly monitored water quality parameters in receiving water studies. Transparency is easily measured using Secchi disks by minimally trained volunteers (Figures 6.58 and 6.59). This has resulted in long-term transparency data being available for many urban lakes. Unfortunately, Secchi disk readings are instantaneous measurements and are usually obtained only during dry weather, with little high-resolution transparency information available. Measurements of water turbidity, however, can be readily obtained from both manual and automatic water sampling efforts, plus from continuous long-term monitoring sondes. Both laboratory and field nephelometers are available for measuring water turbidity (Figures 6.60 and 6.61).

A discussion earlier in this chapter presented the results of a small study conducted along Five-Mile Creek in Jefferson County, AL, where a YSI 6000 sonde, having continuous turbidity monitoring capabilities, was used to indicate the frequency, duration, and severity of wet-weather flow events. Increases in turbidity, along with attendant decreases in specific conductivity, were a much more accurate indicator of the durations of wet-weather flow impacts than flow rate and stream stage. Turbidity immediately increased from base levels (about 10 NTU) to more than 1000 NTU (the upper limit of the instrument) with the initial increases in stream stage. Elevated turbidity levels (greater than 100 NTU) persisted long after the flow subsided. The actual duration of the detrimental effects of the wet-weather flow was two to three times longer than the duration of the elevated flows in the streams. In addition, interstitial water turbidity levels also substantially and rapidly increased (to levels of about 200 NTU) in areas having coarse sediment. The interstitial water turbidity levels remained elevated for a much longer period than the water column turbidity

Figure 6.58 Secchi disk being lowered into lake for transparency measurement.

Figure 6.59 Underwater Secchi disk showing slow disappearance of contrasting disk sectors.

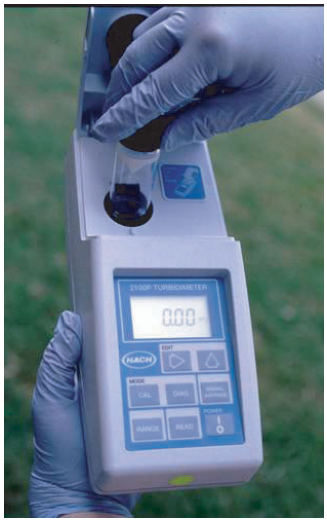


Figure 6.60 HACH 2100P field turbidimeter.

Figure 6.61 HACH turbidity reading.

levels in the creek. There were no indicated interstitial water quality changes in areas having fine-grained (sandy) sediment. Therefore, turbidity can have much more prolonged effects on in-stream (and possibly in-sediment) conditions than is typically assumed, based solely on water flow measurements. The use of continuous turbidity measurements to supplement biological observations in wet-weather receiving water studies is therefore highly desirable.

Dissolved Oxygen

The adverse effects of low dissolved oxygen on aquatic life are well known, and reliable modeling techniques exist that predict DO levels in waters which receive wastewaters (EPA 1986). However, oxygen demand dynamics associated with stormwater events are not well understood. Peak oxygen demand may occur days after storm events, and miles downstream due to BOD and sediment oxygen demand (SOD) loading and transport.

The measurement of SOD is often overlooked in stream surveys and methods are not standardized, but it may be a critical measurement. Research reported by Werblow (2000) has shown that SOD may be a very large sink of DO in Tualatin Basin in Oregon, for example. In systems or reaches where small particle sizes (i.e., silts and clays) dominate and where organic matter and nutrient inputs may be elevated, SOD may be an important stressor. Station selection for SOD measurements should be based on deposition zones and sources of loadings. SOD may be measured in the laboratory or *in situ* (Edberg and Von Hofsten 1973; O'Connor and DiToro 1970; Bowie et al. 1985; Whittemore 1986; Davis et al. 1987). Given the importance of maintaining sediment integrity (Burton 1991; ASTM 1991; Stemmer et al. 1990; Sasson and Burton 1991) in contamination assessments, *in situ* measures are preferred. The precision of SOD measurements is largely a function of the level of operator experience.

The range of diurnal variation must be defined during baseflow and post-event conditions. By sampling three to four times daily over 2 or 3 days, this range may be established (EPA 1986). If DO variations are extreme, then sampling and modeling requirements will be more complex.

Photosynthesis/Respiration (P/R) Rate Analyses

Photosynthesis/respiration measurements are needed to measure local eutrophication problems and to evaluate the potential effects of discharges on receiving waters. Many receiving water quality

models also require photosynthesis and respiration rates in order to calculate dissolved oxygen conditions. Accurate values are important, and "default" values can be very misleading. Therefore, local measurements are strongly recommended. Traditional P/R analyses require the use of light and dark bottles (typically BOD bottles, one set clear, the other set wrapped in aluminum foil). The bottles are filled with the test water, an initial DO is measured, the dark bottles are wrapped, and the bottles are submerged in the waterbody of concern. Every few hours, a set of light and dark bottles (usually at least three of each) is removed and the bottle DO is measured. This is repeated during the day, typically from late morning until midafternoon, obtaining from three to five sets of observations. The DO values are plotted and the trends are measured. Thomann and Mueller (1987) describe the test and data evaluation procedures. The light bottles undergo both photosynthesis and respiration, while the dark bottles only undergo respiration. The P/R rates vary greatly depending on the local conditions. As an example, tests can be conducted in urban streams in full sun, in the shade, in shallow water, and in deep water. Weather conditions (cloud cover, obviously, and temperature) all affect the P/R rates. These variations can all be very important and should be considered when modeling oxygen conditions in urban streams.

Case Study to Measure in Situ *P/R Rates*

The following is a discussion of a more efficient method of measuring P/R rates *in situ*, using a plastic bag test chamber and a continuous water quality monitoring probe, as demonstrated by Easton et al. (1998) as part of an EPA-sponsored project investigating SSO discharge effects in Birmingham, AL. The advantage of this method is that a tremendous amount of data can be collected in a very efficient manner. The only personnel time required is that needed to calibrate the instruments, set up the chambers, retrieve the chambers, and evaluate the data. The probes can be programmed to obtain DO (along with other parameters of interest) every 5 to 15 min for an extended period (up to several weeks). This allows the effects of changing weather (cloud cover, temperature shifts, rains, etc.) on the P/R rates to be directly measured. In addition, numerous replicates of the rates can be easily obtained when the probes are left out for an extended period. These are all significant advantages over conventional light and dark bottle P/R tests. The following case study demonstrates the type of information that can be obtained using this technique, along with the appropriate data analysis procedures.

This study used YSI 6000 UPG sondes. The important aspect of this sonde that allows these tests to be conducted is the rapid-pulse DO probe that consumes very little dissolved oxygen. Measured DO changes in the test chambers are therefore associated with the oxidation of wastes and are not significantly affected by measurement artifacts (including drift). In addition, the long-term monitoring capability of the unit enables many diurnal cycles to be measured efficiently. Also, the other measurements (especially pH, ORP, and conductivity) are very useful in indicating associated water quality changes in the test chamber and offer additional insight into the local P/R process. During this study, the YSI 6000 sondes were used to evaluate *in situ* P/R rates of different mixtures of raw sewage and fresh water. The sondes were calibrated for the following experimental parameters: depth, specific conductance, dissolved oxygen, turbidity, pH, oxidation-reduction potential (ORP), and temperature. The sondes were also programmed to acquire data in unattended mode for 2 weeks at 15-min intervals.

There are several biological processes that were apparent from monitoring the water quality. During the daylight hours, photosynthetic organisms, such as algae, use energy derived from the sun to produce ATP (adenosine triphosphate) and NADPH (reduced nicotine adenine dinucleotide phosphate) — reactions that generate oxygen. Then, the energy (ATP) and reducing power (NADPH) are used to fix carbon dioxide (CO_2) into carbohydrate (Filip and Alberts 1994). Simultaneously, photosynthetic organisms and any other aerobic organism, such as fish and certain types of microorganisms, use oxygen to break down carbohydrates for energy. This process occurs during the daylight and nighttime hours. Therefore, there is a constant drain on levels of dissolved oxygen in the water that

must be replenished by photosynthesis and/or exchange with the atmosphere. The net effect of these processes is that the dissolved oxygen level in the water rises during the daylight and falls at night. In addition, the pH of typical receiving waters is governed by the carbonic acid/bicarbonate/carbonate buffering system. Increases in the dissolved CO_2 concentration causes corresponding decreases in pH, and vice versa. Therefore, the pH increases during the daytime hours because CO_2 is being fixed by photosynthetic organisms and is thereby removed from the water. Then, at night, pH drops because atmospheric CO_2, and CO_2 being produced by respiration, increase the concentration of CO_2 in the water.

Figure 6.62 *In situ* P/R tests being conducted.

The raw sewage was obtained at a local sewage treatment plant. The site for the tests was a small private lake that rarely, if ever, received sanitary sewage. Four different mixtures of sewage and fresh lake water (0/100%, 33/67%, 67/33%, and 100/0% sewage/fresh water) were prepared in their respective test chambers (20-L clear plastic bags containing 15 L of the test water mixture). The sondes were sealed in the bag with as little air trapped inside as possible. The test chambers and sondes were placed on the lake bottom in approximately 1 to 2 ft of water near the shore with full sun during daylight hours (Figure 6.62).

The temperature results showed increasing temperatures with time, consistent with typical spring conditions. The range on day 1 was 20 to 23°C; while the range on day 10 was 23 to 25°C. A diurnal variation of about 3 to 4°C was also observed — again, typical for the day/night solar cycle. It is important to note that the last 2 days were overcast with scattered heavy rains and variable winds, and therefore the diurnal variation was less than it was on days with full sun. The temperature data also show that the results for each of the four probes were quite consistent, except that the 33% sewage chamber did not reach as high a daily peak as the others. It is possible that differences in the temperatures may have been due to differences in the color of the water/algae mixture. The large amount of green biomass observed in the 33% sewage chamber may have acted to moderate the extreme temperature levels found in the other chambers that did not have such a large algal growth.

The pH results were also as predicted. There was a diurnal variation, at least in the test chambers that had photosynthesis occurring: 33% sewage (daily pH change ≅ 1 to 2, after day 7) and 0% sewage (daily pH change ≅ 0.25). This is due to the change in CO_2 concentrations from photosynthesis (removal of dissolved CO_2) and respiration (addition of dissolved CO_2). An increase in dissolved CO_2 causes the pH to decrease from the formation of more carbonic acid, while removal of dissolved CO_2 increases pH.

The results for oxidation-reduction potential (ORP) were also as expected. The test chambers with high oxygen demand, and corresponding reducing environment (67 and 100% sewage), dropped rapidly to less than −400 mV within the first few hours of the experiment, and stayed there. The ORP in the 33% sewage chamber was similar to the 67 and 100% for the first 5 days, but then began to climb, reaching positive ORP values by day 6. This result is well correlated with the DO data, showing that after an initial acclimation period, the algae and other microorganisms began to respire and photosynthesize. The 0% sewage chamber showed a definite diurnal trend and stayed above 300 mV, two factors that correlate well with the diurnal DO cycle resulting from P/R.

The dissolved oxygen data were used to calculate P/R rates for the microorganisms in the test chambers (Figure 6.63). The 0% sewage test chamber contained a 5-day biochemical oxygen demand (BOD_5) of approximately 2.5 mg/L. Therefore, there was a general downward trend in

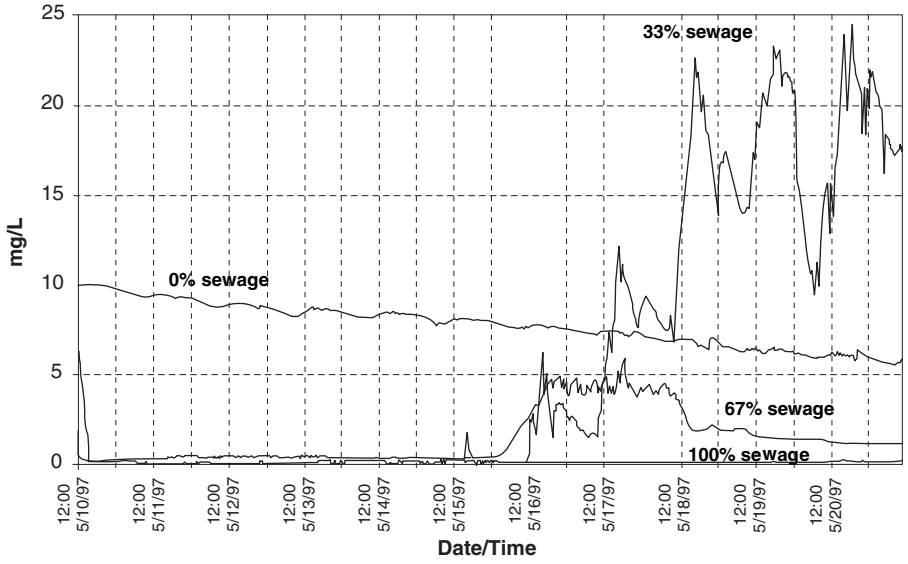


Figure 6.63° Dissolved oxygen data for all four probes over 10-day experiment. (From Easton, J.H., Lalor, M., Pitt, R., and Newman, D.E., The use of a multi-parameter water quality monitoring instrument to continuously monitor and evaluate runoff events. Presented at *Annual Water Resources Conference of the AWRA*, Point Clear, AL. 1988. With permission.)

dissolved oxygen levels over the 10-day period of the experiment, typical for a eutrophic lake. The water body where this study was conducted rarely, if ever, received sanitary sewage. In this case, an acclimation period was expected. However, if the water body had received regular discharges of sewage, the long acclimation period would most likely not be observed. The 33% sewage chamber had initial anoxic conditions, but after acclimating for approximately 5 days, there was a pronounced diurnal P/R variation. Indeed, the DO levels in this chamber were supersaturated during the daylight hours, as photosynthesis rates were very high. When this chamber was pulled at the end of the experiment, there was a large amount of green biomass, indicating the presence of photosynthesizing organisms. The 67% sewage test chamber stayed at anoxic DO levels, as expected. However there was an increase in DO on the 5th and 6th days. Possibly, the organisms in this chamber began photosynthesizing after acclimating to the sewage, but the oxygen demand of the waste quickly drove the DO levels to anoxic levels shortly thereafter. The 100% test chamber stayed anoxic throughout the experiment, as anticipated.

The rates of P/R were analyzed using the following methods. First, after analyzing the data for the entire length of the experiment, it was determined that the data from only the last 5 days would be used to calculate rates of P/R; these days occurred after the acclimation period. An analysis of the dissolved oxygen data given in Figure 6.63 showed that the rates of P/R would be impossible to determine from the 67 and 100% sewage samples because the DO levels were essentially zero. Therefore, the methods were applied only to the 0 and 33% sewage samples. In the future, further experiments should be done to look at sewage dilutions between the 0 and 33% levels. Most examples of raw sewage discharges into receiving waters (such as for SSOs) likely only comprise a few percent of the receiving flow. Plots were then created of the 0 and 33% sewage results for this 5-day period, as shown in Figures 6.64 and 6.65, for detailed analysis.

These plots were inspected visually, and lines were drawn on positive slope portions and negative slope portions of the graphs. The positive slopes (occurring during daylight hours) represented periods of photosynthesis minus respiration (p_{net}), while the negative slopes (occurring during nighttime hours) represented periods of respiration (R). The R rate was then subtracted from the p_{net} rate to obtain an hourly photosynthesis rate. The mean values were:

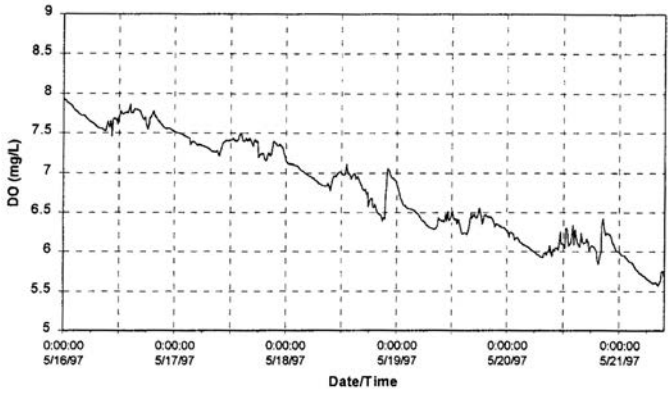


Figure 6.64° Dissolved oxygen data for 0% sewage. (From Easton, J.H., Lalor, M., Pitt, R., and Newman, D.E., The use of a multi-parameter water quality monitoring instrument to continuously monitor and evaluate runoff events. Presented at *Annual Water Resources Conference of the AWRA,* Point Clear, AL. 1998. With permission.)

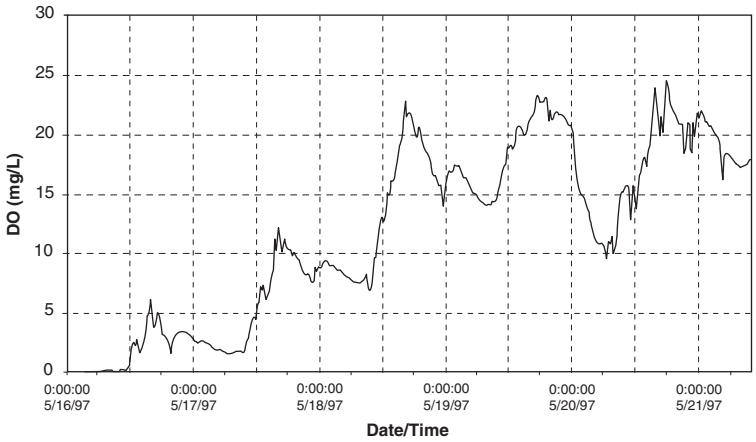


Figure 6.65° Dissolved oxygen data for 33% sewage. (From Easton, J.H., Lalor, M., Pitt, R., and Newman, D.E., The use of a multi-parameter water quality monitoring instrument to continuously monitor and evaluate runoff events. Presented at *Annual Water Resources Conference of the AWRA,* Point Clear, AL. 1998. With permission.)

0% sewage: p_{net} = 0.04 mg/L·hr, R = –0.05 mg/L·hr, P = 0.09 mg/L·hr, and

33% sewage: p_{net} = 1.16 mg/L·hr, R = –0.47 mg/L·hr, P = 1.63 mg/L·hr

The next step in determining the photosynthesis rate was to apply the daily average DO model (Thomann and Mueller 1987). The respiration rate is assumed constant throughout the day. The hourly rates determined previously were multiplied by 24 hours to give a respiration rate in units of mg/L·day. The photosynthetic oxygen production is assumed sinusoidally distributed over the photoperiod from 6:00 am to 7:00 pm for these conditions. These results are given in Table 6.25.

The photosynthesis rates for the 33% sewage were extremely high and variable, ranging from 12 to 30 mg/L·day; and the rates for the 0% sewage (100% lake water) were typical, approximately 1 to 2 mg/L·day. Typical local surface water photosynthesis values of approximately 1 to 4 mg/L·day have been obtained from previous experiments with light and dark bottles in local natural waters (Lake Purdy and the Cahaba River during other student projects at UAB).

Table 6.25 Calculated Values for the Estimated Daily Averaged Photosynthetic Oxygen Production Rate (p_a)

	0% Sewage				33% Sewage			
Date	p_{net} (mg/L·day)	Respir (mg/L·day)	p′ (mg/L·day)	p_a (mg/L·day)	p_{net} (mg/L·day)	Respir (mg/L·day)	p′ (mg/L·day)	p_a (mg/L·day)
5/16/97	1.19	0.91	2.10	1.06	19.37	5.25	24.62	12.47
5/17/97	0.94	0.85	1.79	0.90	28.62	6.30	34.92	17.68
5/18/97	1.19	1.70	2.89	1.46	47.57	12.59	60.17	30.47
5/19/97	0.85	1.00	1.85	0.94	18.32	22.39	40.70	20.61
5/20/97	0.91	1.60	2.51	1.27	25.19	10.40	35.59	18.02
Mean	1.01	1.21	2.23	1.13	27.81	11.39	39.20	19.85
Std. dev.	0.16	0.41	0.47	0.24	11.83	6.84	13.09	6.63
COV	0.16	0.34	0.21	0.21	0.43	0.60	0.33	0.33

From Easton, J.H., Lalor, M., Pitt, R., and Newman, D.E., The use of a multi-parameter water quality monitoring instrument to continuously monitor and evaluate runoff events. Presented at *Annual Water Resources Conference of the AWRA,* Point Clear, AL. 1998. With permission.

Recommendations for P/R Investigations

Site-specific photosynthesis and respiration measurements are needed whenever an in-depth DO investigation (especially to support TMDL analyses) is required. DO has traditionally been one of the most significant indicators of poor receiving water conditions, and many regulatory agencies heavily rely on DO predictions. However, wet-weather flow effects on DO are typically unclear, especially considering the relatively slow effect stormwater has on BOD. Nutrient discharges associated with wet-weather flows can also dramatically affect P/R conditions in a receiving water. Actual measurements of these rates for all of the wastewaters affecting a receiving water can lead to much more accurate in-stream DO predictions.

The *in situ* sonde method for measuring P/R described above is an improved procedure for studying P/R compared to conventional methods (light/dark bottle testing). The data collected are far more useful because they are continuous and collected over multiple day/night cycles. This enables daily variations to be quantified and to account for weather changes. The high-resolution data also enable the identification of periods of questionable data associated with the acclimation period at the beginning of the test period.

WATER AND SEDIMENT ANALYTES AND METHODS

Selection of Analytical Methods

Environmental researchers need to be concerned with many attributes of numerous analytical methods when selecting the most appropriate methods to use for analyses of their samples. The main factors that affect the selection of an analytical method include: cost, reliability (the "data quality objectives," or DQO, discussed earlier in Chapter 5, which includes sensitivity, selectivity, repeatability), and safety. Another factor to be considered is whether the analyses should/can be conducted in the field or in the laboratory. These items can be subdivided into many categories including:

- Capital cost, costs of consumables, training costs, method development costs, age before obsolescence, age when needed repair parts or maintenance supplies are no longer available, replacement costs, other support costs (data management, building and laboratory requirements, waste disposal, etc.)
- Sensitivity, interferences, selectivity, repeatability, quality control, and quality assurance reporting, etc.
- Sample collection, preservation, and transportation requirements, etc.
- Long-term chemical exposure hazards, waste disposal hazards, chemical storage requirements, etc.

Most of these issues are not well documented in the literature for environmental sample analyses. Aspects of analytical reliability have received the most attention in the literature, but most of the other aspects noted above have not been adequately discussed for the many analytical alternatives available, especially for field analytical methods. It is therefore difficult for a water quality analyst to decide which methods to select, or even if a choice exists.

The selection of the appropriate procedure depends on the use of the data and how false negatives or false positives would affect water use decisions or regulatory questions. The QA objectives for the method detection limit (MDL) and precision (RPD) for the compounds of interest have been shown to be a function of the anticipated median concentrations in the samples (Pitt and Lalor 1998). The MDL objectives should generally be about 0.25, or less, of the median value for sample sets having typical concentration variations (COV values ranging from 0.5 to 1.25), based on many Monte Carlo evaluations to examine the rates of false negatives and false positives. The precision goal is estimated to be in the range of 10 to 100% (Relative Percent Difference of duplicate analyses), depending on

Table 6.26 Summary of Quantitative QA Objectives (MDL and RPD) Required for an Example Stormwater Characterization Project

Constituent	Units	Example COV Category[a]	Example Median Conc.	Calculated MDL Requirement	Calculated RPD Requirement
pH	pH units	Very low	7.5	Must be readable to within 0.3 unit	<0.3 unit
Specific conductance	µmhos/cm	Low	100	80	<10%
Hardness	mg/L as $CaCO_3$	Low	50	40	<10%
Color	HACH units	Low	30	24	<10%
Turbidity	NTU	Low	5	4	<10%
COD	mg/L	Medium	50	12	<30%
Suspended solids	mg/L	Medium	50	12	<30%
Particle size	size distribution	Medium	30 µm	7 µm	<30%
Alkalinity	mg/L as $CaCO_3$	Low	35	30	<10%
Chloride	mg/L	Low	2	1.5	<10%
Nitrates	mg/L	Low	5	4	<10%
Sulfate	mg/L	Low	20	16	<10%
Calcium	mg/L	Low	20	16	<10%
Magnesium	mg/L	Low	2	1.5	<10%
Sodium	mg/L	Low	2	1.5	<10%
Potassium	mg/L	Low	2	1.5	<10%
Microtox toxicity screening	I20 or EC50	Medium	I20 of 25%	I20 of 6%	<30%
Chromium	µg/L	Medium	40	9	<30%
Copper	µg/L	Medium	25	6	<30%
Lead	µg/L	Medium	30	7	<30%
Nickel	µg/L	Medium	30	7	<30%
Zinc	µg/L	Medium	50	12	<30%
1,3-Dichlorobenzene	µg/L	Medium	10	2	<30%
Benzo(a)anthracene	µg/L	Medium	30	8	<30%
Bis(2-ethylhexyl)phthalate	µg/L	Medium	20	5	<30%
Butyl benzyl phthalate	µg/L	Medium	15	3	<30%
Fluoranthene	µg/L	Medium	15	3	<30%
Pentachlorophenol	µg/L	Medium	10	2	<30%
Phenanthrene	µg/L	Medium	10	2	<30%
Pyrene	µg/L	Medium	20	5	<30%
Lindane	µg/L	Medium	1	0.2	<30%
Chlordane	µg/L	Medium	1	0.2	<30%

[a]

COV value:	Multiplier for MDL:	RDL Objective:
<0.5 (low)	0.8	<10%
0.5 to 1.25 (medium)	0.23	<30%
>1.25 (high)	0.12	<50%

From Pitt and Lalor 1998.

the sample variability. Table 6.26 lists the typical median stormwater runoff constituent concentrations and the associated calculated MDL and RPD goals, for a typical stormwater monitoring project.

In some cases, field test kits, or especially continuous *in situ* monitors, may be preferred over conventional laboratory methods. Table 6.27 lists some of the benefits and problems associated with each general approach. The advantages of field analytical methods can be very important, but their limitations must be recognized and considered.

The environmental researcher also must be concerned with sampling costs (discussed in Chapter 5), in addition to analytical costs. Most environmental research efforts are not adequately supported to provide the necessary numbers of samples needed for statistically reliable results to support typical (lofty) project goals. Expensive recommendations are therefore commonly made based on too small an analytical investment. The number of samples needed to simply characterize a water quality constituent can be estimated based on the expected variability of the constituent and on the

Table 6.27 Comparisons of Field and Laboratory Analytical Methods

Field Analytical Methods		Conventional Laboratory Methods	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Minimal change in sample character because no transport and storage. Opportunity to collect replacement sample if questionable results, or if sample is damaged. Results generally available soon after sample collection. Continuous *in situ* monitors result in large numbers of observations with fine resolution.	Difficult to control environmental variables affecting analytical measurements and working conditions. Individual samples usually analyzed separately with more time required per sample. Additional time needed to set up equipment and standardize procedure for each location. Analytical hazardous waste (and sharps) management may be a problem. Many field analytical reagent sets are sensitive to storage conditions that may be difficult to meet. Documentation can be incomplete and hazards not described. Generally poor limits of detection and limited working range. Some of the most sensitive tests are very complex with analytical errors common.	Good control of laboratory working conditions and use of in-place hazardous waste management. Can analyze several samples in one batch. More precise equipment generally used for analyses, and less time to set up for analyses. Easier to conduct and meet QA/QC requirements. Usually much lower limits of detection.	Need to preserve samples and conduct analyses in prescribed period of time. Results may not be available for an extended time after sample collection. Minimal opportunity to re-sample due to errors. Generally more expensive and sample numbers are therefore limited. Sample storage space-consuming and requires logging system for sample tracking.

allowable error of the result. As an example, 40 samples are needed to estimate the average concentration with an allowable error of 25%, if the coefficient of variation of the constituent measurements is about 0.8. If only 10 samples are evaluated, the error increases to a possibly unusable 100%. Analyses of toxicants of great interest in many research activities currently can cost many hundreds of dollars per sample for a short list of organic and heavy metal compounds. A simple effort to adequately characterize the conditions at a single location can therefore cost more than $25,000, as shown in Chapter 4. Clearly, there is a great need to be able to afford to collect and analyze a sufficient number of samples. The following discussion therefore presents several methods of collecting the needed data, including continuous *in situ* monitors, simple field test kits, and conventional laboratory analyses.

Use of Field Methods for Water Quality Evaluations

There are many problems with current environmental sampling and analysis programs that can be met by conducting water quality evaluations in the field, especially if continuous, *in situ* procedures are used. Foremost among these problems is the need to collect many samples in order to obtain the desired accuracy of the characteristics of interest. Other concerns involve inadvertent changes that may affect the sample characteristics between sample collection and analysis. The high cost of analyzing trace levels of organic and metallic toxicants using conventional laboratory procedures is also restrictive, but field methods for these analytes are very expensive, complex to use, or not very sensitive. The following discussion covers *in situ* monitoring and the use of field test kits.

In Situ *Physicochemical Monitoring*

One way to collect adequate data is to use simple field analytical methods, preferably continuously recording *in situ* analyses. These methods allow a great amount of data to be collected without sample collection, transportation, or laboratory problems. However, new problems arise, specifically related to long-term reliability and costs of the instrumentation. Many of these instruments are currently available, but they are restricted to only a few of the common constituents (usually temperature, conductivity, dissolved oxygen, and pH, plus turbidity on a few units) and can cost from $3000 to $7000. The newest and most reliable units can be placed in a water body and left unattended for several weeks to months before requiring service. They can continuously record these constituents over this time with very high resolution, enabling a much greater understanding of the dynamics of the pollutant behavior in the water body. Unfortunately, the constituents currently capable of being continuously and automatically monitored do not include many of the most interesting. Some ion-selective electrode (ISE) probes are being offered as options on some of the continuous *in situ* probes. Unfortunately, their reliability is not well established, but they may be very useful for shorter-term and specialized projects.

In situ monitors give continuous and relatively rapid results, in contrast to typical field test kits, which require time and patience to evaluate the chemical parameters of interest. Unfortunately, these are all relatively costly instruments. However, their capabilities cannot be matched using other procedures. These instruments can be separated into two general categories. *In situ* probes, having real-time display capabilities, but with limited data logging capabilities, are designed for real-time monitoring. The other category includes continuously recording probe units that are designed for long-term unattended operation, but are commonly also available with direct read-out displays for real-time use. Examples of both types have been available for more than 20 years.

In Situ *Direct-Reading Probes*

The simplest direct-reading probes that perform their analyses *in situ*, with no sample preparation, include the classical series of field instruments from YSI, such as their DO probe and SCT (salinity, conductivity, and temperature) probe. These are very robust instruments that have been in use at many institutions for decades. The original models of the DO probes did require practice to replace the membranes, and they required relatively frequent (but simple) recalibration. Newer YSI models, especially these utilizing the rapid pulse current probe, exhibit much slower drift and are designed for long-term unattended operation.

Other direct-reading instrumentation includes pH and ORP instruments. These generally are more sensitive to storage conditions and require frequent maintenance or calibrations. Some of the newer dry pH electrodes are very robust and much more reliable and easier to use. Ion selective electrodes (ISE) are sometimes included in this category and various equipment vendors offer them as options for their direct-reading *in situ* probes. It is suggested that careful and frequent evaluations be made of any electrode-equipped equipment (especially pH and ISE) to ensure that the instrument is operating properly and that the probe has not dried out or been damaged by oils or detergents.

Some direct-reading *in situ* probes are available that have the capability to measure several parameters. Most of these are designed for long-term unattended operation, but somewhat less expensive versions are also available that have minimal data logging capabilities and are designed for real-time measurements. The Horiba U-10, for example, was evaluated by Day (1996). It costs about $2500 from Hazco (800-332-0435, catalog # B-H020001) and can simultaneously measure conductivity, temperature, salinity, dissolved oxygen, turbidity, and pH. Hazco also rents the Horiba U-10. It is especially useful for real-time profiling of shallow lakes and small urban streams. Relatively few probes offer turbidity, which is helpful when examining light penetration and algal activity. Solomat and YSI also have hand-held instruments having capabilities similar to those of the Horiba U-10.

Other instrumentation is also available that can monitor hydrocarbon conditions in water on a real-time basis. The Turner 10-AU field fluorometer with “oil in water” optics is extremely sensitive

Figure 6.66 Petrosense being calibrated.

Figure 6.67° Petrosense used to measure hydrocarbons in manhole water.

and is used with no sample preparation. It can be used in a flow-through mode to map hydrocarbon concentrations in real time. It can also be used as a stand-alone instrument for long-term unattended operation, if properly housed. This instrument costs from $8000 to $16,000, depending on housing, data logging, and filter options, and is therefore not likely to be readily available. This instrument is also used for fluorescent tracer analyses (using Rhodamine WT) for primary calibration of water flow equipment. It can also be used for limited real-time chlorophyll *a* analyses, when using appropriate optics and filters.

The Petrosense hydrocarbon probe from FCI Environmental is also available for real-time hydrocarbon analyses (Figures 6.66 and 6.67). This instrument costs about $7000, has a slower response time (about 5 min), and is not nearly as sensitive (about 100 µg/L, as xylene) as the fluorometer. It can also be used in real time to monitor "total" hydrocarbons in water, with no sample preparation. It quantifies hydrocarbons by measuring changes in optical properties caused by hydrocarbon adsorption onto an exposed fiber optic.

Continuously Recording and Long-Term In Situ Measurements of Water Quality Parameters

Several classical instruments have long been available to measure various water quality parameters with unattended instruments for relatively long periods of time. Hydrolab and YSI have long offered equipment that can monitor dissolved oxygen, pH, temperature, and conductivity unattended. The early instruments were plagued with stability problems and were usually most suited for unattended operation over a period of only about a day. This was still a major breakthrough, as it enabled diurnal fluctuations of these important parameters to be obtained accurately and relatively conveniently.

Currently available equipment, in contrast, has been demonstrated to be capable of unattended operation for longer than a month. These are relatively expensive instruments that can cost up to $7000 each, depending on options selected. Examples of equipment currently available include the 803 probe series from Solomat, which can have up to eight sensors installed. These may include pH, ORP, DO, temperature, conductivity, depth, ammonium, nitrite, and other ions by ISE. Several meters and data loggers are available for hand-held real-time measurements, or for long-term unattended operation. YSI also offers several *in situ* probe instruments. The original YSI unit available many years ago (Figure 6.68) was a breakthrough unit that enabled overnight DO measurements. The current 6000 series sonde is much improved (Figure 6.69). It is self-contained, measuring and logging up to nine separate

Figure 6.68 Older YSI DO meter for continuous monitoring. (Courtesy of Wisconsin Department of Natural Resources.)

Figure 6.69 YSI 6000 sonde detail showing several probes.

parameters simultaneously, including DO, conductivity, temperature, pH, depth, ORP, nitrate, ammonium, and turbidity. The rapid pulse DO and self-wiping turbidity sensors enable very long unattended operations (up to 45 days), with minimal fouling or drift. Hazco (800-332-0435) sells the YSI 6000 basic sonde (catalog # B-6001) for about $7000. The unit without the depth sensor is about $500 less. The performance specifications for the more common sensors, provided by the manufacturer, are given in Table 6.28. Appendix E contains detailed instructions for calibrating and setting up this sonde.

These unattended instruments are capable of collecting high-resolution data (typically with observations every 5 to 15 min) over long periods. This is extremely useful in receiving water studies affected by stormwater. Even though few dissolved oxygen problems have ever been associated with stormwater (in contrast to CSOs), these probes are unexcelled in documenting the

Table 6.28 YSI6000 Specifications

Parameter	Sensor Type	Range	Accuracy	Resolution
Dissolved oxygen % saturation	Rapid pulse — Clark-type, polarographic	0 to 200% air saturation	±2% air saturation	0.1% air saturation
Conductivity[a]	4 electrode cell with autoranging	0 to 100 mS/cm	±0.5% of reading + 0.001 mS/cm	0.01 mS/cm
Temperature	Thermistor	–5 to 45°C	±0.15°C	0.01°C
pH	Glass combination electrode	2 to 14 units	±0.2 units	0.01 units
ORP	Platinum ring	–999 to 999 mV	±20 mV	mV
Turbidity	Optical, 90° scatter, mechanical cleaning	0 to 1000 NTU	±5%	0.1 NTU
Depth — Medium	Stainless steel strain gauge	0 to 61 m	±0.12 m	0.001 m
Depth — Shallow	Stainless steel strain gauge	0 to 9.1 m	±0.06 m	0.001 m

[a] Report outputs of specific conductance (conductivity corrected to 25°C).

exposure periods and gross variations in receiving water conditions over many separate storm events. These data are very important when used in conjunction with *in situ* toxicity test chambers that are exposed for relatively long periods of time. In addition, the YSI self-contained probes with rapid-pulse DO sensors (the probes consume very little power and oxygen themselves) can be used in light and dark chambers to conveniently obtain necessary data pertaining to sediment and water photosynthesis and respiration, as previously described.

Field Test Kits

Field test kits cover a wide range of instrumentation and methods. They range from very simple visual comparator tests (which use colored paper, colored solutions in small vials, or color wheels to match against the color developed with the test) to miniaturizations of standard laboratory tests (using small spectrophotometers or other specialized instruments). Appendix E contains listings and photographs of selected field procedures. Appendix E also contains a summary of the tested performance of several representative field test kits, highlighting their performance (limits of detection, repeatability, and recovery), hazards associated with their use, complications and time requirements, approximate costs, and other notes (Day 1996).

The least expensive test kits use small droppers or spoons to measure reagents into a reaction tube where the color is developed. More sophisticated tests use small filter colorimeters to more precisely measure the color developed during the test. HACH also offers continuous wavelength field spectrophotometers that are capable of measuring a wide variety of chemical parameters using a single instrument (Figure 6.70). La Motte has a filter colorimeter that contains several filter sets, also enabling many different chemical analyses to be conducted with the one instrument. HACH also has a field titration kit that is also very flexible, providing additional capabilities not available with spectrophotometric methods. These multiparameter instruments are usually superior to the simple dedicated test kits because of the increased sensitivity and precision that is achievable with the better equipment. They, of course, cost more. If only one or two parameters are to be monitored in the field, then it might be hard to justify the added cost of the more flexible instruments. However, if the best quality data are needed, the cost may be justified, especially if more than a few parameters are to be measured.

Also included in the category of field test kits are very sophisticated methods that are laboratory instrumentation and procedures that have been miniaturized and simplified. Some of these tests even meet the EPA reporting requirements for NPDES permit compliance. However, some of the field procedures skip certain sample cleanup or digestion steps that would be impractical to conduct in the field and are therefore not suitable for compliance monitoring. It is important to check with the field equipment suppliers and the reviewing regulatory agency to verify the current status of a field method for various reporting purposes. Many of the spectrophotometer and titration methods fall into this category of simplified laboratory methods. Several new instruments are also available that permit sensitive and precise heavy metal (especially copper and lead) analyses in the field.

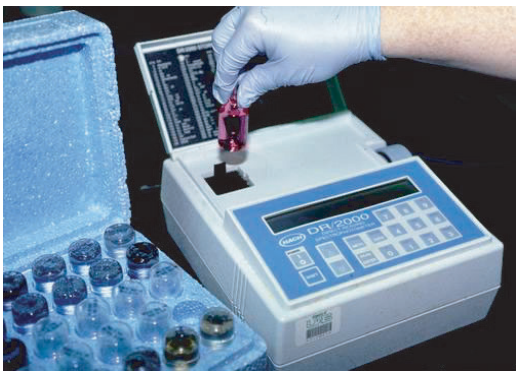


Figure 6.70 HACH DR/2000 field spectrophotometer.

However, these instruments are expensive (equipment costs of $2000 to $4000 and per sample costs of $5 to $15). They are also not sensitive to particulate-bound metals (which may be an advantage, depending on study objectives).

The biggest difficulty with almost all of these field test kits is that they can require a substantial amount of time to evaluate the water sample, especially when only one sample at a time is being analyzed. Continuous and *in situ* monitors eliminate field analytical time. Some of the simple *in situ* monitors are included in this test kit discussion (such as conductivity meters, pH meters, and DO meters), while the more complex continuously recording units were discussed previously. Even though these field test kits enable personnel to evaluate samples at the point of collection, that may not be desirable. Lalor (1993) and Pitt et al. (1994) found that test kit performance was greatly enhanced by bringing the collected samples to a temporary "laboratory" for analyses. This greatly increased sample analytical through-put, as many of the test kits enabled multiple samples to be analyzed at one time. This is especially critical if sampling locations are widely spaced and the alternative is to analyze many parameters at each location before moving to the next sampling location. It may take more than an hour to conduct a relatively few chemical tests at each location, including setting up equipment and restandardizing procedures. However, if many samples are being collected in a small area, the equipment can be left in one place and simultaneous sample analyses would be possible in the field. Indoor facilities should be sought, because protection from weather, available electricity, good lighting, and water enhance analytical performance. Make sure that adequate ventilation is available, however, wherever the tests are conducted. Many of the field test kits are not well labeled, especially concerning hazardous materials in the kit that require special protection and disposal practices.

Safety issues, along with test kit performance, have been examined (Pitt et al. 1994; Day 1996). The test kit evaluations were based on "fatal flaws" of the alternative equipment available for each parameter category. In the series of tests conducted by Day (1996), 50 test kits were subjected to preliminary evaluations with half further subjected to more detailed tests. His results are summarized in the following discussions. Safety hazards, cost, poor detection limits, matrix interferences, limited concentration ranges, poor response factors, and complexity of the test kits were all reasons for rejection. The most suitable test kits in each category were then identified, after rejecting those kits that were much more expensive than alternatives in each category. The comparison of field screening equipment is a somewhat objective process. Some parameters of interest are easily quantified; other features that should be evaluated require more objective evaluation techniques. Therefore, these evaluations were made using both subjective and objective information. The evaluation of the kits was based on five major tests:

1. Subjective evaluations of the health and safety features (kit reagent contents, design features to minimize operator exposure to hazardous reagents, disposal problems, and warnings)
2. Performance using samples spiked with known pollutant additions in "clean" and "dirty" water
3. Comparisons with standard laboratory procedures using parallel analyses of typical samples
4. Repeatability and precision using replicate analyses
5. Complexity of each method

The first tests for each method used spiked samples. The reported ranges for each kit were used to define a gross range of all methods for each parameter. The gross range was bounded by the lowest reported detection limit and the highest upper limit reported by the manufacturers. Two series of samples were prepared, one using reverse osmosis (RO) water and another using a composite of parking lot runoff water. The number of samples prepared varied by parameter depending on the magnitude of the gross range. RO and runoff water blanks were also prepared and tested for each parameter. RO water served as a control for identifying optimal test kit performance (assuming low ionic strength effects did not adversely affect the test). The parking lot runoff water was used to detect any significant matrix interferences. The runoff water was collected from a UAB parking lot.

The spiked standards were evaluated by all methods for each parameter. Due to the large number of methods that were evaluated, no replicate analyses were initially made. In most cases, these kit

methods are used as field screening methods to detect potential problem pollutants in relatively high concentrations. During these analyses, data were collected on "useful" range, capital costs, expendable costs, analysis time, health and safety considerations, and "usability." These parameters are described below:

- Useful range: the range of concentrations that the instrument can measure with some certainty. The lower limit is defined by the detection limit. The upper limit is defined by the highest concentration the method can measure without dilution of the sample. The upper limit values were determined as the lowest spike concentration producing an "over range" error, or the lowest concentration that obviously deviated from the linear range of spike concentration to instrument response. If neither problem was identified, the manufacturer's reported upper limit was reported.
- Capital costs: the initial costs associated with purchasing the capital equipment required to use the method. Prices were obtained from the manufacturers during April 1996.
- Expendable costs: the costs associated with buying replacement reagents for the method. The value reported is per sample. The costs do not include general glassware, tissues, gloves, and other generic equipment required for many of the tests. The prices were obtained from the manufacturers during April 1996. The costs reported are based on list price of the smallest quantity of reagent available, and, therefore, the costs do not reflect bulk discounts which might be available.
- Analysis time: the approximate time to analyze one sample at a time with the method. In some cases, additional time must be allotted to prepare the method for measurement. For example, all analyses assume any needed instrument has been properly calibrated before analysis begins. In some cases, multiple tests can be performed simultaneously.
- Health and safety considerations: the health and safety considerations are a broad scope of factors that represent potential hazards to the user or the environment. The factors considered in this analysis include the hazardous nature of the reagents used, the packaging of the reagents, required disposal of reagent and sample wastes and waste glass, and the potential exposures or any feature of the kit requiring special attention.
- Usability: this ubiquitous term is a subjective evaluation of the expertise required to perform an acceptable analysis. Under this heading, an attempt was made to describe any feature of the kit that may not represent a hazard, but could affect the quality of the test. Examples of factors affecting usability include the number of steps, complexity of the procedure, additional equipment to make the procedure easier, limited shelf life of the kit, or any special skill required to complete the analysis.

From three to seven spiked samples were analyzed using each method for both RO and runoff water sample matrices. For each matrix, a plot of instrument response to spike concentration was made. This was used to estimate the range of linear response of the instrument. Spike responses showing a significant departure from a linear response indicate the range of the method. A regression analysis was performed on the data providing further information about the method. Ideally, the slope generated from these regression analyses (response factor) should be 1. A slope significantly different from 1 indicates a bias in the method. Also, the slope of the regression in the RO water matrix should be the same as the slope of the regression in the runoff water matrix. The difference in the slopes between matrices indicates the magnitude of matrix interference associated with the method. The value of the standard error of the regression was used to estimate the detection limit of the method, using the following equation (McCormick and Roach 1987):

$$D.L. = y_0 + s_y z_a$$

where D.L. = detection limit of the method
y_0 = the intercept of the regression equation
s_y = standard error of the regression
z_a = the area under the normal curve associated with a one-tail probability for a given confidence level (these analyses used the 95% confidence level, with a = 0.05)

Concentrations exceeding the detection limit only indicate the presence of the parameter. The equation may be modified to calculate the limit of quantification. Reported concentrations exceeding the limit of quantification may be used to quantify the results. The modified equation is:

$$LOQ = y_0 + 2s_y z_a$$

Therefore, the LOQ is approximately twice the D.L., if the intercept of the regression line is very small (as it should be). For example, if the D.L. is calculated to be 0.5 mg/L and the LOQ is calculated to be 1.0 mg/L, the following statements are true.

1. A response of 0.25 mg/L does not positively indicate the presence of the pollutant with the desired confidence.
2. A response of 0.75 mg/L does indicate the presence of the pollutant with the desired confidence, but the measured concentration does not have the desired level of confidence.
3. A response of 1.25 mg/L does indicate the presence of the pollutant, and its measured concentration is within the desired level of confidence.

The residuals of the regressions were also examined to identify any evidence of bias. A plot of residual vs. predicted spike concentration should produce a random band of points with an average value representing the concentration of the parameter of interest in the blank sample. Narrow error bands indicate a more precise method. A plot of residuals vs. the order of analysis indicates if a bias is time dependent. For example, the calibration of a pH meter will drift over time. A plot of residuals vs. the order of measurement will show a linear trend if the meter is not regularly recalibrated.

From these analyses, the most suitable set of equipment was identified for further study. These were selected based on the measured detection limits, safety considerations, and shortest analysis time. This subset of methods was then evaluated by parallel analysis for 25 runoff water samples. The test kit results were compared to the results obtained using standard laboratory procedures. This set of analyses was also analyzed by a regression technique to identify the correlation between field measurements and laboratory analyses.

The precision of the selected methods was also evaluated by testing five replicates of a composite polluted water sample. The average, standard deviation, and relative standard deviation (RSD, also known as the coefficient of variation or COV, the ratio of the standard deviation to the mean) for the methods were determined for each test kit.

Assembling an appropriate set of field test kits is obviously dependent on the specific uses of the data. In most cases, several colorimetric analyses will be included in the monitoring program, and the purchase of a good field spectrophotometer or filter colorimeter will be easily justified. The two major choices include the HACH DR/2100 field spectrophotometer (which costs about $1500), or the La Motte Smart Colorimeter (which costs about $800). The use of specific filter colorimeters (which cost from $250 to $400) may only be suited to very simple programs. The use of most manual color comparator tests will limit the utility of the data, but may still be justifiable.

A more important problem, besides cost, is probably associated with the time and expertise needed to conduct the analyses. Many of the analyses can be conducted together (especially those with extensive color development times, such as the immunoassays and the bacteria tests, plus the ammonia, copper, detergents, lead, and potassium tests). However, there will be a limit, as some of the tests are very complex (especially the immunoassays and the LeadTrak, which also require extensive expertise to obtain good results).

Appendix E contains summarizes of the information from the field test kit evaluations conducted by Day (1996), and includes information for the following constituents:

Ammonia
BTEX and PAHs
Chloride

Copper
Detergents
Fluoride
Lead
Nitrate
Potassium
Zinc

Most of the field test kits evaluated performed very well, with significant response factors and recoveries close to 1.0 (slopes of the regression lines when comparing known concentrations with test responses). In addition, the response factors were very close for spiked sample analyses in both RO and runoff sample water, indicating few matrix interference problems. The precision of the tests was generally excellent, with almost all replicate analyses having COV values of less than 20%, and many were much less than 10%. The exceptions were for tests that had very poor detection limits compared to the concentrations in the samples being tested. However, the detection limits of almost all of the analytical methods were much worse than reported by the manufacturers. The limits of quantification are all about twice as large as the detection limits shown in Appendix E. In some cases, this resulted in a very narrow workable range for the method before dilution is needed.

The following comments pertain to several groups of parameters of special interest when using field test kits. These comments stress the need to carefully select and evaluate field test kits used in monitoring programs, especially since there have been few independent evaluations of their capabilities and limitations. Many of the procedures (including some that were relatively inexpensive) were found to be surprisingly good in our tests. In all cases, careful tests, such as performed by Day (1996), should be conducted using samples and conditions representing specific characteristics of the field monitoring program.

Bacteria

Bacteria analysis is an important parameter for many monitoring programs. Unfortunately, conventional laboratory tests are time-consuming (typically requiring at least 24 hours under very controlled temperature conditions). IDEXX supplies a simple procedure for monitoring enterococcus, *E. coli*, and total coliforms for general field work (described later) that can be adapted for field work (Figures 6.71 through 6.74, including Color Figure 6.5). Millipore probably has the most complete selection of field equipment and supplies to conduct bacteria analyses in the field. HACH also supplies suitable field equipment for many types of bacteria tests. However, these tests also require the same standard incubation times as the time-consuming laboratory tests. There are a few procedures that can indicate the presence of very large populations of bacteria in water samples in relatively short periods of time. Most of these require UV light analyses and controlled incubation temperatures. An interesting alternative is the KoolKount Assayer from Industrial Municipal Equipment, Inc. This is a visual colorimetric test that costs about $4 per test. It is unique in that it only requires from 30 min to 13 hours for a determination at “room temperature” incubation. Very high bacteria populations will be evident in a short period of time. This is not a selective test, but sensitive to a mixed microbial population. The test was developed to analyze gross bacterial contamination of cooling waters, but may also be useful in receiving water studies. There are now DNA-based procedures being developed (see a later section on emerging technologies) that offer promise for much more rapid, inexpensive, and easy analysis of bacteria.

In all cases, the user must be aware of the inherent problems in interpreting bacteria data, especially if one is using bacteria as an indicator of sewage contamination. As an example, fecal coliform bacteria are in very high populations is many waters, including stormwater that is not contaminated by sanitary sewage. The use of the fecal streptococci to fecal coliform ratio to indicate sources of contamination is also inherently inaccurate, unless the source of contamination is very

Figure 6.71 Pouring sample into IDEXX analytical tray.

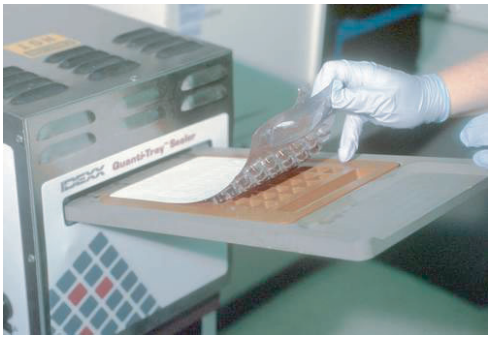


Figure 6.72 Placing analytical tray into heat sealing unit.

recent. O'Shea and Field (1992) reviewed many of these issues for stormwater. A better indication of potential sanitary sewage contamination in surface waters is the use of a small battery of chemical tracer analyses (detergents, fluoride, ammonia, and potassium), as developed and tested by Lalor (1993) and Pitt et al. (1994) and described later.

Organic Compounds

The analysis of organic compounds using field test kits is also of great interest because of the high costs of conventional laboratory analyses and the importance of these compounds. The organic compounds of most interest in studies of receiving waters affected by stormwater include BTEX, PAHs, and pesticides.

The two BTEX test kits evaluated by Day (1996) include the Dexsil PetroFlag (Figures 6.75 and 6.76) and the Dtech immunoassay test kit for BTEX (Figure 6.77). The PetroFlag is a simple solvent extraction test for sediment analyses. It requires a $700 reader that is only used for this test. Each test costs about $10 and requires about 10 min. It has poor detection limits and is not very

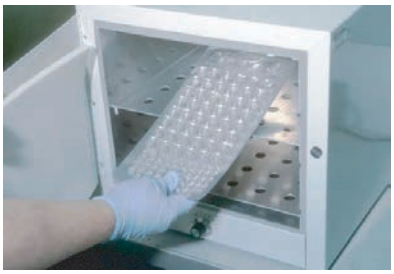


Figure 6.73 Placing prepared tray into incubator.

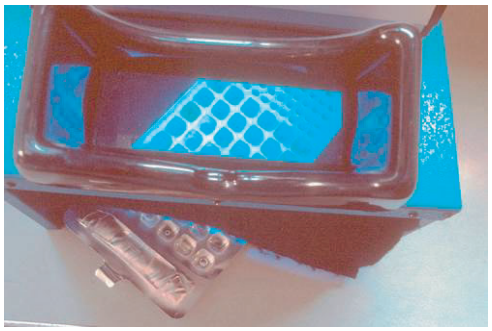


Figure 6.74 IDEXX analysis for *E. coli* in ultraviolet light.

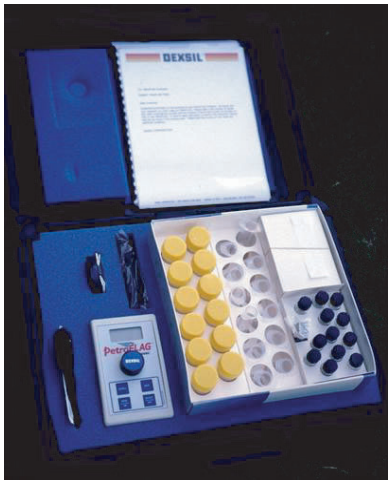


Figure 6.75 PetroFlag kit for field analyses of BTEX.

Figure 6.76 Sample being read using PetroFlag.

selective. An immunoassay test may be the only selective and sensitive option currently available. The Dtech (EM Science) BTEX Test Kit is an example of an immunoassay kit. It has an extremely low detection limit and reasonable selectivity that can be used for both water and sediment BTEX analyses. However, it is very complex and requires up to an hour. An initial cost of $500 for the Dtech reader can be used for both soil and water analyses and for both BTEX and PAH analyses for more precise results. The per-sample cost is about $25 for water samples and about $50 for sediment samples (requiring an additional soil extraction kit). The Dtech reagents have a relatively short shelf life (as little as a few weeks if not refrigerated, to several months if refrigerated).

The only selective option for PAH analyses is probably an immunoassay procedure. One example is the EM Science Dtech PAH Test Kit. Unfortunately, this test is also quite complex, requires more training than most other field test kits, and costs from $25 to $50 per sample. The Dtech reagent also expires in about 1 to 2 months and needs refrigeration.

Strategic Diagnostics, Inc. (www.sdix.com) also offers a number of test tube, magnetic particle immunoassay kits sold under the name RaPID Assay®. Kits are available for the detection of BTEX/TPH in environmental samples ($605/100 samples). Quantitative results can be obtained for BTEX in soil (assay range 0.9 to 30 ppm) or water (0.02 to 3.0 ppm), and if the operator knows the fuel source, total petroleum hydrocarbons (TPH) can be analyzed. The analytical range of this test kit is comparable to EPA GC method 8015 for TPH. Two immunoassay kits for PAHs are available. The PAHs RaPID Assay tests for 16 common PAHs ($1275/100 samples) and is comparable to EPA SW-846 Method #4035 and GC method 8270 or HPLC method 8310, with assay ranges in soil and water of 0.2 to 5.0 ppm and 0.93 to 66.5 ppb, respectively. Results are normalized to phenanthrene. The Carcinogenic RaPID Assay offers increased sensitivity to the seven most carcinogenic PAHs and is normalized to benzo[a]pyrene ($1395/100 samples). As of March 2000, 29 RaPID Assays for commonly used pesticides were available for prices ranging from $435 to $545. These

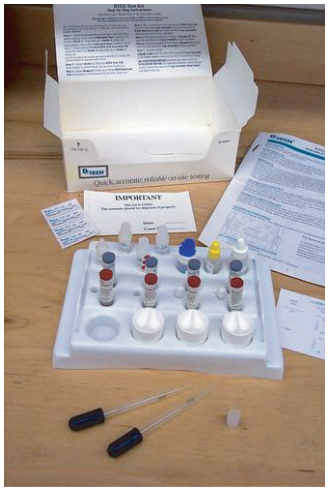


Figure 6.77 Dtech Immunoassay test kit for BTEX.

kits include alachlor, aldicarb, atrazine and five major metabolites, benomy/carbendazim, captan, carbofuran, chlorothalonil, chlorpyrifos, cyanazine, 2,4-D, endothall, fluridone, methomyl, metolachlor, metribuzin, organophosphates/carbamates, paraquat, picloram, procymidone, silvex, simazine, spinosad, TNT, TCP, and trichlopyr. For RaPID Assay kits, an optional soil extraction kit (12 samples), based on a 2-min methanol extraction procedure is available for $120. Kits are often sold for 30 or 100 samples, and results are usually obtained within 60 min. It is not always necessary to purchase the reader, the RPA-I RaPID Analyzer, as many tests can be quantitated on a spectrophotometer.

Two additional instruments were also recently examined at UAB for "total" hydrocarbon analyses. A Turner 10-AU field fluorometer with oil in water optics (see Figure 6.12) is extremely sensitive and is used with no sample preparation. It can be used in a flow-through mode to map hydrocarbon concentrations in real time. It is not very selective for different hydrocarbons. This instrument (which is also used for flow measurements using Rhodamine as a tracer) costs from $8000 to $16,000, depending on options, and is therefore not likely to be readily available to most people conducting field monitoring programs. A Petrosense PHA-100 probe from FCI Environmental, Inc. (see Figures 6.66 and 6.67) was also recently evaluated for real-time hydrocarbon analyses. This instrument costs about $7000 and has a slower response time (about 5 min), and it is not nearly as sensitive (about 100 µg/L, as xylene) as the fluorometer. It can also be used in real time, with no sample preparation.

EnviroLogix, Inc. (www.envirologix.com) offers antibody-based, enzyme-linked immunosorbent assay (ELISA) 96-well plate kits for pesticide detection. Pesticides include acetanilides (alachlor), aldicarb, benomyl/carbendazim, chlorpyrifos, cholinesterases (organophosphates and carbamates), cyanazine, cyclodienes (chlordane), DDT, fenarimol, fluometuron, imidacloprid, iprodione, isoproturon, metalaxyl, methoprene acid, organophosphates (cyclodienes and DDT), paraquat, parathion, synthetic pyrethroids, triazines (atrazine), and 3,5,6-trichloropyridonol. Accessories including soil extraction kits and a miniphotometer are available. The pesticides above cost $396/96-well-plate kit. Broad screening kits for cholinesterase inhibitors and organochlorine pesticides are available for $240 and $340, respectively.

Heavy Metals

Heavy metals are also of great interest in receiving water studies because they are possibly the most important toxic pollutants present. However, most of the metals in stormwater are associated with particulates (Pitt et al. 1995), with the exception of zinc, while all of the field test kits examined are only sensitive to "soluble" forms of the metals.

The HACH Bicinchonate Copper Method using AccuVac ampoules is the most suitable field method available (at a reasonable price) for measuring copper that was evaluated. This test uses the HACH DR/2000 spectrophotometer (at $1495) (or a less expensive dedicated filter spectrophotometer at $400), and the unit test cost is $0.56. It uses AccuVac ampoules that are very easy to use and makes the test very repeatable. However, the glass ampoules do produce glass wastes. The method detects the presence of a copper bicinchonate complex in the sample solution. An AccuVac ampoule is immersed in approximately 50 mL of sample and the tip is broken, which draws a known volume of sample into the ampoule. After a 2-min reaction time, the ampoule is scanned to determine the copper complex concentration, compared to a blank sample. Other metal ions present in large concentrations may also compete with copper for bicinchonate ligands. This interference will most likely produce a reported concentration larger than the true value if the metal complex absorbs in the same range as the copper complex. This method only indicates the presence of ionized copper. Any metallic or chelated copper will not be detected.

The HACH LeadTrak system is by far the most sensitive low-cost lead field test kit available (Figure 6.78). It is capable of detecting lead concentrations as low as 1 µg/L. Unfortunately, it is also quite complex and requires extensive experience. The test also takes about 45 min to conduct, which may be reduced to about 15 min with experience and if conducting several analyses at one

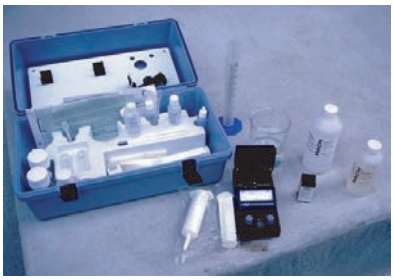


Figure 6.78 HACH LeadTrak field test kit.

time. The initial test kit costs about $400 (including a dedicated filter spectrophotometer) and the per-sample cost is about $5. The LeadTrak system determines lead concentrations through colorimetric determination of a lead complex extracted from the sample. The test procedure is quite complicated, requires a large amount of space compared to the other tests, and uses hazardous chemicals. However, it does produce good results.

The test requires 100 mL of sample, which is treated with an acid preservative (a nitric acid solution buffered with potassium nitrate). The solution is then treated with a solution of trishydroxymethylaminomethane, potassium nitrate, succinic acid, and imidazole. The prepared sample is then filtered through a solid-phase extractor (basically a syringe with a fabric plug). The lead in solution is held by the filter in the extractor. The lead is then removed from the plug with the eluant solution, another nitric acid solution. The eluant is allowed to pass over the plug until it stops flowing. The remaining eluant is forced through with the syringe plunger. This produces approximately 30 mL of extracted solution containing the lead from the sample. The extract is neutralized with a solution of trishydroxyaminomethane, tartaric acid, and sodium hydroxide. One powder pillow, containing potassium chloride and meso-tetra(-4-N-methylpryidyl)-porphine tetratosylate is added to the elutant. Two 10-mL portions are taken. A decolorizing solution is added to one portion; this portion is now the blank. Both sample portions are then analyzed using a spectrophotometer.

The La Motte Zinc test was the only acceptable zinc method investigated. This test uses a dilute solution containing cyanide, whereas the alternative tests use full-strength granular cyanide. The tests cost about $0.60 each and require about 5 min.

UAB recently evaluated two very sensitive electrochemical heavy metal field methods. The Palintest SA-1000 Scanning Analyzer is an anodic stripping voltammeter that uses preprepared electrode cards that come precalibrated (Figure 6.79). The instrument costs $2000 (available from AZUR Environmental), and each analysis for copper and lead costs about $5.50. The test is extremely sensitive (lead

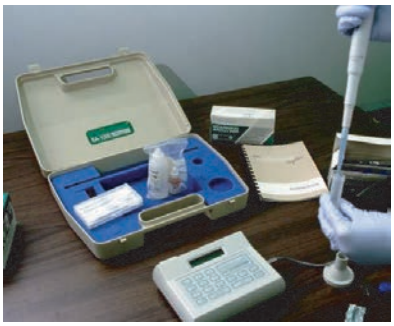


Figure 6.79 Palintest SA-1000 Scanning Analyzer.

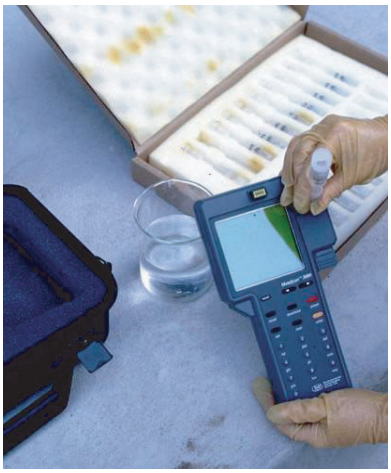


Figure 6.80 Metalyzer 3000 metals analyzer.

to about 5 µg/L and copper to about 75 µg/L) and relatively rapid (3 min). Another field method recently evaluated is the Metalyzer 3000 from Environmental Technologies Group, Inc. (Figure 6.80). This is a potentiometric voltammeter that is also capable of very sensitive simultaneous analyses of copper and lead. This instrument (which includes a built-in data logger) costs about $4200 and each analysis for copper and lead costs about $15. Since neither of these instruments detects particulate-bound heavy metals, their best use may be in evaluating rainwater, most groundwaters, and finished drinking water, where particulate metal forms are not significant. Most surface waters and wastewaters have large fractions of the metals bound to particulates, and any metal analysis procedure that does not include sample digestion will likely severely underreport the total metal content. However, if one is interested only in "dissolved" metal conditions, these procedures may be quite suitable.

Emerging Analytical Methods for Heavy Metals — An important pollutant category that is not represented with any real-time instrumentation is heavy metals. Samples require digestion in order to release all of the particulate-bound heavy metals for analysis. In addition, most metals are not amenable to real-time analyses. Some colorimetric procedures, such as the diethyldithiocarbamate copper method (as available from La Motte) or the bicinchonate copper method (as available from HACH), could be conducted on a real-time basis with an automated chemical mixing and analysis procedure. Recent research at the University of Alabama at Birmingham (in conjunction with the General Physics Institute of the Russian Academy of Sciences and Alabama Laser) sponsored by the National Science Foundation developed and demonstrated a laser-based instrument that may be capable of continuous heavy metal analyses in water (Mirov et al. 1999) (Figure 6.81). This instrument is extremely sensitive, as it is based on atomic fluorescence. The use of lasers enables the specific wavelengths most critical for analysis to be precisely used in the instrument. In addition, automated digestion of the samples may also be possible.

An initial demonstration of the extreme sensitivity of the laser atomic fluorescence (LAF) instrument procedure for copper used selective excitation of the $^2P_{3/2}$ level of Cu and the strong absorption transition $^2S_{1/2}$ ($3d^{10}4s$) $\rightarrow$ $^2P_{3/2}$ ($3d^{10}4p$) at 324.754 nm. The fluorescence signal was detected at the emission transition $^2P_{3/2} \rightarrow {}^2D_{5/2}$ ($3d^94s^2$) at 510.554 nm. The average power of the excitation beam was 10 mW at 324.754 nm (third harmonic of alexandrite laser pumped LiF:F_2^{+}** laser). The repetition rate of the laser was 20 Hz, and the pulse duration was about 50 ns. Spectral resolution of the spectrometer during the experiments was about 0.1 nm. The spectrum accumulation time was set to 5 s (slightly less than the atomization time set by the graphite furnace controller), which allowed for signal collection during approximately 100 laser excitation pulses. Typical examples of the observed fluorescence spectra for water solutions with different concentrations of Cu are shown in Figure 6.82. All three spectra were measured under the same experimental conditions. The graphite furnace was heated to 2800 to 3000°C before and between measurements, in order to clean the graphite tube from possible residuals. The spectral peak at 511.46 nm is due to some scattered light of the third harmonic (255.73 nm) of alexandrite laser in the second diffraction order. This peak is reasonably constant during the experiment, so it was used as an amplitude reference signal. As shown in this figure, it was possible to detect extremely low levels of Cu, even in the RO and distilled water samples (<<10 µg/L).

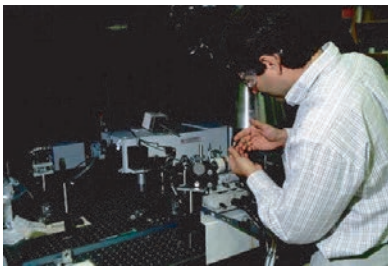


Figure 6.81° Laser fluorescence analysis of heavy metals being adapted for field analyses by UAB Physics Department.

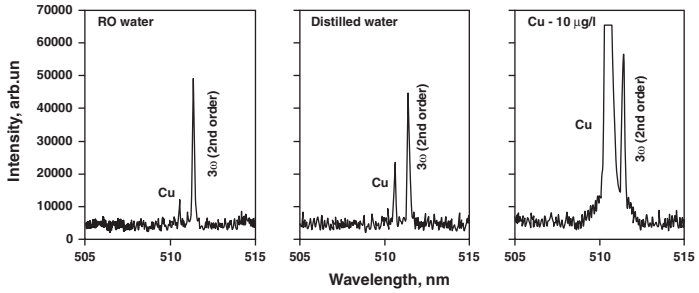


Figure 6.82° Fluorescence of Cu atoms under 324.75 nm laser excitation. (From Mirov, S.B., R.E. Pitt, A. Dergachev, W. Lee, D.V. Martyshkin, O.D. Mirov, J.J. Randolph, L.J. DeLucas, C.G. Brouillette, TT. Basiev, Y.V. Orlovskii, and O.K. Alimiv. A novel laser breakdown spectrophotometer for environmental monitoring. In: *Air Monitoring and Detection of Chemical and Biological Agents,* J. Leonelli and M.L. Althouse, Eds. Society of Photo-Optical Instrumentation Engineers (SPIE). Vol. 3855. pp. 34–41. September 1999. With permission.)

Solids

Analysis of the amount of solids in water samples in the field is another highly desired objective. Unfortunately, that is not practical. However, dissolved solids can be estimated using a simple conductivity meter, while suspended solids may be qualitatively estimated using a field nephelometer. Secchi disk transparency has also been used historically as an indication of suspended solids (especially related to algal activity). An excellent field nephelometer is available from HACH (for $800), while turbidity "probes" (miniaturized nephelometers) are now available on several *in situ* continuously recording multiwater quality probes (the Horiba HU-10 for $2800 and the YSI 6000 series, for about $7000). Numerous pocket conductivity meters are available that have "TDS" scales. These should be avoided in lieu of standard conductivity meters, as site-specific correlations between conductivity and TDS are usually required.

The Horiba Twin is a very small conductivity meter that has done very well in evaluation tests (Figure 6.83). It costs about $250, but the sensor should be replaced every 6 months at a cost of $60. This meter automatically compensates for temperature effects and is suited for very small sample volumes (3 to 4 drops). The meter includes a standard calibration solution. The procedure is to calibrate the meter using the provided standard solution and to select the conductivity mode. The user may partially immerse the probe in the sample or cover the probe with a few drops of sample.

pH

pH is usually considered an easy parameter to measure in the field. Unfortunately, the use of most "pocket" pH meters results in very inaccurate results, as the inexpensive probes included with these meters are not very reliable or robust, especially with storage. Recently available "dry" pH probes offer some hope for better field pH measurements. However, the most common FET transistor-based probes are delicate and can be irrecoverably damaged with abrasion or through contamination with oils and detergents. The Sentron field pH meter (at $600) is very sturdy, stores dry, and can be easily cleaned with a brush. Although the Horiba Twin pH meter is more likely to break, having a thin glass cover, it has worked well and is much less expensive (about $300) (Figures 6.84 and 6.85). Most field pH evaluations

Figure 6.83 Horiba Twin conductivity meter.

Figure 6.84 Horiba Twin pH meter dipped in sample.

Figure 6.85 Horiba Twin pH meter reading sample.

can probably be conducted using standard pH paper, as long as laboratory pH tests are also conducted. Fisher Scientific Alkacid Test Strips, for example, are very simple to use and inexpensive (<$1 per test), but the pH value is only readable to within ±1 pH unit (0.3 would be preferable). However, this sensitivity may be acceptable for many situations.

Dissolved Oxygen

DO is a parameter that is most commonly determined in the field. The YSI line of instruments is probably the best known and most commonly used among DO meters. The newer rapid-pulsed current DO probes from YSI are much superior to the older Clark membranes, especially if long-term monitoring is needed (such as with the *in situ* continuously recording probes). Many companies supply DO probes that work well, but with varying numbers of problems associated with storage, membrane replacement, and calibration. Winkler titration is not commonly used in the field, but HACH's digital field titrator even makes that feasible. The titration procedures work best with BOD analyses, including field titrations of BOD bottles used for *in situ* photosynthesis/respiration tests.

Detergents

The CHEMetrics Detergents (Anionic Surfactants) test kit was the only practical test for detergents investigated (Figure 6.86). The tests cost about $2.38 each and require about 10 min. The test uses a chloroform extraction, but it is very well designed to minimize exposure to the operator and uses a very small amount of chemical (Figures 6.87 through 6.90). The CHEMetrics procedure uses a visual comparator to determine the concentration of the detergents in the sample (Figures 6.91 and 6.92). A small volume of sample (5 mL) is required. An ampoule containing methylene blue and chloroform is mixed with the sample. Anionic detergents complex with the methylene blue and are extracted into the chloroform layer. Cationic detergents and sulfides interfere with the reaction and lead to decreased readings. The method is very quick and easy. However, the it uses chloroform, a known carcinogen. Users must conduct the test in well-ventilated areas. Furthermore, the waste must be disposed of properly. The kit is well designed to minimize the use and exposure of the chloroform. The reagent packs do have a limited shelf life, however. One method that can be used to detect the presence of detergents in outfalls tests for optical brighteners. This method was originally developed by researchers in Massachusetts for detecting inappropriate discharges at outfalls, especially from septic tanks. This method is described at www.thecompass.org/8TB/pages/SamplingContents.html. Untreated cotton

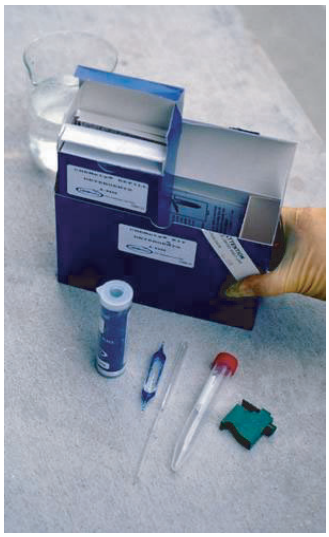


Figure 6.86 CHEMetrics detergent test kit.

Figure 6.87° Extraction step 1 for use of the CHEMetrics detergent test kit.

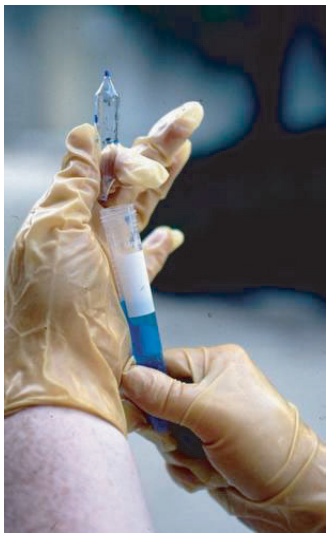


Figure 6.88° Extraction step 2 for use of the CHEMetrics detergent test kit.

Figure 6.89 Extraction step 3 for use of the CHEMetrics detergent test kit.

pads are secured at the test locations where they are left exposed for several days, recovered, and examined under a UV lamp. Optical brighteners adsorb to the cotton, if present in the flowing water. This method is not quantitative but should indicate gross contamination associated with wash waters, septage, and sewage.

Fluoride

The HACH Fluoride SPADNS Reagent test using AccuVac Ampoules is another AccuVac test that shares the DR/2000 (Figure 6.93). The tests cost about $1.17 each and require about 5 min. The test does produce a small amount of glass waste, and the SPADNS reagent is hazardous, requiring special disposal considerations.

Figure 6.90° Extraction step 4 for use of the CHEMetrics detergent test kit.

Figure 6.91 Inserting sample into color comparator.

Figure 6.92° Reading the detergent concentration with the color comparator.

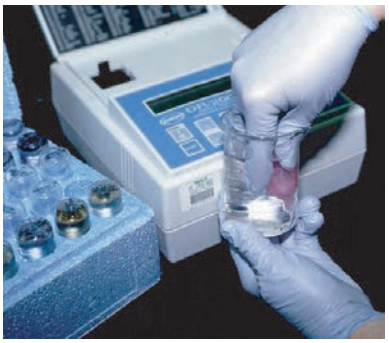


Figure 6.93 HACH AccuVac fluoride method.

Potassium

The La Motte Potassium Reagent Set was tested in the HACH DR/2000 spectrophotometer. This is an example of a hybrid test that was tested successfully by combining the very good La Motte reagents with the very good (and needed for many other tests) HACH DR/2000. The cost per test is about $0.29, and the test should take about 15 min. Potassium (K^+) can be used as an indicator of sewage contamination in water bodies, especially by examining the ratio of ammonia to potassium concentrations (Lalor 1993; Pitt et al. 1994).

The HACH and La Motte kits both determine potassium concentrations using tetraphenylborate salts. These procedures add large doses of sodium tetraphenylborate to the sample. The potassium in the sample reacts with the sodium tetraphenylborate to form insoluble potassium tetraphenylborate.

The insoluble potassium tetraphenylborate increases the turbidity of the sample solution. The presence of magnesium (Mg^{2+}), ammonium (NH_4^+) and calcium (Ca^{2+}) ions can interfere with the reaction by competing in the reaction with tetraphenylborate. These salts will result in a reported potassium concentration larger than is actually present in the sample. Both methods measure this increase in turbidity, using a spectrophotometer. To compensate for not using a nephelometer to measure this turbidity, both procedures include very specific timing requirements. The reaction and settling times must be followed exactly in order to obtain repeatable results.

Nutrients

The most common nutrient tests are for ammonia nitrogen, nitrate nitrogen, and phosphorus. The HACH Nitrate, MR test also shares the DR/2100 spectrophotometer and uses AccuVacs. The test is therefore very simple and quick, but produces glass debris and a hazardous reagent waste. The test costs about $0.56 per test and takes about 7 min.

The HACH Ammonia method using salicylate without distillation is a colorimetric determination of ammonia using salicylate. This method requires a DR/2100 spectrophotometer (usable for several other parameters) and a per sample cost of $2.88. It is also available as a self-contained test kit with a colorimeter for about $400.

Numerous simple field test kits are available for phosphorus. HACH, for example, has eight separate spectrographic tests and 11 colorimetric tests available for different forms and concentration ranges for phosphorus. Reactive phosphorus (orthophosphate) is probably of greatest interest for most simple environmental monitoring activities. The HACH AccuVac ascorbic acid method with the DR/2100 spectrophotometer is probably the simplest test procedure available. The tests cost about $0.56 each, after purchase of the spectrophotometer.

Figure 6.94 Horiba Cardy ion selection electrode for potassium.

Two ion selective electrode (ISE) probes were also evaluated for fluoride analyses, with disappointing results. Probe problems were mostly associated with the lack of stability of the probe, especially with storage, and time-consuming standardization. Similar problems were found with ISE probes for ammonia, detergents, and potassium. ISE probes may work well in controlled laboratory settings, especially with proper care of the probes, but their use in the field is probably restricted to trained electrochemists who know how to take proper care of the probes and who know how to calibrate them more efficiently. Exceptions were the Horiba Cardy ISE probes for potassium (Figure 6.94) and nitrates that have worked very well in the field, although they are not very sensitive.

Selection of Appropriate Field Test Kits

The most appropriate field test kit for a specific use can be selected based on the criteria presented earlier in this section, and in Chapter 5, and summarized in Appendix E. In most cases, the limits of detection are the most critical criteria. It is quite possible that the simplest field test kits may be useful for some studies, as most were found to be generally free from interferences (Day 1996). However, during tests using actual stormwater samples and spiked waters, their sensitivity was found to be generally poor, even less sensitive than typically advertised. This will likely lead to false negatives if actual limits of detection are not determined through sensitivity tests using local waters. The needed limits of detection must be known before analytical methods are selected, using methods presented earlier in Chapter 5.

The field test kits highlighted in the above discussion were selected based on our (Pitt et al. 1993; Day 1996) laboratory and field comparison tests and have been found to generally best meet our needs during investigations of stormwater problems, although other field test kits are also likely suitable.

If field test kits or *in situ* methods are suitable and available to meet the project objectives, other criteria must also be considered, especially the amount of time required for analyses, complexity and training needs, hazardous wastes and sharps produced, and cost. As indicated in Appendix E, some analyses are virtually instantaneous, while other tests may require almost an hour. Obviously, if multiple samples can be evaluated at the same time, the longer times required for some of the tests may not be as critical. A more serious concern is the use and production of hazardous reagents and wastes, and glass sharps. Unless personnel are especially well trained and have suitable facilities, these field test kits need to be avoided. The complex tests, such as the immunoassay kits for organics, may also require specialized training, as indicated in Appendix E, also eliminating their use except for the most patient and skilled analysts. If the field test kits are suitable for the needed monitoring activity, conventional laboratory procedures, discussed in the following section, are available.

The following example illustrates how this information can be used to select the most appropriate field testing methods, or to rely on conventional laboratory analyses. Table 4.37 was a simple matrix showing which parameters would be of greatest concern when evaluating receiving waters having different beneficial uses. In this example, biological life and integrity uses are of concern. Table 6.29 lists the water quality parameters of most interest for this use, expected concentrations of most concern (from Appendix G, a discussion of water quality criteria) and their associated assumed variation, and corresponding needed limits of detection. Obviously, the listed parameters shown on this table are only a portion of the needed field study for this assessment, as habitat destruction, high/low flow durations, inappropriate discharges, benthic macroinvertebrate and fish sampling, sediment investigations, and bioaccumulation of toxicants should also be considered (as listed in Table 4.37).

The only other primary water quality constituents noted on Table 4.37 of great interest for receiving water assessments include the microorganisms. These currently cannot be analyzed in the field, although portable sample preparation and field incubators are available from HACH and others. Because of the long incubation periods required (typically 18 to 24 hours for preliminary results), these methods are not really considered field methods here. Therefore, the analyses that might be conducted using field test kits that meet basic sensitivity requirements include:

Conventional Constituents:
- Hardness (using field titration equipment)
- Alkalinity (using field titration equipment)
- Turbidity (possible using moderately expensive field nephelometer, or expensive *in situ* recording probes)
- pH (easily conducted using electrodes, or expensive *in situ* recording probes)
- Conductivity (easily conducted using electrodes, or expensive *in situ* recording probes)
- DO (easily conducted using electrodes, or expensive *in situ* recording probes)
- Temperature (easily conducted using electrodes, thermometers, or expensive *in situ* recording probes)

Nutrients:
- Ammonia (several simple field test kits available)
- Nitrates (several simple field test kits available)
- Phosphates (several simple field test kits available)

Toxicants:
- Lead (but difficult, time-consuming, or expensive)
- Toxicity screening (expensive instrument)
- Pesticides, PAHs, PCBs, etc., by immunoassays (but difficult, time-consuming, and expensive)

Of these, DO (field probe preferred to titration in most cases), temperature (mandatory), and pH (within a few hours) may need to be conducted in the field to meet QA/QC requirements, while conductivity is very easy to measure in the field (and therefore commonly done). The decision to measure the other constituents listed above in the field should be based on other considerations,

Table 6.29 Water Quality Measurements of Interest and Expected Analytical Requirements for Hypothetical Receiving Water Investigation Assessing Aquatic Life Use Impairments

Water Quality Parameter	Example Water Quality Objectives Associated with Aquatic Life Beneficial Uses (short-term exposures)	Expected Coefficient of Variation (COV) Category[a]	Estimated Needed MDL[a]	Suitable Field Measurement Methods Providing Estimated Needed MDL (mostly from Table E-2, from Day 1996, also from text).
Zinc	<120 µg/L (CMC[2])	Medium	28 µg/L	No available field method could approach this desired MDL. The lowest MDL found was about 140 µg/L for Zn. Most of the field test methods also require toxic (cyanide) reagents.
Copper	<13 µg/L (CMC)	Medium	3 µg/L	No available field method could approach this desired MDL. The lowest MDL found was about 100 µg/L for Cu.
Lead	<65 µg/L (CMC)	Medium	15 µg/L	The HACH LeadTrak system has a MDL of about 5 µg/L, although it is a time-consuming test and relatively expensive. The Metalyzer 3000 and Palintest SA-1000 both have lead MDLs of about 5 µg/L and would therefore be suitable, but are expensive instruments.
Microtox screening test	n/a: indicative of toxicants that may be present (such as pesticides), desire low value; I20 of <25%.	Medium	I20 of 6%	Deltatox (expensive instrument, but field portable).
Hardness	Narrative (want moderate to hard water conditions to reduce effect of some toxicants), would like to detect hardness to at least 50 mg/L.	Low	40 mg/L	HACH Digital Titrator and CHEMetrics EDTA titration methods would both likely be suitable field methods.
Alkalinity	n/a (would like moderate to high levels of alkalinity to reduce effects of some toxicants), would like to detect alkalinity to at least 25 mg/L.	Low	20 mg/L	Field titration methods available, but not evaluated.
Ammonia	<3.8 mg/L (2.5 × chronic at 30°C)	Low	3 mg/L	All 4 field test kits investigated have limits of detection lower than this estimated needed MDL. However, one requires refrigeration, and others contain mercury in waste.
Nitrates	n/a (rarely toxic to aquatic life in natural streams, but indicative of potential eutrophication problems in nitrogen limited streams), would like to detect NO_3 to at least 1 mg/L.	Low	0.8 mg/L	The La Motte and CHEMetrics nitrate tests, and likely the HACH low range nitrate test, can meet this MDL objective. Sharps and cadmium containing wastes are common with these methods.
Phosphates	Narrative, <25 µg/L to prevent eutrophication.	Low	20 µg/L	Numerous phosphate field test kits are available, although not reviewed by Day (1996). It is expected that there are several that can meet these performance objectives.

Table 6.29 Water Quality Measurements of Interest and Expected Analytical Requirements for Hypothetical Receiving Water Investigation Assessing Aquatic Life Use Impairments *(Continued)*

Water Quality Parameter	Example Water Quality Objectives Associated with Aquatic Life Beneficial Uses (short-term exposures)	Expected Coefficient of Variation (COV) Category[a]	Estimated Needed MDL[a]	Suitable Field Measurement Methods Providing Estimated Needed MDL (mostly from Table E-2, from Day 1996, also from text).
Suspended solids	Narrative: <100 mg/L settleable fraction to prevent smothering of stream bed.	Large	12 mg/L	No field instruments known for measuring suspended solids (requires drying ovens and analytical balance), but can be predicted/tracked using turbidity.
COD	n/a (indication of organic matter), would like to be <5 mg/L.	Medium	1 mg/L	No field instruments known for measuring COD (requires digestion).
pH	Between 6.5 and 9 desired (harmless to fish in this range).	Very low	Readable to 0.3 pH units	All of the pH electrode methods investigated should meet this readability objective, but the pH paper methods are not likely suitable.[c]
Conductivity	n/a (variation should be minimal), would like to determine conductivity at 100 µS/cm.	Low	80 µS/cm	All three conductivity probes investigated had limits of detection about equal to this objective and would be suitable.[c]
Turbidity	Narrative: <50 NTU increase above background conditions.	Large	6 NTU	The HACH portable nephelometer, or the Horiba HU-10 and YSI in-situ probes can measure turbidity in the field, although these are all moderate to very expensive options.[c]
DO	>5.0 mg/L	Low	Readable to 0.25 mg/L	Most modern field DO meters could be used to meet these objectives.[c]
Temperature	Narrative (variation from natural conditions should be minimal).	Low	Readable to 0.5°C	Most modern field DO meters also have temperature readouts and would be suitable, alternatively, simple pocket thermometers could be used.[c]

[a] If the COV is low (<0.5), the multiplier for the MDL is 0.8 × the desired median value of the observations, in this case taken to be the water quality objectives. If the COV is medium (0.5 to 1.25), the multiplier is 0.23, and if the COV is large (>1.25), the multiplier is 0.12; see Table 6.26 and corresponding discussion.
[b] CMC: criterion maximum concentration (exposure period of 1 hr)
[c] The combination probes (such as the YSI 6000) should be considered as they can monitor several needed constituents: pH, conductivity, turbidity, DO, and temperature).

mainly safety, cost, time, and difficulty. In many cases, it is not practical to conduct field measurements at the time of sample collection due to the time needed to set up equipment, standardize the procedures, and conduct the individual constituent analyses at each sampling location. However, it might be very reasonable to use these field methods in a temporary field laboratory when conducting sampling in remote areas. In this case, samples collected over a short period of time (such as during the day) can be analyzed together, minimizing the time requirements. In addition, the use of continuous recording *in situ* probes should be seriously considered for turbidity, conductivity, pH, DO, and temperature, in addition to possibly ORP and stream stage (depth). Although expensive (can be rented for short periods), these probes have been extremely useful when monitoring these key constituents over several weeks that include both wet and dry periods. The high resolution data (measurements typically are taken and logged every 15 min) dramatically illustrate the variabilities of these constituents over short periods of time (as discussed in the narratives for some of the water quality criteria) and help to understand the duration of exposure to wet-weather-related discharges.

The earlier Table 4.37 also included additional water quality measurements that were not listed as primary constituents and therefore not discussed above. Many of these should also be periodically evaluated as part of an assessment project, but few are amenable to safe, inexpensive, and rapid field measurements. These other constituents (such as the PAHs and pesticides, other metals, and microorganisms), plus those listed above that are not generally suited (or selected) for field measurements, must be analyzed using conventional laboratory methods. Of course, a good QA/QC plan would also require that samples being analyzed in the field be periodically split and analyzed using conventional laboratory methods for comparison. In some cases, it may be appropriate to use some of the more difficult field test kits (such as the immunoassay tests) due to the lack of conventional laboratory facilities, or for faster turn-around time.

Conventional Laboratory Analyses

Table 6.30 lists standard analytical methods that may be used for stormwater analyses. Several methods need to be modified to effectively analyze stormwater samples, especially if only small sample volumes are available (such as from pore water from stream sediments, from bench-scale treatability tests, or to reduce sample shipping costs). Modifications to the standard methods are described in Appendix E and are necessary because of the large particulate fractions of the organic toxicants which interfered with conventional extraction methods. Reducing the sample volumes (especially for the organic analyses) also significantly reduces the volumes of hazardous laboratory wastes. Appendix E also contains information pertaining to heavy metal analysis options and laboratory safety. This table should not be considered as a complete listing of laboratory methods for stormwater analyses, but is an example of some analyses and the associated standard methods.

Quality control and quality assurance activities (see Chapter 5 and Appendix E) require a substantial effort in most analytical laboratories. EPA analytical guidelines published in the *Federal Register* for the various tests specify the types and magnitude of QA/QC analyses. These analyses supplement the standardization efforts as they are used to measure the efficiency of the sample preparation and analysis procedures. Blanks are used to identify possible contamination problems, while matrix spikes added to the samples prior to any preparation steps indicate the efficiency of the complete analytical process. Spikes added to the samples prior to analyses are also used to identify interferences, mainly associated with other compounds in the sample. In heavy metal analyses, for example, it is not uncommon to increase the sample analysis effort by an extra 50% for standards and QA/QC samples in production work. Method development activities require an even greater additional analytical effort.

Appendix E contains descriptions of the modifications to the standard methods for the organic toxicants noted in the above table that are needed for effective measurements of stormwater characteristics. These modifications are needed to obtain necessary levels of recovery of the organics that are bound to particulates in the stormwater. The following discussions present summaries of special aspects of laboratory tests of possible interest in receiving water investigations, especially

Table 6.30 Typical List of Standard and Modified Methods for Wet-Weather Flow Analyses

Parameter	Method
Physical Analyses	
Color, spectrophotometric	EPA 110.3
Conductance, specific conductance	EPA 120.1
Particle size analysis by Coulter Counter Multi Sizer IIe	Coulter method
pH, Electrometric	EPA 150.1
Residue, filterable, gravimetric, dried at 180°C	EPA 160.1
Residue, nonfilterable, gravimetric, dried at 103–105°C	EPA 160.2
Residue, total, gravimetric, dried at 103–105°C	EPA 160.3
Residue, volatile, gravimetric, ignition at 550°C	EPA 160.4
Turbidity, nephelometric	EPA 180.1
Inorganic Analyses	
Hardness, total (mg/L as $CaCO_3$), Titrimetric EDTA	EPA 130.2
Aluminum, arsenic, cadmium, chromium, copper, iron, lead, nickel, and zinc	EPA 200.9
Chloride, fluoride, nitrate, nitrite, phosphate, and sulfate	EPA 300.0
Ammonium, calcium, lithium, magnesium, potassium, and sodium	EPA 300.0 modified
Alkalinity, titrimetric (pH 4.5)	EPA 310.1
Organic Analyses	
Chemical oxygen demand, colorimetric	EPA 410.4
Aldrin, Chlordane-alpha, Chlordane-gamma, 4,4′-DDD, 4,4′-DDE, 4,4′-DDT, Dieldrin, Endosulfan I, Endosulfan II, Endosulfan sulfate, Endrin, Endrin aldehyde, Endrin ketone, HCH-alpha, HCH-beta, HCH-gamma (Lindane), Heptachlor, Heptachlor epoxide, and Methoxychlor	EPA 608 modified
Acenaphthene, Acenaphthylene, Anthracene, Azobenzene, Benzo(a)anthracene, Benzo(b)fluoranthene, Benzo(g,h,i)perylene, Benzo(k)fluoranthene, Benzo(a)pyrene, 4-Bromophenyl-phenylether, Bis-(2-chloroethyl)ether, Bis-(2-chloroethoxy)methane, Bis-(2-ethylhexyl)phthalate, Butylbenzyl phthalate, Carbazole, 4-Chloro-3-methylphenol, 2-Chloronaphthalene, 2-Chlorophenol, 4-Chlorophenyl-phenylether, Chrysene, Coprostanol, Dibenzo(a,h)anthracene, 1,2-Dichlorobenzene, 1,3-Dichlorobenzene, 1,4-Dichlorobenzene, 2,4-Dichlorophenol, Diethyl phthalate, 2,4-Dimethylphenol, Dimethyl phthalate, Di-n-butyl phthalate, 2,4-Dinitrophenol, 2,4-Dinitrotoluene, 2,6-Dinitrotoluene, Di-n-octyl phthalate, Fluoranthene, Fluorene, Hexachlorobenzene, Hexachlorobutadiene, Hexachlorocyclopentadiene, Hexachloroethane, Indeno(1,2,3-cd)pyrene, Isophorone, 2-Methylnaphthalene, 2-Methylphenol, 4-Methylphenol, Naphthalene, Nitrobenzene, 2-Nitrophenol, 4-Nitrophenol, N-Nitroso-di-n-propylamine, N-Nitroso-diphenylamine, Pentachlorophenol, Phenanthrene, Phenol, Pyrene, 1,2,4-Trichlorobenzene, 2,4,5-Trichlorophenol, and 2,4,6-Trichlorophenol	EPA 625 modified
Toxicity Analyses	
Microtox 100% toxicity screening analysis (using reagent salt for osmotic adjustments)	Azur Environmental method

methods suitable for large numbers of samples, particle size analyses, and laboratory tests to identify associations of metal compounds that determine their effects on receiving water uses.

Automated Methods Suitable for Large Numbers of Samples

There are a number of laboratory instruments suitable for rapidly analyzing large numbers of samples for common constituents. Two instruments that have been especially helpful in the Environmental Engineering Laboratories at the University of Alabama at Birmingham have been a Dionex Ion Chromatograph (we use an older DX-100 with an autosampler) and a Bran + Luebbe TRAACS 2000 Continuous-Flow Analyzer (we use a basic 2-channel unit, with XYZ autosampler and syringe diluter). These instruments are relatively expensive and are most suitable for rapidly analyzing many samples for a few constituents at one time. The sample volume requirements are very small (less than 10 mL) and expendable analytical cost per analysis is also very small (typically less than $0.10).

Figure 6.95 3 TRAACS 2000 instrument showing older linear auto sampler and main module.

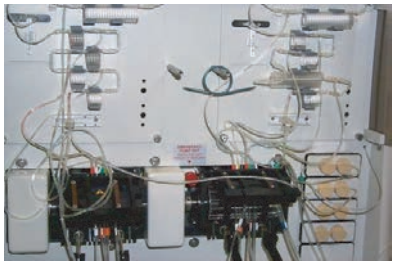


Figure 6.96 TRAACS manifold.

Unfortunately, required sample cleanup for the ion chromatograph adds several dollars per sample, and required filtration of surface water samples for the TRAACS also adds several dollars per sample. However, if many samples are to be analyzed in a short time, especially when working with small sample volumes, these instruments are very cost effective. However, necessary operator training and skill is much more than required for most conventional manual analyses.

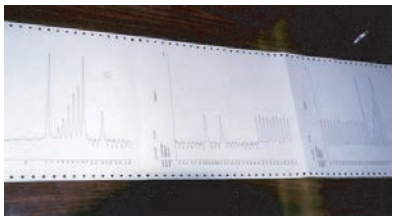


Figure 6.97 Quality control output for TRAACS.

Bran + Luebbe TRAACS 2000 Continuous-Flow Analyzer — This is a new instrument in our laboratory, and we are still learning its capabilities (and requirements). We are using the TRAACS mostly for dissolved nutrient analyses (phosphate, ammonia, nitrate, and nitrite), plus hardness and alkalinity (Figures 6.95 through 6.97). The instrument is capable of analyzing many other analytes, requiring a several-hour period to switch reagents and tubing, and for other initial setup activities. A block digester is available that would also enable total forms of the nutrients (specifically total Kjeldahl nitrogen and total phosphorus) to be rapidly analyzed. The instrument draws samples and needed reagents through specialized manifolds where mixing takes place before colorimetric determinations. The basic two-channel instrument enables two different analytes to be determined simultaneously. It is possible to add more channels, allowing additional simultaneous analyses. There are some restrictions on the analytes that can be simultaneously analyzed on the parallel channels, however. The XYZ autosampler, containing samples, plus standard solutions and blanks, allows several hundred samples to be evaluated at one setup. The syringe diluter automatically adjusts sample strength if a sample goes over-range.

Most analyses require only a few minutes per sample and very small amounts of standard reagents. The cost per analysis is therefore very low, but the setup time and other maintenance requirements make the instrument most suitable when a relatively large number of samples are to be analyzed at one time.

Dionex DX-100 Ion Chromatograph — We have much more experience with the DX-100, having used it for several years in support of many of our recent stormwater research projects. It can be used for the determination of the following common inorganic ions in drinking water, surface water, mixed domestic and industrial wastewaters, groundwater, reagent waters, solids (after extraction), and leachates (when no acetic acid is used):

- Anions: fluoride, chloride, nitrate-N, nitrite-N, ortho-phosphate-P, and sulfate
- Cations: lithium, sodium, potassium, ammonium, magnesium, and calcium

A small volume of sample (0.5 mL) is introduced into the ion chromatograph using the autosampler. The ions of interest are separated and measured, using a system comprised of a guard

Table 6.31 Anion Chromatographic Conditions and Detection Limits in Water

Analyte	Peak No.	Retention Time (min)	MDL (mg/L)
Fluoride	1	1.2	0.027
Chloride	2	1.7	0.080
Nitrite-N	3	2.0	0.111
Nitrate-N	4	3.2	0.040
o-Phosphate-P	5	5.4	0.084
Sulfate	6	7.0	0.083

Standard conditions: pump rate 2.0 mL/min, sample loop 25 µL.

Table 6.32 Cation Chromatographic Conditions and Detection Limits in Water

Analyte	Peak No.	Retention Time (min)	MDL (mg/L)
Lithium	1	1.3	0.0138
Sodium	2	2.0	0.454
Ammonium	3	3.2	0.123
Potassium	4	4.8	0.081
Magnesium	5	5.7	0.055
Calcium	6	7.9	0.318

Standard conditions: pump rate 1.0 mL/min, sample loop 25 µL.

column, analytical column, suppressor device, and conductivity detector. The difference between the methods for determining anion and cation concentrations are the separator columns, guard columns, and sample preparation procedures.

Tables 6.31 and 6.32 give the single laboratory method detection limit (MDL) for each ion (based on analyses at UAB).

These detection limits can be easily improved by changing sample loop lengths (and therefore the sample volume introduced into the IC), but resolution may suffer (and the ability to separate some ions) with increased volumes, and the upper limits also decrease correspondingly when the detection limits are improved.

Substances with retention times that are similar to and/or overlap those of the ion of interest can interfere with the analysis. Any ion that is not retained by the column or only slightly retained will elute in the area of fluoride or lithium and interfere. Known co-elution is caused by carbonate and small organic ions. At concentrations of fluoride and lithium above 1.5 mg/L, this interference is likely not significant. However, quality control is required to show whether this interference occurred. Do not attempt to quantify unretained peaks, such as low-molecular-weight organic acids (formate, acetate, propionate, etc.) which are conductive and co-elute with or near fluoride. These will bias the fluoride measurement in some drinking water and most wastewaters. The acetate anion elutes early during the chromatographic run, and the retention times of the anions seem to differ when large amounts of acetate are present. Therefore, do not use this method for leachates of solid samples when acetic acid is used. Residual chlorine dioxide present in the sample will result in the formation of additional chlorite. If chlorine dioxide is suspected in the sample, purge the sample with an inert gas (argon or nitrogen) for about 5 min or until no chlorine dioxide remains. The water dip or negative peak that elutes near, and can interfere with, the fluoride peak can usually be eliminated by the addition of the equivalent of 1 mL of concentrated eluant to 100 mL of each standard and sample.

Large amounts of an ion can interfere with the peak resolution of an adjacent ion. Sample dilution and/or fortification can be used to solve most interference problems associated with retention times. However, this method is not recommended for samples containing snowmelt runoff where chloride is used as a deicer. Samples that contain particles larger than 0.45 µm and reagent solutions that contain particles larger than 0.20 µm require filtration to prevent damage to instrument columns and flow systems.

Particle Size Measurements

Knowing the settling velocity characteristics associated with stormwater particulates is necessary when designing numerous stormwater control devices. In addition, particle size can be critical when understanding the effects and sources of stormwater sediments. There is a wide range of methods for determining particle size based on different principles and assumptions. No one method is ideal for all applications. For a review of sediment grain size methods, see ASTM Standard E 1391-94 (1994).

Particle size is directly related to settling velocity (using Stokes law, for example, and using appropriate shape factors, specific gravity, and viscosity values) and is usually used in the design of detention facilities. Particle size can also be much more rapidly measured in the laboratory than settling velocities. Settling tests for stormwater particulates need to be conducted over a period of about 3 days in order to quantify the smallest particles that are of interest in stormwater. If designing rapid treatment systems (such as grit chambers or vortex separators for CSO treatment), then much more rapid settling tests can be conducted. Probably the earliest description of conventional particle settling tests for stormwater samples was made by Whipple and Hunter (1981).

Randall et al. (1982), in settleability tests of urban runoff, found that nonfilterable residue (suspended solids) behaves liked a mixture of discrete and flocculant particles. The discrete particles settled rapidly, while the flocculent particles were very slow to settle. Therefore, simple particle size information may not be sufficient when flocculant particles are also present. Particle size analyses should include identification of the particle by microscopic examination to predict the extent of potential flocculation.

Figure 6.98 shows approximate stormwater particle size distributions derived from several upper Midwest and Ontario analyses, from all of the U.S. EPA's National Urban Runoff Program (NURP) data (Driscoll 1986), and for several eastern sites that reflect various residue concentrations (Grizzard and Randall 1986). Pitt and McLean (1986) microscopically measured the particles in selected stormwater samples collected during the Humber River Pilot Watershed Study in Toronto. The upper Midwest data sources were two NURP projects: Terstriep et al. (1982), in Champaign/Urbana, IL, and Akeley (1980) in Washtenaw County, MI. Figure 6.99 also shows the particle size distributions of stormwater particulates from the Monroe St. detention pond study in Madison, WI.

The Monroe St. project was a joint effort of the Wisconsin Department of Natural Resources and the U.S. Geological Survey. It obtained a number of stormwater particle size distributions for 46 storms having a wide range of characteristics. Bedload samplers were also used to obtain measurements representing the larger particles that are commonly not sampled by most researchers. The observed median particle sizes ranged from about 2 to 26 μm, with a median size of about 9 μm.

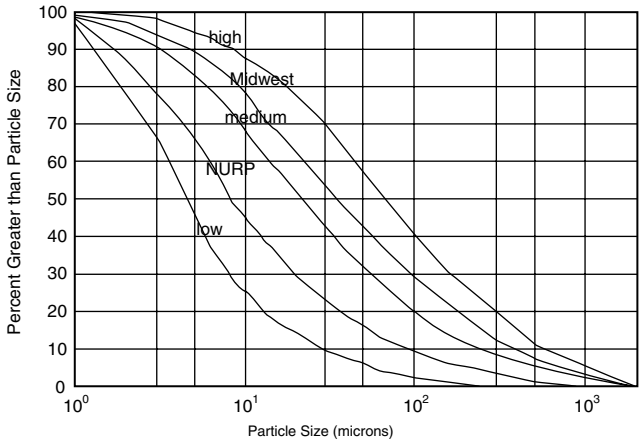


Figure 6.98 3 Particle size distributions for various stormwater sample groups.

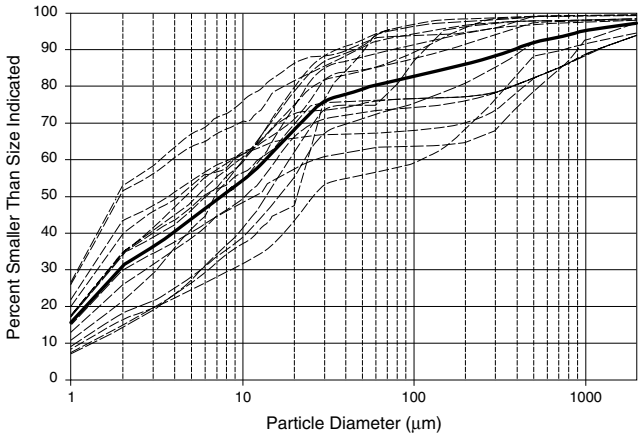


Figure 6.99 Monroe St. particle size distributions.

These distributions included bedload material that was also sampled and analyzed during these tests. This distribution is generally comparable to the "all NURP" particle size distribution presented previously. The 50th and 90th percentile particle size values are as follows for the different data groups:

	90%	50%
Monroe St.	0.8	9.1 µm
All NURP	1	8
Midwest	3.2	34
Low solids concentration	1.4	4.4
Medium solids concentration	3.1	21
High solids concentration	8	66

For many urban runoff conditions, the median stormwater particle size is estimated to be about 30 µm (which can be much smaller than the median particle size of some source area particulates). Very few particles larger than 1000 µm are found in stormwater, but particles smaller than 10 µm are expected to make up more than 20% of the stormwater total residue weight.

Specific conditions (such as source area type, rain conditions, and upstream controls) have been shown to have dramatic effects on particle size distributions. Randall et al. (1982) monitored particle size distributions in runoff from a shopping mall that was cleaned daily (by street cleaning). Their data (only collected during the rising limb of the hydrographs) showed that about 80% of the particles were smaller than 25 µm, in contrast to about 40% which were smaller than 25 µm during the outfall studies. They also only found about 2% of the runoff particles in sizes greater than 65 µm, while the outfall studies found about 35% of the particles in sizes greater than 65 µm.

Limited data are available concerning the particle size distribution of erosion runoff from construction sites. Hittman (1976) reported erosion runoff having about 70% of the particles (by weight) in the clay fraction (less than 4 µm), while the exposed soil being eroded had only about 15 to 25% of the particles (by weight) in the clay fraction. When the available data are examined, it is apparent that many factors affect runoff particle sizes. Rain characteristics, soil type, and on-site erosion controls are all important.

The particle size distributions of stormwater at different locations in an urban area greatly affect the ability of different source area and inlet controls to reduce the discharge of stormwater pollutants. A series of U.S. Environmental Protection Agency-funded research projects has examined the sources and treatability of urban stormwater pollutants (Pitt et al. 1995). This research included particle size analyses of 121 stormwater inlet samples from three states (southern New Jersey; Birmingham, AL; and at several cities in Wisconsin) that were not affected by stormwater controls. Particle sizes were measured using a Coulter® Multisizer™ IIe and

verified with microscopic, sieve, and settling column tests. In all cases, the New Jersey samples had the smallest particle sizes associated with specific occurrence frequencies (even though they were collected using manual "dipper" samplers and not automatic samplers, which might miss the largest particles), followed by Wisconsin, and then Birmingham, AL, which had the largest particles (which were collected using automatic samplers). The New Jersey samples were obtained from gutter flows in a residential neighborhood that was xeriscaped; the Wisconsin samples were obtained from several source areas, including parking areas and gutter flows mostly from residential, but from some commercial areas, while the Birmingham samples were collected from a long-term parking area on the UAB campus.

The median particle sizes ranged from 0.6 to 38 μm and averaged 14 μm. The 90th percentile sizes ranged from 0.5 to 11 μm and averaged 3 μm. These particle sizes are all substantially smaller than have been typically assumed for stormwater. Stormwater particle size distributions typically do not include bedload components because automatic sampler intakes are usually located above the bottom of the pipe where the bedload occurs. During the Monroe St. (Madison, WI) detention pond monitoring, the USGS and WI DNR installed special bedload samplers that trapped the bedload material for analysis. This additional bedload comprised about 10% of the annual total solids loading. This is not a large fraction of the solids, but it represents the largest particle sizes flowing in the stormwater, and it can be easily trapped in most detention ponds or catchbasins. The bedload component in Madison was most significant during the early spring rains when much of the traction control sand that could be removed by rains was being washed from the streets.

The settling velocities of discrete particles are shown in Figure 6.100, based on Stokes' and Newton's settling relationships. Probably more than 90% of all stormwater particulates are in the 1 to 100 μm range, corresponding to laminar flow conditions. This figure also shows the effects of different specific gravities on the settling rates. In most cases, stormwater particulates have specific gravities in the range of 1.5 to 2.5. This corresponds to a relatively narrow range of settling rates for a specific particle size.

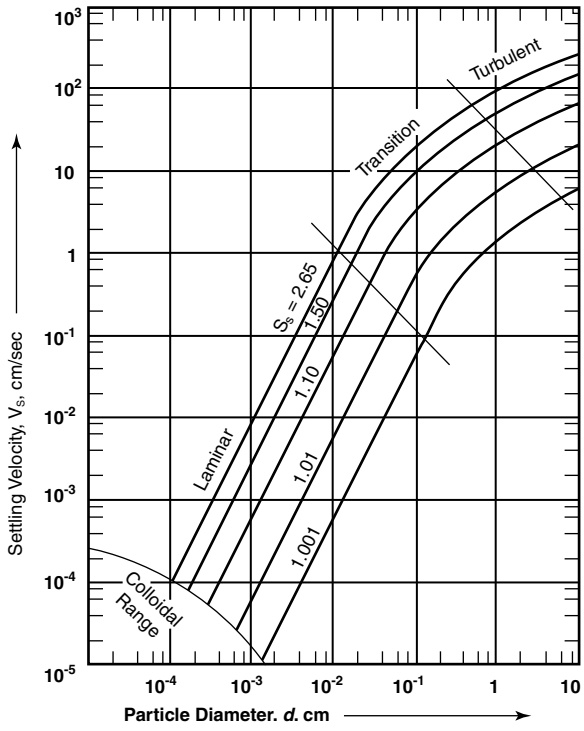


Figure 6.100 Settling rates of stormwater particulates.

Methods to Measure Stormwater Particle Sizes and Settling Velocities

Particle size is much easier to measure than settling rates. Automated (but expensive) particle sizing equipment is recommended because it enables very fast and accurate measurements, especially if supplemented with periodic settling column tests to determine deviations from standard settling theory. The following paragraphs briefly describe some of the particle settling options that have been used successfully for stormwater analyses. The most critical aspect of these analyses is obtaining an accurate sample, representing all particle sizes of interest. Automatic water samplers are suitable for obtaining samples having particles up to several hundred µm in size, but they cannot adequately sample particles much larger than about 1 mm in size. These large sizes are rare in stormwater, but they should be included in analyses in order to make suitable conclusions based on the data analyses. Automatic samplers can be supplemented using bedload samplers, as described in Chapter 5. However, the bedload samplers normally have to collect samples over an extended period of time to obtain sufficient samples for analysis. Manual sampling is usually easiest for representative sediment sampling, but is representative of only very short periods of time. Effective manual sampling must represent the complete water column, including bedload. This is easiest to accomplish if a "dipper" or "bucket" is used to collect flowing stormwater as it drops from an outfall or into an inlet.

Figure 6.101 Sieve analysis for stormwater particle determinations.

Sieve Analyses — This is probably the best procedure for laboratories that do not have access to expensive automated equipment, but have typical solids analysis balances, drying ovens, etc. (Figure 6.101). The basic procedure is as follows, using a 1- to 2-L well-mixed stormwater sample and a set of small sieves (usually about eight sieves, from 25 to 2000 µm, each having about one half the sieve opening as the next largest sieve):

1. Remove 100 mL of the sample for standard TDS and suspended solids analysis. The TDS sample is obtained by filtering the 100 mL through a 0.45-µm glass fiber filter. The filtrate, after passing through the filter, is placed in a dried and preweighed crucible for evaporation and final weighing. The filter is placed in a clean and preweighed small aluminum foil dish for drying and final weighing for the suspended solids analysis. Another 100-mL sample is placed directly in a preweighed crucible for evaporation and final weighing for a total solids analysis (and to check for errors associated with the separate TDS and SS analyses).
2. The remaining complete sample is then poured through the largest-sized sieve (the 2000 µm), and collected in another beaker. The sieved water captured in the second beaker is then sampled for total solids. After another 100-mL sample is removed for analysis, the remaining water is poured through the next smallest sieve, and another sample for total solids is removed. This process is repeated until water has been poured through all of the sieves and appropriate samples have been obtained for total solids analysis for each fraction.
3. All of the total solids samples are then oven dried, placed in a desiccator for cooling, and then weighed. The total solids content of each size fraction is then calculated, using the amount of water sample evaporated. The TDS content of the sample is subtracted from each total solids value, resulting in the suspended solids concentration for each particle size. An accumulative particle size distribution can then be prepared for the sample.

Unfortunately, this straightforward procedure requires a lot of time per sample and is limited as to the smallest particle size that can be measured, because the smallest sieve size available is

about 25 µm. There is therefore a large gap between this particle size and the 0.45-µm "TDS" size, and much of the sample may be in this size range. It is possible to obtain higher resolution data in this range by using a series of Teflon or nylon filters (mounted on a vacuum filtering setup, as for the TDS filtration) in this size range. These are relatively expensive filters.

If the filtered water is to be analyzed for other pollutants (usually heavy metals, COD, and nutrients are the primary constituents of concern for particle size analysis of stormwater), stainless steel sieves and plastic or Teflon membrane filters should be used on a plastic filter stand. Standard glass fiber filters used for suspended solids analyses and glass filter stands cause zinc contamination from the glass, and standard brass sieves cause contamination of many heavy metals. In all cases, blank water should be subjected to the sampling processing apparatus and tested for contamination potential.

Automated Particle Size Analyses — This is the fastest, easiest, but most expensive (in terms of equipment) procedure for determining particle sizes in stormwater. There are many instruments capable of automated particle size analyses, but most are designed for high concentration suspensions and slurries that are not suitable for stormwater analyses, unless extraordinary sample preparation significantly concentrates the sample. The most common methods used for stormwater samples are laser-based diffraction instruments and the "electrical sensing zone method" (the Coulter Multisizer, Figure 6.102). The following briefly describes the features of the Coulter method used in the UAB Environmental Engineering Laboratory. This method is intended to characterize particles and agglomerated state particles in water. This technique uses the Electrical Sensing Zone Method, which has been utilized and verified for many decades in the medical and health services industries.

Figure 6.102 Coulter Multisizer for stormwater particle size analyses.

The Coulter Multisizer method determines the number and size of particles suspended in a conductive liquid (a saline solution containing several mL of the sample) by monitoring the electrical current between two electrodes immersed in the conductive liquid (Isoton) on either side of a small aperture. The continuously stirred liquid containing the sample is forced to flow through the aperture by a pump in the unit. As a particle passes through the aperture, it changes the impedance between the electrodes and produces an electrical pulse of short duration having a magnitude proportional to the particle volume. The series of pulses is electronically scaled, counted, and accumulated in a number of size-related channels which, when their contents are displayed on an integral visual display, produces a size distribution curve.

This method provides accurate particle size distribution curves within a 30:1 dynamic range by diameter from any one aperture. Size distributions from 0.4 to 1200 µm can be evaluated, depending on the orifice tube aperture size. Aperture sizes larger than 200 µm require a modification of sample viscosity using Karo corn syrup to prevent the particles from settling during the test. Each aperture allows the measurement of particles in the nominal diameter range of 2 to 60% of the aperture diameter.

When more than one particle passes through the aperture at the same time, it is called *coincidence*. Coincidence is detected by the Multisizer II by the unique properties of coincident signals. The instrument reports the level of coincidence as the measurement is being made. Coincidence levels of 5 to 10% are normal and acceptable. The Multisizer II reports coincidence level, raw count, and coincidence corrected count as part of the size distribution report. If coincidence levels are too high, the sample must be diluted. If there is no coincidence, the sample is not concentrated enough and a larger aliquot of sample must be pipetted into the Isoton solution.

We have found it most accurate to prefilter the sample before analyses with our Coulter Counter. We separate the sample into three size fractions: <0.45 µm, >120 µm, and 0.45 to 120 µm, with

the intermediate size fraction further analyzed on the Coulter Counter using both the 50- and 200-µm aperture tubes. This results in four particle size distributions for each sample. These are manually combined (based on particle mass values for each size increment) using a spreadsheet. The most size data (highest resolution) is obtained from the intermediate sample fraction, which represents the majority of the particles (by mass) found in normal stormwater samples. This multistep approach is needed to ensure that the sample portions outside the normal working range of the Coulter Counter are included in the final size distribution. The sample is prepared as follows (about 300 to 500 mL of sample are needed for this analysis):

1. Remove 100 mL of the sample for standard TDS and SS analysis. The TDS sample is obtained by filtering 100 mL of sample through a 0.45-µm glass fiber filter (precleaned and preweighed). The filtrate (the sample after passing through the filter) is placed in a dried and preweighed crucible for evaporation, and final weighing (for the TDS determination). The filter is placed in a clean and preweighed small aluminum foil dish for drying and final weighing for the suspended solids analyses.
2. Another 100-mL sample is placed directly in a preweighed crucible for evaporation and final weighing for a total solids analysis (to check for errors associated with the separate TDS and SS analyses.)
3. The remaining sample (several hundred mL) is then poured through a moderate-sized sieve (with about 120-µm openings), and collected in another beaker. The sieved water captured in the second beaker is then sampled for total solids by removing another 100 mL sample for evaporation in a clean and preweighed crucible.
4. Finally, a sample is removed from the sieved water for the Coulter Counter analysis. This sieved sample should contain only particles up to about 1 to 120 µm, the range for the 50- and 200-µm aperture tubes that we commonly use.

The total solids fractions representing the three main sample portions are therefore known. The mid-fraction is further divided into very small increments using the data from the Coulter Counter tests. The final distribution of particle sizes is therefore well known over the entire range of particulates in the collected sample.

Settling Column Tests for Settling Velocity — Small-scale settling columns (using 50-cm-diameter Teflon columns about 0.7 m long) can be used to directly measure settling rate distributions of particles using basic engineering test procedures described in most wastewater and water treatment texts. Type one (discrete) settling is the predominant settling process for discrete stormwater particulates, and a simple settling column can be used with only a single sample port near the bottom of the tube. If Type two (hindered) settling is expected (due to high concentrations of flocculant particulates near the settling zone), then multiple sampling ports are needed along the settling column. For a simple settling apparatus, extended settling periods are needed to obtain information for the small particles that may be of most interest in stormwater. The test typically lasts about 3 days, with frequent samples (for total solids analyses) taken near the beginning of the test, tapering off as the test progresses. This is therefore a time-consuming (and expensive) test, but it should be conducted in conjunction with more frequent simpler particle size tests to confirm the relationship between size and settling velocity.

Much simpler hydrometer analyses of stormwater may not be effective because these procedures are intended for solutions having very high concentrations of particulates (Figure 6.103); however, they are useful for quantifying the clay size component of sediments (ASTM 1994). Some of the most polluted construction site runoff water (having suspended solids concentrations of several tens of thousands of mg/L) can be used with this method. Other settling rate monitoring methods, such as the Andreason pipette, are also rarely useful for the same reason (Figure 6.104). These are normally soil texture procedures where high concentrations of the soil particles can be mixed with water for the tests.

Figure 6.103 Hydrometer analyses for particle size determinations.

Figure 6.104 Andreason pipette for particle size determinations.

Visual Observations of Particle Characteristics — Microscopic observations of stormwater and receiving water particulates can yield much information. Standard laboratory microscopes, especially if equipped with a CCD camera and connected to a computer having particle analysis software (such as SigmaScan Pro by SPSS Software) can be used to measure particle sizes, particle morphology, and even origin (Figure 6.105). *The Particle Atlas,* both in print and the software version, from McCrone Assoc., Chicago, has a wealth of information to enable identification of particles. Most particles in stormwater are of erosion (mineral) origin that have become contaminated. As shown in Figure 6.106 (a typical microscopic view of stormwater particulates), relatively few particulates are from plants, and some are obviously from asphalt degradation and automobile exhaust. This photograph covers an area of about 600 by 800 µm, so the largest particles noted are about 100 µm in length. The polarized light images show asphalt particles dark, while minerals are generally much lighter.

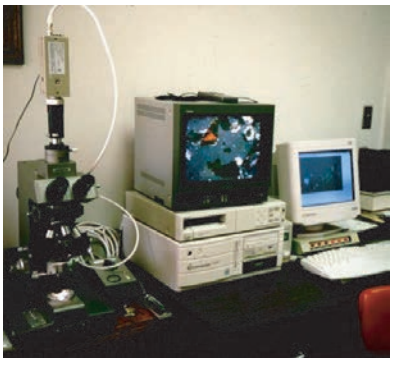


Figure 6.105 Microscope, video camera, and computer analyses of stormwater particles.

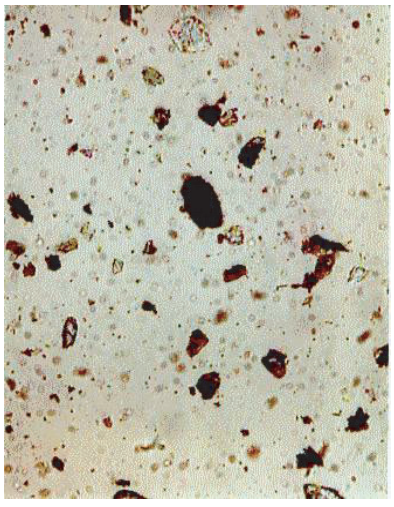


Figure 6.106 Typical microscopic view of particles in stormwater.

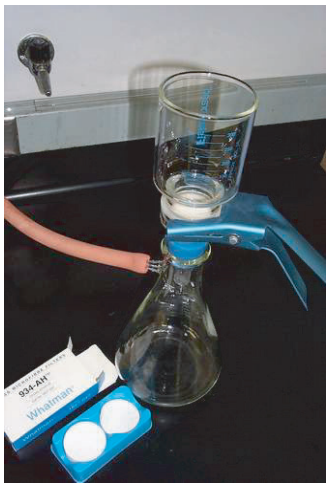


Figure 6.107 Standard filtration setup used with different membrane filters.

Figure 6.108 UV light digestion for controlled photooxidation.

Special Analytical Methods and Sample Preparation Procedures for Identifying Specific Forms of Metals

Sequential extraction has been used to separate the metals in a sample into various forms, such as separating the fraction bound to organic material from the fraction bound to mineral particulates, and to identify the fraction of the metals that may accumulate in aquatic organisms (Florence and Batley 1980). Figures 6.107 through 6.109 show various equipment used in the UAB environmental labs for treating samples for sequential analyses.

Several types of sequential extraction procedures were summarized by Bott (1995) to identify the form of heavy metals that may exist in a water sample (Figures 6.110 from Figura and McDuffie 1980; 6.111 from Florence and Batley 1980; and 6.112 from Nurnberg 1985). These procedures are useful to supplement the Toxicity Identification Evaluation (TIE) scheme noted later if metals are found to be the causative agent for stormwater toxicity (highly likely). The TIE scheme resulted

Figure 6.109 Solid phase extraction manifold for resin exchange experiments.

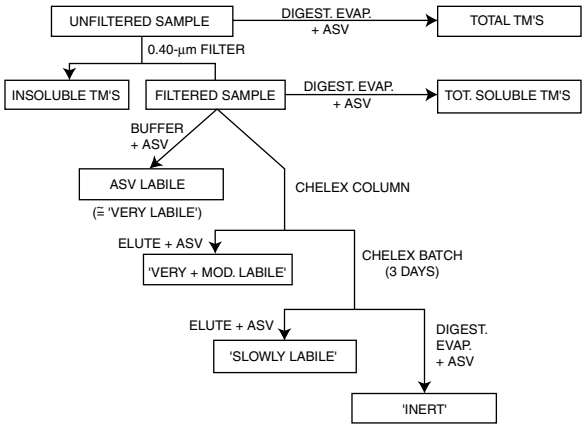


Figure 6.110 Figura and McDuffie scheme for chemical speciation analyses of trace metals. (From Figura, P. and B. McDuffie. *Anal. Chem.*, 52, 1433, 1980. Copyright 1980. American Chemical Society. Reprinted with permission.)

in sample components having specific toxicities. The most toxic sample components can then be subjected to further analyses to measure the toxicant concentrations. Organic analyses using GC/MSD or HPLC technology are very sensitive and can identify specific organic compounds present in the water. Unfortunately, the heavy metal analysis methods are only capable of measuring the total and filterable forms of each metal. However, heavy metals have greatly varying toxicities depending on their form. These sequential extraction procedures can result in a better understanding of the forms of the metals present in the sample and can identify the likely toxic forms present. These schemes typically separate the metals into functional categories, depending on the sample handling. As an example, Figure 6.111 shows a 0.45-µm filtration step to separate particulate from "soluble" forms. The soluble forms are further subjected to acetate buffer digestion (at pH 4.9) to identify labile forms of the metals, then to Chelex-100 extraction columns to identify forms that are sorbed onto inorganics or organics, and finally to UV digestion to identify the organic bound fraction. Anodic stripping voltammetric (ASV) methods are available to further identify the oxidation state of many of the metals of interest and can result in much more information than if graphite furnace atomic adsorption spectrophotometry is used for the metal analyses with these schemes. The sequential extraction procedures have been widely reported for studies of nutrient and metal availability in agricultural soils and for studies of sediments and dredged materials (for example see Tessier and Turner 1999).

Use of Tracers to Identify Sources of Inappropriate Discharges to Storm Drainage and Receiving Waters

Sources of Inappropriate Discharges into Storm Drainage

The need to identify inappropriate sources of discharges to storm drainage is critical for all stormwater management activities and is required by the EPA's stormwater discharge permits. Prior research (as summarized in EPA 1983a; Lalor 1993; Pitt et al. 1993) has shown that dry-weather flows from storm drainage may contribute a larger annual discharge mass for many pollutants than stormwater. These dry-weather sources may include direct connections to the storm drainage and sources that enter the drainage mainly through infiltration. Direct connections refer to physical

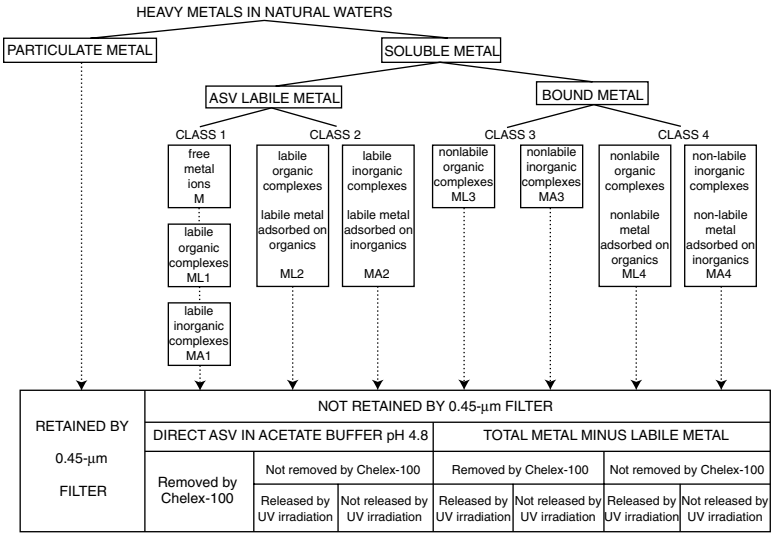


Figure 6.111 Florence and Batley scheme for chemical speciation analyses of trace metals. (From Bott, A. Voltammetric determination of trace concentrations of metals in the environment. *Current Separations,* 14(1): 24–30. July 1995. With permission.)

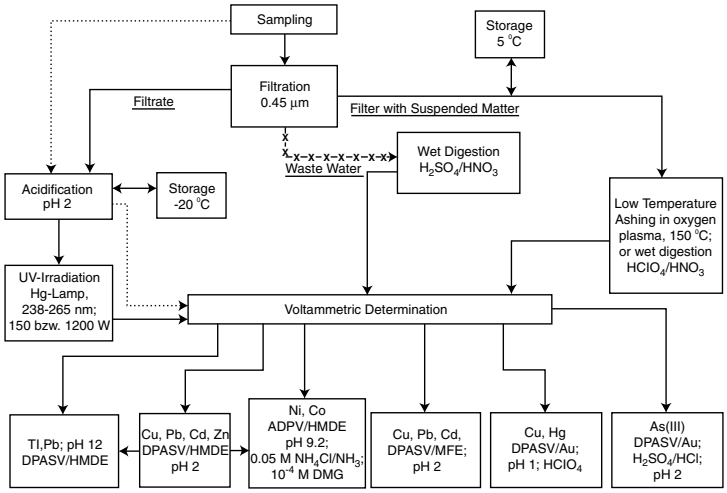


Figure 6.112 3 Nurnberg scheme for chemical speciation analyses of trace metals. Note: the last row of boxes contains notations describing the typical anodic stripping voltammetry (ASV) methods for each group of heavy metals. (From Nurnberg, H.W. Applications and potentialities of voltammetry in environmental chemistry of ecotoxic metals. In *Electrochemistry in Research and Development.* Edited by R. Kalvoda and R. Parsons. Plenum Publishing. pp. 121. New York. 1985. With permission.)

connections of sanitary, commercial, or industrial piping carrying untreated or partially treated wastewaters to a separate storm drainage system. These connections are usually unauthorized. They may be intentional, or may be accidental due to mistaken identification of sanitary sewer lines. They represent the most common source of entries to storm drains by industry. Direct connections can result in continuous or intermittent dry-weather entries of contaminants into the storm drain. Some common situations are:

- Sanitary sewers that tie into a storm drain.
- Foundation drains or residential sump-pump discharges that are frequently connected to storm drains. While this practice may be quite appropriate in many cases, it can be a source of contamination when the local groundwater is contaminated, as for example by septic tank failures.
- Commercial laundries and car wash establishments that may route process wastewaters to storm drains rather than sanitary sewers.

Continuous dry-weather flows may be caused by groundwater infiltration into storm drains when the storm sewers are located below the local groundwater table. These continuous discharges generally are not a pollution threat to surface waters, since most groundwaters which infiltrate into storm sewers are not contaminated, but these flows will have variable flow rates due to fluctuations in the level of the water table and percolation from rainfall events. Underground potable water main breaks are a potential clean source of releases to storm drains. While such occurrences are not a direct pollution source, they should obviously be corrected. However, when groundwater pollution does occur, such as from leaky underground storage tanks, storm drains may become a method of conveyance for these contaminants to the surface waters. Infiltration into storm drains most commonly occurs through leaking pipe joints and poor connections to catchbasins, but can also be due to other causes, such as damaged pipes and subsidence. Storm drains, as well as natural drainage channels, can therefore intercept and convey subsurface groundwater and percolating waters. Groundwater may be contaminated, either in localized areas or on a relatively widespread basis. In cases where infiltration into the storm drains occurs, it can be a source of excessive contaminant levels in the storm drains. Potential sources of groundwater contamination include, but are not limited to:

- Failing or nearby septic tank systems
- Exfiltration from sanitary sewers in poor repair
- Leaking underground storage tanks and pipes
- Landfill seepage
- Hazardous waste disposal sites
- Naturally occurring toxicants and pollutants due to surrounding geological or natural environment

Leaks from underground storage tanks and pipes are a common source of soil and groundwater pollution and may lead to continuously contaminated dry-weather entries. These situations are usually found in commercial operations, such as gasoline service stations, or industries involving the piped transfer of process liquids over long distances and the storage of large quantities of fuel, e.g., petroleum refineries. Pipes that are plugged or collapsed, as well as leaking storage tanks, may cause pollution when they release contaminants underground which can infiltrate through the soil into stormwater pipes.

The most common potential nonstormwater entries, which have been identified by a review of documented case studies for commercial and residential areas by Lalor (1993) and Pitt et al. (1993) included:

- Sanitary wastewater sources:
  - Raw sanitary wastewater from improper sewerage connections, exfiltration, or leakage
  - Effluent from improperly operating, designed, or nearby septic tanks
- Automobile maintenance and operation sources:
  - Car wash wastewaters
  - Radiator flushing wastewater
  - Engine degreasing wastes
  - Improper oil disposal
  - Leaky underground storage tanks
- Relatively clean sources:
  - Lawn runoff from over-watering
  - Direct spraying of impervious surfaces
  - Infiltrating groundwater
  - Water routed from preexisting springs or streams
  - Infiltrating potable water from leaking water mains
- Other sources:
  - Laundry wastewaters
  - Noncontact cooling water
  - Metal plating baths
  - Dewatering of construction sites
  - Washing of concrete ready-mix trucks
  - Sump pump discharges
  - Improper disposal of household toxic substances
  - Spills from roadway and other accidents

From the above list, sanitary wastewater is the most significant source of bacteria, while automobile maintenance and plating baths are the most significant sources of toxicants. Waste discharges associated with the improper disposal of oil and household toxicants tend to be intermittent and low volume. These wastes may therefore not reach the stormwater outfalls unless carried by higher flows from another source, or by stormwater during rains.

Human Health Problems Caused by Inappropriate Discharges

There are several mechanisms through which exposure to stormwater can cause potential human health problems. These include exposure to stormwater contaminants at swimming areas affected

by stormwater discharges, drinking water supplies contaminated by stormwater discharges, and the consumption of fish and shellfish that have been contaminated by stormwater pollutants. In receiving waters having only stormwater discharges, it is well known that inappropriate sanitary and other wastewaters are also discharging through the storm drainage system. The most serious problems appear to be associated with the presence of potential pathogens in problematic numbers. Contact recreation in pathogen-contaminated waters has been studied at many locations. The sources of the pathogens are typically assumed to be sanitary sewage effluent or periodic industrial discharges from certain food preparation industries (especially meat packing and fish and shellfish processing). However, several studies have investigated pathogen problems associated with stormwater discharges. It has generally been assumed that the source of the pathogens in the stormwater is inappropriate sanitary connections. However, stormwater unaffected by these inappropriate sources still contains high counts of pathogens that are also found in surface runoff samples from many urban surfaces. Needless to say, sewage contamination of urban streams is an important issue that needs attention during an urban water assessment investigation. Obviously, inappropriate discharges must be identified and corrected as part of any effort to clean up urban streams. If these sources are assumed to be nonexistent in an area and are therefore not considered in the stormwater management activities, incorrect and inefficient management decisions are likely, with disappointing improvements in the receiving waters.

A number of issues emerged from the individual projects of the U.S. EPA's NURP (EPA 1983a). One of these issues involved illicit connections to storm drainage systems and was summarized as follows in the Final Report of the NURP executive summary: "A number of the NURP projects identified what appeared to be illicit connections of sanitary discharges to stormwater sewer systems, resulting in high bacterial counts and dangers to public health. The costs and complications of locating and eliminating such connections may pose a substantial problem in urban areas, but the opportunities for dramatic improvement in the quality of urban stormwater discharges certainly exist where this can be accomplished. Although not emphasized in the NURP effort, other than to assure that the selected monitoring sites were free from sanitary sewage contamination, this BMP (best management practice) is clearly a desirable one to pursue." The illicit discharges noted during NURP were especially surprising, because the monitored watersheds were carefully selected to minimize factors other than stormwater. Presumably, illicit discharge problems in typical watersheds would be much worse. Illicit entries into urban storm sewerage were identified by flow from storm sewer outfalls following substantial dry periods. Such flow could be the result of direct "illicit connections" as mentioned in the NURP final report, or could result from indirect connections (such as contributions from leaky sanitary sewerage through infiltration to the separate storm drainage). Many of these dry-weather flows are continuous and would therefore also occur during rain-induced runoff periods. Pollutant contributions from the dry-weather flows in some storm drains have been shown to be high enough to significantly degrade water quality because of their substantial contributions to the annual mass pollutant loadings to receiving waters.

In many cases, sanitary sewage was an important component (although not necessarily the only component) of the dry-weather discharges from storm drainage systems that have been investigated. From a human health perspective (associated with pathogens), it may not require much raw or poorly treated sewage to cause a receiving water problem. However, at low discharge rates, the DO receiving water levels may be minimally affected. The effects these discharges have on the receiving waters is therefore highly dependent on many site-specific factors, including frequency and quantity of sewage discharges and the creek flows. In many urban areas, the receiving waters are small creeks in completely developed watersheds. These creeks are the most at risk from these discharges as dry baseflows may be predominantly dry-weather flows from the drainage systems. In Tokyo (Fujita 1998), for example, numerous instances were found where correcting inappropriate sanitary sewage discharges resulted in the urban streams losing all of their flow. In cities adjacent to large receiving waters, these discharges likely have little impact (such as DO impacts from Nashville CSO discharges on the Cumberland River; Cardozo et al. 1994). The presence of pathogens from

raw or poorly treated sewage in urban streams, however, obviously presents a potentially serious public health threat. Even if the receiving waters are not designated as water contact recreation, children often play in small city streams.

Assessment Strategies for Identifying Inappropriate Discharges to Storm Drainage

The following is a summary of the strategy developed by Lalor (1993) and Pitt et al. (1993) for the EPA to support the outfall screening activities required by the National Pollutant Discharge Elimination System (NPDES) Stormwater Permit Program to identify and correct inappropriate discharges to storm drainage systems. Those documents should be consulted for more detailed information. The methods summarized here require the use of multiple indicators used in combination. The evaluation procedures outlined range from the most basic, requiring minimal information, to more complex, requiring additional analyses.

The detection and identification of flow components require the quantification of specific characteristics of the observed combined flow. Lalor (1993) developed a simple test suite that tested very reliably in field verification trials. This method requires the analysis of detergents, fluoride, ammonia, and potassium, plus noting obvious indicators. The characteristics of most interest should be relatively unique for each potential flow source. This will enable the presence of each flow source to be indicated, based on the presence (or absence) of these unique characteristics. The selected characteristics are termed *tracers*, because they have been selected to enable the identification of the sources of these waters. These methods can be used in many areas, although the selection of the specific tracers might vary if the likely source flows are different. This section also discusses other methods used to indicate sources of contaminants, such as fingerprinting hydrocarbon residuals and newly available analytical methods that are very specific to individual sources.

Investigations designed to determine the contribution of urban stormwater runoff to receiving water quality problems have led to a continuing interest in inappropriate connections to storm drainage systems. Urban stormwater runoff is traditionally defined as that portion of precipitation which drains from city surfaces and flows via natural or man-made drainage systems into receiving waters. In fact, urban stormwater runoff also includes waters from many other sources which find their way into storm drainage systems. Sources of some of this water can be identified and accounted for by examining current NPDES permit records for permitted industrial wastewaters that can be legally discharged to the storm drainage system. However, most of the water comes from other sources, including illicit and/or inappropriate entries to the storm drainage system. These entries can account for a significant amount of the pollutants discharged from storm sewerage systems (Pitt and McLean 1986).

In response to the early studies that indicated the importance of stormwater discharge effects on receiving waters, provisions of the Clean Water Act (1987) now require NPDES permits for stormwater discharges. Permits for municipal separate storm sewers include a requirement to effectively prohibit problematic nonstormwater discharges, thereby placing emphasis on the elimination of inappropriate connections to urban storm drains. Section 122.26 (d)(1)(iv)(D) of the rule specifically requires an initial screening program to provide means for detecting high levels of pollutants in dry-weather flows, which should serve as indicators of illicit connections to the storm sewers. To facilitate the application of this rule, the EPA's Office of Research and Development's Storm and Combined Sewer Pollution Control Program and the Environmental Engineering & Technology Demonstration Branch, along with the Office of Water's Nonpoint Source Branch, supported research for the investigation of inappropriate entries to storm drainage systems (Pitt et al. 1993). This research was designed to provide information and guidance to local agencies by (1) identifying and describing the most common potential sources of nonstormwater pollutant entries into storm drainage systems; and (2) developing an investigative methodology that would allow a user to determine whether significant nonstormwater entries are present in a storm drain, and then to identify the type of source, as an aid to determining the location of the source. An

important premise for the development of this methodology was that the initial field screening effort would require minimal effort and expense, but would have little chance of missing a seriously contaminated outfall. This screening program would then be followed by a more in-depth analysis to more accurately determine the significance and source of the nonstormwater pollutant discharges.

The approach presented in this research was based on the identification and quantification of clean baseflow and the contaminated components during dry weather. If the relative amounts of potential components are known, then the importance of the dry-weather discharge can be determined. As an example, if a baseflow is mostly uncontaminated groundwater, but contains 5% raw sanitary wastewater, it is likely an important source of pathogenic bacteria. Typical raw sanitary wastewater parameters (such as BOD_5 or suspended solids) would be in low concentrations and the sanitary wastewater source would be difficult to detect. Fecal coliform bacteria measurements would not help much because they originate from many possible sources, in addition to sanitary wastewater. Expensive unique microorganism or biochemical measurements would probably be needed to detect the presence of the wastewater directly. However, a tracer may be identified that can be used to identify relatively low concentrations of important source flows in storm drain dry-weather baseflows.

The ideal tracer should have the following characteristics:

- Significant difference in concentrations between possible pollutant sources
- Small variations in concentrations within each likely pollutant source category
- A conservative behavior (i.e., no significant concentration change due to physical, chemical, or biological processes)
- Ease of measurement with adequate detection limits, good sensitivity, and repeatability

In order to identify tracers meeting the above criteria, literature characterizing potential inappropriate entries into storm drainage systems was examined. Several case studies which identified procedures used by individual municipalities or regional agencies were also examined. Though most of the investigations resorted to expensive and time-consuming smoke or dye testing to locate individual illicit pollutant entries, a few provided information regarding test parameters or tracers. These screening tests were proven useful in identifying drainage systems with problems before the smoke and dye tests were used. The case studies also revealed the types of illicit pollutant entries most commonly found in storm drainage systems.

Selection of Parameters for Identifying Inappropriate Discharge Sources

Table 6.33 is an assessment of the usefulness of candidate field survey parameters in identifying different potential nonstormwater flow sources. Natural and domestic waters should be uncontaminated (except in the presence of contaminated groundwaters entering the drainage system, for example). Sanitary sewage, septage, and industrial waters can produce toxic or pathogenic conditions. The other source flows (wash and rinse waters and irrigation return flows) may cause nuisance conditions or degrade the ecosystem. The parameters marked with a plus sign can probably be used to identify the specific source flows by their presence. Negative signs indicate that the potential source flow probably does not contain the listed parameter in adverse or obvious amounts, and may help confirm the presence of the source by its absence. Parameters with both positive and negative signs for a specific source category would probably not be very helpful due to expected wide variations.

Fecal Coliform Bacteria as Indicators of Inappropriate Discharges of Sanitary Sewage

Several investigations have relied on fecal coliform measurements as indicators of sanitary sewage contamination of stormwater. However, the use of fecal coliforms has been shown to be

Table 6.33 Candidate Field Survey Parameters and Associated Non-Stormwater Flow Sources

Parameter	Natural Water	Potable Water	Sanitary Sewage	Septage Water	Indus. Water	Wash Water	Rinse Water	Irrig. Water
Fluoride	–	+	+	+	+/–	+	+	+
Hardness change	–	+/–	+	+	+/–	+	+	–
Surfactants	–	–	+	–	–	+	+	–
Fluorescence	–	–	+	+	–	+	+	–
Potassium	–	–	+	+	–	–	–	–
Ammonia	–	–	+	+	–	–	–	+/–
Odor	–	–	+	+	+	+/–	–	–
Color	–	–	–	–	+	–	–	–
Clarity	–	–	+	+	+	+	+/–	–
Floatables	–	–	+	–	+	+/–	+/–	–
Deposits and stains	–	–	+	–	+	+/–	+/–	–
Vegetation change	–	–	+	+	+	+/–	–	+
Structural damage	–	–	–	–	+	–	–	–
Conductivity	–	–	+	+	+	+/–	+	+
Temperature change	–	–	+/–	–	+	+/–	+/–	–
pH	–	–	–	–	+	–	–	–

Note: – implies relatively low concentration; + implies relatively high concentration; +/– implies variable conditions.

From Pitt et al. 1993.

an inadequate indicator of sewage except in gross contamination situations (see also Chapter 3). Low fecal coliform levels may also cause false negative findings, as was indicated during the Inner Grays Harbor study where a storm drain outfall with a confirmed domestic sewage connection was not found to have elevated fecal coliform levels (Pelletier and Determan 1988). High fecal coliform bacteria populations were observed at storm sewer outfalls at all times in both industrial and residential/commercial areas during a study in Toronto (Pitt and McLean 1986). During the warm-weather storm sampling period, surface sheetflows were shown to be responsible for most of the observations of bacteria at the outfalls. However, during cold weather, very few detectable surface snowmelt sheetflow or snow pack fecal coliform observations were obtained, while the outfall observations were still quite high. High fecal coliform bacteria populations were also observed during dry-weather flow conditions at the storm sewer outfalls during both warm and cold weather. Leaking, or cross-connected, sanitary sewerage was therefore suspected at both study areas. Contaminated sump-pump water (from poorly operating septic tank systems in medium-density residential areas) in the Milwaukee area has been noted as a potentially significant source of bacteria to storm drainage systems (R. Bannerman, WI DNR, personal communication).

The presence of bacteria in stormwater runoff, dry-weather flows, and in urban receiving waters has caused much concern, as described in Chapter 3. However, there are many potential sources of fecal coliforms in urban areas, besides sanitary sewage. Research projects conducted in Toronto, Ontario (Pitt and McLean 1986), and in Madison, WI (R. Bannerman, WI DNR, personal communication) have investigated the abundance of common indicator bacteria, potential pathogenic bacteria, and bacterial types that may indicate the source of bacterial contamination. The monitoring efforts included sampling from residential, industrial, and commercial areas. As in many previous studies, fecal coliforms were commonly found to exceed water quality standards by large amounts during the Toronto investigations. Fecal coliform populations were very large at all land uses investigated during warm weather (typical median outfall values were 10,000 to 30,000 organisms per 100 mL). Dry-weather baseflow fecal coliform populations were found to be statistically similar to the stormwater runoff populations. The cold-weather fecal coliform populations were much lower (300 to 10,000 per 100 mL), but still exceeded the water quality standards.

Samples were obtained from many potential sources, in addition to the outfall, during the Toronto study (Pitt and McLean 1986). Source area fecal coliform populations were very similar for different land uses for the same types of areas, but different source areas within the watersheds

varied significantly. Generally, roof runoff had the lowest fecal coliform populations, while roads and roadside ditches had the largest populations.

The types and concentrations of different bacteria biotypes vary for different animal sources. Quresh and Dutka (1979) found that pathogenic bacteria biotypes are present in urban runoff and are probably from several different sources. The sources (nonhuman vs. human) of bacteria in urban runoff are difficult to determine. Geldreich and Kenner (1969) caution against using the ratio of fecal coliform to fecal streptococci as an indicator, unless the waste stream is known to be "fresh." Unfortunately, urban runoff bacteria may have been exposed to the environment for some time before rain washed it into the runoff waters. Delays may also be associated with some dry-weather bacteria sources. This aging process can modify the fecal coliform to fecal streptococci ratio to make the bacteria appear to be of human origin. In fact, samples collected in runoff source areas usually have the lowest FC/FS ratio in a catchment, followed by urban runoff, and finally the receiving water (Pitt 1983). This transition probably indicates an aging process and not a change in bacteria source.

Debbie Sargeant of the Washington State Department of Ecology has prepared a summary of different methods for fecal contamination source identification. Her report is available at www.ecy.wa.gov/biblio/99345.html. She concluded that there is no easy, low-cost method for differentiating between human and nonhuman sources of bacterial contamination. Genetic fingerprinting and newly emerging PCR methods, plus combinations of indicators, are some of the recommendations made in this report to further investigate bacterial sources.

Therefore, bacteria are usually poor indicators of the presence of sanitary sewage contamination. Past use of fecal strep to fecal coliform ratios to indicate human vs. nonhuman bacteria sources in mixed and old wastewaters (such as most nonpoint waters) has not been successful and should be used with extreme caution. There may be some value in investigating specific bacteria types, such as fecal strep biotypes, but much care should be taken in the analysis and interpretation of the results. A more likely indicator of human wastes may be the use of certain molecular markers, specifically the linear alkylbenzenes and fecal sterols, such as coprostanol and epicoprostanol (Eaganhouse et al. 1988), although these may also be discharged by other carnivores (especially dogs) in a drainage ditch. Recent discussions of specific tracers for indicating sanitary sewage contamination is presented later in this discussion. The following discussion presents a more generally useful approach for identifying inappropriate discharges to storm drainage, relying on easily evaluated chemical tracers and visual observations.

Tracer Characteristics of Local Source Flows

Table 6.34 is a summary of tracer parameter measurements for Birmingham, AL by Pitt et al. (1993). This table is a summary of the "library" that describes the tracer conditions for each potential source category. The important information shown on this table includes the median and coefficient of variation (COV) values for each tracer parameter for each source category. The COV is the ratio of the standard deviation to the mean. A low COV value indicates a much smaller spread of data compared to a data set having a large COV value. It is apparent that some of the generalized relationships shown in Table 6.33 did not exist during the demonstration project. This emphasizes the need for obtaining local data describing likely source flows.

The fluorescence values shown in Table 6.34 are direct measurements from a fluorometer having general-purpose filters and lamps and at the least sensitive setting (number 1 aperture). The toxicity screening test results are expressed as the toxicity response noted after 25 minutes of exposure using an Azur Environmental Microtox unit which measures toxicity using the light output from phosfluorescent algae. The I_{25} values are the percentage light output decreases observed after 25 minutes of exposure to the sample, compared to a reference. Fresh potable water has a relatively high toxicity response because of the chlorine levels present. Dechlorinated, potable water has much smaller toxicity responses.

Table 6.34 Tracer Concentrations Found in Birmingham, AL, Waters (Mean, Standard Deviation, and Coefficient of Variation, COV)

	Spring Water	Treated Potable Water	Laundry Wastewater	Sanitary Wastewater	Septic Tank Effluent	Car Wash Water	Radiator Flush Water
Fluorescence	6.8	4.6	1020	250	430	1200	22,000
(% scale)	2.9	0.35	125	50	100	130	950
	0.43	0.08	0.12	0.20	0.23	0.11	0.04
Potassium (mg/L)	0.73	1.6	3.5	6.0	20	43	2800
	0.070	0.059	0.38	1.4	9.5	16	375
	0.10	0.04	0.11	0.23	0.47	0.37	0.13
Ammonia (mg/L)	0.009	0.028	0.82	10	90	0.24	0.03
	0.016	0.006	0.12	3.3	40	0.066	0.01
	1.7	0.23	0.14	0.34	0.44	0.28	0.3
Ammonia/potassium	0.011	0.018	0.24	1.7	5.2	0.006	0.011
(ratio)	0.022	0.006	0.050	0.52	3.7	0.005	0.011
	2.0	0.35	0.21	0.31	0.71	0.86	1.0
Fluoride (mg/L)	0.031	0.97	33	0.77	0.99	12	150
	0.027	0.014	13	0.17	0.33	2.4	24
	0.87	0.02	0.38	0.23	0.33	0.20	0.16
Toxicity	<5	47	99.9	43	99.9	99.9	99.9
(% light decrease	n/a	20	<1	26	<1	<1	<1
after 25 min, I_{25})	n/a	0.44	n/a	0.59	n/a	n/a	n/a
Surfactants	<0.5	<0.5	27	1.5	3.1	49	15
(mg/L as MBAS)	n/a	n/a	6.7	1.2	4.8	5.1	1.6
	n/a	n/a	0.25	0.82	1.5	0.11	0.11
Hardness (mg/L)	240	49	14	140	235	160	50
	7.8	1.4	8.0	15	150	9.2	1.5
	0.03	0.03	0.57	0.11	0.64	0.06	0.03
pH (pH units)	7.0	6.9	9.1	7.1	6.8	6.7	7.0
	0.05	0.29	0.35	0.13	0.34	0.22	0.39
	0.01	0.04	0.04	0.02	0.05	0.03	0.06
Color (color units)	<1	<1	47	38	59	220	3000
	n/a	n/a	12	21	25	78	44
	n/a	n/a	0.27	0.55	0.41	0.35	0.02
Chlorine (mg/L)	0.003	0.88	0.40	0.014	0.013	0.070	0.03
	0.005	0.60	0.10	0.020	0.013	0.080	0.016
	1.6	0.68	0.26	1.4	1.0	1.1	0.52
Specific conductivity	300	110	560	420	430	485	3300
(μS/cm)	12	1.1	120	55	311	29	700
	0.04	0.01	0.21	0.13	0.72	0.06	0.22
Number of samples	10	10	10	36	9	10	10

From Pitt et al. 1993.

Appropriate tracers are characterized by having significantly different concentrations in flow categories that need to be distinguished. In addition, effective tracers also need low COV values within each flow category. Table 6.33 shows the expected changes in concentrations per category, and Table 6.34 indicates how these expectations compared with the results of an extensive local sampling effort. The study indicated that the COV values were quite low for each category, with the exception of chlorine, which had much greater COV values. Chlorine is therefore not recommended as a quantitative tracer to estimate the flow components. Similar data should be collected in each community where these procedures are to be used. Recommended field observations include color, odor, clarity, presence of floatables and deposits, and rate of flow, in addition to chemical measurements for fluoride, potassium, ammonia, and detergents (or fluorescence).

Collection of Samples and Field Analyses

All outfalls should be evaluated, not just those larger than a certain size. Lalor (1994) found that the smallest outfalls were typically the most contaminated because they were likely to be

associated with creek-side small automotive businesses that improperly disposed of their wastes through small pipes. Figure 6.113 illustrates the simple sample collection methods used. The creeks are walked and all outfalls observed are evaluated. Generally, three-person crews are used, two walking the creek with waders, sampling equipment, and notebooks, while the third person drives the car to the next downstream meeting location (typically about 1/2 mile). It requires several (typically at least three) trips along a stream to find all the outfalls. Multiple sampling visits are also needed throughout the year to verify changing discharge conditions. Outfalls may be dry during some visits, but flowing during others.

We have found it to be much more convenient and efficient to collect samples in the field and return them to the laboratory where groups of samples can be evaluated together. Some simple field analyses are appropriate. Figure 6.114 shows a portable gas analyzer that can indicate explosive conditions, lack of oxygen, and the presence of H_2S. This is important from a safety standpoint in areas having little ventilation, and the H_2S can also be used to indicate sewage problems. Most of the field test kits examined during this research (and as summarized earlier in this chapter) would take much too long to conduct correctly and safely in the field.

Simple Data Evaluation Methods to Indicate Sources of Contamination

Negative Indicators Implying Contamination

Indicators of contamination (negative indicators) are clearly apparent visual or physical parameters indicating obvious problems and are readily observable at the outfall during the field screening activities. Relying only on these indicators can lead to an unacceptably high rate of false negatives and false positives and must therefore be supplemented with additional confirmatory methods. However, these indicators are easy to measure, are useful for indicating gross contamination, are easy to describe to nontechnical decision makers, and are therefore highly recommended as an important part of a field screening effort.

These observations are very important during the field survey because they are the simplest method of identifying grossly contaminated dry-weather outfall flows. The direct examination of outfall characteristics for unusual conditions of flow, odor, color, turbidity, floatables, deposits/stains, vegetation conditions, and damage to drainage structures is therefore an important part of these investigations. Table 6.35 presents a summary of these indicators, along with narratives of the descriptors to be selected in the field.

Figure 6.113 Collecting outfall samples for inappropriate discharge evaluations.

Figure 6.114 Portable gas analyzer for H_2S and explosive conditions.

Table 6.35 Interpretations of Physical Observation Parameters and Possible Associated Flow Sources

Odor — Most strong odors, especially gasoline, oils, and solvents, are likely associated with high responses on the toxicity screening test. Typical obvious odors include gasoline, oil, sanitary wastewater, industrial chemicals, decomposing organic wastes, etc.

sewage: smell associated with stale sanitary wastewater, especially in pools near outfall or septic system drainage.

sulfur ("rotten eggs"): industries that discharge sulfide compounds or organics (meat packers, canneries, dairies, etc.).

oil and gas: petroleum refineries or many facilities associated with vehicle maintenance or petroleum product storage.

rancid-sour: food preparation facilities (restaurants, hotels, etc.).

Color — Important indicator of inappropriate industrial sources. Industrial dry-weather discharges may be of any color, but dark colors, such as brown, gray, or black, are most common.

yellow: chemical plants, textile and tanning plants.

brown: meat packers, printing plants, metal works, stone and concrete, fertilizers, and petroleum refining facilities.

green: chemical plants, textile facilities.

red: meat packers or iron oxide from groundwater seeps, e.g., acid mine drainage.

gray: dairies, sewage.

Turbidity — Often affected by the degree of gross contamination. Dry-weather industrial flows with moderate turbidity can be cloudy, while highly turbid flows can be opaque. High turbidity is often a characteristic of undiluted dry-weather industrial discharges or soil erosion.

cloudy: sanitary wastewater, concrete or stone operations, fertilizer facilities, automotive dealers.

opaque: food processors, lumber mills, metal operations, pigment plants.

Floatable Matter — A contaminated flow may contain floating solids or liquids directly related to industrial, sanitary wastewater pollution, or agricultural feed lots. Floatables of industrial origin may include animal fats, spoiled food, oils, solvents, sawdust, foams, packing materials, or fuel.

oil sheen: petroleum refineries or storage facilities and vehicle service facilities.

sewage: sanitary wastewater.

Deposits and Stains — Refers to any type of coating near the outfall and are usually of a dark color. Deposits and stains often will contain fragments of floatable substances. These situations are illustrated by the grayish-black deposits that contain fragments of animal flesh and hair which often are produced by leather tanneries, or the white crystalline powder which commonly coats outfalls due to nitrogenous fertilizer wastes.

sediment: construction site or agricultural soil erosion.

oily: petroleum refineries or storage facilities, vehicle service facilities, and large parking lot runoff.

Vegetation — Vegetation surrounding an outfall may show the effects of industrial pollutants. Decaying organic materials coming from various food product wastes would cause an increase in plant life, while the discharge of chemical dyes and inorganic pigments from textile mills could noticeably decrease vegetation. It is important not to confuse the adverse effects of high stormwater flows on vegetation with highly toxic dry-weather intermittent flows.

excessive growth: food product facilities, sewage, or agricultural operations.

inhibited growth: high stormwater flows, beverage facilities, printing plants, metal product facilities, drug manufacturing, petroleum facilities, vehicle service facilities and automobile dealers, pesticide spraying.

Damage to Outfall Structures — Another readily visible indication of industrial contamination. Cracking, deterioration, and spalling of concrete or peeling of surface paint, occurring at an outfall are usually caused by severely contaminated discharges, usually of industrial origin. These contaminants are usually very acidic or basic in nature. Primary metal industries have a strong potential for causing outfall structural damage because their batch dumps are highly acidic. Poor construction, hydraulic scour, and old age may also adversely affect the condition of the outfall structure.

concrete cracking: industrial flows.

concrete spalling: industrial flows.

peeling paint: industrial flows.

metal corrosion: industrial flows.

From Pitt et al. 1993.

This method does not allow quantifiable estimates of the flow components, and it will very likely result in many incorrect negative determinations (missing outfalls that have important levels of contamination). These simple characteristics are most useful for identifying gross contamination. Only the most significant outfalls and drainage areas would therefore be recognized from this method. The other methods, requiring chemical determinations, can be used to quantify the flow contributions and to identify the less obviously contaminated outfalls.

Indications of intermittent flows (especially stains or damage to the structure of the outfall) could indicate serious illegal toxic pollutant entries into the storm drainage system that will be very difficult to detect and correct. Highly irregular dry-weather outfall flow rates or chemical characteristics could indicate industrial or commercial inappropriate entries into the storm drain system.

Correlation tests were conducted to identify relationships between outfalls that were known to have severe contamination problems and the negative indicators (Lalor 1994). Pearson correlation tests indicated that high turbidity (lack of clarity) and odors appeared to be the most useful physical indicators of contamination when contamination was defined by toxicity and the presence of detergents. Lack of clarity best indicated the presence of detergents, with an 80% correlation. As noted later, the detergent test was the single most useful of the chemical tests for distinguishing between contaminated and uncontaminated flows. The Pearson correlation tests also showed that noticeable odor was the best indicator of toxicity, with a 77% correlation. There is no theoretical connection between the physical indicators and these problems. High turbidity was noted in 74% of the contaminated source flow samples. This represented a 26% false negative rate (indication of no contamination when contamination actually exists), if one relied on turbidity alone as an indicator of contamination. High turbidity was noted in only 5% of the uncontaminated source flow samples. This represents the rate of false positives (indication of contamination when none actually exists) when relying on turbidity alone. Noticeable odor was indicated in 67% of flow samples from contaminated sources, but in none of the flow samples from uncontaminated sources. This translates to 37% false negatives, but no false positives. Obvious odors identified included gasoline, oil, sanitary wastewater, industrial chemicals or detergents, decomposing organic wastes, etc. A 65% correlation was also found to exist between color and Microtox toxicity. Color is an important indicator of inappropriate industrial sources, but it was also associated with some of the residential and commercial flow sources. Color was noted in 100% of the flow samples from contaminated sources, but it was also noted in 40% of the flow samples from uncontaminated sources. This represents 60% false positives, but no false negatives. Finally, a 63% correlation between the presence of sediments (assessed as settleable solids in the collection bottles of these source samples) and Microtox toxicity was also found. Sediments were noted in 34% of the samples from contaminated sources and in none of the samples from uncontaminated sources.

False negatives are more of a concern than a reasonable number of false positives when working with a screening methodology. Screening methodologies are used to direct further, more detailed investigations. False positives would be discarded after further investigation. However, a false negative during a screening investigation results in the dismissal of a problem outfall for at least the near future. Missed contributors to stream contamination may result in unsatisfactory in-stream results following the application of costly corrective measures elsewhere.

The method of using physical characteristics to indicate contamination in outfall flows does not allow quantifiable estimates of the flow components and, if used alone, will likely result in many incorrect determinations, especially false negatives. However, these simple characteristics are most useful for identifying gross contamination: only the most significantly contaminated outfalls and drainage areas would therefore be recognized using this method.

Detergents as Indicators of Contamination

Results from Mann–Whitney U tests (at the $\alpha = 0.05$ confidence level) indicated that samples from any of the dry-weather flow sources could be correctly classified as clean or contaminated

based only on the measured value of any one of the following parameters: detergents, color, or conductivity (Lalor 1994). Color and high conductivity were present in samples from clean sources as well as contaminated sources, but their levels of occurrence were significantly different between the two groups. If samples from only one source were expected to make up outfall flows, the level of color or conductivity could be used to distinguish contaminated outfalls from clean outfalls. However, since multisource flows occur, measured levels of color or conductivity could fall within acceptable levels because of dilution, even though a contaminating source was contributing to the flow. Detergents (anionic surfactants), on the other hand, can be used to distinguish between clean and contaminated outfalls simply by their presence or absence, using a detection limit of 0.06 mg/L. All samples analyzed from contaminated sources contained detergents in excess of this amount (with the exception of three septage samples collected from homes discharging only toilet flushing water). No clean source samples were found to contain detergents. Contaminated sources would be detected in mixtures with uncontaminated waters if they made up at least 10% of the mixture.

The HACH detergents test was used during these analyses and was found to work very well. Unfortunately, this test uses a large amount of benzene for sample extractions and so great care is needed with the analysis and waste disposal. Only the most highly trained analysts, understanding the dangers of using benzene, should be allowed to use this test. An alternative method examined by CHEMetrics uses relatively small amounts of chloroform (well contained) for sample extractions and is therefore much safer, although care is also needed during the test and in disposal of waste. However, this method has a poorer detection limit (about 0.15 mg/L) than the HACH method, leading to less sensitivity (and possible false negatives).

Because of the hazardous problems associated with using these simple detergent (anionic surfactant) tests, we have investigated numerous alternative, but related, tests. Standard tests for boron are relatively simple, safe, and sensitive. Historically, boron was an important component in laundry detergents and tests were conducted to see if this analysis would be a suitable substitute for the detergent tests. Unfortunately, boron appears to have been replaced in most U.S. detergents, as numerous tests of commercial laundry products found little boron. In addition, boron tests of sewage mixtures and from numerous mixed wastewaters from throughout the country also indicated little boron. Fluorescence of test waters, using an extremely sensitive, but expensive, fluorometer (Turner 10-AU), was also evaluated, but with mixed results. The analyses of sewage and detergents found highly variable fluorescence values because of the highly variable amounts of fabric whiteners found in detergents. However, it is possible to use fluorescence as a good presence/absence test, like the initial detergent evaluations. The previous discussion of optical brighteners (as a field test kit) indicated the potential usefulness of this method, but more work is needed to determine its sensitivity. As indicated later, more sophisticated tests for detergent components (LAS and perfumes, especially) have been successfully used as sewage tracers in many waters, but these analyses require expensive and time-consuming HPLC analyses.

Simple Checklist for Major Flow Component Identification

Table 6.36 is a simplification of the analysis strategy to separate the major nonstormwater discharge sources for areas having no industrial activity. The first indicator is the presence or absence of flow. If no dry-weather flow exists at an outfall, then indications of intermittent flows must be investigated. Specifically, stains, deposits, odors, unusual stream-side vegetation conditions, and outfall structural damage can all indicate intermittent nonstormwater flows. However, multiple visits to outfalls over long time periods are needed to confirm that only stormwater flows occur. The following paragraphs summarize the rationale used to distinguish between treated potable water and sanitary wastewater, the two most common dry-weather flow sources in storm drainage systems in residential and commercial areas.

Table 6.36 Simplified Checklist to Identify Residential Area Non-Stormwater Flow Sources

1. Flow? If yes, go to 2; if no, go to 3.
2. Fluorides (or different hardness)? If yes, probably treated water (may be contaminated), go to 4; if no, then untreated natural water (probably uncontaminated), or untreated industrial water (may be toxic).
3. Check for intermittent dry-weather flow signs (may be contaminated). If yes, recheck outfall at later date; if no, then not likely a significant non-stormwater source.
4. Surfactants (or fluorescence)? If yes, may be sanitary wastewater, laundry water, or other wash water (may be pathogenic, or nuisance), go to 5; if no, then may be domestic water line leak, irrigation runoff, or rinse water (probably not a contaminated non-stormwater source, but may be a nuisance).
5. Elevated potassium (or ammonia)? If yes, then likely sanitary wastewater source (pathogenic); if no, likely wash water (probably not a contaminated non-stormwater source, but may be a nuisance).

From Pitt et al. 1993.

Treated Potable Water — A number of tracer parameters may be useful for distinguishing treated potable water from natural waters:

- Major ions or other chemical/physical characteristics of the flow components can vary substantially, depending on whether the water supply sources are groundwater or surface water, and whether the sources are treated or not. Specific conductance may also serve as an indicator of the major water source.
- Fluoride can often be used to separate treated potable water from untreated water sources. This latter group may include local springs, groundwater, regional surface flows, or nonpotable industrial waters. If the treated water has no fluoride added, or if the natural water has fluoride concentrations close to potable water fluoride concentrations, then fluoride may not be an appropriate indicator. Water from treated water supplies (that test positive for fluorides or other suitable tracers) can be relatively uncontaminated (domestic water line leakage or irrigation runoff), or it may be heavily contaminated. If the drainage area has industries that have their own water supplies (quite rare for most urban drainage areas), further investigations are needed to check for industrial nonstormwater discharges. Toxicity screening methods would be very useful in areas known to have commercial or industrial activity, or to check for intermittent residential area discharges of toxicants. Fluoride can be very high in some commercial wash waters and industrial wastewaters.
- Hardness can also be used as an indicator if the potable water source and the baseflow are from different water sources. An example would be if the baseflow is from hard groundwater and the potable water is from softer surface supplies.
- If the concentration of chlorine is high, then a major leak of disinfected potable water is probably close to the outfall. Because of the rapid loss of chlorine in water (especially if some organic contamination is present), it is not a good parameter for quantifying the amount of treated potable water observed at the outfall.

Water from potable water supplies (that test positive for fluorides, or other suitable tracers) can be relatively uncontaminated (domestic water line leakage or irrigation runoff) or heavily contaminated (sanitary wastewater).

Sanitary Wastewaters — In areas containing no industrial or commercial sources, sanitary wastewater is probably the most important dry-weather source of storm drain flows. In addition, septic systems often do not operate properly and might be a significant source of contamination in rural areas. The following parameters can be used for quantifying the sanitary wastewater components of the treated domestic water portion:

- Surfactant (detergent) analyses may be useful in determining the presence of sanitary wastewaters, as noted previously. However, surfactants present in water originating from potable water sources could indicate sanitary wastewaters, laundry wastewaters, car washing wastewater, or any other waters containing surfactants. If surfactants are not present, then the potable water could be relatively uncontaminated (domestic water line leaks or irrigation runoff).

- The presence of fabric whiteners (as measured by fluorescence) can also be used in distinguishing laundry and sanitary wastewaters.
- Sanitary wastewaters often exhibit predictable trends during the day in flow and quality. In order to maximize the ability to detect direct sanitary wastewater connections into the storm drainage system, it would be best to survey the outfalls during periods of highest sanitary wastewater flows (mid to late morning hours).
- The ratio of surfactants to ammonia or potassium concentrations may be an effective indicator of the presence of sanitary wastewaters or septic tank effluents. If the surfactant concentrations are high, but the ammonia and potassium concentrations are low, the contaminated source may be laundry wastewaters. Conversely, if ammonia, potassium, and surfactant concentrations are all high, sanitary wastewater is the likely source. Some researchers have reported low surfactants in septic tank effluents. Therefore, if surfactants are low but potassium and ammonia are both high, septic tank effluent may be present. However, research in the Birmingham, AL, area found high surfactant concentrations in septic tank effluent, further stressing the need to obtain local characterization data for potential contaminating sources.
- Obviously, odor and other physical appearances such as turbidity, coarse and floating "tell-tale" solids, foaming, color, and temperature would also be very useful in distinguishing sanitary wastewater from wash water or laundry wastewater sources, as noted previously. However, these indicators may not be very obvious for small levels of sanitary wastewater contamination.

Flowchart for Most Significant Flow Component Identification

A further refinement of the above checklist is the flowchart shown on Figure 6.115. This flow chart describes an analysis strategy that may be used to identify the major component of dry-weather flow samples in residential and commercial areas. This method does not attempt to distinguish among all potential sources of dry-weather flows identified earlier, but rather the following four major groups of flow are identified: (1) tap waters (including domestic tap water, irrigation water, and rinse water), (2) natural waters (spring water and shallow groundwater), (3) sanitary wastewaters (sanitary sewage and septic tank discharge), and (4) wash waters (commercial laundry waters, commercial car wash waters, radiator flushing wastes, and plating bath wastewaters). The use of this method would not only allow outfall flows to be categorized as contaminated or uncontaminated, but would also allow outfalls carrying sanitary wastewaters to be identified. These outfalls could then receive the highest priority for further investigation leading to source control. This flowchart was designed for use in residential and/or commercial areas only. Investigations in industrial or industrial/commercial land use areas must be approached in an entirely different manner.

In residential and/or commercial areas, all outfalls should be located and examined. The first indicator is the presence or absence of dry-weather flow. If no dry-weather flow exists at an outfall, indications of intermittent flows must be investigated. Specifically, stains, deposits, odors, unusual stream-side vegetation conditions, and damage to outfall structures can all indicate intermittent nonstormwater flows. However, frequent visits to outfalls over long time periods, or the use of other monitoring techniques, may be needed to confirm that only stormwater flows occur. If intermittent flow is not indicated, the outfall probably does not have a contaminated nonstormwater source. The other points on the flowchart serve to indicate if a major contaminating source is present, or if the water is uncontaminated. Component contributions cannot be quantified using this method, and only the "most contaminated" type of source present will be identified.

If dry-weather flow exists at an outfall, then the flow should be sampled and tested for detergents. If detergents are not present, the flow is probably from a noncontaminated nonstormwater source. The lower limit of detection for detergent should be about 0.06 mg/L.

If detergents are not present, fluoride levels can be used to distinguish between flows with treated water sources and flows with natural sources in communities where water supplies are fluoridated and natural fluoride levels are low. In the absence of detergents, high fluoride levels

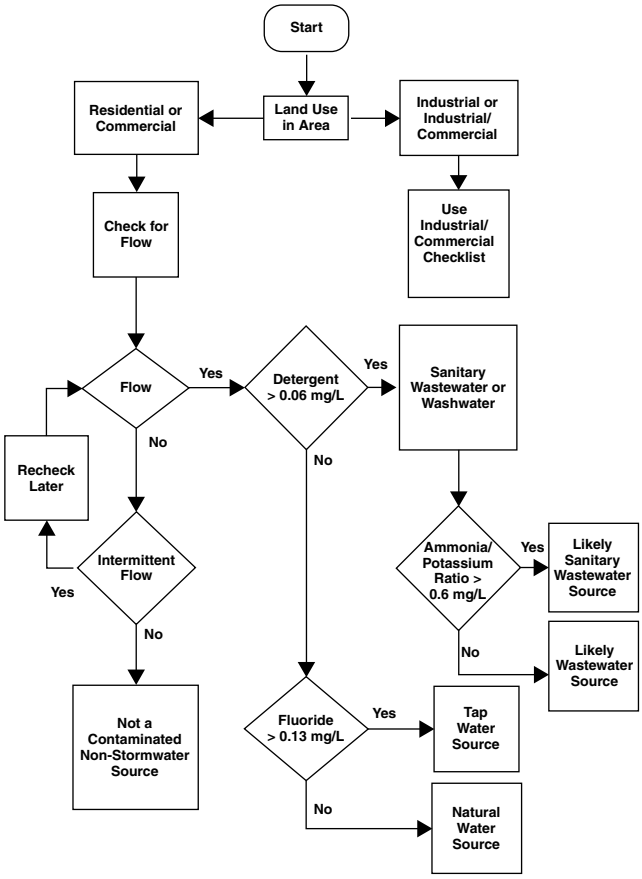


Figure 6.115 Simple flowchart method to identify significant contaminating sources. (From Lalor, M. *An Assessment of Non-Stormwater Discharges to Storm Drainage Systems in Residential and Commercial Land Use Areas.* Ph.D. dissertation. Department of Civil and Environmental Engineering. Vanderbilt University. 1994. With permission.)

would indicate a potable water line leak, irrigation water, or wash/rinse water. Low fluoride levels would indicate waters originating from springs or shallow groundwater. Based on the flow source samples tested in this research (Table 6.34), fluoride levels above 0.13 mg/L would most likely indicate that a tap water source was contributing to the dry-weather flow in the Birmingham, AL, study area.

If detergents are present, the flow is probably from a contaminated nonstormwater source, as indicated on Table 6.34. The ratio of ammonia to potassium can be used to indicate whether or not the source is sanitary wastewater. Ammonia/potassium ratios greater than 0.60 would indicate likely sanitary wastewater contamination. Ammonia/potassium ratios were above 0.9 for all septage and sewage samples collected in Birmingham (values ranged from 0.97 to 15.37, averaging 2.55). Ammonia/potassium ratios for all other samples containing detergents were below 0.7, ranging from 0.00 to 0.65, averaging 0.11. One radiator waste sample had an ammonia/potassium ratio of 0.65.

Noncontaminated samples collected in Birmingham had ammonia/potassium ratios ranging from 0.00 to 0.41, with a mean value of 0.06 and a median value of 0.03. Using the mean values for noncontaminated samples (0.06) and sanitary wastewaters (2.55), flows comprised of mixtures containing at least 25% sanitary wastes with the remainder of the flow from uncontaminated sources would likely be identified as sanitary wastewaters using this method. Flows containing a smaller percentage of contributions from sanitary wastewaters might be identified as having a wash water source, but would not be identified as uncontaminated.

General Matrix Algebra Methods to Indicate Sources of Contamination through Fingerprinting

Other approaches can also be used to calculate the source components of mixed outfall flows. One approach is the use of matrix algebra to simultaneously solve a series of chemical mass balance equations. This method can be used to predict the most likely flow source, or sources, making up an outfall sample. It is possible to estimate the outfall source flow components using a set of simultaneous equations. The number of unknowns should equal the number of equations available, resulting in a square matrix. If there are seven likely source categories, then there should be seven tracer parameters used. If there are only four possible sources, then only the four most efficient tracer parameters should be used. Only tracers that are linearly related to mixture components can be used. As an example, pH cannot be used in these equations, because it is not additive.

Further site-specific statistical analyses may be needed to rank the usefulness of the tracers for distinguishing different flow sources. As an example, chlorine is generally not useful for these analyses because the concentration variability within many source categories is high (it is also not a conservative parameter). Chlorine may still be a useful parameter, but only to identify possible large potable water line leaks. Another parameter having problems for most situations is pH. The variation of pH between sources is very low (they are all very similar). pH may still be useful to identify industrial wastewater problems, but it cannot be used to quantify flow components. Toxicity is another parameter used during this research that was found not to be linearly additive.

This method estimates flow contributions from various sources using a "receptor model," based on a set of chemical mass balance equations. Such models, which assess the contributions from various sources based on observations at sampling sites (the receptors), have been applied to the investigation of air pollutant sources for many years (Lee et al. 1993; Cooper and Watson 1980). The characteristic "signatures" of the different types of sources, as identified in the library of source flow data, allow the development of a set of mass balance equations. These equations describe the measured concentrations in an outfall's flow as a linear combination of the contributions from the different potential sources. A major requirement for this method is the physical and chemical characterization of waters collected directly from potential sources of dry-weather flows (the "library"). This allows concentration patterns (fingerprints) for the parameters of interest to be established for each type of source. Theoretically, if these patterns are different for each source, the observed concentrations at the outfall would be a linear combination of the concentration patterns from the different component sources, each weighted by a source strength term (m_n). This source strength term would indicate the fraction of outfall flow originating from each likely source. By measuring a number of parameters equal to, or greater than, the number of potential source types, the source strength term could be obtained by solving a set of chemical mass balance equations of the type:

$$C_p = \sum_n m_n x_{pn}$$

where C_p is the concentration of parameter p in the outfall flow and x_{pn} is the concentration of parameter p in source type n.

As an example of this method, consider eight possible flow sources and eight parameters, as presented in Table 6.37. The number of parameters evaluated for each outfall must equal the number of probable dry-weather flow sources in the drainage area. Mathematical methods are available which provide for the solution of over-specified sets of equations (more equations than unknowns), but these are not addressed here.

The selection of parameters for measurement should reflect evaluated parameter usefulness. Evaluation of the Mann–Whitney U Test results (Lalor 1994) suggested the following groupings

of parameters, ranked by their usefulness for distinguishing between all the types of flow sources sampled in Birmingham, AL:

- First set (most useful): potassium and hardness
- Second set: fluorescence, conductivity, fluoride, ammonia, detergents, and color
- Third set (least useful): chlorine

If parameter variations within the sources are not accounted for, the equations would take the form presented in Table 6.38. Here, the x terms, representing parameter concentrations within the specified source, have been replaced with the mean concentrations noted in the source library. After measured values are substituted into the equations for parameter concentrations in the outfall flow (C_p), this set of simultaneous equations can be solved using matrix algebra. The use of mean concentration values in the equation set was evaluated by entering the potential dry-weather flow source samples from Birmingham as unknowns (as if they were outfall samples) and solving for fractions of flow (the m terms in Table 6.38). This exercise resulted in four false negatives (6%) and 27 false positives (73%). The results of these simple preliminary tests indicated that there was too much variation of parameter concentrations within the various source types to allow them to be adequately characterized by simple use of the mean concentrations alone. Another method, recognizing variations in source flow characteristics in a Monte Carlo model, is presented by Lalor (1994). Both of these methods listed the likely multiple contaminating sources and estimated their relative contributions. Unfortunately, confirmation testing indicated inaccurate results much of the time, implying the greater usefulness of the simpler methods described previously. However, these matrix algebra methods may be very useful in other situations or locations and should be investigated as part of a local screening project to identify inappropriate discharges to storm drainage.

There are numerous other statistical analysis methods suitable for identifying sources of flows. Salau et al. (1997) present a review of several advanced statistical methods also derived from air pollution source identification research (see Chapter 7 for illustrations from his paper). Principal component analysis and hierarchical cluster analysis are shown as tools that can identify common sources of contamination by examining a set of well-selected tracer compounds (in northwest Mediterranean marine sediments in their example). These are used to develop the alternating least squares approach, similar to Lalor's (1994) use of these same techniques to identify the best parameters for the simultaneous equation solutions described above.

Table 6.37 Set of Chemical Mass Balance Equations

	Source 1	Source 2	Source 3	Source 4	Source 5	Source 6	Source 7	Source 8 Outfall
Parameter 1:	(m1)(x11)	+ (m2)(x12)	+ (m3)(x13)	+ (m4)(x14)	+ (m5)(x15)	+ (m6)(x16)	+(m7)(x17)	+ (m8)(x18) = C1
Parameter 2:	(m1)(x21)	+ (m2)(x22)	+ (m3)(x23)	+ (m4)(x24)	+ (m5)(x25)	+ (m6)(x26)	+(m7)(x27)	+ (m8)(x28) = C2
Parameter 3:	(m1)(x31)	+ (m2)(x32)	+ (m3)(x33)	+ (m4)(x34)	+ (m5)(x35)	+ (m6)(x36)	+(m7)(x37)	+ (m8)(x38) = C3
Parameter 4:	(m1)(x41)	+ (m2)(x42)	+ (m3)(x43)	+ (m4)(x44)	+ (m5)(x45)	+ (m6)(x46)	+(m7)(x47)	+ (m8)(x48) = C4
Parameter 5:	(m1)(x51)	+ (m2)(x52)	+ (m3)(x53)	+ (m4)(x54)	+ (m5)(x55)	+ (m6)(x56)	+(m7)(x57)	+ (m8)(x58) = C5
Parameter 6:	(m1)(x61)	+ (m2)(x62)	+ (m3)(x63)	+ (m4)(x64)	+ (m5)(x65)	+ (m6)(x66)	+(m7)(x67)	+ (m8)(x68) = C6
Parameter 7:	(m1)(x71)	+ (m2)(x72)	+ (m3)(x73)	+ (m4)(x74)	+ (m5)(x75)	+ (m6)(x76)	+(m7)(x77)	+ (m8)(x78) = C7
Parameter 8:	(m1)(x81)	+ (m2)(x82)	+ (m3)(x83)	+ (m4)(x84)	+ (m5)(x85)	+ (m6)(x86)	+(m7)(x87)	+ (m8)(x88) = C8

Equations of the form $C_p = \sum_n m_n x_{pn}$

where: C_p = the concentration of parameter p in the outfall flow

m_n = the fraction of flow from source type n

x_{pn} = the mean concentration of parameter p in source type n

From Lalor, M. *An Assessment of Non-Stormwater Discharges to Storm Drainage Systems in Residential and Commercial Land Use Areas.* Ph.D. dissertation. Department of Civil and Environmental Engineering. Vanderbilt University. 1994. With permission.

Once sources are identified, it is important to confirm their source and to ensure that corrective action is undertaken. Figures 6.116 and 6.117 show TV surveying being conducted in Boston to confirm the likely source of inappropriate discharges. Normally, the TV camera is remotely operated and pulled through small pipes (Figure 6.116). However, in the coastal area and in large pipes, crews were required to conduct the surveys manually (Figure 6.117).

Emerging Tools for Identifying Sources of Discharges

Coprostanol and Other Fecal Sterol Compounds Utilized as Tracers of Contamination by Sanitary Sewage

A more likely indicator of human wastes than fecal coliforms and other "indicator" bacteria may be the use of certain molecular markers, specifically the fecal sterols, such as coprostanol and epicoprostanol (Eaganhouse et al. 1988). However, these compounds are also discharged by other carnivores (especially dogs) in a drainage. A number of research projects have used these compounds to investigate the presence of sanitary sewage contamination. The most successful application may be associated with sediment analyses instead of water analyses. As an example, water analyses of coprostanol are difficult due to the typically very low concentrations found, although the concentrations in many sediments are quite high and much easier to quantify. Unfortunately, the long persistence of these compounds in the environment easily confuses recent contamination with historical or intermittent contamination.

Particulates and sediments collected from coastal areas in Spain and Cuba receiving municipal sewage loads were analyzed by Grimalt et al. (1990) to determine the utility of coprostanol as a chemical marker of sewage contamination. Coprostanol cannot by itself be attributed to fecal matter inputs. However, relative contributions of steroid components can be useful indicators. When the relative concentrations of coprostanol and coprostanone are higher than their 5α epimers, or more realistically, other sterol components of background or natural occurrence, they can provide useful information.

Sediment cores from Santa Monica Basin, CA, and effluent from two local municipal wastewater discharges were analyzed by Venkatesan and Kaplan (1990) for coprostanol to determine the degree of sewage addition to sediment. Coprostanols were distributed throughout the basin sediments in association with fine particles. Some stations contained elevated levels, either due to their proximity to outfalls or because of preferential advection of fine-grained sediments. A noted decline of coprostanols relative to total sterols from outfalls seaward indicated dilution of sewage by biogenic sterols.

Other chemical compounds have been utilized for sewage tracer work. Saturated hydrocarbons with 16 to 18 carbons, and saturated hydrocarbons with 16 to 21 carbons, in addition to coprostanol, were chosen as markers for sewage in water, particulate, and sediment samples near the Cocoa, FL, domestic wastewater treatment plant (Holm et al. 1990). The concentration of the markers was highest at points close to the outfall pipe and diminished with distance. However, the concentration of C16 to C21 compounds was high at a site 800 m from the outfall, indicating that these compounds were unsuitable markers for locating areas exposed to the sewage plume. The concentrations for the other markers were very low at this station.

The range of concentrations of coprostanol found in sediments and mussels of Venice, Italy, were reported by Sherwin et al. (1993). Raw sewage is still discharged directly into the Venice lagoon. Coprostanol concentrations were determined in sediment and mussel samples from the lagoon using gas chromatography/mass spectroscopy. Samples were collected in interior canals and compared to open-bay concentrations. Sediment concentrations ranged from 0.2 to 41.0 μg/g (dry weight). Interior canal sediment samples averaged 16 μg/g compared to 2 μg/g found in open-bay sediment samples. Total coprostanol concentrations in mussels ranged from 80 to 620 ng/g (wet weight). No mussels were found in the four most polluted interior canal sites.

Table 6.38 Chemical Mass Balance Equations with Parameter Means

Parameters	Spring Water 1	Ground-Water 2	Tap Water 3	Irrigation Water 4	Sanitary Sewage 5	Septic Tank 6	Car Wash 7	Laundry Water 8	Unknown Sample
Potassium	(m1)(0.73)	+ (m2)(1.19)	+ (m3)(1.55)	+ (m4)(6.08)	+ (m5)(5.97)	+ (m6)(18.82)	+ (m7)(42.69)	+ (m8)(3.48)	= (1)(C_p)
Hardness	(m1)(240)	+ (m2)(27)	+ (m3)(49)	+ (m4)(40)	+ (m5)(143)	+ (m6)(57)	+ (m7)(157)	+ (m8)(36)	= (1)(C_p)
Fluorescence	(m1)(6.8)	+ (m2)(29.9)	+ (m3)(4.6)	+ (m4)(214)	+ (m5)(251)	+ (m6)(382)	+ (m7)(1190)	+ (m8)(1024)	= (1)(C_p)
Conductivity	(m1)(301)	+ (m2)(51)	+ (m3)(112)	+ (m4)(105)	+ (m5)(420)	+ (m6)(502)	+ (m7)(485)	+ (m8)(563)	= (1)(C_p)
Fluoride	(m1)(0.03)	+ (m2)(0.06)	+ (m3)(0.97)	+ (m4)(0.90)	+ (m5)(0.76)	+ (m6)(0.93)	+ (m7)(12.3)	+ (m8)(32.82)	= (1)(C_p)
Ammonia	(m1)(0.01)	+ (m2)(0.24)	+ (m3)(0.03)	+ (m4)(0.37)	+ (m5)(9.92)	+ (m6)(87.21)	+ (m7)(0.24)	+ (m8)(0.82)	= (1)(C_p)
Detergents	(m1)(0.00)	+ (m2)(0.00)	+ (m3)(0.00)	+ (m4)(0.00)	+ (m5)(1.50)	+ (m6)(3.27)	+ (m7)(49.00)	+ (m8)(26.90)	= (1)(C_p)
Color	(m1)(0.0)	+ (m2)(8.0)	+ (m3)(0.0)	+ (m4)(10.0)	+ (m5)(37.9)	+ (m6)(70.6)	+ (m7)(221.5)	+ (m8)(46.7)	= (1)(C_p)

Equations of the form $C_p = \sum_n m_n x_{pn}$

where: C_p = the concentration of parameter p in the outfall flow

m_n = the fraction of flow from source type n

x_{pn} = the mean concentration of parameter p in source type n

From Lalor, M. *An Assessment of Non-Stormwater Discharges to Storm Drainage Systems in Residential and Commercial Land Use Areas.* Ph.D. dissertation. Department of Civil and Environmental Engineering. Vanderbilt University. 1994. With permission.

Figure 6.116 Remotely operated TV camera surveys of storm sewers in Boston to locate inappropriate discharges.

Figure 6.117 Manual surveys conducted in Boston in large tidally influenced storm drains.

Nichols et al. (1996) also examined coprostanol in stormwater and the sea-surface microlayer to distinguish human vs. nonhuman sources of contamination. Other steroid compounds in sewage effluent were investigated by Routledge et al. (1998) and Desbrow et al. (1998), who both examined estrogenic chemicals. The most commonly found were 17β-estradiol and estrone, which were detected at concentrations in the tens of nanograms per liter range. These were identified as estrogenic through a toxicity identification and evaluation approach, where sequential separations and analyses identified the sample fractions causing estrogenic activity using a yeast-based estrogen screen. GC/MS was then used to identify the specific compounds.

Estimating Potential Sanitary Sewage Discharges into Storm Drainage and Receiving Waters Using Detergent Tracer Compounds

As described above, detergent measurements (using methylene blue active substance, MBAS, test methods) were the most successful individual tracers to indicate contaminated water in storm sewerage dry-weather flows. Unfortunately, the MBAS method uses hazardous chloroform for an extraction step. Different detergent components, especially linear alkylbenzene sulfonates (LAS) and linear alkylbenzenes (LAB), have also been tried to indicate sewage dispersal patterns in receiving waters. Boron, a major historical ingredient of laundry chemicals, can also potentially be used. Boron has the great advantage of being relatively easy to analyze using portable field test kits, while LAS requires chromatographic equipment. LAS can be measured using HPLC with fluorescent detection, after solid-phase extraction, to very low levels. Fujita et al. (1998) developed an efficient enzyme-linked immunosorbent assay (ELISA) for detecting LAS at levels from 20 to 500 µg/L.

LAS from synthetic surfactants (Terzic and Ahel 1993) which degrade rapidly, as well as nonionic detergents (Zoller et al. 1991) which do not degrade rapidly, have been utilized as sanitary sewage markers. LAS was quickly dispersed from wastewater outfalls except in areas where wind was calm. In these areas, LAS concentrations increased in fresh water but were unaffected in saline water. After time, the lower alkyl groups were mostly found, possibly as a result of degradation or settling of longer alkyl chain compounds with sediments. Chung et al. (1995) also describe the distribution and fate of LAS in an urban stream in Korea. They examined different LAS compounds having carbon ratios of C12 and C13 compared to C10 and C11, plus ratios of phosphates to MBAS and the internal to external isomer ratio (I/E) as part of their research. González-Mazo et al. (1998)

examined LAS in the Bay of Cádiz off the southwest coast of Spain. They found that LAS degrades rapidly. Fujita et al. (1998) found that complete biodegradation of LAS requires several days and is also strongly sorbed to particulates. In areas close to shore and near the untreated wastewater discharges, there was significant vertical stratification of LAS: the top 3 to 5 mm of water had LAS concentrations about 100 times greater than those found at 0.5 m.

Zeng and Vista (1997) and Zeng et al. (1997) describe a study off San Diego where LAB was measured, along with polycyclic aromatic hydrocarbons (PAHs) and aliphatic hydrocarbons (AHs) to indicate the relative pollutant contributions of wastewater from sanitary sewage, nonpoint sources, and hydrocarbon combustion sources. They developed and tested several indicator ratios (alkyl homologue distributions and parent compound distributions) and examined the ratios of various PAHs (such as phenanthrene to anthracene, methylphenanthrene to phenanthrene, fluoranthene to pyrene, and benzo(a)anthracene to chrysene) as tools for distinguishing these sources. They concluded that LABs are useful tracers of domestic waste inputs to the environment due to their limited sources. They also describe the use of the internal to external isomer ratio (I/E) to indicate the amount of biodegradation that may have occurred to the LABs. They observed concentrations of total LABs in sewage effluent of about 3 µg/L, although previous researchers have seen concentrations of about 150 µg/L in sewage effluent from the same area.

The fluorescent properties of detergents have also been used as tracers by investigating the fluorescent whitening agents (FWAs), as described by Poiger et al. (1996) and Kramer et al. (1996). HPLC with fluorescence detection was used in these studies to quantify very low concentrations of FWAs. The two most frequently used FWAs in household detergents (DSBP and DAS 1) were found at 7 to 21 µg/L in primary sewage effluent and at 3 to 9 µg/L in secondary effluent. Raw sewage contains about 10 to 20 µg/L FWAs. The removal mechanisms in sewage treatment processes is by adsorption to activated sludge. The type of FWAs varies from laundry applications to textile finishing and paper production, making it possible to identify sewage sources. The FWAs were found in river water at 0.04 to 0.6 µg/L. The FWAs are not easily biodegradable, but they are readily photodegraded. Photodegradation rates have been reported to be about 7% for DSBP and 71% for DAS 1 in river water exposed to natural sunlight, after 1-hour exposure. Subsequent photodegradation is quite slow.

Other Compounds Found in Sanitary Sewage That May Be Used for Identifying Contamination by Sewage

Halling-Sørensen et al. (1998) detected numerous pharmaceutical substances in sewage effluents and in receiving waters. Their work addressed human health concerns of these low-level compounds that can enter downstream drinking water supplies. However, the information might also be used to help identify sewage contamination. Most of the research has focused on clofibric acid, a chemical used in cholesterol-lowering drugs. It has been found in concentrations ranging from 10 to 165 ng/L in a Berlin drinking water sample. Other drugs commonly found include aspirin, caffeine, and ibuprofen. Current FDA guidance mandates that the maximum concentration of a substance or its active metabolites at the point of entry into the aquatic environment be less than 1 µg/L (Hun 1998).

Caffeine has been used as an indicator of sewage contamination by several investigators (Shuman and Strand 1996). The King County, WA, Water Quality Assessment Project is examining the impacts of CSOs on the Duwamish River and Elliott Bay. They are using both caffeine (representing dissolved CSO constituents) and coprostanol (representing particulate-bound CSO constituents), in conjunction with heavy metals and conventional analyses, to help determine the contribution of CSOs to the river. The caffeine is unique to sewage, while coprostanol is from both humans and carnivorous animals and is therefore also in stormwater. They sampled upstream of all CSOs, but with some stormwater influences, 100 m upstream of the primary CSO discharge (but downstream of other CSOs), within the primary CSO discharge line, and 100 m downriver of the CSO discharge location. The relationship between caffeine and coprostanol was fairly consistent

for the four sites (coprostanol was about 0.5 to 1.5 µg/L higher than caffeine). Similar patterns were found between the three metals, chromium was always the lowest and zinc was the highest. King County is also using clean transported mussels placed in the Duwamish River to measure the bioconcentration potential of metal and organic toxicants and the effects of the CSOs on mussel growth rates (after 6-week exposure periods). Paired reference locations are available near the areas of deployment, but outside the areas of immediate CSO influence. *U.S. Water News* (1998) also described a study in Boston Harbor that found caffeine at levels of about 7 µg/L in the harbor water. The caffeine content of regular coffee is about 700 mg/L, in contrast.

DNA Profiling to Measure Impacts on receiving water Organisms and to Identify Sources of Microorganisms in Stormwater

This rapidly emerging technique seems to have great promise in addressing a number of nonpoint source water pollution issues. Kratch (1997) summarized several investigations on cataloging the DNA of *E. coli* to identify their source in water. The procedure, developed at the Virginia Polytechnic Institute and State University, has been used in Chesapeake Bay. In one example, it was possible to identify a large wild animal population as the source of fecal coliform contamination of a shellfish bed, instead of suspected failing septic tanks. DNA patterns in fecal coliforms vary among animals and birds, and it is relatively easy to distinguish between human and nonhuman sources of the bacteria. However, some wild animals have DNA patterns that are not easily distinguishable. Some researchers question the value of *E. coli* DNA fingerprinting, believing that there is little direct relationship between *E. coli* and human pathogens. However, this method should be useful to identify the presence of sewage contamination in stormwater or in a receiving water.

One application of the technique, as described by Krane et al. (1999) of Wright State University, used randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) generated profiles of naturally occurring crayfish. They found that changes in the underlying genetic diversity of these populations were significantly correlated with the extent to which they have been exposed to anthropogenic stressors. They concluded that this rapid and relatively simple technique can be used to develop a sensitive means of directly assessing the impact of stressors on ecosystems. These Wright State University researchers have also used the RAPD-PCR techniques on populations of snails, pill bugs, violets, spiders, earthworms, herring, and some benthic macroinvertebrates, finding relatively few obstacles in its use for different organisms. As noted above, other researchers have used DNA profiling techniques to identify sources of *E. coli* bacteria found in coastal waterways. It is possible that these techniques can be expanded to enable rapid detection of many different types of pathogens in receiving waters, and the most likely sources of these pathogens.

Stable Isotope Methods for Identifying Sources of Water

Stable isotopes had been recommended as an efficient method to identify illicit connections to storm sewerage. A demonstration was conducted in Detroit as part of the Rouge River project to identify sources of dry-weather flows in storm sewerage (Sangal et al. 1996). Naturally occurring stable isotopes of oxygen and hydrogen can be used to identify waters originating from different geographical sources (especially along a north–south gradient). Ma and Spalding (1996) discuss this approach by using stable isotopes to investigate recharge of groundwaters by surface waters. During water vapor transport from equatorial source regions to higher latitudes, depletion of heavy isotopes occurs with rain. Deviation from a standard relationship between deuterium and ^{18}O for a specific area indicates that the water has undergone additional evaporation. The ratio is also affected by seasonal changes. As discussed by Ma and Spalding (1996), the Platte River water is normally derived in part from snowmelt from the Rocky Mountains, while the groundwater in parts of Nebraska is mainly contributed from the Gulf air stream. The origins of these waters are

sufficiently different and allow good measurements of the recharge rate of the surface water to the groundwater. In Detroit, Sangal et al. (1996) used differences in origin between the domestic water supply, local surface waters, and the local groundwater to identify potential sanitary sewage contributions to the separate storm sewerage. Rieley et al. (1997) used stable isotopes of carbon in marine organisms to distinguish the primary source of carbon being consumed (sewage sludge vs. natural carbon sources) in two deep sea sewage sludge disposal areas.

Stable isotope analyses would not be able to distinguish between sanitary sewage, industrial discharges, wash waters, and domestic water, as they all have the same origin. Nor would it be possible to distinguish sewage from local groundwaters if the domestic water supply was from the same local aquifer. This method works best for situations where the water supply is from a distant source and where separation of waters into separate flow components is not needed. It may be an excellent tool to study the effects of deep well injection of stormwater on deep aquifers having distant recharge sources (such as in the Phoenix area). Few laboratories can analyze for these stable isotopes, requiring shipping the samples and a long wait for the analytical results. Sangal et al. (1996) used Geochron Laboratories, in Cambridge, MA.

Dating of sediments using ^{137}Cs was described by Davis et al. (1997). Arsenic-contaminated sediments in the Hylebos Waterway in Tacoma, WA, could have originated from numerous sources, including a pesticide manufacturing facility, a rock-wool plant, steel slags, powdered metal plant, shipbuilding facilities, marinas and arsenic-based boat paints, and the Tacoma Smelter. Dating the sediments, combined with knowing the history of potential discharges and conducting optical and electron microscopic studies of the sediments, was found to be a powerful tool to differentiate the metal sources to the sediments.

Comparison of Parameters That Can Be Used for Identifying Inappropriate Discharges to Storm Drainage

In almost all cases, a suite of analyses is most suitable for effective identification of inappropriate discharges. An example was reported by Standley et al. (2000), where fecal steroids (including coprostanol), caffeine, consumer product fragrance materials, and petroleum and combustion by-products were used to identify wastewater treatment plant effluent, agricultural and feedlot runoff, urban runoff, and wildlife sources. They studied numerous individual sources of these wastes from throughout the United States. A research-grade mass spectrophotometer was used for the majority of the analyses in order to achieve the needed sensitivities, although much variability was found when using the methods in actual receiving waters affected by wastewater effluent. This sophisticated suite of analyses did yield much useful information, but the analyses are difficult to conduct and costly and may be suitable for special situations, but not for routine survey work.

Another series of tests examined several of these potential emerging tracer parameters, in conjunction with the previously identified parameters, during a project characterizing stormwater that had collected in telecommunication manholes, funded by Tecordia (previously Bellcore), AT&T, and eight regional telephone companies throughout the country (Pitt and Clark 1999). Numerous conventional constituents, plus major ions, and toxicants were measured, along with candidate tracers to indicate sewage contamination of this water. Boron, caffeine, coprostanol, *E. coli*, enterococci, fluorescence (using specific wavelengths for detergents), and a simpler test for detergents were evaluated, along with the use of fluoride, ammonia, potassium, and obvious odors and color. About 700 water samples were evaluated for all of these parameters, with the exception of bacteria and boron (about 250 samples), and only infrequent samples were analyzed for fluorescence. Coprostanol was found in about 25% of the water samples (and in about 75% of the 350 sediment samples analyzed). Caffeine was found in very few samples, while elevated *E. coli* and enterococci (using IDEXX tests) were observed in about 10% of the samples. Strong sewage odors in water and sediment samples were also detected in about 10% of the samples. Detergents and fluoride (at >0.3 mg/L) were found in about 40% of the samples and are expected to have been

contaminated by industrial activities (lubricants and cleansers) and not sewerage. Overall, about 10% of the samples were therefore expected to have been contaminated with sanitary sewage, about the same rate previously estimated for stormwater systems.

Additional related laboratory tests, funded by the University of New Orleans and the EPA (Barbé et al. 2000), were conducted using many sewage and laundry detergent samples, and it was found that the boron test was a poor indicator of sewage, possibly due to changes in formulations in modern laundry detergents. Laboratory tests did find that fluorescence was an excellent indicator of sewage, especially when using specialized "detergent whitener" filter sets, but this was not very repeatable. Researchers also examined several UV absorbance wavelengths as sewage indicators and found excellent correlations with 228 nm, a wavelength having very little background absorbance in local spring waters, but with a strong response factor with increasing strengths of sewage.

Table 6.39 summarizes the different measurement parameters discussed above. We recommend that our originally developed and tested protocol (including measurement of obvious indicators, detergents, fluoride, ammonia, and potassium) still be used as the most efficient routine indicator of sewage contamination of stormwater drainage systems, with the possible addition of specific *E. coli* and enterococci measurements and UV absorbance at 228 nm. The numerous exotic tests requiring specialized instrumentation and expertise do not appear to warrant their expense and long analytical turn-around times, except in specialized research situations, or when special confirmation is economically justified (such as when examining sewer replacement or major repair options).

Hydrocarbon Fingerprinting for Investigating Sources of Hydrocarbons

Fingerprinting to identify the likely source of hydrocarbon contamination is a unique process that recognizes degradation of the material by examining a wide variety of parameters, usually by sophisticated chromatography methods. There are numerous experts who have developed and refined the necessary techniques. The following is a list of some of these expert groups, from recommendations from the Internet environmental engineering list serve group, enveng-L:

- Friedman & Bruya, Seattle, WA
- Arthur D. Little, Inc., Cambridge, MA
- GW/S Environmental Consulting, Tulsa, OK
- Public & Environmental Affairs, Indiana University, Bloomington, IN
- Graduate School of Oceanography, University of Rhode Island, Narragansett, RI
- Louisiana State University, Baton Rouge, LA
- Geological and Environmental Research Group, Texas A&M University, College Station, TX
- Trillium, Inc., Coatesville, PA
- McLaren/Hart, Inc., Albany, NY
- Phoenix Laboratories, Chicago, IL
- Golder Assoc., Mississauga, Ontario, Canada
- Daniel B. Stephens & Assoc., Albuquerque, NM
- Global Geochemistry Corp., Canoga Park, CA
- Fluor Daniel GTI, Kent, WA
- Battelle, Inc., Duxbury, MA

In addition, the University of Wisconsin, Madison, Department of Engineering Professional Development (608-262-1299) periodically offers extension classes specifically on hydrocarbon pattern recognition and dating, led by experts in the field. The IBC Group (Southborough, MA, 508-481-6400) also offers an executive forum on environmental forensics, also led by many of the above experts, that addresses many issues pertaining to the legal implications of hydrocarbon tracing.

Stout et al. (1998) prepared an overview of environmental forensics, describing how systematic investigation of a contaminated site or an event can make it possible to determine the true origin and nature of complex chemical conditions. Chemical fingerprinting, generally using high-resolution gas

Table 6.39 Comparison of Measurement Parameters Used for Identifying Inappropriate Discharges into Storm Drainage

Parameter Group	Comments	Recommendation
Fecal coliform bacteria and/or use of fecal coliform to fecal streptococci ratio	Commonly used to indicate presence of sanitary sewage.	Not very useful as many other sources of fecal coliforms are present, and ratio not accurate for old or mixed wastes.
Physical observations (odor, color, turbidity, floatables, deposits, stains, vegetation changes, damage to outfalls)	Commonly used to indicate presence of sanitary and industrial wastewater.	Recommended due to easy public understanding and easy to evaluate, but only indicative of gross contamination, with excessive false negatives (and some false positives). Use in conjunction with chemical tracers for greater sensitivity and accuracy.
Detergents presence (anionic surfactant extractions)	Used to indicate presence of wash waters and sanitary sewage.	Recommended, but care needed during hazardous analyses (only for well-trained personnel). Accurate indicator of contamination during field tests.
Fluoride, ammonia and potassium measurements	Used to identify and distinguish between wash waters and sanitary sewage.	Recommended, especially in conjunction with detergent analyses. Accurate indicator of major contamination sources and their relative contributions.
TV surveys and source investigations	Used to identify specific locations of inappropriate discharges, especially in industrial areas.	Recommended after outfall surveys indicate contamination in drainage system.
Coprostanol and other fecal sterol compounds	Used to indicate presence of sanitary sewage.	Possibly useful. Expensive analysis with GC/MSD. Not specific to human wastes or recent contamination. Most useful when analyzing particulate fractions of wastewaters or sediments.
Specific detergent compounds (LAS, fabric whiteners, and perfumes)	Used to indicate presence of sanitary sewage.	Possibly useful. Expensive analyses with HPLC. A good and sensitive confirmatory method.
Fluorescence	Used to indicate presence of sanitary sewage and wash waters.	Likely useful, but expensive instrumentation. Rapid and easy analysis. Very sensitive.
Boron	Used to indicate presence of sanitary sewage and wash waters.	Not very useful. Easy and inexpensive analysis, but recent laundry formulations in U.S. have minimal boron components.
Pharmaceuticals (colfibric acid, aspirin, ibuprofen, steroids, illegal drugs, etc.)	Used to indicate presence of sanitary sewage.	Possibly useful. Expensive analyses with HPLC. A good and sensitive confirmatory method.
Caffeine	Used to indicate presence of sanitary sewage.	Not very useful. Expensive analyses with GC/MSD. Numerous false negatives, as typical analytical methods not suitably sensitive.
DNA profiling of microorganisms	Used to identify sources of microorganisms	Likely useful, but currently requires extensive background information on likely sources in drainage. Could be very useful if method can be simplified, but with less specific results.
UV absorbance at 228 nm	Used to identify presence of sanitary sewage.	Possibly useful, if UV spectrophotometer available. Simple and direct analyses. Sensitive to varying levels of sanitary sewage, but may not be useful with dilute solutions. Further testing needed to investigate sensitivity in field trials.
Stable isotopes of oxygen	Used to identify major sources of water.	May be useful in area having distant domestic water sources and distant groundwater recharge areas. Expensive and time consuming procedure. Cannot distinguish between wastewaters if all have common source.
E. coli and enterococci bacteria	More specific indicators of sanitary sewage than coliform tests.	Recommended in conjunction with chemical tests. Relatively inexpensive and easy analyses, especially if using the simple IDEXX methods.

chromatography coupled with mass spectroscopy, is usually supplemented with site information on soils and groundwater conditions. The presentation of masses of data is usually highly visually oriented to make complex patterns and associations easier to comprehend. In addition to GC/MS, stable isotope analyses may be conducted to identify origins of very similar materials. Historical records also need to be reviewed to understand the changes that a site has undergone over the years ("corporate archaeology"). Sanborn Fire Insurance Maps (Geography and Map Division, Library of Congress) are commonly used to identify site activities during the second half of the 19th century, for example. This type of approach can be used to identify sources of contaminated sediments in urban streams, especially in areas having historical industrial activities.

Other techniques can be used to date deposits and to indicate the extent of the weathering of petroleum (Whittaker and Pollard 1997). The weathered state of spilled (or discharged) hydrocarbons can be determined using biomarkers (pristane, phthane, hopanes, and steranes) which are quite resistant to weathering processes (biotransformations and evaporation). These are therefore relatively conservative materials and can be compared to less stable oil components to indicate the extent of weathering that has occurred, and hence the approximate time since the petroleum was deposited. Other biomarkers can also be used as unique fingerprints to identify the likely source of the oil. Hurst et al. (1996) also describe how lead isotopes ($^{206}Pb/^{207}Pb$ ratio) can be used to age spilled gasoline, based on changes in gasoline additives with time.

MICROORGANISMS IN STORMWATER AND URBAN RECEIVING WATERS

As discussed in Chapter 3, microorganisms frequently interfere with beneficial uses in urban receiving waters. The use of conventional indicator organisms may be helpful, but investigations of specific pathogens is also becoming possible with new analytical technologies. The following discussion contains some background on the development of water quality standards for indicator organisms, describes some new analytical procedures, and presents an approach that measures organism die-off *in situ*, which is important for assessing the public health risk associated with water contact in urban receiving waters.

Pathogens in stormwater and urban receiving waters are a significant concern potentially affecting human health. The use of indicator bacteria is controversial for stormwater, as is the assumed time of typical exposure of swimmers to contaminated receiving waters. However, recent epidemiological studies have shown significant health effects associated with stormwater-contaminated marine swimming areas. Protozoan pathogens, especially those associated with likely sewage-contaminated stormwater, is also a public health concern.

Human health standards for body contact recreation (and for fish and water consumption) are based on indicator organism monitoring. Monitoring for the actual pathogens, with few exceptions, requires an extended laboratory effort, is very costly, and not very accurate. Therefore, the use of indicator organisms has become established. Dufour (1984a) presents an excellent overview of the history of indicator bacterial standards and water contact recreation.

Total coliforms were initially used as indicators for monitoring outdoor bathing waters, based on a classification scheme presented by W.J. Scott in 1934. Total coliform bacteria, refers to a number of bacteria including *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter.* They are able to grow at 35°C and ferment lactose. They are all Gram-negative asporogenous rods and have been associated with feces of warm-blooded animals. They are also present in soil.

The fecal coliform test is not specific for any one coliform type, or groups of types, but instead has an excellent positive correlation for coliform bacteria derived from the intestinal tract of warm-blooded animals (Geldreich et al. 1968). The fecal coliform test measures *Escherichia coli* as well as all other coliforms that can ferment lactose at 44.5°C and are found in warm-blooded fecal discharges. Geldreich (1976) found that the fecal coliform test represents over 96% of the coliforms derived from human feces and from 93 to 98% of those discharged in feces from other

warm-blooded animals, including livestock, poultry, cats, dogs, and rodents. In many urban runoff studies, all of the fecal coliforms were *E. coli* (Quresh and Dutka 1979). *E. coli*, a member of the fecal coliform group, has been used as a better indicator of fresh fecal contamination, compared to fecal coliforms. Table 6.40 indicates the species and subspecies of the *Streptococcus* and *Enterococcus* groups of bacteria that are used as indicators of fecal contamination.

Table 6.40 Streptococcus Species Used as Indicators of Fecal Contamination

Indicator Organism	Enterococcus Group	Streptococcus Group
Group D antigen		
Streptococcus faecalis	X	X
S. faecealis subsp. *liquifaciens*	X	X
S. faecalis subsp. *zymogenes*	X	X
S. faecium	X	X
S. bovis		X
S. equinus		X
Group Q antigen		
S. avium		X

Fecal streptococci bacteria are all of the intestinal streptococci bacteria from warm-blooded animal feces (Geldreich and Kenner 1969). The types and concentrations of different bacteria biotypes vary for different animal sources. Fecal streptococci bacteria are indicators of fecal contamination. The enterococci group is a subgroup that is considered a better indication of human fecal contamination. *S. bovis* and *S. equinus* are considered related to feces from nonhuman warm-blooded animals (such as from meat processing facilities, dairy wastes, and feedlot and other agricultural runoff), indicating that enterococcus may be a better indication of human feces contamination. However, *S. faecalis* subsp. *liquifaciens* is also associated with vegetation sources, insects, and some soils.

The EPA's evaluation of the bacteriological data indicated that using the fecal coliform indicator group at the maximum geometric mean of 200 organisms per 100 mL, as recommended in *Quality Criteria for Water* would cause an estimated eight illnesses per 1000 swimmers at freshwater beaches. Additional criteria, using *E. coli* and enterococci bacteria analyses, were developed using these currently accepted illness rates. See Appendix G for specific details of these criteria. These bacteria are assumed to be more specifically related to poorly treated human sewage than the fecal coliform bacteria indicator. It should be noted that these indicators only relate to gastrointestinal illness, and not other problems associated with waters contaminated with bacterial or viral pathogens. Common swimming beach problems associated with contamination by stormwater include skin and ear infections caused by *Pseudomonas aeruginosa* and *Shigella*.

Viruses may also be important pathogens in urban runoff. Very small amounts of a virus are capable of producing infections or diseases, especially when compared to the large numbers of bacterial organisms required for infection (Berg 1965). The quantity of enteroviruses which must be ingested to produce infections is usually not known (Olivieri et al. 1977b). Viruses are usually detected at low levels in urban receiving waters and storm runoff. Researchers have stated that even though the minimum infective doses may be small, the information available indicates that stormwater virus threats to human health are small. Because of the low levels of virus necessary for infection, dilution of viruses does not significantly reduce their hazard.

States et al. (1997) examined *Cryptosporidium* and *Giardia* in river water serving as Pittsburgh's water supply. They collected monthly samples from the Allegheny and Youghiogheny Rivers for 2 years. They also sampled a small stream flowing through a dairy farm, treated sanitary sewage effluent, and CSOs. The CSO samples had much greater numbers of the protozoa than any of the other samples. No raw sewage samples were obtained, but they were assumed to be very high because of the high CSO sample values. The effluent from the sewage treatment plant was the next highest, at less than half the CSO values. The dairy farm stream was not significantly different

from either of the two large rivers. The water treatment process appeared to effectively remove *Giardia*, but some *Cryptosporidium* was found in the filtered water. Settling the river water seemed to remove some of the protozoa, but the removal would not be adequate by itself. States et al. (1997) also reviewed *Giardia* and *Cryptosporidium* monitoring data. Raw drinking water supplies were shown to have highly variable levels of these protozoa, typically up to several hundred *Giardia* cysts and *Cryptosporidium* oocysts per 100 L, and were found in 5 to 50% of the samples evaluated. Conventional water treatment appeared to remove about 90% of the protozoa.

A microorganism monitoring program for stormwater-impacted urban receiving waters could therefore be very complex and expensive if all the above organisms were to be evaluated. The bacteria (especially total coliforms, fecal coliforms, *E. coli*, enterococci, and hopefully *Pseudomonas aeruginosa*) should probably all be adequately covered in a monitoring program. Total coliforms are of most interest in marine environments based on epidemiological studies conducted in Santa Monica Bay (see case study in Chapter 4). In most cases, total coliform data could be misleading because of its ubiquitous nature (see Chapter 8). Protozoa, and especially viruses, require highly specialized analytical skills and are therefore not likely to be routinely investigated. However, protozoa are being more commonly monitored, especially with new federal regulations to protect drinking water supplies.

Sampling for microorganism evaluations is more challenging than for most constituents, requiring sterile sample containers and tools, plus rapid shipment of the samples to the laboratory and immediate initiation of analyses. Bacteriological analyses are becoming much more simplified with special procedures and methods developed by HACH, Millipore, and IDEXX Corp., for example. Available methods require little more than mixing a freeze-dried "reagent" with a measured amount of sample, pouring the mixture into special incubation trays and sealing them, and finally placing them into incubators for the designated time (usually 18 to 48 hours).

The IDEXX method for *E. coli*, Colilert-18 (see Figures 6.71 through 6.74), is used by many state agencies for EPA reporting purposes. It is used for the simultaneous detection, specific identification, and confirmation of total coliforms and *E. coli* in water. It is based on IDEXX's patented Defined Substrate Technology® (DST™). It is a most probable number (MPN) method. Colilert-18 utilizes nutrient indicators that produce color and/or fluorescence when metabolized by total coliforms and *E. coli.* When the Colilert-18 reagent is added to a sample and incubated, it can detect these bacteria at 1 cfu in 100 mL within 18 hours with as many as 2 million heterotrophic bacteria per 100 mL present. The required apparatus includes the Quanti-tray sealer, an incubator, a 6-watt 365-nm UV light, and a fluorescence comparator. This procedure requires 100 mL of sample, which should be analyzed ASAP after sampling. Marine water samples must be diluted at least tenfold with sterile fresh water to reduce the salinity. Quality control includes testing with cultures of *E. coli, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*.

The Enterolert procedure, also from IDEXX, is very similar to the Colilert method outlined above. Enterolert is used for the detection of enterococci such as *Enterococcus faecium* or *E. faecalis* in fresh and marine water. When the Enterolert reagent is added to a sample and incubated, bacteria down to 1 CFU in a 100 mL sample can be detected within 24 hours. This method also has a quality control procedure that should be conducted on each lot of Enterolert, using test cultures of *E. faecium*, *Serratia marcescens* (Gram-negative), and *Aerococcus viridans* (Gram-positive).

Determination of Survival Rates for Selected Bacterial and Protozoan Pathogens

The following discussion was prepared by John Easton while he was a Ph.D. student at the University of Alabama at Birmingham and describes some of the experiments he has conducted concerning the survival of wet-weather flow bacteria and pathogens after being discharged to urban receiving waters (Easton 2000). This section is not intended to be a comprehensive review of survival of microorganisms in the environment, but is intended to illustrate how actual site-specific survival rates can be determined, especially for unusual conditions (affected by water temperature,

turbidity, natural predation, local sources and receptors, etc.). This information is necessary for human health assessments when predicting resulting downstream pathogen conditions. Much of the literature on microorganism survival is based on laboratory investigations that might not be applicable to actual field conditions. The simple tests described in this section allow more accurate in-stream predictions.

Pathogenic organisms found in sewage can adversely impact public health when the sewage is discharged to waters that humans come in contact with when wading, swimming, fishing, drinking, etc. UAB is conducting research to develop a risk assessment methodology for evaluating varying degrees of risk related to human contact with pathogenic microorganisms found in sewage-contaminated waters, especially those caused by separate sanitary sewer overflows (SSOs). One component of this research is to study the fate and transport of these microorganisms in the environment. The survivability, or die-off, rates for these organisms are critical to understanding their fate in the environment, e.g., from an SSO discharge through a receiving water.

Microorganisms have varying degrees of stability within the environment. Their numbers are dependent upon population dynamics, which is controlled by several criteria (McKinney 1992): (1) competition for food (limited food sources limit microbial numbers), (2) predator–prey relationships (some organisms consume others for food sources), (3) nature of organic matter (carbohydrates, organic acids, and proteins all stimulate different organisms), and (4) environmental conditions (oxygen concentration, nutrient levels, temperature, pH, etc.). Since there are a multitude of factors that contribute to microorganism survivability, the use of an *in situ* method to characterize the rates of growth and death is necessary to account for variable environmental conditions.

Several experiments were conducted to evaluate the rate of die-off, or decay, for the study microorganisms. These *in situ* experiments were conducted in specially designed chambers (Figure 6.118). These were designed to allow passage of water and nutrients between the inside of the chamber and the outside environment (Five-Mile Creek in Jefferson County, AL), while sequestering the microorganisms inside to allow enumeration at various times during the experiment.

These experiments included exposures over a 21-day period. A polyethersulfone (Supor®, Gelman Sciences) membrane filter, which is not susceptible to biological degradation, was used. This membrane material was clamped onto either end of a piece of acrylic tubing in a design devised by researchers at UAB (Figure 6.119). The membrane pore size is 0.22 μm, allowing exchange of ions with the surrounding water while sequestering the microorganisms inside the test chamber.

Multiple chambers containing sewage samples were placed in the creek and removed after 0, 1, 3, 7, 10, 14, and 21 days. For each time point, three separate chambers were removed and composited for analysis. Once the samples were composited, they were blended (Waring blender for 2 min) to minimize agglomeration of the microorganisms.

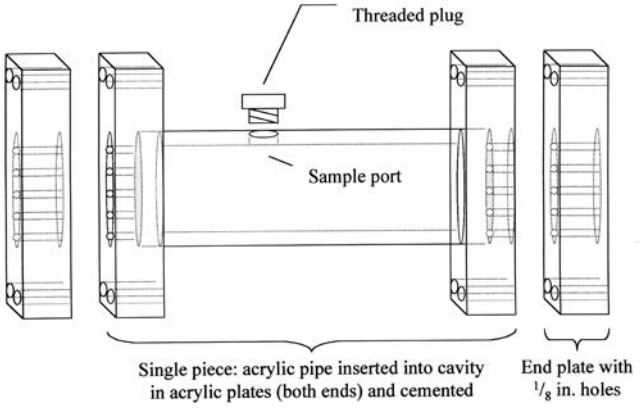


Figure 6.118 Acrylic components of *in situ* chamber. (From Easton, J. *The Development of Pathogen Fate and Transport Parameters for Use in Assessing Health Risks Associated with Sewage Contamination.* Ph.D. dissertation, the Department of Civil and Environmental Engineering, University of Alabama at Birmingham. 2000. With permission.)

The experiments conducted to evaluate degradation of *G. lamblia* were conducted *in situ*. The sewage matrix was spiked with approximately 10,000 cysts per liter to enable detection after significant die-off. These cysts were formalinized in order not to risk releasing a potentially infectious pathogen into the environment. Since these organisms are in cyst form, i.e., relatively inert, it was hypothesized that the mechanism of die-off would be predation by other organisms and formalinized organisms would be a suitable surrogate for "live" ones.

The results of these experiments show that the microorganisms die off at a constant, rapid rate (assumed in most receiving models) only for an initial short period. As time progressed, the die-off rate slows. Figure 6.120 is a plot of the levels of *Giardia* cysts vs. time. The method used to enumerate these organisms (EPA method 1623) requires a presumptive test followed by a confirmed test. The presumptive test consists of identifying objects of the correct size and shape which are stained by a *Giardia*-specific antibody bound to a fluorescent probe. Next, the organisms are confirmed by identification of internal structures stained by the nuclear stain DAPI (4′,6-diamindino-2-phenylindole). Unfortunately, problems were encountered with the confirmation test in these

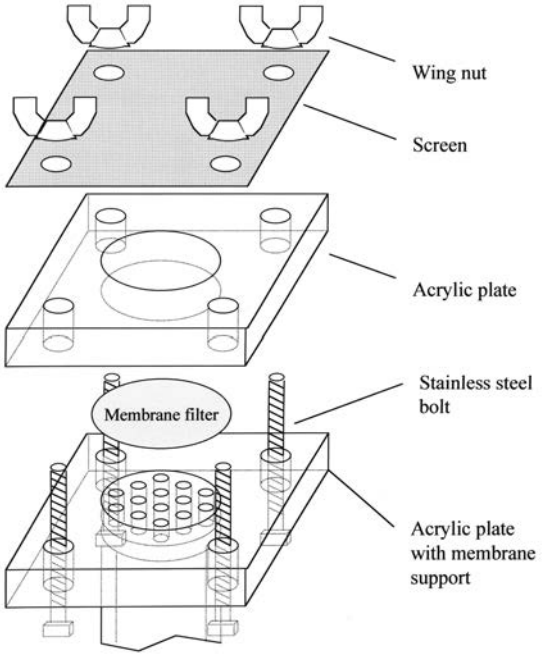


Figure 6.119 End-plate of *in situ* chamber showing the location of membrane filter. (From Easton, J.H. et al. The use of a multi-parameter water quality monitoring instrument to continuously monitor and evaluate runoff events. Presented at *Annual Water Resources Conference of the AWRA,* Point Clear, AL. 1998. With permission.)

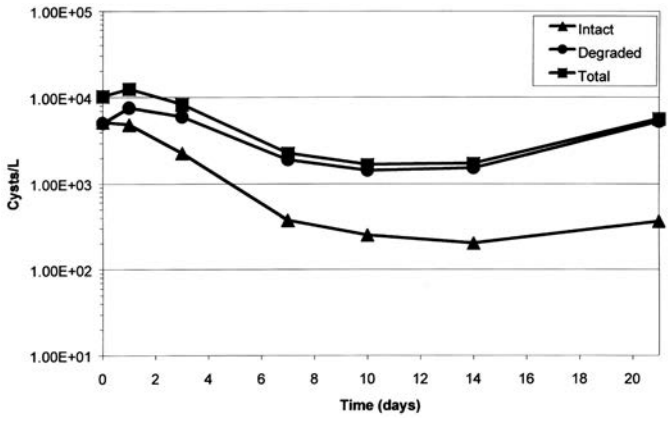


Figure 6.120 Degradation plot of *Giardia* cysts. (From Easton, J.H. et al. The use of a multi-parameter water quality monitoring instrument to continuously monitor and evaluate runoff events. Presented at *Annual Water Resources Conference of the AWRA,* Point Clear, AL. 1998. With permission.)

experiments (the DAPI stain of the background was too intense to enable identification of internal structures). However, using the presumptive stain, which binds to the cyst cell wall, it was possible to detect differences in these presumptive *Giardia* cysts. Some cysts were intact (i.e., the stain covered the cell wall continuously), and some cysts were present but degraded (i.e., the staining of the cell wall was less intense and not continuous). The levels of the former, "intact cysts," are plotted along with the levels of the latter, "degraded cysts," in Figure 6.120.

Since the microorganisms' rate of die-off seems to decrease over time, the regression model was applied stepwise, starting with the first three data points and adding one additional point until the entire 21-day, or 7-point, data set was used. In general, the die-off rates decreased, and T_x values correspondingly increased as data over longer time periods are included in the regression analyses. The T_{90} values (time needed for 90% die-off) for the indicator bacteria, total coliforms and *E. coli*, are in accordance with conventional wisdom. Many studies have shown T_{90} values for these organisms to be in the range of several hours to a few days (Droste and Gupgupoglu 1982; Geldreich et al. 1968; Geldreich and Kenner 1969). The initial, rapid die-off occurred, generally, within the first 7 days of the experiment. Table 6.41 gives a first-order die-off constant, k ($days^{-1}$), and its associated 95% confidence interval, for each of the microorganisms. In addition, the results of the Mann–Kendall Test (a nonparametric test for trend) are given. All of the die-off constants (slope of the regression line) are statistically significant except for enterococci.

Table 6.41 Die-off Rates Determined Using Day 0 to Day 7 Data

Organism	Die-off Rate (day^{-1})	95% CI	Mann–Kendall Trend[a]
Total coliforms	–0.310	± 0.152	p = 0.042
E. coli	–0.331	± 0.049	p = 0.042
Enterococci	–0.078	± 0.189	p = 0.375[b]
Giardia	–0.171	± 0.074	p = 0.042

[a] $p < 0.05$ indicates significant downward trend.
[b] Not significant, no trend (die-off).

From Easton, J. *The Development of Pathogen Fate and Transport Parameters for Use in Assessing Health Risks Associated with Sewage Contamination.* Ph.D. dissertation, the Dept. of Civil and Environmental Engineering, University of Alabama at Birmingham. 2000. With permission.

The data generated by this study suggest that if one were using die-off constants from indicator bacteria studies, then one may tend to underpredict the length of time or distance downstream in which adverse health effects due to pathogens in sewage are present. In addition, these data indicate that assumptions regarding the constancy of die-off rates may be invalid. There seems to be a modulation of the rate of die-off with increased time, as all of the test organisms showed a pattern of leveling off toward some equilibrium level with increasing time.

The *Enterococcus* results are quite different from the others, with no rapid initial die-off, as generally reported in the literature (Facklam and Sahm 1995). This persistence is due to the enterococci being Gram-positive and is therefore a better indicator of virus survival. For these reasons, the EPA has selected enterococci as an indicator organism in their new guidance documents.

The *Giardia* results were not as expected. The descriptions of this organism found in the literature seem to predict that *Giardia* will persist much longer than observed in these tests. This study seems to show that *Giardia*, and perhaps other protozoan pathogens, exhibits die-off characteristics similar to the bacteria included in this study. However, these cysts were treated with formalin and therefore may have been less resistant to degradation in the environment.

There are many stormwater microorganisms of interest when conducting a receiving water study. However, besides characterizing microorganism conditions, it is also necessary to understand population dynamics when predicting fate and exposures. This section briefly described some of

the currently used analytical methodologies for measuring microorganism counts, along with an example *in situ* die-off experiment.

BENTHOS SAMPLING AND EVALUATION IN URBAN STREAMS

Ecosystem degradation via water, sediment, and habitat alteration affects food resources, reproduction, growth, and survival of aquatic biota, thereby altering the structure and functioning of the system. Structural indicators include the number and kinds of individuals, species, population, and communities as measured by a variety of metrics. The structural alterations may impact ecosystem functions such as productivity, respiration, organic matter degradation, nutrient cycling, and energy flow, which, unfortunately, are often difficult to quantify and are resource-demanding. A useful way to measure functional changes is an indirect method whereby organisms are placed into trophic categories (e.g., predators/consumers, producers, omnivores, detritivores), which allows production and consumption dynamics to be measured. This concept has been described by Cummins (1974, 1975) and Vannote et al. (1980) in stream ecosystems as a predictable and continuous gradient of interrelated physical, structural, and functional characteristics (Table 6.42). When conditions deviate from those in reference streams of a similar stream order for that ecoregion, then impacts may be occurring.

Bottom-dwelling organisms comprise all the major trophic levels including decomposers, photosynthetic organisms (algae and macrophytes), and herbivorous and carnivorous animals. These communities live on or in the sediment or other solid surfaces (e.g., roots, decaying wood, rocks) for significant parts of their life cycle. The fauna and flora studied in environmental quality assessments have ranged from small to large, using bacteria, phytoplankton, macrophytes, protozoa, worms, crustaceans, molluscs, insects, and fish (Burton 1991). Fish will be discussed in a following subsection. The major component of benthic fauna is often the bacteria, segmented worms (e.g., oligochaetes), microcrustacea (e.g., ostracods), macrocrustacea (e.g., isopods, decapods, amphipods), and immature insects (e.g., chironomids, plecoptera, trichoptera, and ephemeroptera). Of these major groups, the immature insects have received the greatest amount of study. Consequently, there is a large database concerning life history information and relative pollution sensitivity. The major aquatic insect groups are Odonata (dragonflies), Ephemeroptera (mayflies), Plecoptera (stoneflies), Trichoptera (caddisflies), Coleoptera (beetles), and Diptera (flies, midges, mosquitoes). Each group varies in its pollution sensitivity. Each goes through multiple life stages and molts, often emerging from the water as adults. Life cycles range from a few weeks to 2 years. See Pennak (1989) and Merritt and Cummins (1984) for more information on life histories. The sedentary (nontransitory) nature of most benthic species makes them ideal chronic, long-term pollution indicators, as compared to migratory fish or other species, such as zooplankton.

The micro-, meiofauna and flora may play a major role in the aquatic ecosystem's functioning, such as photosynthetic production by periphyton, and organic matter and nutrient processing–cycling by a variety of microbial populations and communities. These groups have temporal spatial dynamics and microhabitat requirements that are much different from the macrofauna and flora, and in many respects are more difficult to study (Burton 1991). For holistic, integrative ecosystem assessments of stormwater impacts, it is necessary to define effects on the benthic microbial communities, which will require additional expertise and resources. Further information is available from Burton et al. (2000) and the annual review issue of *Water Environment Research*. Most studies, however, whose objectives are to assess stormwater effects on receiving waters, will focus on the macroinvertebrate component of the benthos. This is not because they are more important than the meio- or microbenthos, but rather because they are more effectively used in pollution assessments. The following discussions highlight some of the important benthic groups and the characteristics one should consider in their sampling and evaluation.

Table 6.42 General Characteristics of Running Water Ecosystems According to Size of Stream

Stream Size	Primary Energy Source	Production (trophic) State	Light and Temperature Regime	Trophic Status of Dominant Insects	Fish
*Small headwater streams (stream order 1–3)	Coarse particular organic matter (CPOM) from the terrestrial environmental	Heterotrophic	Heavily shaded	Shredders	Invertivores
	Little primary production	P/R < 1	Stable temperatures	Collectors	
*Medium-sized streams (4–6)	Fine particulate organic matter (FPOM),mostly	Autotrophic	Little shading	Collectors	Invertivores
	Considerable primary production	P/R < 1	High daily temperature variation	Scrapers (grazers)	Piscivores
Large rivers (7–12)	FPOM from upstream	Heterotrophic	Little shading	Planktonic	Planktivores
		P/R < 1	Stable temperatures	Collectors	

* Streams are typically subdivided into three size classes based on the stream order classification system of Kuehne (1962).

Modified from Cummins, K.W. Ecology of running waters: theory and practice, in *Proc. Sandusky River Basin Symposium.* Edited by D.B. Baker. Heidelburg College, Tiffin, OH. 1975.

Periphyton Sampling

Periphyton is a general descriptor which can encompass epipelic (sediment surface), epilithic (stone surface), and epiphytic (plant surface) algae and other benthic meio-, microorganisms. Most periphyton studies have focused on the diatom group, which frequently dominates. Green algae, blue-green algae (cyanobacteria), and flagellates are also dominant species in some sediments, with diatoms favoring calcareous sediments (Wetzel 1975). The animal communities which may be present include protozoa, rotifers, nematodes, and bryozoans. A major controlling factor is light. In turbid, eutrophic, shaded, or deep waters, the low light levels may restrict photosynthetic activity (Wetzel 1975). Some epipelic algae appear to have a diurnal migration pattern through the top few centimeters of sediment in response to light availability. Their photosynthetic activity causes a diurnal change in oxygen concentrations with the upper few millimeters of sediment (Carlton and Klug 1990), which may affect metal bioavailability. They serve as an important transformation link for nitrogen, assimilating pore water ammonia and excreting organic nitrogen to overlying waters, and may be the primary productivity source (Wetzel 1975). These communities have less temporal fluctuation in a lake than planktonic algae and may have one to two biomass peaks per year (Wetzel 1975). Some algae present on sediment surfaces may have settled from the water column and can resuspend to overlying waters.

The algal community is not only extremely important in aquatic ecosystems, but has several attributes as a monitoring tool. Algae have short life cycles. Therefore, they indicate recent-to-present water quality conditions. They are directly affected by physical and chemical conditions since they are primary producers. Sampling of indigenous algae is nondestructive, easy, and inexpensive, and traditional assessment methods exist. Finally, they represent a unique level of biological organization and are sensitive to contaminants which may not be detected with nonalgal surrogates.

Periphyton is difficult to study in a quantitative manner when collecting from natural substrates, as small particle size-surface area differences between samples or sites can have significant effects. Often-used taxonomic references for algae and diatoms include Smith (1950), Prescott (1962, 1970), and Patrick and Reimer (1966). The use of artificial substrates for periphyton and other benthic communities removes the substrate variable. Natural substrates may be sampled using the methods of Stevenson and Lowe (1986) or Hamala et al. (1981). A commonly used artificial sampler (diatometer) consists of multiple glass slides suspended from a floating holding frame (APHA 1985; Figure 6.121; also see Figure 4.11 illustrating the use of a diatometer in Coyote Creek, San Jose, CA). Not all species will colonize the glass slides, but the advantages of / efficient and precise evaluations outweigh this / disadvantage in most cases. Valid station comparisons are only possible when the key variables / affecting periphyton communities are similar; these include flow, turbidity, temperature, dissolved / oxygen, alkalinity, hardness, conductivity, nutrients (APHA 1985), and photosynthetically active/ radiation (LiCor 1979). /

Figure 6.121 Diatometer for artificial substrate pe phyton sampling.

The periphyton community can be evaluated for stormwater effects using several endpoints. When using a diatometer, slides should be left *in situ* for 6 to 14 days, then placed in formalin upon collection. Evaluation endpoints may include: number, richness, relative abundance, diversity, chlorophyll *a*, and other community or productivity indices (APHA 1985; Crossey and LaPoint

1988). Stevenson and Lowe (1986) recommend counting 200 cells for dominant, 500 for uncommon, and 1000 cells for rare species, or an additional 100 cells for each new species encountered (EPA 1989a,b,c, 1999).

Periphyton community analyses may be of a structural and functional nature. Structural measures include diversity indices, taxa richness, indicator species, and biomass (Rodgers et al. 1979; Wetzel 1979; Palmer 1977; Patrick 1973). Functional measures which have been used are primary productivity (e.g., chlorophyll *a*), or respiration (Rodgers 1979). Integrating the structural and functional characteristic provides the best means of evaluating ecosystem health, as demonstrated in the macroinvertebrate and fish approaches below.

Protozoan Sampling

Protozoans, like algae, exist in the planktonic and benthic communities. Because their biomass is relatively low compared to that of other aquatic communities, their contribution as a food source to higher trophic levels is probably limited; however, their function as predators or decomposers may fill important ecosystem niches and assist in maintaining–stabilizing decomposition and cycling processes. When protozoan cropping of bacteria is removed, the sediments can function as a carbon sink and microbial community structure–function relationships could alter, affecting nutrient availability to higher trophic levels (Griffiths 1983; Porter et al. 1987).

Several studies have shown the effective use of artificial (polyurethane) substrates in water and sediment pollution studies (Pontasch et al. 1989; Henebry and Ross 1989). This approach allows the foam substrates to colonize at reference sites for several days. Then they are exposed to toxicants either in the laboratory or *in situ* to test sample waters and compared to reference responses. The test endpoints of this multispecies assay include decolonization, protozoan abundance, taxa number, phototroph and heterotroph abundance, respiration, and island-epicenter colonization rates. Both stimulatory and inhibitory results are observed, and careful interpretation is required (Henebry and Ross 1989).

Macroinvertebrate Sampling

This group is operationally defined as those invertebrates retained on sieve mesh sizes greater than 0.2 mm (Hynes, 1970); however, the larger size of 0.5 or 0.95 mm (U.S. Standard No. 30) is used routinely (EPA 1989c). More representative benthos samples may be collected using smaller mesh sizes, such as 0.25 mm (U.S. Standard No. 60), which collect early life stages, chironomids, and nadid and tubificid oligochaetes (EPA 1990b). The major freshwater taxonomic groups may be separated into the trophic levels — functional feeding group descriptors of herbivores, omnivores, carnivores; or deposit and detritus feeders, collectors, shredders, grazers; or scrapers, parasites, scavengers, and predators (EPA 1990b). In most studies of high-to-medium-quality waters, species level identification will be necessary, with tolerant species only dominating in polluted systems. Each taxonomic group may contain a variety of functional feeding groups (Table 6.43). Some common pollution indicators are shown in Figure 6.122.

The benthic macroinvertebrate community has been used for many years to qualitatively and, more recently, to quantitatively assess water quality and pollution effects. There are advantages and disadvantages in using macrobenthos in water quality assessments (Table 6.44). However, except in cases of extreme and obvious pollution, they should always be a component of a stormwater effect assessment.

There is a wealth of reference information available to assist in the use of macroinvertebrates as monitoring tools, including Armitage (1978), Benke et al. (1984), Brinkhurst (1974), Cairns (1979), Cummins et al. (1984), Cummins and Wilzbach (1985), Edmondson and Winberg (1971), Goodnight and Whitley (1960), Hart and Fuller (1974), Hellawell (1978, 1986), Hilsenhoff (1977), Howmiller and Scott (1977), Hynes (1960, 1970), Holme and McIntyre (1971), Hulings and Gray (1971),

Table 6.43 Trophic Mechanisms and Food Types of Aquatic Insects

General Category Based on Feeding Mechanism	General Particle Size Range of Food (μm)	Subdivision Based on Feeding Mechanisms	Subdivision Based on Dominant Food	Aquatic Insect Taxa Containing Predominant Examples
Shredders	>103	Chewers and miners	Herbivores: living vascular plant tissue	Trichoptera (*Phryganeidae, Leptoceridae*) Lepidoptera Coleoptera (*Chrysomelidae*) Diptera (*Tipulidae, Chironomidae*)
		Chewer and miners	Detritivores (large particle detritivores): decomposing vascular plant tissue	Plecoptera (*Filipalpia*) Trichoptera (*Limnephilidae, Lepidostomatidae*) Diptera (*Tipulidae, Chironomidae*)
Collectors	<103	Filter or suspension feeders	Herbivores-detritivores: living algal cells, decomposing vascular plant tissue	Ephemeroptera (*Siphionuridae*) Trichoptera (*Philopotamidae, Psychomyidae, Hydropsychidae, Brachycentridae*) Lepidoptera Diptera (*Simuliidae, Chironomidae, Culicidae*)
		Sediment or deposit (surface) feeders	Detritivores (fine particle detritivores): decomposing organic particulate matter	Ephemeroptera (*Caenidae, Ephemendae, Leptophlebiidae, Baetidae, Ephemerellidae, Heptageniidae*) Hemiptera (*Gerridae*) Coleoptera (*Hydrophilidae*) Diptera (*Chironomidae, Ceratopogonidae*)
Scrapers	<103	Mineral scrapers	Herbivores: algae and associated microflora attached to living and nonliving substrates	Ephemeroptera (*Heptageniidae, Baetidae, Ephemerellidae*) Triochoptera (*Glossosomatidae, Helicopsychidae, Molannidae, Odontoceridae, Goreridae*) Lepidoptera Coleoptera (*Elmidae, Psephenidae*) Diptera (*Chironomidae, Tabanidae*)
		Organic scrapers	Herbivores: algae and associated attached microflora	Ephemeroptera (*Caenidae, Leptophlebiidae, Heptageniidae, Baetidae*) Hemiptera (*Corixidae*) Trichoptera (*Leptoceidae*) Diptera (*Chironomidae*)
Predators	>103	Swallowers	Carnivores: whole animals (or parts)	Odonata Plecoptera (*Setipalpia*) Megaloptera Trichoptera (*Rhyacophilidae, Polycentropidae, Hydropsychidae*) Coleoptera (*Dytiscidae, Gyrinnidae*) Diptera (*Chironomidae*)
		Piercers	Carnivores: cell and tissue fluids	Hemiptera (*Belastomatidae, Nepidae, Notonectidae, Naucoridae*) Diptera (*Chironomidae*)

Lenat (1983), Lind (1985), Merritt and Cummins (1984), Mason (1981), Metcalfe (1989), Milbrink (1983), Meyer (1990), Neuswanger et al. (1982), Pennak (1989), Posey (1990), Resh (1979), Resh and Roseberg (1984), Resh and Unzicker (1975), Reynoldson et al. (1989), Ward and Stanford (1979), Warren (1971), Waters (1977), Welch (1948), Welch (1980), Winner et al. (1975), EPA (1989a,c, 1990a,c, 1999), and OEPA (1989). Previous discussions highlighted the importance of attempting to control habitat (e.g., substrate), flow dynamics, and seasonal variables when monitoring — particularly the benthic macroinvertebrate community. Other, obviously critical issues, include the sampling procedure's precision and accuracy, taxonomic identification, and data evaluation.

Substrates can be sampled with nets, grab (dredge), core, and vegetation collection devices (Table 6.45). The Hess and Surber samples are often used to sample stream riffle habitats, whereby the substrates within a confined 0.1 m^2 area are vigorously disrupted and scrubbed down to a depth of approximately 10 cm. A flow velocity of at least 0.5 m/s is required for effective use of these net samplers. See also ASTM (1987) for additional information.

Sampling is frequently of a qualitative to semiquantitative nature that is relatively easy to conduct. The objective here is to determine differences between sites. Semiquantitative methods incorporate a level-of-effort constant or use quantitative methods in a nonrandom manner (EPA 1990b). Quantitative methods sample unit areas or volumes of habitat in a random manner. The approach chosen should depend on the data quality objectives (DQOs).

Semi- and quantitative sampling may use grab samplers (see Chapter 5 and Table 6-45), stream net samplers (Figure 6.123 and Table 6.46), and artificial substrates (Figures 6.124 through 6.127 and Table 6.47).

In large streams, deep waters, and areas of slow current velocities, it is necessary to use core or dredge samplers, which are also used for sediment sampling as discussed previously (Chapter 5). See also ASTM (1987, 1991), Lind (1979), APHA (1985), Downing (1984), and Wetzel and Likens (1991), for additional sampler information. The Ekman and Ponar grab samplers are commonly used in relatively soft sediments of clay to gravel size, with relatively good efficiency (Elliott and Drake 1981). The hand and gravity corers are preferred in soft sediments because pressure waves and loss of surficial fines are reduced, variance can be determined horizontally and vertically, sieving volume is reduced, precision is increased, and sediment structure-integrity is maintained to

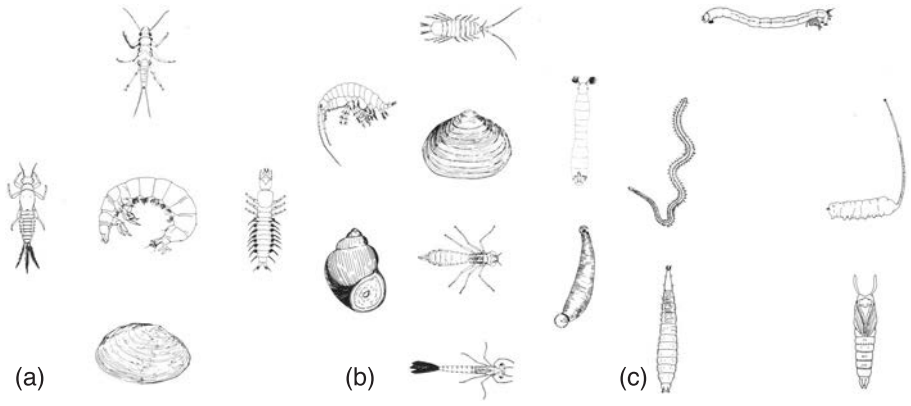


Figure 6.122 Representatives of stream bed animals commonly associated with various degrees of organic pollution. (a) The clean water (sensitive) group (from left): stonefly nymph, mayfly naiad, caddisfly larvae, hellgrammite, unionid clam. (b) The intermediately tolerant group (from left): scud, sowbug, blackfly larvae, fingernail clam, snail, dragonfly nymph, leech, damselfly nymph. (c) The very tolerant group (from left): bloodworm or midge larvae, sludgeworm, rattailed maggot, sewage fly larvae, sewage fly pupae. (From *The Practice of Water Pollution Biology.* U.S. Department of the Interior. Washington, D.C. 1969.)

Table 6.44 Advantages and Disadvantages of Using Macroinvertebrates and Fish in Evaluation of the Biotic Integrity of Freshwater Aquatic Communities

Advantages	Disadvantages
Macroinvertebrates	
Fish, highly valued by humans, are dependent on benthic invertebrates as a food source.	They require taxonomic expertise for identification, which is also time-consuming.
Many species are sensitive to pollution	Background life-history information is lacking for some species and groups.
Bottom fauna often have a complex life cycle of a year or more, and, therefore, represent long-term exposure periods to water and sediment conditions.	Results are difficult to translate into values meaningful to the general public.
Many have an attached or sessile mode of life and are not subject to rapid migrations, therefore serve as resident monitors of test site quality.	May not detect short-term or recent chronic pollution events.
	Not as sensitive a pollution indicator in large rivers, bays, lakes, and marine systems.
	Natural levels of spatial and temporal variation may make detection of significant effects difficult.
Fish	
Life history information is extensive for most species.	Sampling fish communities is selective in nature.
Fish communities generally include a range of species that represent a variety of trophic levels (omnivores, herbivores, insectivores, planktivores, piscivores) and utilize foods of both aquatic and terrestrial origin. Their position at the top of the aquatic food web also helps provide an integrated view of the watershed environment.	Fish are highly mobile. This can cause sampling difficulties and also creates situations of preference and avoidance. Fish also undergo movements on diurnal and seasonal time scales. This increases spatial and temporal variability, which makes detection of significant effects or trends difficult.
Fish are highly valued by the public.	There is a high requirement for manpower and equipment for field sampling.
Fish are relatively easy to identify. Most samples can be sorted and identified in the field, and then released.	
Both lethality and stress effects (depressed growth, lesions, abnormalities, and reproductive success) can be evaluated. Careful examination of recruitment and growth dynamics among ages of fish can help pinpoint periods of unusual stress.	

Modified from Cairns, J., Jr. and K.L. Dickson. A simple method for the biological assessment of the effects of waste discharges on aquatic bottom-dwelling organisms. *J. Water Pollut. Control Fed.,* 43: 755–772. 1971; Karr, J.R. and D.R. Dudley. Ecological perspective on water quality goals. Ecological perspective on water quality goals. *Environ. Manage.*, 5: 55–68. 1981. With permission.

Table 6.45 Sampling Methods for Macroinvertebrates

Method	Habitat	Substrate Type	Effort Required[a]		Ref.
			Persons	Time (hr)	
Hess, Surber	Stream riffle (<0.5 m deep)	Sand, gravel, cobble	1	0.50	ASTM (1987)
Ponar grab	Rivers, lakes, estuaries	Mud, silt, sand, fine gravels	2	0.50	ASTM (1987)
Ekman grab	Stream pools, shallow lakes	Mud, silt, sand	1	0.25	ASTM (1987)
Corers	Rivers, lakes	Mud, silts	1–2	0.25	Downing (1984)
Sweep net	Littoral	Vegetation	1	0.25	Downing (1984)
Macan McCauley Minto Wilding	Littoral	Vegetation	1	0.50	Downing (1984)
Standardized substrates	All	All	1	0.25–1.0[b]	APHA (1985)

[a] Effort includes time spent in field to collect, sieve, and isolate one sample. Laboratory time required to remove and identify organisms ranges from 1 to 5 per sample, depending on expertise level, and taxonomic resolution sought.

[b] Two- to six-week colonization time ended before sample is removed.

Modified from EPA. *Ecological Assessment of Hazardous Waste Sites.* Environmental Research Laboratory. U.S. Environmental Protection Agency, Corvallis, OR. EPA 600/3-89/013. 1989a.

a much higher degree than in dredge samples. The principal disadvantages, however, are their ineffective sampling of coarse, large-grained sediments and the small volumes that are collected.

The efficiency of benthic collection samplers has been compared, and, in general, the grab samplers are less efficient than the corers (ASTM 1991a). The Ekman dredge is the most commonly used sampler for benthic investigations (Downing 1984). The Ekman is limited to less compacted, fine-grained sediments, as are the corer samplers. However, these are usually the sediments of greatest concern in toxicity assessments. The most commonly used corer is the Kajak–Brinkhurst, or hand corer. In more resistant sediments, the Petersen, Ponar, Van Veen, and Smith–McIntyre dredges are used most often (Downing 1984). Based on studies of benthic macroinvertebrate populations, the sediment corers are the most accurate samplers, followed by the Ekman dredge, in most cases (Downing 1984). For consolidated sediments, the Ponar dredge was identified as the most accurate, while the Petersen was the least effective (Downing 1984).

Quantitative benthic macroinvertebrate sampling of small streams may be improved by also using small to large emergence traps. These samplers trap the dominant stream insects as they leave the water as flying adults. In this way, effects from habitat heterogeneity are reduced, time-consuming "bug" picking from substrate samples is avoided, and most adult stages can be identified to the species level. See also Wetzel and Likens (1991), Illies (1971), Hall et al. (1980), and Peckarsky (1984).

Semiquantitative methods also include the traveling kick method (Horning and Pollard 1978) and the Rapid Bioassessment Protocols II and III (kicknets) (EPA 1990b). Readers should note that the EPA Rapid Bioassessment Protocol manual has been revised (EPA 1999) and no longer differentiates Protocols I through III (EPA 1989c). As with other sediment-associated components, quantitative evaluations are complicated by often high degrees of variability. By using multimetric (indice) assessment endpoints, the impact of population variability can be reduced (EPA 1990b). Nevertheless, it is essential that replicate sampling of each habitat niche be conducted at each site, allowing measures of precision. Precision may also be increased by collecting larger samples, thus the influence of reducing small patches. Three to five replicates are a minimum requirement. Use of a transect to select replicate sites may result in different habitats being selected.

A number of artificial substrate samplers have been used to assess benthic macroinvertebrate conditions, i.e., flow, depth, light, and temperature. These samplers remove the substrate variable and provide known sampling areas and exposure times. Unfortunately, there are some disadvantages which may be significant, including some taxa may not utilize the substrate; proportional relationships may be altered; substrates are colonized primarily by upstream "drift" organisms, and effects from contact with possibly contaminated bed sediments is reduced or eliminated; they require 4- to 8-week exposures and two sampling trips; and they may be lost due to high flow or vandalism (see Figures 6.123 to 6.127). As with the periphyton samplers, care must be taken to ensure uniformity.

For most studies, semiquantitative approaches using the EPA's Rapid Bioassessment Protocols II or III (RBP) and the Ohio EPA Hester–Dendy samplers (Figure 6.127) are preferred, with habitat evaluations. The RBP II method samples 1 m^2 riffle areas, and 100 organisms are randomly picked and identified to the family level (EPA 1989c). The Ohio EPA method uses 10 metrics (nine based on Hester–Dendy results and one based on dip net sampling) to compute an Invertebrate Community Index in wadeable streams (Ohio 1989). In streams where rocks are the dominant habitat, it may be useful to use a basket sampler (Figure 6.124) containing approximately 30 rocks of equal size or a particle size distribution similar to the test or reference site. This approach is used by the State of Maine and by other investigators (e.g., Clements et al. 1996). It is the most realistic artificial substrate method. When high quantities of biomass are needed, such as for tissue residue analyses, the grill-basket sampler containing 3M polyethylene mesh is useful (Stauffer et al. 1974). All of the artificial substrates are set out in triplicate and secured to concrete blocks in shallow waters for 4 to 8 weeks. The metrics vary in their ability to detect organic material or toxicant-related impacts. They overlap in ranges of sensitivity and thereby reinforce final conclusions regarding the condition of the system's biological communities (EPA 1989c). The RBP II methods, organism pollution tolerance levels, and indices calculated in the RBP and Ohio EPA methods are described in detail in Appendix B. Note that in many states, special collection permits are required to collect macroinvertebrates.

Table 6.46 Comparison of Stream-Net Samplers

Type	Habitats and Substrates Sampled	Effectiveness of Device	Advantages	Limitations
Surber sampler	Shallow, flowing streams, less than 32 cm in depth with good current; rubble substrate, mud, sand, gravel	Relatively quantitative when used by experienced biologist; performance depends on current and substrate	Encloses area sampled; easily transported or constructed; samples a unit area	Difficult to set in some substrate types, that is, large rubble; cannot be used efficiently in still slow-moving streams
Portable invertebrate box sampler, Hess stream bottom sampler, and stream-bed fauna sampler	Same as Surber	Same as Surber	Same as above except completely enclosed with stable platform; can be used in weed beds	Same as Surber
Drift nets	Flowing rivers and streams; all substrate types	Relatively quantitative and effective in collecting all taxa which drift in the water column; performance depends on current velocity and sampling period	Low sampling error; less time, money, effort; collects macroinvertebrates from all substrates, usually collects more taxa	Unknown where organisms come from; terrestrial species may make up a large part of sample in summer and periods of wind and rain; does not collect nondrifting organisms

From EPA. *Macroinvertebrate Field and Laboratory Methods for Evaluating the Biological Integrity of Surface Waters.* Office of Research and Development, U.S. Environmental Protection Agency, Washington, D.C., EPA 600/4-90/030. 1990.

Table 6.47 Comparison of Substrate Samplers

Type of Substrate	Advantages	Limitations
	Artificial	
General characteristics	Reduce habitat substrate variability influence Eliminate subjectivity in collection process Patchiness reduced Skill level required is less Long exposure periods (6–8 weeks) Discriminate between sediment and water toxicity	Habitats may be different, thus promotes growth of different species, not representative of site. Two trips needed Long exposure periods (6–8 wks) Sediment substrate effects, including toxicity, reduced Sampler loss through vandalism or sedimentation
Modified	Reduces compounding effects of substrate differences, multiplate sampler	Long exposure time, difficult to anchor, easily vandalized
Fullner	Wider variety of organisms	Same as modified Hester–Dendy
Basket Type	Comparable date, limited extra material for quick lab processing. Large amount of biomass.	No measure of pollution on strata, only community formed in sampling period, long exposure time, difficult to anchor, easily vandalized
Periphyton	Floats on surface, easily anchored, glass slides exposed just below surface	May be damaged by craft or flows, easily vandalized
	Natural	
Any bottom or sunken material	Indicate effects of pollution, gives indication of long-term pollution	May be difficult to quantitate; possible lack of growth, not knowing previous location or duration of exposure

Modified from EPA. *Handbook for Sampling and Sample Preservation of Water and Wastewater.* Environmental Monitoring and Support Lab, U.S. Environmental Protection Agency, Cincinnati, OH, EPA 600/4-82/029. 1982; EPA. *Ecological Assessment of Hazardous Waste Sites.* Environmental Research Laboratory, U.S. Environmental Protection Agency, Corvallis, OR. EPA 600/3-89/013. 1989a.

Figure 6.123 Stream net sampler.

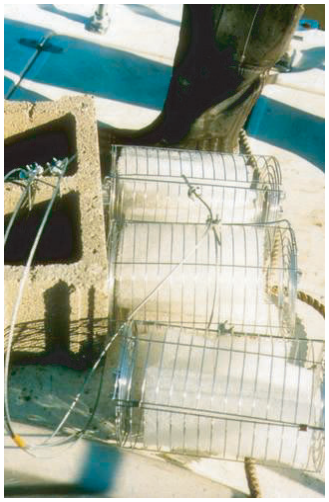


Figure 6.124 Artificial substrates (polyethylene mesh) in BBQ baskets secured to cinder blocks.

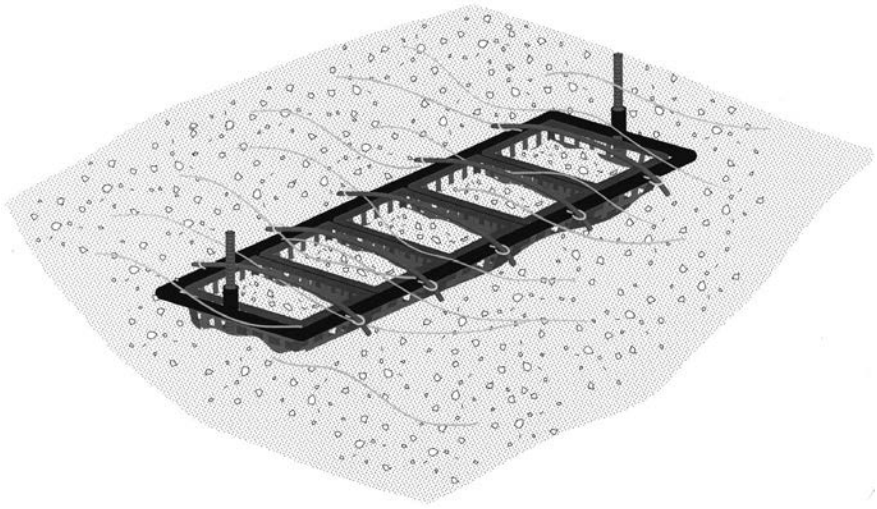


Figure 6.125 Colonization trays buried to stream sediment surface and secured with iron rods. Baskets are filled with cleaned substrates representative of the reference or test site.

Figure 6.126 Periphyton sampler, two styrofoam floats with eight glass microscope slides in rack.

Figure 6.127 Periphyton sampler in place, plus Hester–Dendy samplers.

Table 6.48 Comparison of Net Sampling Devices

Devices	Application	Advantages	Disadvantages
Wisconsin net	Zooplankton	Efficient shape concentrates samples	Qualitative
Clarke-Bumpus	Zooplankton	Quantitative	No point sampling, difficult to measure depth of sample accurately

From EPA. *Handbook for Sampling and Sample Preservation of Water and Wastewater.* Environmental Monitoring and Support Lab, U.S. Environmental Protection Agency, Cincinnati, OH, EPA 600/4-82/029. 1982.

ZOOPLANKTON SAMPLING

The zooplankton community plays a major role in the food web and aquatic ecosystem dynamics. Its use as an indicator of pollution in lotic systems has been limited. Studies are more common in lentic systems; however, they are complicated by a high degree of spatial and temporal variability, and less knowledge of pollution tolerances as compared to the benthos. The cladocerans, *Daphnia magna*, *Daphnia pulex,* and *Ceriodaphnia dubia*, have been useful as sensitive toxicity surrogate species. If an intensive lake–reservoir ecosystem effect study is to be conducted, they should be included. Commonly used sampling nets are listed in Table 6.48 and Figure 6.128.

Figure 6.128 Net sampler for plankton.

FISH SAMPLING

The fish community is perhaps the most important component of the ecosystem as viewed by public opinion, commercial interests, and regulatory requirements. In reality, however, it is no more important than any other major level of biological organization and is not as useful as other groups when evaluating stormwater effects. Fish, by nature, are in general a more transitory species than other aquatic organisms and, therefore, produce more variable results in biosurveys. Because they are mobile, they are often able to avoid polluted waters. This avoidance behavior makes evaluations of site-specific sources and problems more difficult. Sampling methods vary in their degree of efficiency and selectivity and compound data variance problems (EPA 1989c). They do, however, possess many advantages in the assessment process:

- Beneficial uses of stream segments characterized in terms of fisheries
- Many endangered species exist
- Effective collection methods exist
- Effective quality assessments are possible using community structure and functional metrics to form an index of integrity
- Used as regulatory and monitoring tools for decades; an extensive database exists on life history, distribution, and effects
- Indicators of long-term exposures and watershed conditions
- Comprise multiple trophic levels
- Drive ecosystem dynamics in the "top-down" approach theory and may integrate effects from lower trophic levels ("bottom-up" approach)
- Contaminant source to humans
- Useful for sublethal, chronic pollutant exposure effect studies

Many fish communities contain multiple trophic levels, such as invertivores, planktivores, herbivores, omnivores, and piscivores (Table 6.49; Karr et al. 1983). Trophic guild information is useful for evaluating system alterations at a functional and structural level. The omnivore component typically increases as water quality declines. Streams with fewer than 20% omnivores are often of good quality, and poor if greater than 45% are true omnivores (Karr 1981). There is also often a strong inverse correlation between the abundance of insectivorous cyprinids (minnows) and water quality (more abundant minnow populations indicate worse water quality). Another generality of feeding type and water quality is the presence/absence of top carnivores, which are at the top of

the aquatic food chain and thereby integrate lower trophic level effects. They are most likely to show biomagnified toxicants in their tissue, but might not necessarily show effects from those toxicants. The validity of these generalizations has been well documented in the agricultural Midwest. However, there are exceptions nationwide. Tissue residues are good indicators of exposure for some nonpolar organics and methyl mercury; however, many metals and organics that can be metabolized cannot be assessed well with tissue information.

Sampling of fish communities is relatively labor intensive, often requiring special equipment and expertise. But, given the importance of fish in ecosystem structure and functioning, sport and commercial fishing, and public perceptions, they should be monitored.

Generally, the preferred sampling season is mid to late summer, when stream and river flows are moderate to low, and less variable than during other seasons (EPA 1990b). Although some fish species are capable of extensive migration, fish populations and individual fish may remain in the same area during summer (Funk 1957; Gerking 1959; Cairns and Kaesler 1971). However, large river, lake, and harbor habitats promote greater migration ranges. Ross et al. (1985) and Matthews (1986) found that stream fish assemblages were stable and persistent for 10 years, recovering rapidly from droughts and floods, indicating that large population fluctuations are unlikely to occur in response to purely natural environmental phenomena. However, comparison of data collected during different seasons is discouraged, as are data collected during or immediately after major flow changes (EPA 1989a).

Although various collection methods are routinely used to sample fish; electrofishing (Figures 6.129 through 6.131), seines (Figure 6.132), and rotenone (a poison) are the most commonly used methods in freshwater habitats (Tables 6.50 and 6.51). Each method has advantages and disadvantages (Nielsen and Johnson 1983; Hendricks et al. 1980). However, electrofishing is recommended for most fish field surveys because of its greater applicability and efficiency, and the good recoverability of stunned fish that are returned to the water (EPA 1989a,c).

Table 6.49 Trophic Guilds Used by Schlosser (1981, 1982a, 1982b) to Categorize Fish Species

Herbivore–Detritivores (HD)	HD species feed almost entirely on diatoms or detritus.
Omnivores (OMN)	OMN species consume plant and animal material. They differ from GI species in that, subjectively, greater than 25% of their diet is composed of plant or detritus material.
Generalized insectivores (GI)	GI species feed on a range of animal and plant material including terrestrial and aquatic insects, algae, and small fish. Subjectively, less than 25% of their diet is plant material.
Surface and water column insectivores (SWI)	WSI species feed on water column drift or terrestrial insects at the water surface.
Benthic insectivores (BI)	BI species feed predominantly on immature forms of benthic insects.
Insectivore–Piscivores (IP)	IP species feed on aquatic invertebrates and small fish. Their diets range from predominantly fish to predominantly invertebrates.

Figure 6.129 Electrofishing with backpack unit in main stream reach (notice nearby seine to capture stunned fish).

Figure 6.130 Electrofishing with backpack unit in near-shore areas.

Figure 6.131 Boat electrofishing unit. (Courtesy of Wisconsin Department of Natural Resources.)

Figure 6.132 Fish seining.

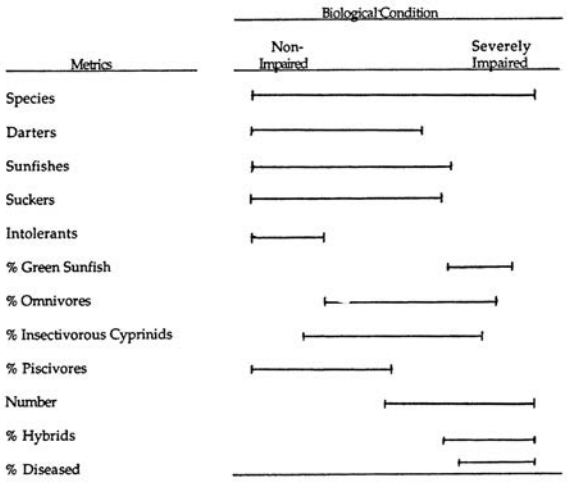


Figure 6.133 Range of sensitivities of Rapid Bioassessment Protocol V fish metrics in assessing biological condition. (Modified from EPA. *Ecological Assessment of Hazardous Waste Sites.* Environmental Research Laboratory. U.S. Environmental Protection Agency, Corvallis, OR. EPA 600/3-89/013. 1989a.)

Indices of Fish Populations

Perhaps the most popular index is the IBI. A slightly modified version is used in the EPA Rapid Bioassessment Protocols for fish. The IBI is weighted on the basis of individual species' tolerances to water and habitat quality. The IBI is comprised of 12 metrics, as follows:

A. Species richness and composition
   1. Species number
   2. Darter species number
   3. Sucker species number
   4. Sunfish species number
   5. Intolerant species number
   6. Green sunfish proportion

B. Abundance and condition
   1. Individual numbers
   2. Hybrid proportion
   3. Proportion with disease anomalies

C. Trophic composition
   1. Omnivore proportion
   2. Insectivorous cyprinid proportion
   3. Piscivore proportions

Table 6.50 Fish Sampling Methods

Methods	Advantages	Disadvantages
Electrofishing	Greater standardization of catch per unit of effort Less time and manpower than some sampling methods Less selective than seining (although it is selective toward size and species) Adverse effects on fish are minimized Appropriate in a variety of habitats	Sampling efficiency is affected by turbidity and conductivity. Initial cost of equipment Although less elective than seining, electrofishing is size and species selective. Effects of electrofishing increase with body size. Species specific behavioral and anatomical differences also determine vulnerability to electroshocking A hazardous operation that can injure field personnel if proper safety procedures are ignored
Reformed seining	Relatively inexpensive Lightweight and are easily transported and stored Repair and maintenance are minimal and can be accomplished on-site Restricted water quality parameters Effects on the fish population are minimal because fish are collected alive and are generally unharmed	Previous experience and skill, knowledge of fish habitats and behavior, and sampling effort are probably more important in seining than in the use of any other gear Sample effort and results are more variable than sampling with electrofishing or rotenoning Generally restricted to slower water with smooth bottoms, and is most effective in small streams or pools with little cover Standardization of unit of effort to ensure data comparability is difficult
Rotenoning	Effective use independent of habitat complexity Greater standardization of unit of effort than seining Provides more complete censusing of the fish population than seining or electrofishing	Kills all fish and possibly nontarget species, should only be used if other methods are not appropriate and if the data are essential Prohibited in many states Application and detoxification can be time and manpower intensive Effective use affected by temperature, light, dissolved oxygen, alkalinity, and turbidity High environmental impact; concentration miscalculations can produce substantial fish kills downstream of the study site

From EPA. *Ecological Assessment of Hazardous Waste Sites.* Environmental Research Laboratory, U.S. Environmental Protection Agency, Corvallis, OR. EPA 600/3-89/013. 1989a.

Table 6.51 Sampling Methods for Fish[a]

Method	Habitat	Persons	Time (hr)
Electrofishing	Small streams	2	0.25–1
	Large streams, rivers, lakes	2	0.25–1
Seining	Small streams or impoundments	2–3	0.50–1
Hoop net	Streams or rivers	2–3	2[b]
Gill, trammel nets	Lakes[d]	2–3	2–4[c]
Fyke net	Lakes[d]	2–3	2[c]

[a] Taken from Lagler (1978); Hendricks et al. (1980); Hubert (1983); Nielsen and Johnson (1985).

[b] Time for obtaining fish sample; time for stationary netting techniques includes time spent setting and receiving nets. It does not include time required to process sample (weighing, measuring, or taxonomic identification), which can range from 1 to 4 hours depending on taxonomic resolution and number of fish obtained.

[c] Time for hoop, gill, trammel, and fyke nets does not include 24 hours or period for which net is left in water to obtain sample.

[d] Gill, trammel, and fyke nets can also be used in some cases in flowing water if properly anchored; however, debris usually makes these applications troublesome.

From EPA. *Protocols for Short-Term Toxicity Screening of Hazardous Waste Sites.* Environmental Research Laboratory, U.S. Environmental Protection Agency, Corvallis, OR. EPA 600.3-88/029. 1989b.

Each metric is scored as 1 (worst), 3 (moderate), or 5 (best) as compared to the reference site or other data (see Fausch et al. 1984) showing regional norms (Table 6.52). Therefore, the index may range from 12 to 60 after all metric scores are totaled. Regional modifications have been developed by Hughes and Gammon (1987), Leonard and Orth (1986), Steedman (1988), and Wade and Stalaup (1987). The IBI is shown generally in Figure 6.134 and described in detail in Appendix C.

The Index of Well-Being (IWB), developed by Gammon (1976), was also developed in the midwestern United States to evaluate environmental stress effects on riverine fish. It is simpler than the IBI, using four measures: numbers of individuals, biomass, and the Shannon diversity index based on numbers and weight. Unfortunately, in some systems, high numbers and biomass of pollution-tolerant species produce a high index value, yet quality is reduced. To deal with this problem the Ohio EPA (1989) and Gammon (1989) developed a modified IWB which eliminates highly tolerant species, exotic species, or hybrids from the numbers and biomass components of the IWB, but retained in the Shannon index calculations. This modification has proven to be an effective assessment tool, which is consistent and sensitive to a wide range of environmental stresses. These equations are listed below:

Index of Well-Being:

$$IWB = 0.5 \ln N + 0.5 \ln B + H (no.) + H (wt.)$$

where N = relative number of all species
B = relative weight of all species
H (no.) = Shannon index based on relative numbers
H (wt.) = Shannon index based on relative weight

Shannon Diversity Index:

$$\overline{H} = -\sum\left(\frac{n_i}{N}\right)\ln\left(\frac{n_i}{N}\right)$$

where n_i = relative numbers or weight of the *i*th species
N = total number or weight of the sample

The IBI and mIWB require that indigenous fish species be classified in terms of environmental tolerance (to both natural and anthropogenic stressors). Tolerance levels (Appendix C) vary with each species, between ecoregions, seasonally, at different life stages, and they depend on the presence of other stressors, organism health, and the type of stressor. This group of critical variables makes any "tolerance" classification crude and tenuous. Nonetheless, the use of these classifications has been effective in evaluating ecosystem impairment. For many systems, shifts in dominant species and trophic classification away from sensitive, nonomnivores (e.g., trout, walleye) to tolerant omnivores (e.g., carps), clearly and easily show impairment exists. In other areas, where impairment is just beginning, as in a stream reach downstream of acute effects ("gray" zone), and where ecosystem recovery is beginning, the species tolerance levels will be uncertain.

TOXICITY AND BIOACCUMULATION

Why Evaluate Toxicity?

Toxicity and bioaccumulation evaluations are important and often essential components of stormwater impact assessments. They produce information that cannot be accurately determined or extrapolated from other assessment components. Toxicity tests have strengths and weaknesses that must be recognized (Table 6.53). If there is a clear understanding of the test responses and associated assumptions, and if proper QA/QC is followed, toxicity testing will allow for sensitive, meaningful, and efficient assessments of ecosystem quality and will identify stressor magnitude frequency, and duration. The science of aquatic toxicology has progressed rapidly in recent years and is now an integral component of many EPA regulatory programs. Toxicity testing may evaluate effects and address a wide variety of study objectives, using any of several general and specific monitoring approaches (Table 6.54). This variety of approaches allows for a high number of different component combinations, with each possibly providing unique information and having different assumptions associated with them. Many different approaches and organisms have been used for toxicity testing, and these will be discussed in the following section. Figures 6.135 through 6.138 show several test setups used in the Environmental Health Sciences laboratories at Wright State University, while Figures 6.139 and 6.140 are two of the Azur Environmental procedures, using phosphorescent phytoplankton, used in the environmental engineering labs at the University of Alabama at Birmingham.

Odum (1992) stated that stress is usually first detected in sensitive species at the population level. Natural population and community responses are not measured directly with whole effluent toxicity (WET) tests (La Point et al. 1996, 2000). The traditional surrogates (*P. promelas* and *C. dubia*) may not be as sensitive as indigenous species (Cherry et al. 1991). Indirect effects of toxicity on species, population, and community interactions can be important (Clements et al. 1989; Clements and Kiffney, 1996; Day et al. 1995; Fairchild et al. 1992; Giesey et al. 1979; Gonzalez and Frost 1994; Hulbert 1975; La Point et al. 2000; Schindler 1987; Wipfli and Merritt 1994), and may not be detected by WET testing. A huge ecological database exists showing the importance of species interactions in structuring communities (e.g., Dayton 1971; Power et al. 1988; Pratt et al. 1981).

It is less likely that strong relationships will exist between WET test responses and indigenous communities at sites where there are other pollutant sources, where effluent toxicity is low to moderate, or where dilution is high. Based on fish and benthic invertebrate responses, several studies suggest that WET tests are not always predictive of receiving water impacts (Clements and Kiffney 1994; Cook et al. 1999; Dickson et al. 1992, 1996; Niederlehner et al. 1985; Ohio EPA 1987); however, many studies have shown WET tests to be predictive of aquatic impacts (e.g., Birge et al. 1989; Diamond et al. 1997; Dickson et al. 1992, 1996; Eagleson et al. 1990; Schimmel and Thursby 1996; Waller et al. 1996). These differences should not be surprising however, as it is likely a result of WET test organisms and field populations experiencing different exposures (Burton et al. 2000; EPA 1991e). In an effluent-dominated system, the in-stream exposure is very similar to a WET test. A less degraded watershed, or one that is not dominated by point sources, may have sensitive indigenous populations that are exposed to "toxic" effluents at nontoxic concentrations. Conversely, if sensitive species have already been lost from a watershed, a toxic effluent may be inhibiting their return. In highly degraded sites, virtually any traditional assessment tool (acute toxicity testing, chemical concentrations, indigenous communities) will show effects and strong correlations with other tools. The WET tests were not developed to evaluate all natural and anthropogenic stressors nor to show all biological responses (such as mutagenicity, carcinogenicity, teratogenicity, endocrine disruption, or other important subcellular responses). In addition, highly nonpolar compounds may elicit an effect in short-term exposures. These issues dictate that additional assessment tools be utilized in order to protect aquatic ecosystems (Waller et al. 1996).

Table 6.52 Regional Variations of IBI Metrics

Variations in IBI Metrics	Midwest	New England	Ontario	Central Appalachia	Colorado Front Range	Western Oregon	Sacramento San Joaquin
1. Total Number of Species	X	X		X	X		X
# native fish species			X			X	
# salmonid age classes						X	X
2. Number of Darter Species							
# sculpin species						X	
# benthic insectivore species		X					
# darter and sculpin species			X				
# salmonid yearlings (individuals)						X	X
% round-bodied suckers	X						
# sculpins (individuals)							X
3. Number of Sunfish Species	X				X		
# cyprinid species						X	
# water column species		X					
# sunfish and trout species			X				
# salmonid species							X
# headwater species	X						
4. Number of Sucker Species	X	X				X	
# adult trout species						X	X
# minnow species	X				X		
# sucker and catfish species			X				
5. Number of Intolerant Species	X	X			X	X	
# sensitive species	X						
# amphibian species							X
Presence of brook trout			X				X
6. % Green Sunfish							
% common carp						X	
% white sucker		X			X		
% tolerant species	X						
% creek chub				X			
% dace species			X				

7. % Omnivores	X	X	X	X	X	X	
% yearling salmonids					X	X	
8. % insectivorous Cyprinids	X						
% insectivores		X				X	
% specialized insectivores				X	X		
# juvenile trout							X
% insectivorous species	X						
9. % Top Carnivores	X	X	X				
% catchable salmonids						X	
% catchable wild trout							X
% pioneering species	X						
Density catchable wild trout							X
10. Number of Individuals	X		X	X	X	X	X
Density of individuals		X					
11. % Hybrids	X	X					
% introduced species					X	X	
# simple lithophils	X						
% simple lithophilic species	X						
% native species							X
% native wild individuals							X
12. % Diseased Individuals	X	X	X	X	X	X	
13. Total Fish Biomass						X	

Note: X = metric used in region. Many of these variations are applicable elsewhere.

From EPA. *Rapid Bioassessment Protocols for Use in Streams and Rivers: Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 444/4-89/001. 1989c.

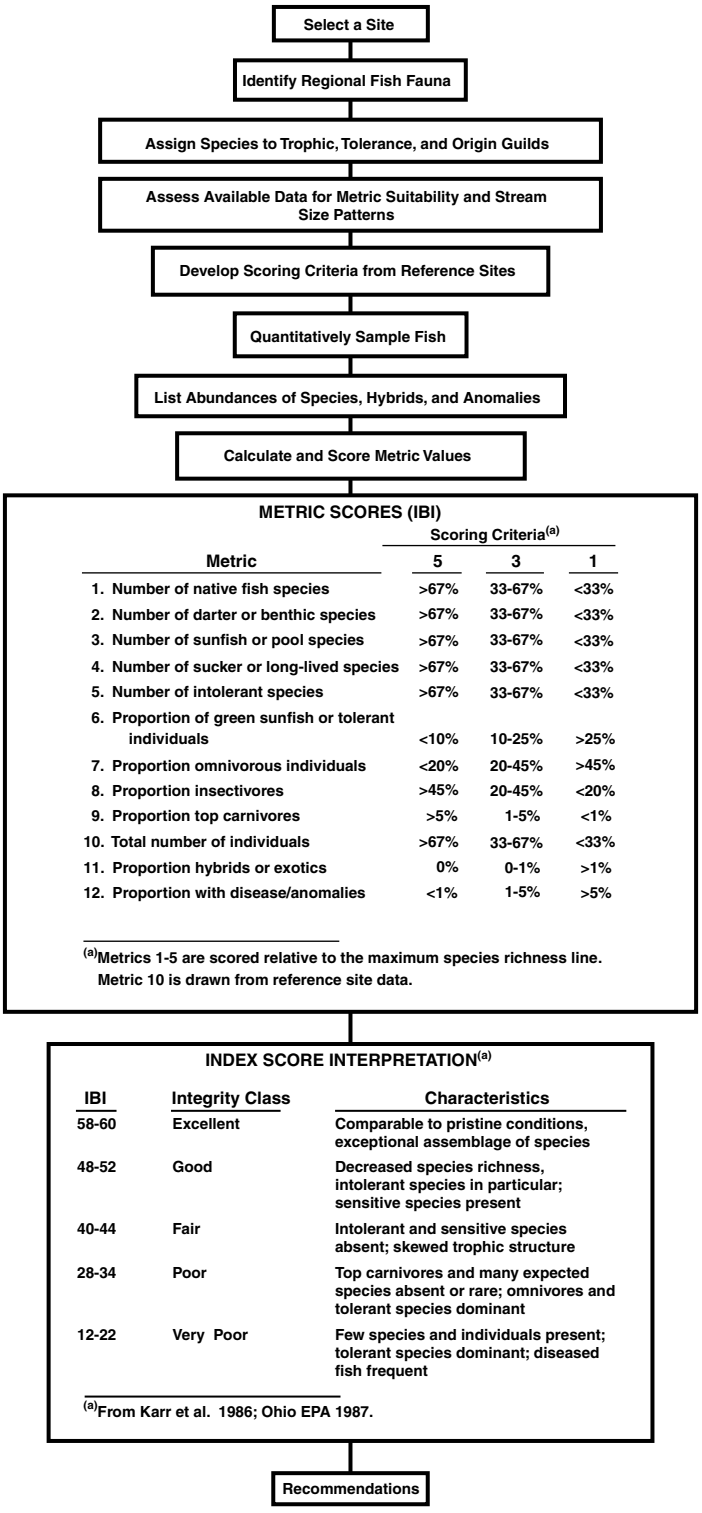


Figure 6.134 Flowchart of bioassessment approach advocated for Rapid Bioassessment Protocol V. (From EPA. *Rapid Bioassessment Protocols for Use in Streams and Rivers: Benthic Macroinvertebrates and Fish.* Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 444/4-89/001. 1989c.)

Table 6.53 Strengths and Weaknesses of Toxicity Tests in Stormwater Assessments

Strengths	Weaknesses
Toxicity can be quantified and linked to the presence of specific or multiple contaminants, sources, or affected media (i.e., soil, water, sediment, vegetation, aquatic biota); an important assessment component needed to establish causality.	Measure of potential toxic effects on resident biota at the test site; however, cannot always be directly translated into an expected magnitude of effects on populations in the field.
Response is an integrated index of bioavailable contamination, whereas chemical analyses measure only total concentrations of specific compounds.	Results are dependent on specific techniques, e.g., test species, collection method, water or sediment quality, test duration, etc.
More sensitive than biosurvey methods.	If surrogate species used, there is a question of their response relationship to indigenous species.
Sensitive in all types of aquatic ecosystems.	Single species test responses may not relate to community structure and ecosystem function impacts.
Results are specific to the location at which the sample was collected; thus they can be used to develop maps of the extent and distribution of bioavailable contamination and toxic conditions.	May not detect long-term toxicity, bioaccumulation, sublethal effects, or persistent, hydrophobic contaminants.
Temporal toxicity dynamics of stormwater events can be quantified and correlated with flow and other physicochemical characteristics.	Laboratory exposure conditions in toxicity tests are not directly comparable to field exposures; additional confounding variables and other stresses are important in the field.
Indigenous species may be tested in the laboratory or *in situ.*	
Approach effectively used by the EPA and many states to regulate point source pollution.	
Multiple species, multiple trophic levels, and multiple levels of biological organization (e.g., plant, bacteria to fish) may be evaluated.	
Results are easily interpreted and amenable to QA/QC; within- and among-laboratory precision estimates are already available for several tests.	
May be tested *in situ,* thus reducing laboratory-sample handling related artifacts.	
Acute toxicity tests are relatively quick, easy, and inexpensive to conduct; results from acute tests are used as a guide in the design of chronic toxicity tests.	
Chronic and short-term chronic toxicity tests are generally more sensitive than are acute tests, and can be used to define “no effect” levels; in addition, chronic tests provide a better index of field population responses and more closely mimic actual exposures in the field.	
In situ and laboratory exposures may be used to assess bioaccumulation.	
May reveal recent short-term toxicity events that are not detected in biosurveys.	
Have a long regulatory use in the NPDES program	

Table 6.54 Problem Definition: Toxicity Test Approaches

Assessment Component	Monitoring Approach
Test media	Effluent (e.g., point source discharges of wastewater or runoff)
	Ambient water
	Sediment
	Interstitial water
	Extractable fraction (e.g., elutriate)
	Soil
	Sludge
	Sample fractionation (e.g., the EPA's Toxicity Identification Evaluation procedures)
Test organism	Surrogate
	Indigenous to ecoregion
	Resident
	Single species
	Multiples of single species
	Communities or populations
	Multitrophic and/or multiple levels of biological organization
Effect level	Acute (lethality endpoint)
	Short-term chronic (e.g., growth or reproduction during partial life cycle
	Chronic (sublethal endpoint during full life cycle)
	Biomarker (sublethal endpoint in short-term exposure)
	Concentration response defined (e.g., LC50, NOEL[a]) vs. exposure to undiluted (100%) sample
Test environment	Laboratory:
	Static, static-renewal, recirculating, or flow-through
	Water only
	Water (reconstituted or site water)[b] and sediment (suspended[c] or bedded[d])
	In situ:
	Effluent mixing zone
	Ambient water only
	Sediment only
	Water and sediment
	Artificial substrate
Measured endpoints	Functional
	Population-community structure
	Organism
	Cellular or molecular

[a] Sample concentration with 50% lethality, no observable effect level.
[b] Allows separation of water and sediment toxicity.
[c] Suspended solids concentration physically maintained or fluctuates.
[d] Mixed, sieved, or intact core.

Figure 6.135 Fathead minnow rearing tanks at Environmental Health Sciences laboratories at Wright State University.

Figure 6.136 Adult fathead minnow rearing tank at Wright State.

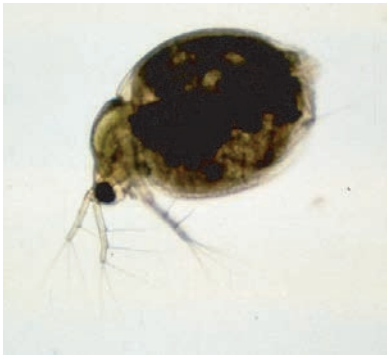


Figure 6.137 *Ceriodaphnia dubia* used for toxicity tests at Wright State.

Figure 6.138 Sediment toxicity tests at Wright State.

Figure 6.139 Microtox screening toxicity test at environmental engineering labs at UAB.

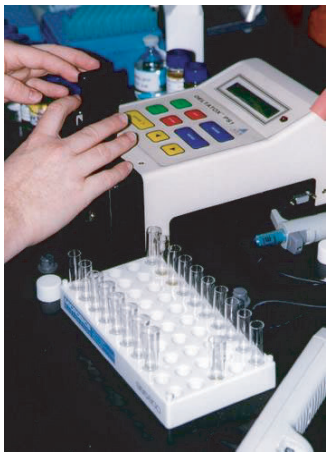


Figure 6.140 Deltatox screening toxicity test at environmental engineering labs at UAB.

Stormwater Toxicity

The water quality of stormwater, or of ambient waters immediately following high flow events, has been shown to be degraded in many studies with chemical concentrations which may exceed toxicity thresholds (e.g., Horner et al. 1994; Makepeace et al. 1995; Morrison et al. 1993; Waller et al. 1995a). Stormwater toxicants are primarily associated with particulate fractions and are typically assumed to be "unavailable." Toxicity tests with sediment removed have found reduced levels of toxicity in stormwater, compared to stormwater that has not undergone sediment removal (Crunkilton et al. 1996), as described in Chapter 3.

Also confusing is that typically short and intermittent runoff events cannot be easily compared to the criteria or standards developed and tested for traditional "long" duration point source discharges. Chemical analyses, without biological analyses, typically underestimate the severity of the problems because the water column quality varies rapidly, while the major problems were associated with sediment quality and effects on macroinvertebrates (Lenat and Eagleson 1981; Lenat et al. 1981).

Standardized toxicity tests have been used for many years in the United States to evaluate effluents in the National Pollutant Discharge Elimination System (NPDES) (EPA 1991e). These

whole-effluent toxicity (WET) tests have been shown to be useful for evaluating stormwaters. The use of toxicity tests on stormwater and receiving waters, especially *in situ* and side-stream tests that also reflect changing conditions for extended periods, has added greatly to our knowledge of toxicant problems associated with stormwater. While some stormwaters may not be toxic, there is a large body of evidence that suggests many are. Laboratory testing of runoff samples has shown acute and chronic toxicity to a variety of species (Connor 1995; Cooke et al. 1995; Dickerson et al. 1996; Hatch and Burton 1999; Ireland et al. 1996; Katznelson et al. 1995; Kuivila and Foe 1995; McCahon and Pascoe 1990, 1991; McCahon et al., 1990, 1991; Medeiros and Coler 1982; Medeiros et al. 1984; Mote Marine Laboratory 1984; Tucker and Burton 1999; Werner et al. 2000; Vlaming et al. 2000; Bailey et al. 2000). Pesticide pulses have been followed through watersheds, remaining toxic for days to weeks from runoff (Kuivila and Foe 1995; Werner et al. 2000). Samples from urban streams in southern California showed 85% exceeded diazinon criteria and 80% exceeded chlorpyrifos criteria. Of these samples, 76.6% produced 100% *C. dubia* mortality within 72 hours of exposure. Toxicity Identification Evaluations (TIE) confirmed the toxicity was due to the pesticides. Diazinon has been implicated as the primary toxicant in runoff causing acute toxicity to *C. dubia*, *P. promelas*, and *in situ Corbicula fluminea* assays (Kuivila and Foe 1995; Connor 1995; Waller et al. 1995a,b; Cooke et al. 1995). Organophosphate (chlorpyrifos, diazinon, malathion) and carbamate (carbofuran, carbaryl) pesticides in a delta draining urban and agricultural areas were the primary toxicants causing acute toxicity in 9.8 to 19.6% of water samples sampled between 1993 and 1995 (Werner et al. 2000). *C. dubia* reproduction and growth of *C. fluminea in situ* closely paralleled the health of the indigenous communities (Dickson et al. 1992; Waller et al. 1995b). A simulation of farm waste effluent (increased ammonia and reduced dissolved oxygen) found amphipod precopula disruption to be the most sensitive indicator of stress (McCahon et al. 1991). Mortality occurred only when D.O. fell to 1 to 2 mg/L and feeding rates recovered after exposure to ammonia (5 to 7 mg/L) ended. Elevations of major ion concentrations were toxic to *C. dubia* and *P. promelas* in some irrigation drainage waters (Dickerson et al. 1996).

Toxicity may also be reduced in runoff. When turbidity increased during high flow, photoinduced toxicity of PAHs was reduced *in situ,* as compared to baseflow conditions (Ireland et al. 1996). A recent study of the chronic toxicity of fenoxycarb to *Daphnia magna* showed a realistic single-pulse exposure resulted in an MATC of 26 µg/L, as compared to 0.0016 µg/L from a standard, constant-exposure study (Hosmer et al. 1998).

WET tests have also been used to evaluate the toxicity of effluents from stormwater runoff treatment systems. An evaluation of an urban runoff treatment marsh found strong relationships between *C. dubia* time-to-death, conductivity, and storm size, and time from storm flow initiation (Katznelson et al. 1995). Airport runoff containing glycol-based deicer/anti-icer mixtures was toxic to *P. promelas* and *D. magna* during high-use winter months; however, during summer months runoff toxicity only coincided with fuel spills (Fisher et al. 1995). Anti-icer was more toxic to *P. promelas*, *D. magna, D. pulex,* and *C. dubia* than deicer. Additives were more toxic than glycols (Hartwell et al. 1995). Stormwater detention ponds reduced *P. promelas* and Microtox toxicity 50 to 90% when particles greater than 5 µm were removed (Crunkilton et al. 1996; Pitt et al. 1999a).

Pulse Exposures

Some have suggested that relatively short periods of exposure to the toxicant concentrations in stormwater are not sufficient to produce the receiving water effects that are evident in urban receiving waters, especially considering the relatively large portion of the toxicants that are associated with particulates (Lee and Jones-Lee 1995a,b). Lee and Jones-Lee (1995b) suggest that the biological problems evident in urban receiving waters are mostly associated with illegal discharges and that the sediment-bound toxicants are of little risk. Mancini and Plummer (1986) have long been advocates of numeric water quality standards for stormwater that reflect the partitioning of the toxicants and the short periods of exposure during rains. Unfortunately, this approach attempts

to isolate individual runoff events and does not consider the cumulative adverse effects caused by the frequent exposures of receiving water organisms to stormwater (Davies 1986, 1991, 1995; Herricks 1995; Herricks et al. 1996).

A growing preponderance of data, however, is showing that toxicity is commonly observed during stormwater runoff and that short-term, pulse exposures can be more toxic than long-term, continuous exposures (e.g., Brent and Herricks 1998; Crunkilton et al. 1996; Curtis et al. 1985). Short pulse exposures in stormwater produced lethality several days to weeks later (Abel 1980; Bascombe 1988; Bascombe et al. 1989; Brent and Herricks 1998; Ellis et al. 1992). Some of this apparent response delay may be a result of uptake and accumulation kinetics (Bascombe et al. 1989, 1990; Borgmann and Norwood 1995; Borgmann et al. 1993). Recent investigations have identified acute toxicity problems and the importance of an adequate post-exposure observation period in side-stream studies with *P. promelas* in urban streams (Crunkilton et al. 1996), and in laboratory spiking studies (Cd, Zn, phenol) with *Ceriodaphnia dubia, Pimephales promelas,* and *Hyalella azteca* (Brent and Herricks 1998; Van Der Hoeven and Gerritsen 1997). Other laboratory studies have also shown acute and chronic toxicity of short-term exposures using fish and amphipods exposed to chloroamines, metals, and pesticides (Abel 1980; Abel and Gardner 1986; Holdway et al. 1994; Jarvinen et al. 1988a,b; McCahon and Pascoe 1991; Meyer et al. 1995; Parsons and Surgeoneer 1991a,b; Pascoe and Shazili 1986). In general, it appears that exposure to higher concentrations of toxicants for brief periods is more important that exposure to lower concentrations for longer periods (Brent and Herricks 1998; McCahon and Pascoe 1990; Meyer et al. 1995). However, increased amphipod depuration or metallothionein induction in the presence of Zn allowed greater tolerance (Borgmann and Norwood 1995; Brent and Herricks 1998).

Not all pulsed exposures are more toxic. If there is adequate time for organism recovery between pulsed exposures to toxicants, the effects of the pulsed exposure of some toxicants are diminished (Brent and Herricks 1998; Kallander et al. 1997; Mancini 1983; Wang and Hanson 1985). This difference may be attributed to the mechanism of toxicity. For example, organophosphates are relatively irreversible inhibitors of acetylcholinesterase (AChE), while carbamate inhibition may be reversible (Kuhr and Dorough 1976; Matsumura 1985). So little difference is observed between continual exposures and pulsed exposures (Kallander et al. 1997). Trout were observed to acclimate to ammonia if pulsed exposures were below their toxicity threshold (Thurston et al. 1981). Fenoxycarb was four orders of magnitude less toxic in a single pulsed exposure to *Daphnia magna* compared to a standard WET exposure (Hosmer et al. 1998). Complicating predictions of effects are synergistic interactions that occur between some contaminants such as pesticides and metals (Forget et al. 1999) and between herbicides and insecticides (Pape-Lindstrom and Lydy 1997). Organisms recovered to varying degrees given adequate time in clean water following pulsed exposures to phenol, permethrin, fenitothion, and carbamates (Brent and Herricks 1998; Green et al. 1988; Kallander et al. 1997; Kuhr and Dorough 1976; Parsons and Surgeoner 1991a,b).

Measuring Effects of Toxicant Mixtures in Organisms

Toxicant exposure is dependent on toxicant, organism, and habitat characteristics, such as toxicant partitioning (fugacity), the organisms' direct contact with substrates, and their feeding mechanisms. The toxicant target site and effect within the organism will be toxicant, species, and life stage dependent. The mixed function oxygenase (MFO) system and metallothionein production are well-known metabolic processes which often detoxify compounds, converting them to excretable metabolites (Rand and Petrocelli 1985). These metabolic systems vary dramatically among aquatic species, so it is difficult to predict aquatic toxicity to multiple species without actual testing each species. All of the above uncertainties associated with toxicant differences and interactions, exposure pathways, and organism responses support the use of multiple species in stormwater assessments.

There are mixtures of chemicals in stormwaters. Since chemical water quality criteria and standards only consider effects from one chemical, the question arises as to what effects may result

to organisms when they are exposed to a mixture of potentially toxic chemicals. Mixture effects have been studied for decades. Sprague and Ramsay (1965) proposed a toxic unit (TU) that defined the strength of a toxicant. One toxic unit is equal to the incipient LC50 (the level of a toxicant that is lethal to 50% of the individuals exposed for a period of time where acute lethal effects have ceased). The strength of a toxicant, or the TU, is calculated as actual toxicant concentration in solution divided by the LC50. If the calculated sum of toxic units in a mixture of chemicals is one or larger, the mixture is said to be lethal.

The EPA (1991e) assumes that chemical toxicants act in an additive fashion, as opposed to being antagonistic (less toxicity than predicted) or synergistic (greater toxicity than predicted). A great deal of experimentation has been completed in this area, and some general principles have emerged. Overall, it appears that joint toxicity often occurs among chemicals with a similar mode of action. Within similar modes of action, the concentration-addition model (often called the TU concept) often describes the interaction

$$\text{TU mixture} = \sum_{i=1}^{n} TU_i$$

Additivity or near additivity has been demonstrated for many groups of chemicals, such as narcotics, organophosphate pesticides, pyrethroid pesticides, polynuclear aromatic hydrocarbons, major ions, and metals (Sprague 1968; Sprague and Ramsay 1965; Broderius and Kahl 1985; Carder and Hoagland 1998; Deneer et al. 1988a; Hermens and Leewangh 1982; Hermens et al. 1984a,b,c; Konemann 1981; Muska and Weber 1977).

In contrast to mixtures of chemicals with similar modes of action, chemicals with dissimilar modes of action (e.g., zinc and diazinon) show antagonistic, little, or no interaction, such that the toxicity of a binary mixture shows toxicity equal to or less than that of the most toxic component (Howell 1985; Herbes and Beauchamp 1977; Schultz and Allison 1979; Deneer et al. 1988b; Spehar and Fiandt 1986; Alabaster and Loyd 1982).

Extreme interactions of chemical mixtures, such as synergy (TU mixture >> $\sum TU_i$) have also been frequently reported (Sprague and Ramsay 1965; Spehar and Fiandt 1986; Sharma et al. 1999; Christensen 1984; Vasseur et al. 1988; Marking 1977; Christen 1999; Marking and Dawson 1975; Anderson and Weber 1977; Doudoroff 1952; Wink 1990; Pape-Linstrom and Lydy 1997; Forget et al. 1999). One mechanism for synergism is where one chemical has a potentiating effect on the physiological pathway that is the target of a second toxicant. The classic example is piperonyl butoxide and pyrethroid pesticides; piperonyl butoxide blocks the detoxification pathway for pyrethroids, thereby greatly exacerbating their toxicity. In fact, this interaction is used intentionally in pyrethroid pesticide formulations.

While laboratory experiments have demonstrated approaches for mixture assessment, the test of the approach lies in its effectiveness when applied to mixtures occurring in the field, and experience suggests that the approach of assuming addition *within* modes of action and independence *between* different modes of action is adequate in many cases. For example, in studies of over 80 municipal and industrial effluents, toxicity identification studies showed no instances where observed toxicity was greater than would be predicted by this approach (D.R. Mount and J.R. Hockett, unpublished data).

The finding that mixture models are necessary to account for the potency of PAHs and dioxin-like compounds in the field provides excellent insights into the circumstances necessary for the expression of interactive toxicity in the environment. In addition to sharing a common mode of action (narcosis for PAHs; Ah-receptor agonism for dioxins/furans/PCBs), the sources for these contaminants and their environmental fate are such that they occur in mixture compositions where multiple components contribute meaningfully to the toxicity. The absence of the latter attribute greatly simplifies the assessment of many mixtures. In cases where one component of the mixture

dominates, ignoring toxic interactions within the mixture adds little uncertainty to the overall assessment. Metals provide an excellent example. In practice, many metal mixtures are dominated by a particular metal. Hence, assessing the potency of the mixture on the basis of its most potent component is often effective. In the case of PAHs, however, multiple individual PAHs contribute substantially to toxicity, and the additive toxicity must be taken into account to adequately assess the mixture.

Unfortunately, the many studies cited above suggest that toxicity resulting from stressor mixtures cannot be accurately predicted simply based on additivity or chemical type. A number of studies have shown that interactions of chemical mixtures can change from antagonistic to synergistic based on the life stage of the organisms, concentrations or levels of the contaminants, or length of exposures (Sprague and Ramsay 1965; Eaton 1973; Spehar and Fiandt 1986; Marking and Dawson 1975; Munawar et al. 1987; Sharma et al. 1999; Cairns et al. 1978). This suggests that site-specific *in situ* assessments of toxicity and biological communities, as discussed later in this section, are necessary for establishing the effects of stormwater runoff.

Standard Testing Protocols: Waters

As with any of the preceding assessment methods and approaches, it is usually important that standard methods and proper QA/QC practices be followed. This helps ensure the production of valid data that are comparable to other similar study results, are reproducible, and may be usable in enforcement actions. For many of the toxicity test applications, standard methods exist, either as EPA, state, APHA, or ASTM methods. However, the absence of a standard method, such as for *in situ* or multispecies assays, does not preclude their use. These "nonstandard" assays should be based on methods published in peer-reviewed scientific periodicals that have been demonstrated as valid and useful. Since this science is relatively young, the standardization process is also young and ongoing. Standard test species have been shown to represent the sensitive range of ecosystems analyzed (EPA 1991e). In addition, resident species testing is more difficult and subject to variability than standardized testing, and many important quality assurance–quality control requirements (e.g., same life stage, sensitive life stage, reference toxicant testing, interlaboratory variation, acclimation) cannot be met (EPA 1991e).

The preferred assessment design is to have toxicity tests as a screening and definitive tool, using acute and short-term chronic toxicity measures from multiple levels of biological organization. This approach has been the foundation of chemical-specific water quality criteria development and modification. Most toxicity test requirements in NPDES permits require the use of the fathead minnow (*Pimephales promelas*) and cladoceran *Ceriodaphnia dubia* (Figures 6.141 through 6.143). However, the EPA recommends that three species be tested in whole-effluent toxicity (WET) calculations including a fish, an invertebrate, and an algae (EPA 1991e). EPA guidance on hazardous waste site evaluations suggested the fish, *Daphnia*, and green algal (*Selenastrum capricornutum*) assays (Figure 6.144), along with terrestrial testing of seed germination and root elongation,

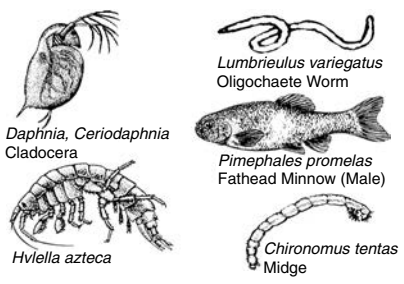


Figure 6.141 Common freshwater toxicity test organisms in the United States.

Figure 6.142 *Ceriodaphnia dubia*, the water flea.

Figure 6.143 Test setup for the *C. dubia* short-term chronic toxicity test.

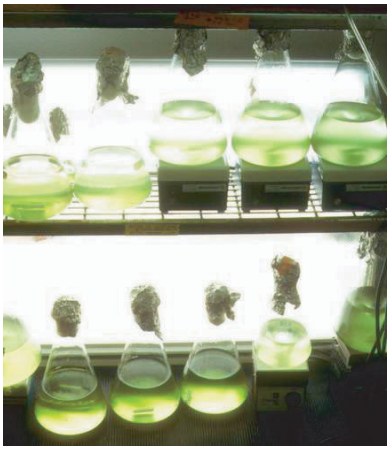


Figure 6.144 Culturing *Selenastrum capricornutum.*

earthworm survival, and soil respiration (Table 6.55; EPA 1989a,b; Porcella 1983). The ASTM and EPA now have standardized methods for sediment toxicity and bioaccumulation evaluations using benthic macroinvertebrates (EPA 2000c; ASTM 2000). They recommend a multispecies approach that is essential, as no one organism can serve as a surrogate for all species. An analysis of species sensitivity ranges observed in the National Water Quality Criteria documents found that when four or more species were tested, the LC50 of all was within one order of magnitude for 71 of the 73 pollutants tested (EPA 1991e). No one species was consistently the most sensitive (EPA 1991e).

A wide variety of useful and sensitive assays exists for toxicity evaluations of waters (Table 6.56) and sediments (Table 6.57). The optimal assay(s) is dependent on several issues, which will vary with the geographic area, study objectives, and pollutant problem (Table 6.58). For typical stormwater assessments, a tiered assessment approach is warranted, where the initial runoff is tested using a toxicity screening technique using the water flea (*D. magna, D. pulex,* or *C. dubia*) in 24- to 48-hour exposures. Additionally, if depositional (clay-silt) sediments exist downstream of stormwater outfalls, they should be evaluated for toxicity using EPA 10-day whole-sediment methods. If no toxicity is detected, however, the community indices of the benthic macroinvertebrate or fish communities indicate impairment, additional toxicity testing should be conducted, such as short-term chronic toxicity (EPA 7-day assays) and/or *in situ* toxicity exposures (described below and Appendix D). If toxicity problems are identified in the stormwater samples from the screening tests, definitive testing is conducted that may consist of acute to chronic laboratory, on-site, and/or *in situ* exposures; testing whole sediment, ambient water, or effluent; testing additional species such as bacteria (e.g., Microtox), photosynthetic organisms (e.g., duckweed, green algae), and fish (e.g., fathead minnow); and/or TIE evaluations to identify specific toxicants.

Defining stormwater toxicity at both a spatial and temporal scale may require large numbers of samples, which would surpass the resource capabilities of most projects if attempting to run conventional EPA-approved surrogate species (e.g., *P. promelas* and *C. dubia*). Stressor variability, as discussed previously, will be substantial through the course of a storm event and the return to baseflow conditions. The EPA recommends that for sampling of effluents and for annual monitoring of effluents using grab sampling, a minimum of four to six samples be collected in 1 day, once per month, to better define short-term variation. Sewage treatment plant effluents typically have shown coefficients of variation (COV) for acute toxicity of 20 to 42% and 0 to 88% for chronic toxicity. Among oil refinery effluents, the COVs ranged from 19 to 54% for acute and 30 to 60% for chronic data. Other manufacturing facility effluents had acute toxicity COVs of 20 to 100% (EPA 1991e). It may be useful to split definitive samples and run Microtox in tandem with the macrofaunal assays. If a consistent relationship is observed, i.e., few false positive or false negatives using

Table 6.55 Toxicity Evaluation Categories for Hazardous Waste Sites

Assay	Activity Measured	Sample Type[a]	MAD[b]	Units	Response Levels for LC50 or EC50 Concentrations[c]		
					High	Moderate	Low or Not Detectable
Freshwater fish	96-hr LC50 (lethality)	S	1	g/L	<0.01	0.01–0.1	0.1–1
		L	100	%	<20	20–75	75–100
Freshwater invertebrate	46-hr EC50 (immobilization)	S	1	g/L	<0.01	0.01–0.1	0.1–1
		L	100	%	<20	20.75	75–100
Freshwater algae	96-hr EC50 (growth inhibition)	S	1	g/L	<0.01	0.1–01	0.1–1
		L	100	%	<20	20–72	75–100
Seed germination and root elongation	115-hr EC50 (inhibited root elongation)	L	100	%	<20	20–75	75–100
Earthworm test	336-hr LC50	S	500	g/kg	<50	50–500	500
Soil respiration test	336-hr EC50	S	500	g/kg	<50	50–500	500
		L	100	%	<50	20–75	75–100

[a] S = solid, L = aqueous liquid, includes water samples and elutriate or leachate. Nonaqueous liquids are evaluated on an individual basis because of variations in samples, such as vehicle, percent organic vehicle, and percent solids.
[b] MAD = Maximum applicable dose.
[C] LC50 = Calculated concentration expected to kill 50% of population within the specified time interval. EC50 = Calculated concentration expected to produce effect in 50% of population within the specified time interval.

From Porcella, D.B. *Protocol for Bioassessment of Hazardous Waste Sites,* prepared for Environmental Research Laboratory, U.S. Environmental Protection Agency, Corvallis, OR, EPA 600/2-83/054, NTIS Publ. No. PB83-241737. 1983.

Table 6.56 Useful Species and Life Stages for Aqueous Phase Testing

	Species	Life Stage
	Fish	
Cold Water		
Brook trout	*Salvelinus fontinalis*	30 to 90 days
Coho salmon	*Oncorhynchus kisutch*	30 to 90 days
Rainbow trout	*Salmo gairdneri*	30 to 90 days
Warm Water		
Bluegill	*Lepomis macrochirus*	1 to 90 days
Channel catfish	*Ictalurus punctatus*	1 to 90 days
Fathead minnow	*Pimephales promelas*	Embryo to 90 days
	Benthic Invertebrates	
Cold Water		
Stoneflies	*Pteronarcys* spp.	Larvae
Crayfish	*Pacifastacus leniusculus*	Juveniles
Mayflies	*Baetis* spp. or *Ephemerella* spp.	Nymphs
Warm Water		
Amphipods	*Hyalella azteca*	Juveniles (<.250 mm)
	Gammarus lacustris, G. fasciatus, or *G. pseudolimnaeus*	Juveniles Juveniles
Cladocera	*Daphnia magna* or *D. pulex,* *Ceriodaphnia* spp.	1 to 24 hours 1 to 24 hours
Crayfish	*Orconectes* spp., *Cambarus* spp., *Procambarus* spp.	Juveniles Juveniles
Mayflies	*Hexagenia limbata* or *H. bilineata*	Nymphs
Midges	*Chironomus tentans* or *C. riparius*	Larvae (1st or 2nd instar)
	Algae	
Green algae	*Selenastrum capricornutum*	Log-phase growth
	Bacteria	
Microtox	*Photobacterium phosphoreum*	Log-phase growth (freeze-dried culture)

Modified from EPA. *A Compendium of Superfund Field Operations Methods.* Office of Emergency and Remedial Response, U.S. Environmental Protection Agency, Washington, D.C., EPA 540/P-87/001. 1987.

Microtox, then the assumption may be made that Microtox responses are related (noting statistical confidence) to the other surrogate responses. This will allow for the analysis of many more samples, because Microtox requires a few hours rather than days to complete, and many samples can conveniently be evaluated at one time.

When conducting ecotoxicity evaluations, it is important that one understand what effects sample collection, processing manipulation, and exposure design have on the observed toxicity response. Is this response similar to what is occurring in the field or is it simply an artifact of the method? A thorough discussion of this critical issue is beyond the scope of this book. See ASTM (1991) and Burton (1991) for additional information. For sediment testing, these effects are particularly significant, as sample integrity is easily disrupted, altering bioavailability and partitioning of toxicants. Sediment test phases include whole sediments, interstitial water, elutriate, or other extractable phases. Each has associated strengths and weaknesses (Table 6.57) (Burton 1991).

Table 6.57 Sediment Phases Used in Toxicity Tests

Phase	Strengths	Weaknesses	Routine Uses
Extractable phase (XP) (solutes vary)	• Use with all sediment types • Sequentially extract different degrees of bioavailable fractions • Greater variety of available assay endpoints • Determine dose response	• Ecosystem realism: Bioavailability unknown, chemical alteration	• Rapid screen • Unique endpoints component of test battery
Elutriate phase (EP) (water extractable)	• Use with all sediment types • Readily available fraction • Mimics anoxic toxic environmental process • Large variety of available assay endpoints • Methods relatively standardized • Determine dose response	• Ecosystem realism: Only one oxidizing condition used; only one solid: water ratio; exposure for extended period of one-phase condition that never occurs *in situ* or never occurs in equilibrium *in situ* • Extract conditions vary with investigator • Filtration affects response, sometimes used	• Rapid screen • Endpoints not possible with WS • Dredging evaluations
Interstitial water (IW)	• Direct route of uptake for some species • Semi-direct exposure phase for some species • Large variety of available assay endpoints • Methods of exposure relatively standardized • Determine dose response • Sediment quality criteria	• Cannot collect IW from some sediments • Limited volumes can be collected efficiently • Optimal collection method unknown, constituents altered by all methods • Exposure phase altered chemically and physically when isolated from WS • Flux between overlying water and sediment unknown • Relationship to and between some organisms uncertain: burrowers, epibenthic, water column species, filter feeders, selective filtering, life cycle vs. pore water exposure	• Rapid screen • Endpoints not possible with WS • Initial surveys • Sediment criteria
Whole sediment (WS)	• Use with all sediment • Relative realism high • Determine dose response • Holistic (whole) versus reductionist toxicity approach (water, IW, EP, and XP) • Use site or reconstituted water to isolate WS toxicity	• Some physical/chemical/microbiological alteration from field collection • Dose–response methods tentative • Testing more difficult with some species and some sediments • Few standard methods • Indigenous biota may be present in sample	• Rapid screen • Chronic studies • Initial surveys • Sediment criteria
In situ[a] (NS)	• Real measure integrating all key components, eliminating extraneous influences • Sediment quality criteria may be determined • Resuspension/suspended solids effects assessed.	• Fee methods and endpoints • Not as rapid as some assay systems • Mesocosms variable • Predation by indigenous biota	• Resuspension effects • Intensive system monitoring • Sediment criteria

[a] Organism exposed *in situ* in natural systems, pond/stream mesocosms, or lake limnocorrals.

Reprinted with permission from Burton, G.A., Jr. Assessing freshwater sediment toxicity. *Environ. Toxicol. Chem.,* 10: 1585–1267, 1991. © SETAC, Pensacola, FL, U.S.A.

Table 6.58 Optimal Toxicity Assay Considerations

1. Verification components
Ecosystem relevance
Species sensitivity patterns
Appropriate test phase
Short or long exposure period
Definitive response dynamics
2. Resource components
Organism availability
Laboratory availability
Expertise required
Expense and time required
3. Standardization components
Approved standard methods
Reference database
Interlaboratory validation
Quality assurance and quality control criteria

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Case Study: Example Use of Microtox to Identify Sources and Controllability of Stormwater Toxicants

A series of projects were sponsored by the EPA to investigate sources and treatability of toxicants in stormwater (Pitt et al. 1995, 1999). The first project phase investigated typical toxicant concentrations in stormwater, the origins of these toxicants, and storm and land-use factors that influenced these toxicant concentrations. The second project phase investigated the control of stormwater toxicants using a variety of conventional bench-scale treatment processes. The Microtox 100% sample toxicity screening test by Azur Environmental (was Microbics, Inc.) was selected for this research because of its unique capabilities: it is a rapid procedure (requiring about 1 hour) and only requires small (<40 mL) sample quantities. The Microtox toxicity test uses marine bioluminescence bacteria and monitors the light output for different sample concentrations. About 1 million bacteria organisms are used per sample, resulting in highly repeatable results. The more toxic samples produce greater stress on the bacteria test organisms, which results in a greater light attenuation compared to the control sample. It must be stressed that the Microtox toxicity screening test was not used to indicate the absolute toxicities of the samples nor to predict the toxic effects of the stormwater runoff on receiving waters during this research. It was used as a control parameter to indicate relative toxicities of different source flows and to measure relative benefits of different control options. The precision and bias of the Microtox test were easy to measure and control during these tests, which also strongly favored its use for our purposes. The following paragraphs describe the results of these tests and indicate the types of information that can be obtained using a toxicity screening procedure, such as the Microtox test.

Phase 1 — Sources of Stormwater Toxicants

The first project phase included the collection and analysis of 87 urban stormwater runoff samples from a variety of source areas under different rain conditions. All of the samples were analyzed in filtered (0.45-µm filter) and nonfiltered forms to enable partitioning of the toxicants into particulate and filterable forms. The samples were all obtained from the Birmingham, AL, area. Samples were obtained from shallow flows originating from homogeneous sources. These

data were used to evaluate the effects of different land uses and source areas, plus the effects of rain characteristics, on sample toxicant concentrations and toxicity. Organic pollutants were analyzed using two gas chromatographs, one with a mass selective detector (GC/MSD) and another with an electron capture detector (GC/ECD). The pesticides were analyzed according to EPA method 505, while the base neutral compounds were analyzed according to EPA method 625 (but using only 100-mL samples). The pesticides were analyzed on a Perkin Elmer Sigma 300 GC/ECD using a J&W DB-1 capillary column (30 m by 0.32 mm ID with a 1-µm film thickness). The base neutrals were analyzed on a Hewlett Packard 5890 GC with a 5970 MSD using a Supelco DB-5 capillary column (30 m by 0.25 mm ID with a 0.2-µm film thickness).

Metallic toxicants were analyzed using a graphite furnace-equipped atomic absorption spectrophotometer (GFAA). EPA methods 202.2 (Al), 213.2 (Cd), 218.2 (Cr), 220.2 (Cu), 239.2 (Pb), 249.2 (Ni), and 289.2 (Zn) were followed in these analyses. A Perkin Elmer 3030B atomic absorption spectrophotometer was used after nitric acid digestion of the samples. Previous research (Pitt and McLean 1986; EPA 1983a) indicated that low detection limits were necessary in order to measure the filtered sample concentrations of the metals, which would not be achieved by use of a standard flame atomic absorption spectrophotometer. Low detection limits would enable partitioning of the metals between the solid and liquid phases to be investigated, an important factor in assessing the fates of the metals in receiving waters and in treatment processes.

Comparison of Microtox with Other Toxicity Tests — The Microtox procedure was compared with about 20 different laboratory bioassay tests using 20 stormwater and CSO samples. Conventional bioassay tests were conducted using freshwater organisms at the EPA's Duluth, MN, laboratory and using marine organisms at the EPA's Narragansett Bay, RI, laboratory. In addition, other toxicity tests were also conducted at the Environmental Health Sciences Laboratory at Wright State University, Dayton, OH. The comparison tests were all short-term tests. However, some of the tests were indicative of chronic toxicity (life cycle tests and the marine organism sexual reproduction tests, for example), whereas the others are classically considered as indicative of acute toxicity (Microtox and the fathead minnow tests, for example). The following list shows the major tests that were conducted by each participating laboratory:

- University of Alabama at Birmingham, Environmental Engineering Laboratory
  - Microtox bacterial luminescence tests (10-, 20-, and 35-min exposures) using the marine *Photobacterium phosphoreum*
- Wright State University, Biological Sciences Department
  - Macrofaunal toxicity tests:
    - *Daphnia magna* (water flea) survival
    - *Lemma minor* (duckweed) growth
    - *Selenastrum capricornutum* (green alga) growth
  - Microbial activity tests (bacterial respiration):
    - Indigenous microbial electron transport activity
    - Indigenous microbial inhibition of β-galactosidase activity
    - Alkaline phosphatase for indigenous microbial activity
    - Inhibition of β-galactosidase for indigenous microbial activity
    - Bacterial surrogate assay using *O*-nitrophenol-β-D-galactopyranside activity and *Escherichia coli*
- EPA Environmental Research Laboratory, Duluth, MN
  - *Ceriodaphnia dubia* (water flea) 48-hour survival
  - *Pimephales promelas* (fathead minnow) 96-hour survival
- EPA Environmental Research Laboratory, Narraganset Bay, RI
  - *Champia parvula* (marine red alga) sexual reproduction (formation of cystocarps after 5 to 7 days exposure)
  - *Arbacua punctulata* (sea urchin) fertilization by sperm cells

Therefore, the tests represented a range of organisms that included fish, invertebrates, plants, and microorganisms.

Table 6.59 summarizes the results of the toxicity tests. The *C. dubia. P. promelas,* and *C. parvula* tests experienced problems with the control samples, and those results are therefore uncertain. The *A. pustulata* tests on the stormwater samples also had a potential problem with the control samples. The CSO test results (excluding the fathead minnow tests) indicated that from 50 to 100% of the samples were toxic, with most tests identifying the same few samples as the most toxic. The toxicity tests for the stormwater samples indicated that 0 to 40% of the samples were toxic. The Microtox screening procedure gave rankings similar to those of the other toxicity tests.

All of the Birmingham samples represented separate stormwater. However, as part of the Microtox evaluation, several CSO samples from New York City were also tested to compare the different toxicity tests.

Table 6.59 Fraction of Samples Rated as Toxic

Sample Series	Combined Sewer Overflows, %	Stormwater, %
Microtox marine bacteria	100	20
C. dubia	60	0[a]
P. promelas	0[a]	0[a]
C. parvula	100	0[a]
A. punctulata	100	0[a]
D. magna	63	40
L. minor	50[a]	0

[a] Results uncertain, see text.

Source Area Sampling Results — Thirteen organic compounds, out of more than 35 targeted compounds analyzed, were detected in over 10% of all samples. The greatest detection frequencies were for 1,3-dichlorobenzene and fluoranthene, which were each detected in 23% of the samples. The organics most frequently found in these source area samples (i.e., PAHs, especially fluoranthene and pyrene) were similar to the organics most frequently detected at outfalls in prior studies (EPA 1983a). Roof runoff, parking area, and vehicle service area samples had the greatest detection frequencies for the organic toxicants. Vehicle service areas and urban creeks had several of the observed maximum organic compound concentrations. Most of the organics were associated with the nonfiltered sample portions, indicating an association with the particulate sample fractions. The compound 1,3-dichlorobenzene was an exception, having a significant dissolved fraction.

In contrast to the organics, the heavy metals analyzed were detected in almost all samples, including the filtered sample portions. The nonfiltered samples generally had much higher concentrations, with the exception of zinc, which was associated mostly with the dissolved sample portion (i.e., not associated with the suspended solids). Roof runoff generally had the highest concentrations of zinc, probably from galvanized roof drainage components, as previously reported by Bannerman et al. (1983). Parking and storage areas had the highest nickel concentrations, while vehicle service areas and street runoff had the highest concentrations of cadmium and lead.

Replicate samples were collected from several source areas at three land uses during four different storm events to statistically examine toxicity and pollutant concentration differences due to storm and site conditions. These data indicated that variations in Microtox toxicities and organic toxicant concentrations may be better explained by rain characteristics than by differences in sampling locations. As an example, high concentrations of many of the PAHs were more likely associated with long antecedent dry periods and large rains, than by any other storm or sampling location parameter.

Phase 2 — Laboratory-Scale Toxicant Reduction Tests

The Phase 2 tests examined toxicant treatability for a variety of conventional bench-scale treatment processes. The data from Phase 1 identified the critical source areas (storage/parking and vehicle service areas, which generally had the highest toxicant concentrations) for study during the second research phase.

The objective of the second research phase was to obtain relative measurements of sample toxicity improvements for different stages of each bench-scale treatment method to indicate the relative effectiveness of different treatment efforts and processes. To meet this objective and considering resource restraints on cost and time, the Microtox screening toxicity test was chosen to indicate relative changes in toxicity.

The selected source area runoff samples all had elevated toxicant concentrations compared to other urban source areas, allowing a wide range of laboratory partitioning and treatability analyses to be conducted. The treatability tests conducted were:

1. Settling column (37 mm × 0.8 m Teflon column)
2. Flotation (series of eight glass, narrow-neck, 100-mL volumetric flasks)
3. Screening and filtering (series of 11 stainless steel sieves, from 20 to 106 μm, and a 0.45-μm membrane filter).
4. Photodegradation (2-L glass beaker with a 60-watt, broad-band, incandescent light placed 25 cm above the water, stirred with a magnetic stirrer with water temperature and evaporation rate also monitored)
5. Aeration (the same beaker arrangement as above, without the light, but with filtered compressed air keeping the test solution supersaturated and well mixed)
6. Photodegradation and aeration combined (the same beaker arrangement as above, with compressed air, light, and stirrer)
7. Undisturbed control sample (a sealed and covered glass jar at room temperature)

Each test (except for filtration, which was an "instantaneous" test) was conducted over a duration of 3 days. Plots of the toxicity reductions observed during each treatment procedure examined, including the control measurements, were prepared. The plots were grouped according to source area sampling location and the treatment type. Figures 6.145 through 6.147 are plots of toxicity reductions associated with filtering selected samples through different sized screens. Significant and important toxicity reductions are associated with screening using the smaller apertures.

The highest toxicant reductions were obtained by settling for at least 24 hours (providing at least 50% reductions for all but two samples), screening through at least a 40-μm screen (20 to 70% reductions), and aeration and/or photodegradation for at least 24 hours (up to 80% reductions). Increased settling, aeration or photodegradation times, and screening through finer meshes, all reduced sample toxicities further. The flotation tests produced floating sample layers that generally increased in toxicity with time and lower sample layers that generally decreased in toxicity with time, as expected; however, the benefits were quite small (less than 30% reduction).

These tests indicate the wide-ranging behavior of these related samples for the different treatment tests. Some samples responded poorly to some tests, while other samples responded well to all of the treatment tests. Any practical application of these treatment unit processes would therefore require a treatment train approach, subjecting critical source area runoff to a combination of processes in order to obtain relatively consistent overall toxicant removal benefits.

Phase 3 – Pilot-Scale Demonstration of the Multichambered Treatment Train (MCTT)

The last research phase included a pilot-scale test of the most promising treatment processes suitable for small critical source areas. This device consists of a series of chambers, including an initial grit and aeration chamber, an intermediate tube settler with oil sorbents, and a final mixed

sand/peat filter. Extensive testing of PAHs, phthalate esters, phenols, pesticides, metals, toxicity screening, chemical oxygen demand, pH, conductivity, turbidity, hardness, sodium adsorption ratio, major ions, particle sizes, solids, and nutrients was performed on filtered and unfiltered samples during 12 rains at the inlets and outlets of each component of the treatment train. The results from this pilot-scale test were confirmed by full-scale installations in Wisconsin constructed and monitored by the WI DNR. The MCTT units have been shown to be extremely effective, with >90%

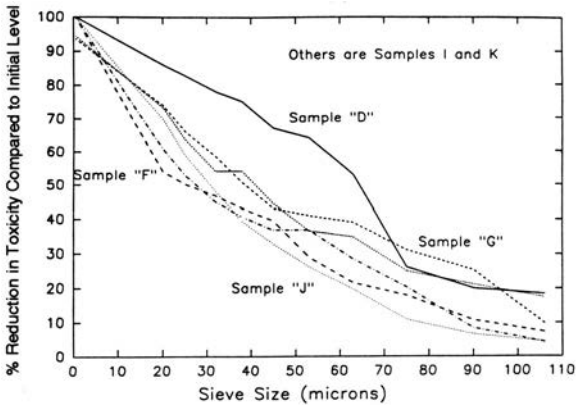

Figure 6.145 Toxicity reductions during sieving of industrial loading and parking area runoff samples. (From Pitt, R., B. Robertson, P. Barron, A. Ayyoubi, and S. Clark. *Stormwater Treatment at Critical Areas: The Multi-Chambered Treatment Train (MCTT).* U.S. Environmental Protection Agency, Wet Weather Flow Management Program, National Risk Management Research Laboratory. EPA/600/R-99/017. Cincinnati, OH. 505 pp. March 1999b.)

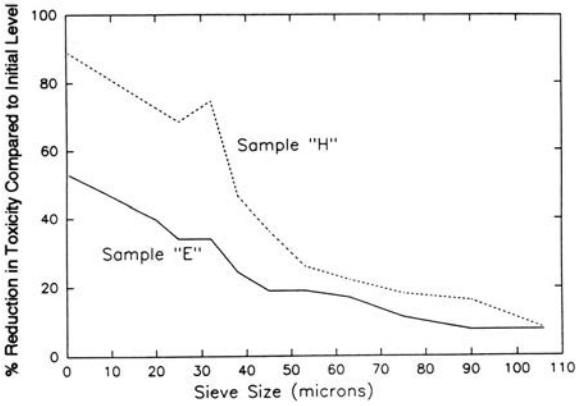

Figure 6.146 Toxicity reductions during sieving of automobile service facility runoff samples. (From Pitt, R., B. Robertson, P. Barron, A. Ayyoubi, and S. Clark. *Stormwater Treatment at Critical Areas: The Multi-Chambered Treatment Train (MCTT).* U.S. Environmental Protection Agency, Wet Weather Flow Management Program, National Risk Management Research Laboratory. EPA/600/R-99/017. Cincinnati, OH. 505 pp. March 1999b.)

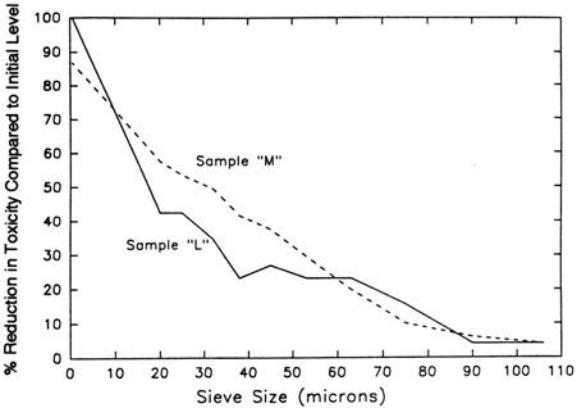

Figure 6.147 Toxicity reductions during sieving of automobile salvage yard runoff samples. (From Pitt, R., B. Robertson, P. Barron, A. Ayyoubi, and S. Clark. *Stormwater Treatment at Critical Areas: The Multi-Chambered Treatment Train (MCTT).* U.S. Environmental Protection Agency, Wet Weather Flow Management Program, National Risk Management Research Laboratory. EPA/600/R-99/017. Cincinnati, OH. 505 pp. March 1999b.)

removal of heavy metals and most organic toxicants. Caltrans (California Department of Transportation) is currently constructing and monitoring three MCTT units for treatment of runoff from a maintenance area and from parking lots in Los Angeles.

This research showed the usefulness of a toxicity screening test in evaluating sources of stormwater toxicants and in developing and testing control technologies. It would have been prohibitively expensive to base this research solely on chemical analyses of specific metallic and organic toxicants, although toxicants were specifically monitored as part of the demonstration projects to show applicability of results.

Standard Testing Protocols: Sediments

The release of the EPA Contaminated Sediment Management Strategy and Sediment Quality Inventory compiled the limited sediment data (only 4% of monitored sites had toxicity data) and documented that adverse effects are probable from sediments at 26% (>5000) of sites surveyed (EPA 1998). A recent random survey of sediments in North Carolina's estuaries found from 19 to 36% had contaminant levels known to cause toxicity and 13% had few to no living organisms (Pelly 1999). These areas are dominated by agricultural watershed inputs. The paucity of sediment toxicity information and the focus of past sediment surveys on industrialized waterways raises the question of whether the extent of sediment contamination is actually much greater than envisioned. Since chemicals, nutrients, and pathogens readily sorb to sediments, sediment contamination is likely in depositional areas of urban and agricultural watersheds (Burton 1992a,b; Burton et al. 1987). Contaminated sediments have been shown to severely impact aquatic ecosystems (e.g., Burton 1992a,b; EPA 1998) and are the source of fish contamination and advisories in many parts of the nation (EPA 1998). For this reason, it is essential that their contribution to use impairment be determined.

By the mid-1990s, standardized methods for whole-sediment toxicity testing occurred within the EPA, American Society for Testing and Materials (ASTM), and Environment Canada. These tests measured acute (short-term ≤ 10 days) toxicity in benthic macroinvertebrates such as the amphipods *Hyalella azteca, Rhepoxynius abronius, Ampelisca abdita, Eohaustorius estuarius,* and *Leptocheirus plumulosus,* and the midges *Chironomus tentans* and *Chironomus riparius* (EPA 2000c). The primary response measured was mortality, but in the case of the midge, growth was included and reburial was an additional endpoint for the *Rhepoynmius abronius*. These whole-sediment tests have been useful at testing sediment contamination (Figure 6.148) and provide information on chemical bioavailability. A large number of other species have been used for determining the toxicity of sediments, ranging from bacteria to fish and amphibians (Burton 1991). Comparisons of their sensitivities have shown a wide range of responses to different types of sediment contamination, with an equally wide range of discriminatory power (ability to detect differences between samples) (Burton et al. 1996a). This reality suggests that more than one or two species may be necessary to determine with certainty whether or not sediment contamination is ecologically significant (EPA 1994c).

Unfortunately, most of the test methods are focused on acute and not chronic toxicity. The measures of acute toxicity are often not adequate to detect the impacts on benthic communities. For instance, the 10-day test with *Rhepoxynius abronius* was not sensitive enough to describe the loss of amphipods from the Lauritizen Channel in San Francisco Bay (Swartz et al. 1994). In reality, chronic toxicity is the more pervasive problem, and it is the chronic responses, such as changes in reproduction, that lead to population level responses. Late in 1999, the EPA released its first standardized methods for determining chronic toxicity, specifically focused on growth and reproduction in *Hyalella azteca* and *Chironomus tentans* (as described in Benoit et al. 1997; Ingersoll et al. 1998). While these methods greatly aid our ability to determine if sediments are chronically toxic, their long duration and increased costs may impede their widespread adoption by state agencies.

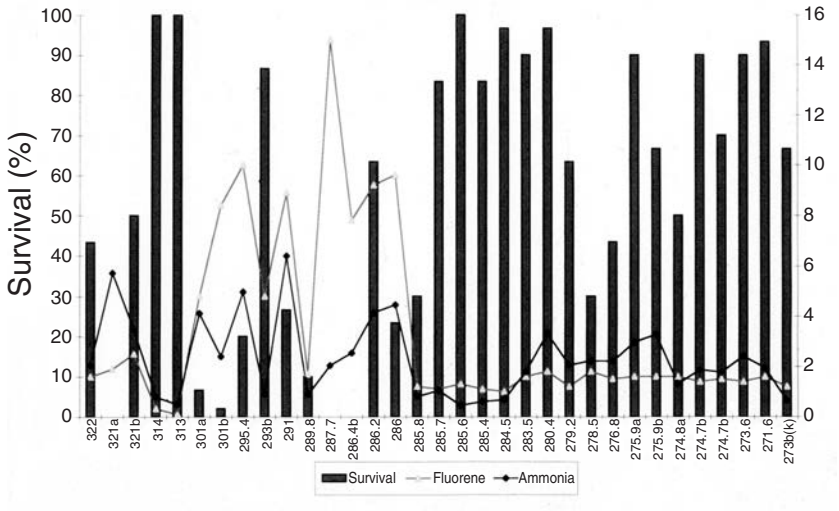

Figure 6.148 *Chironomus tentans* response in sediments from the DuPage River below Chicago in 10-day EPA exposures. Significant relationships with ammonia and fluorene sediment concentrations.

Beyond the standard tests, there have been a large number of tests with a wide range of marine benthos that may lead to better, or at least more effective, measures of chronic toxic response. For example, tests with marine amphipods have already been described in the literature to optimize the conditions for a 28-day test to examine growth and reproduction with *Leptochierius plumolosus* (Gray et al. 1998). Additional tests make use of organisms with shorter life spans, such as marine copepods, and can sort out differential response to different life stages (Green et al. 1996). These copepods are also useful in more community structure-based assessments, such as in the use of microcosms (Chandler et al. 1997). These meiobenthos may well be useful for developing standardized chronic tests since life cycle tests can be completed in 15 to 25 days and the organisms have been found to be sensitive to sediment-associated toxicants under laboratory and field conditions (Coull and Chandler 1992). Tests with organisms having shorter life spans and methods that include mixed assemblages in microcosms linked with single species tests provide insight into the functioning of communities. These new methods will help bridge the gap between our field observations and the cause–effect links that can be established in the laboratory.

There are several reasons the "water column" species used in WET tests are useful for assessments of sediments. Aquatic organisms rarely exclusively inhabit one media during their life cycle. Many "pelagic" organisms may graze on surficial sediments and even encounter pore waters. For example, the often-used "water column" surrogate, the fathead minnow (*Pimephales promelas*) is an omnivore, ingesting a mixture of detritus and invertebrates (Lemke and Bowan 1998) and frequently feeding on sediment surfaces. The zooplankton, *Daphnia magna,* grazes on surficial sediments in whole-sediment toxicity assays. The responses of WET tests have been highly predictive of indigenous benthic community responses at many sites (Dickson et al. 1996; Eagleson et al. 1990). Many vertebrate and invertebrate species have some link to sediments and have been shown to be adversely affected by sediment contamination through toxicity and effects of bioaccumulation (e.g., Baumann and Harshbarger 1995; Benson and Di Giulio 1992; Burgess and Scott 1992; Burton 1989, 1991, 1992a,b, 1999; Burton and Scott 1992; Burton and Stemmer 1988; Burton et al. 1989, 1996a,b,c; Chapman et al. 1992; Lamberson et al. 1992; Lester and McIntosh 1994; Ludwig et al. 1993; Mac and Schmitt 1992; Maruya and Lee 1998).

For most stormwater effect evaluations, sediment toxicity determinations should focus on sampling surficial sediments (approximately to 2 cm) during low flow conditions and use whole-sediment exposures. During high flow conditions, suspended-sediment assays can be conducted in

the laboratory or *in situ*. The EPA and ASTM has developed standard guides for whole-sediment toxicity and bioaccumulation testing using invertebrates (ASTM 2000; EPA 2000c). Specific species guidance exists for *H. azteca*, *C. tentans*, and *C. riparius* (Figures 6.149 through 6.151). ASTM methods are also available for *Daphnia* and *Ceriodaphnia* spp. and resuspension testing. For additional test method references, see Burton (1991). Appendix D includes summaries of toxicity test methods for aqueous samples, using fish, cladocerans, algae, benthic invertebrates, and Microtox, which may be modified for sediment testing (Burton 1991). Testing suspended-sediment toxicity in the laboratory presents a logistical challenge. It is difficult to maintain a constant suspended solids concentration yet keep flow velocity and mixing turbulence reduced so as not to overly stress the test species, such as *Daphnia* sp. or *P. promelas* larvae. Relatively simple recirculation systems have been described by Hall (1986), Schuytema et al. (1984), and Schrap and Opperhuizen (1990). A preferred method of testing suspended solids is either with on-site mobile laboratories (using a flow-through pump system) or with *in situ* exposure chambers (Sasson and Burton 1991; Ireland et al. 1996; Burton and Moore 1999).

Standardized test methods have been developed for chronic toxicity testing of freshwater sediments. The EPA and ASTM have nearly identical methods (EPA 2000c; ASTM 2000). These methods are for *H. azteca* and *C. tentans* and extend for 42 to 60 days.

Hyalella azteca are routinely used to assess the toxicity of chemicals in sediments (e.g., Burton et al. 1989, 1996c; Burton 1991). Test duration and endpoints recommended in previously developed standard methods for sediment testing with *H. azteca* include 10-day survival and 10- to 28-d survival and growth. Short-term exposures, which only measure effects on survival, can be used to identify high levels of contamination, but may not be able to identify moderately contaminated sediments.

This method can be used to evaluate potential effects of contaminated sediment on survival, growth, and reproduction of *H. azteca* in a 42-day test. The sediment exposure starts with 6- to 8-day-old amphipods. On Day 28, amphipods are isolated from the sediment and placed in water-only chambers where reproduction is measured on Day 35 and 42. Typically, amphipods are first in amplexus at about Day 21 to 28 with release of the first brood between Day 28 to 42. Endpoints measured include survival (Day 28, 35, and 42), growth (dry weight measured on Day 28 and 42), and reproduction (number of young/female produced from Day 28 to 42). The EPA and ASTM state that a subset of endpoints may be measured with minor method modifications.

Reproduction in amphipods is measured by exposing them in sediment until a few days before the release of the first brood. The amphipods are then sieved from the sediment and held in water to determine the number of young produced. This test design allows a quantitative measure of reproduction. One limitation to this design is that amphipods might recover from effects of sediment exposure during this holding period in clean water; however, amphipods are exposed to sediment during critical developmental stages before release of the first brood in clean water.

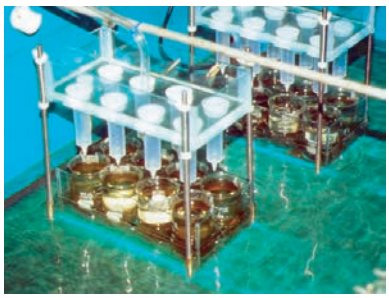

Figure 6.149 EPA whole sediment, overlying water renewal design.

Figure 6.150 The amphipod *Hyalella azteca*, also known as the scud.

The midge *Chironomus tentans* has been used extensively in the short-term assessment of chemicals in sediments (e.g., Burton 1991; Burton et al. 1996c), and standard methods have been developed for testing with this midge using 10-day exposures (EPA 2000c). *Chironomus tentans* is a good candidate for long-term toxicity testing because it normally completes its life cycle in a relatively short period of time (25 to 30 days at 23°C), and a variety of developmental (growth, survivorship) and reproductive (fecundity) endpoints can be monitored. In addition, emergent adults can be readily collected, so it is possible to transfer organisms from the sediment test system to clean, overlying water for direct quantification of reproductive success. In Europe and Canada, the chronic midge method ends after emergence.

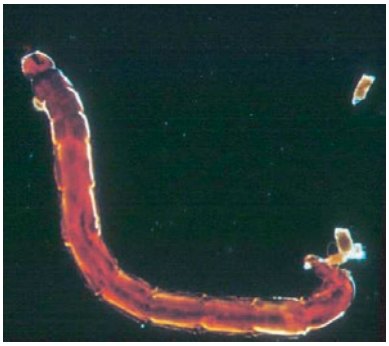

Figure 6.151 The midge *Chironomus tentans.*

The long-term sediment toxicity test with the midge, *Chironomus tentans*, is a life-cycle test in which the effects of sediment exposure on survival, growth, and emergence are measured. In addition, reproduction endpoints may be assessed. Survival is determined at 20 days and at the end of the test (about 50 to 65 days). Growth is determined at 20 days, which corresponds to the 10-day endpoint in the 10-day *C. tentans* growth test started with 10-day-old larvae. From Day 23 to the end of the test, emergence is monitored daily. Each treatment of the life-cycle test is ended separately when no additional emergence has been recorded for 7 consecutive days (the 7-day criterion). When no emergence is recorded from a treatment, ending of that treatment should be based on the control sediment using this 7-day criterion. EPA and ASTM state that minor modifications to the basic methods and a subset of endpoints may be used.

In Situ Toxicity Testing

An effective and accurate way to determine stormwater effects is through *in situ* toxicity testing. This may be done by placement of either artificial substrates (e.g., Hester–Dendy [OEPA 1989], rock- or mesh-filled baskets [EPA 1990b], foam [Henebry and Ross 1989], glass slides [APHA 1985]), side-stream chambers, or placing chambers-cages containing the test species into the stream or lake. The substrates or chambers must be secured to the stream bottom and be able to withstand high flow conditions. Some form of protective barrier might be necessary which might complicate flow-related effects on colonization.

In situ assessments of toxicity using confined organisms, while not new, have not been used traditionally in contaminant assessments (Burton et al. 1996b). A limited number of *in situ* exposures have been conducted to assess water or effluent toxicity. These assays have utilized adult fish, phytoplankton, amphipods, oligochaetes, and protozoans. Recent studies have shown the usefulness of *in situ* toxicity testing (Burton et al. 1996b; Chappie and Burton 1997; Crane et al. 1995; Monson et al. 1995; Sasson-Brickson and Burton 1991; Ireland et al. 1996; Bascombe et al. 1990; Ellis et al. 1995; Maltby et al. 1995; Sarda and Burton 1995; Schulz 1996; Nichols et al. 1999; Pereira et al. 1999; Maltby et al. 2000; Schulz and Liess 1999; Sibley et al. 1999). Determining the significance of sediment-associated contaminants requires an assessment of overlying water toxicity as organisms are exposed to both. This water-column exposure includes low and high flow conditions, in which water quality can vary markedly (Figure 6.152). Laboratory testing of wet-weather runoff samples has shown acute and chronic toxicity to a variety of species (e.g., Portele et al. 1982; Medeiros and Coler 1982; Medeiros et al. 1984; Ireland et al. 1996; Tucker and Burton 1999; Bailey et al. 2000). However, it is difficult to extrapolate results of these constant exposures with actual time-scale events

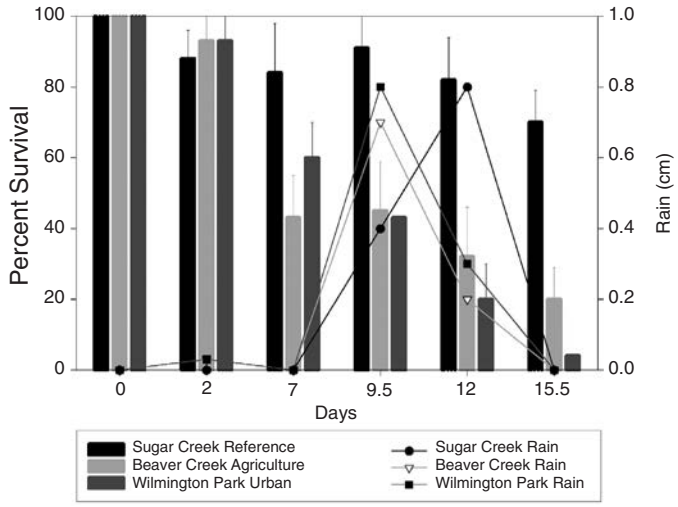

Figure 6.152 Decreased survival in urban and agricultural waters following a rain event.

in the field (Burton et al. 1996b; Tucker and Burton 1999; Burton and Moore 1999). Other *in situ* studies which have been used successfully in runoff studies include exposure of fish eggs (Pitt and Bissonnette 1984), artificial substrates for benthic invertebrate colonization and protozoa (e.g., Sayre et al. 1986), and use of transplants (Cherry 1996; Malley 1996).

There are several advantages to *in situ* testing. This approach removes sampling and laboratory-related errors from the assessment process, negating laboratory-to-field extrapolation uncertainties. Field conditions which may affect organism response and toxicity (and which are difficult to simulate in the laboratory) include sunlight, diurnal effects of temperature and oxygen, suspended solids, stressor(s) magnitude, frequency and duration, sediment integrity, spatial and temporal variation of physicochemical constituents, resident meio–microfaunal interactions, and other unknowns. Significant differences have been observed between laboratory and field testing. For example, acute toxicity to *C. dubia* in 48-hour exposures (Figure 6.153) was increased and overlying water reduced in the laboratory as compared to simultaneous *in situ* exposures (Figures 6.154 and 6.155) (Sasson-Brickson and Burton 1991). Ellis et al. (1992) observed acute and chronic toxicity to the amphipod, *Gammarus* sp., following storm event exposures in an urban stream. Death occurred up to 3 weeks following the storm and was related to elevated zinc concentrations in high-flow waters. Effects could also be correlated with *Gammarus* tissue levels of Zn. Kocan and Landolt (1990) exposed herring embryos both in the laboratory and *in situ* by placing 20 to 25 eggs on five glass slides, covering the slide holder with mesh and placing *in situ*. This system was not tested in fresh waters or in flowing waters.

Artifacts associated with sampling and manipulation (e.g., sieving and mixing of sediments) of the test samples are reduced in *in situ* assays. Such manipulations may disrupt sediment vertical contaminant gradients, thereby altering the contaminant exposure regime that organisms face in

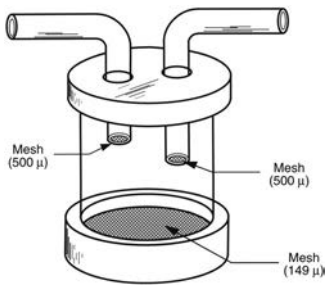

Figure 6.153 Sediment exposure chamber for invertebrates. (Reprinted with permission from Sasson-Brickson, G. and Burton, G.A., Jr. *In situ* and laboratory toxicity testing with *Ceriodaphnia dubia*. *Environ. Toxicol. Chem.,* 10: 201–207, 1991. © SETAC, Pensacola, FL, U.S.A.

the field (Sasson-Brickson and Burton 1991). *In situ* collection of interstitial water by deploying "peeper" devices has shown chemistry differences when compared to traditional collection methods using grab or core sampling (e.g., Adams 1991; Sarda and Burton 1995) and also when used for organism exposures (Fisher 1992; Figure 6.156).

In situ toxicity tests are more realistic than laboratory tests at integrating stressors (both measured and unmeasured), and can be used to study a variety of effects, such as photoinduced toxicity of PAHs (interactions with sunlight, solids, and contaminants), stormwater runoff (interactions of contaminants, suspended and dissolved solids, flow, and food), sediment-associated contaminants and physicochemical stressors, point source effluents, and contaminant gradients (Sasson-Brickson and Burton 1991; Ireland et al. 1996; Jones et al. 1995; Absil et al. 1996; Postma et al. 1994; Dickson et al. 1992; Roper and Hickey 1995; Hickey et al. 1995). Worms, bivalves, and fish have all been used *in situ* in bioaccumulation studies (e.g., Monson et al. 1995; Warren et al. 1995) with a need for linking critical body burdens to biological responses (Borgman 1996). Multiple stressors in the field usually occur in nonlinear, nonorthogonal combinations, challenging

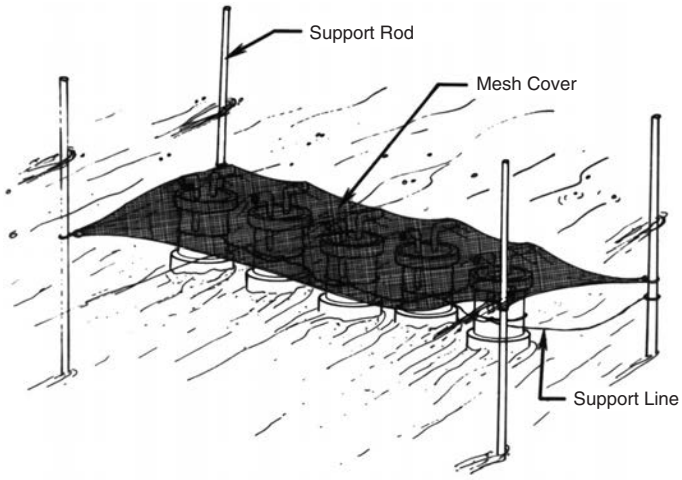

Figure 6.154 Sediment exposure chamber units secured in stream bed. (Reprinted with permission from Sasson-Brickson, G. and Burton, G.A., Jr. *In situ* and laboratory toxicity testing with *Ceriodaphnia dubia. Environ. Toxicol. Chem.,* 10: 201–207, 1991. © SETAC, Pensacola, FL, U.S.A.)

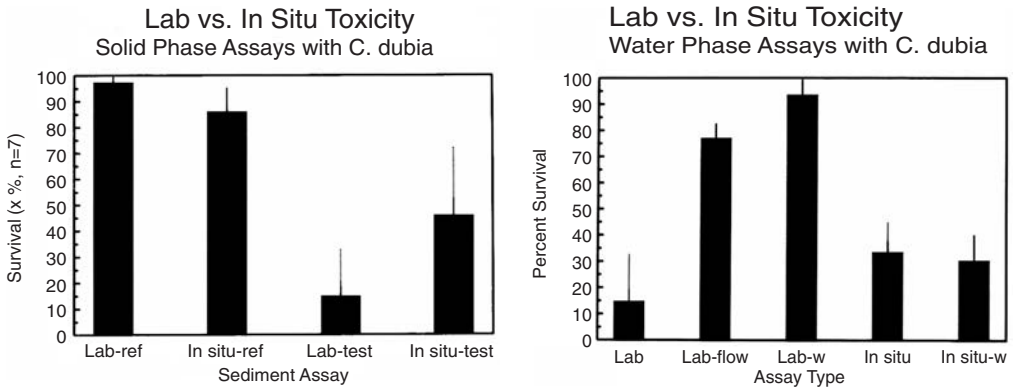

Figure 6.155 *Ceriodaphnia dubia* survival in laboratory (static and flow-through) whole sediment and site water (W) exposures; as compared to *in situ* exposures (whole sediment and overlying water, and overlying water (W) only).

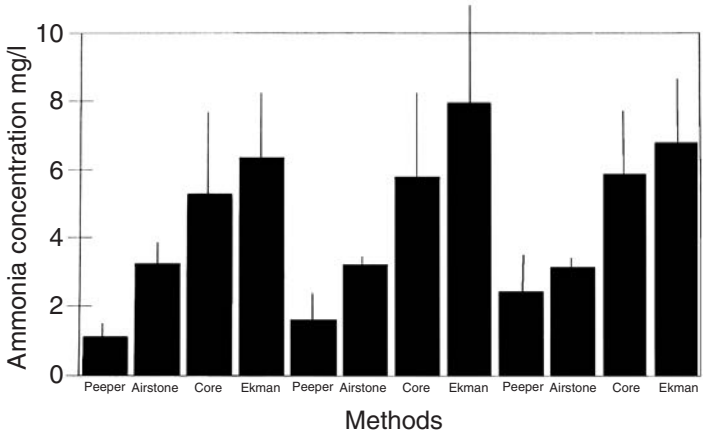

Figure 6.156 Differences in ammonia concentrations associated with various water collection methods.

biological systems in ways that are difficult at best to reproduce in the laboratory. So methods for teasing out the relative contributions of each stressor are often best conducted using a combination of *in situ* and laboratory-based experiments.

The integration of time-varying stressors (such as those related to wet-weather flow, pesticide runoff, or tidal inundation) is best conducted with field-deployed tests allowing continual exposure, as opposed to the grab sampling, static-type exposures of the laboratory. The first-flush of stormwater or pesticide runoff produces acute to sublethal responses to organisms exposed *in situ* (e.g., Herricks et al. 1994; Maltby et al. 1995; Crane et al. 1995; Waller et al. 1995b). Bivalve gape monitoring appears to be useful as an early warning indicator of effluent or stormwater toxicity (Waller et al. 1995a).

In situ methodologies can be extended to examine toxicological responses at the community level, for which they are much more cost effective than mesocosm studies (i.e., the laboratory analog). Typically, these experiments have been carried out by placing dosed sediments into the field (Berge 1990; Watzin et al. 1994) or by carrying out contaminant dosing *in situ* (Pridmore et al. 1991; Morrisey et al. 1996).

At the same time, the limitations of *in situ* toxicity tests should be recognized. Laboratory tests control variability of nontreatment factors much better than their *in situ* analogs. Deployment of caged organisms introduces the possibility of acclimation and transportation stress. If this is not monitored, data interpretation could be flawed. The *in situ* tests incorporate spatial and temporal variation, so the appropriate sampling design and analytical methods must be adapted to ensure there is adequate sensitivity and discriminatory power. The ease and practicality of *in situ* testing is site dependent. Deployment in intertidal or shallow water systems is easier than in deeper waters. Shallow subtidal deployment has the advantages of its inaccessibility to the public and reduced disturbance of sediment, especially in the case of very soft muds where trampling of intertidal sites can be a major problem. However, subtidal studies may be impacted by fishing trawls (e.g., Morrisey et al. 1996). In some areas, destruction of cages by vandals is problematic.

Primary considerations in the design and analysis of *in situ* testing approaches are the availability of food and potential starvation associated with exposures. The bioaccumulation and toxicity of contaminants is strongly influenced by food or feeding (Absil et al. 1996; Postma et al. 1994). Laboratory feeding often cannot duplicate either the quality or quantity of food present in the field. Stimulatory or inhibitory effects in these situations will likely be most marked for filter- or deposit-feeding organisms (Roper and Hickey 1995; Hickey et al. 1995).

Stressor exposures may be altered due to caging effects. Primary among these would be reduced flow, altered suspended solids or food, and interactions with predators, communities, or the food web. Depending on the organisms and the flow dynamics, cage design restricts flow to varying

degrees associated with flow-through screens (Nowell and Jumars 1984). It is essential in stormwater evaluations to reduce flow velocity to protect cages and organisms. This, however, increases the uncertainty concerning flow-related interactions in the receiving water (Vogel 1994). Predator–prey effects, suspended solids concentration, and settling within the cage may be increased or reduced depending on the mesh size. Artifacts associated with *in situ* experiments are further discussed by DeWitt et al. (1996).

Other important issues with *in situ* toxicity testing are the controls and reference sites. Selection of the appropriate controls and references is partially dictated by the questions being addressed in the study. In order to ascertain where stressors exist, site controls may be needed as well as reference sites. *A priori* impressions of what constitutes a "good" reference site may be incorrect. Multiple reference sites may be desirable to adequately interpret the impact data and accommodate unexpected loss of *in situ* devices. Artificial (formulated) sediments are also useful tools for investigating effects of food and bioavailability controls in conjunction with *in situ* deployments.

In situ testing provides unique information that may not be provided by laboratory testing or community surveys. The laboratory environment is superior for mechanistic and single-stressor effect delineation. However, complex exposure dynamics and stressor interactions are difficult or impossible to reproduce in the laboratory and may best be studied *in situ*. Significant advancements in understanding ecotoxicological processes and in conducting site assessments will come from the creative use of laboratory and *in situ* testing, and community survey approaches. When properly used in an integrated weight-of-evidence approach, *in situ* testing should help reduce the uncertainties associated with evaluating contaminant and natural stressor effects in complex ecosystems.

Bioaccumulation

Why Evaluate Bioaccumulation?

Aquatic organisms are exposed to chemicals through their contact with water and sediment and ingestion of food. Many inorganic and organic chemicals have been found to accumulate in organisms. These chemicals may accumulate to levels that cause chronic toxicity or even death. One of the most common sources of tissue contamination is sediment-associated contaminants. This contamination has been linked via food web transfer to impacts on upper trophic levels. Such transfer occurs with mercury and some organochlorines, such as PCBs and DDT, that are not well biotransformed and are hydrophobic; however, with other chemicals, these connections are more difficult to establish. Some organics such as PAHs are metabolized by many organisms, so detection in tissues may indicate recent exposures. Metals are difficult to evaluate in tissues since many are essential and can be regulated by organisms. Bioconcentration factors cannot be used with metals (with the exception of methyl mercury) because they can be high or low depending on the organism, their surrounding media, the metals, and their adaptation — most of which are not clearly defined in a study. From modeling exercises, food web transfer of persistent contaminants is important for maintaining the chemical concentrations observed in upper tropic levels, and the benthic component is essential in accounting for the observed concentrations (Thomann et al. 1992; Morrison et al. 1996; see Chapter 8). Further, trophic transfer of sediment-associated contaminants has been documented in both freshwater systems (e.g., Lester and McIntosh 1994) and marine systems (e.g., Maruya and Lee 1998). This food web transfer does not have to be limited to the aquatic environment and connections have been made to terrestrial species, particularly birds (Froese et al. 1998). In Saginaw Bay, Lake Huron, tree swallows were found to accumulate PCBs from sediments. In some areas of the Great Lakes and in the Hudson River, NY, system reproductive damage has been observed for this species directly linked to PCBs (Bishop et al. 1999; McCarty and Secord 1999).

Determining Bioaccumulation

A useful way to establish a link between beneficial use impairment and contamination is by showing that exposure to sediment or stormwater runoff contaminants results in tissue residue and adverse effects in organisms. Because many factors appear to alter the bioavailability of contaminants in sediments and stormwaters, approaches to establish links between the body-residue concentrations and effects in aquatic organisms provide the insight to better link the toxic response directly to contaminants. The concept is based on the understanding that it is the dose at the receptor that is responsible for the toxic response and that the receptor concentration is proportional to the contaminant concentration in the organism. This leads to development of a database of the concentrations of contaminants responsible for toxic responses in organisms (McCarty and Mackay 1993). Data have been amassed over the course of the past several years that allow the direct comparison of some residue levels with acute and chronic effects (McCarty and Mackay 1993; Jarvinen and Ankley 1999; www.wes.army.mil/el/ered). However, the database is very limited at this time, and there is still need to establish a weight-of-evidence approach for developing the link between the observed response and the presence of contaminants in sediments. Currently, there is only one standardized EPA test for sediment bioaccumulation. It is a 28-day test with the oligochaete *Lumbriculus variegatus* (Figure 6.157).

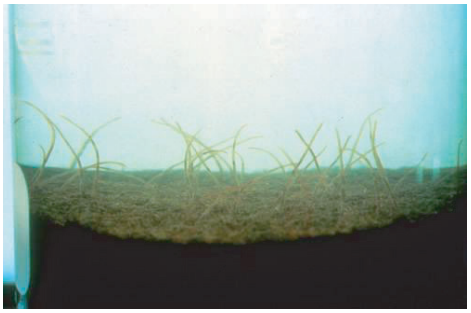

Figure 6.157 *Lumbriculus variegatus* 28-day bioaccumulation test.

Bioaccumulation has often been assessed with *in situ* studies to determine site-specific effects. These studies have primarily used caged mussels (marine) or fish (EPA 1987; Mac et al. 1990). In one approach, adult fish (*P. promelas*) are placed in mesh cages (10 fish per compartment, 4 compartments per cage) and exposed for 10 days *in situ*. This may also be done with benthic invertebrates (e.g., mussels, amphipods, and oligochaetes (e.g., *Lumbriculus variegatus*), providing there is adequate biomass for chemical analyses. Caution should be exercised when formulating conclusions from these studies because the organisms are not exposed for extended periods, they may not be able to ingest foods and surficial sediments due to their mesh-cage barrier, and they may be stressed due to caging. These factors alter toxicokinetics. These weaknesses can be addressed by also collecting resident target species (Table 6.60) and analyzing tissues (EPA 2000a,b). Target species should be large adults that are upper trophic level (top predator) and/or bottom feeders, and they should be collected prior to winter yet well after spawning. Nonmigratory species are preferred, and their commercial or sport fishing importance should be considered. Samples should be processed as described in Appendix D. The decision of whether to analyze whole fish or select target organs (e.g., gills, liver, kidneys) depends on the study objective and concerns over food chain or human health effects.

Residue information should be interpreted with caution (as discussed above with metals); however, guidance exists for calculating fish consumption advisories (EPA 2000a). There is little information available on what constitutes a significant tissue concentration, and correlations with adverse effects are usually lacking. Many contaminants are present for days or less (e.g., synthetic pyrethroids), rapidly metabolized (e.g., synthetic pyrethroids, organophosphates), biotransformed (e.g., polycyclic aromatic hydrocarbons), or only present in the environment seasonally (e.g., herbicides, insecticides). The U.S. Food and Drug Administration and the U.S. Fish and Wildlife Service have some effect-level information for a few common contaminants. For further information, see EPA (1982, 2000a), Carlton and Klug (1990), and Mac and Schmidt (1992).

Table 6.60 Target Fish Species for Use in Tissue Analysis

I. Target Species (East of Appalachian Mountains)

***Brook trout (*Salvelinus fontinalis*)	**Bluegill (*Lepomis macrochirus*)
***Small mouth bass (*Micropterus dolomieui*)	**Pumpkinseed (*Lepomis gibbosus*)
***Large mouth bass (*Micropterus salmoides*)	**Black crappie (*Pomoxis nigromaculatus*)
***Channel catfish (*Ictalurus punctatus*)	**Striped bass (*Morone saxatilis*)
**Brown trout (*Salmo trutta*)	*Carp (*Cyprinus carpio*)
**Rainbow trout (*Salmo gairdnerii*)	

II. Target Species (West of Appalachian Mountains and East of Rocky Mountains)

***Rainbow trout (*Salmo gairdnerii*)	**Yellow perch (*Perca flavescens*)
***Brook trout (*Salvelinus fontinalis*)	**Walleye (*Stizostedion vitreum*)
***Small mouth bass (*Micropterus dolomieui*)	**Bluegill (*Lepomis macrochirus*)
***Large mouth bass (*Micropterus salmoides*)	**Brown trout (*Salmo trutta*)
***Channel catfish (*Ictalurus punctatus*)	*Carp (*Cyprinus carpio*)
**Striped bass (*Morone saxatilis*)	

III. Target Species (West of and including Rocky Mountains)

***Rainbow trout (*Salmo gairdnerii*)	**Bluegill (*Lepomis macrochirus*)
***Brook trout (*Salvelinus fontinalis*)	**Striped bass (*Morone saxatilis*)
***Small mouth bass (*Micropterus dolomieui*)	*Cutthroat trout (*Salmo clarki*)
***Large mouth bass (*Micropterus salmoides*)	*Brown trout (*Salmo trutta*)
***Channel catfish (*Ictalurus punctatus*)	*Carp (*Cyprinus carpio*)

*** Preferred target species.
** Good target species.
* Acceptable target species.

From Freed, J. et al. *Sampling Protocol for Analysis of Toxic Pollutants in Ambient Water, Bed Sediments, and Fish, Interim Final Report.* Office of Water Planning and Standards, U.S. Environmental Protection Agency, Washington, D.C. 1980.

Emerging Tools for Toxicity Testing

Semipermeable Membrane Devices (SPMDs)

While no standard methods exist, SPMDs have been reported widely in recent years as an excellent passive, *in situ* sampling device for organic contaminants in water and in air (Huckins et al. 1999; Axelman et al. 1999; Peterson et al. 1995; Sabaliunas et al. 1998; Petty et al. 1998; Woolgar and Jones 1999; Zabik et al. 1992; Prest et al. 1995, 1992). Granmo et al. (2000) recently conducted tests in marine waters in Sweden using SPMDs for comparison with bioaccumulation of organochlorine compounds (chlorobenzenes, chlorophenols, and PCBs) in feral and caged mussels and the concentrations found in sediment and the associated water column. Short-term exposures (30-day) of SPMDs and caged mussels were used to find whether the high pollutant concentrations found in the sediments were associated with recent or older industrial discharges. Feral mussels were also analyzed after longer exposure periods. They found that the test approach using the combination of SPMDs and mussels allowed the detection of short-term changes of discharges of these organochlorine compounds, especially considering that the SPMDs were found to be more effective at concentrating some of the target compounds.

The devices are generally polymeric (such as low-density polyethylene) tube bags containing a neutral lipid (such as triolein, iso-octane, 2,2,4-trimethylpentane). These bags are placed in receiving waters for a period of days and then recovered and the contents analyzed using gas chromatography/mass spectrometry, or high-pressure liquid chromatography for target compounds. The concentrations accumulated in the bags have been found to be relatively similar to what is

accumulated in resident fish and shellfish. However, the concentrations may be higher or lower by several-fold and vary in their relationship to each other. This method has the advantage of being easy to deploy and retrieve, and can sample compounds found at a specific site that are in the water column during a specified period of time (unlike fish, which migrate to different areas). In addition, biological organisms are not sacrificed for the analyses. Extended exposures may result in biofouling of the bag and care must be taken to ensure adequate field blanks are used to assess that no water-related contamination has occurred.

DNA Fingerprinting

Another novel assessment tool to measure stress is genetic markers (as discussed above). Randomly amplified polymorphic DNA (RAPD) markers have proven promising for determining differences in genetic variability in populations (Williams et al. 1990, 1991). Other studies (Sternberg et al. 1996; Ellis et al. 1997) showed highly significant differences in DNA profiles between benthic invertebrates from stressed and nonstressed sites. This inexpensive and quick assay shows the number and size of distinctive DNA profiles of genomic DNA from each organism. Because RAPD-PCR primers are not designed to amplify specific target sequences, the amplified loci are anonymous, scattered throughout the genome, and are not associated with stressor adaptation (neutral markers) (Williams et al. 1990). RAPD-PCR products are often highly polymorphic within naturally occurring populations and have proven to be excellent indicators of genetic diversity (Clark and Lanigan 1993).

Biological Toxicity Fractionations

After toxicity is identified in receiving waters, researchers commonly attempt to identify the toxicants responsible for the observed effects through toxicity identification evaluation (TIE) studies. Numerous TIE protocols have been used. Figure 6.158, from Lopes et al. (1995), is one example that was used in association with a stormwater toxicity study conducted in Phoenix, AZ. Acute toxicity of stormwater was found to occur, especially to fathead minnows, and was likely to degrade the quality of the receiving water (the Salt River).

This test protocol involved first conducting toxicity tests to identify stormwater that was toxic (>20% mortality after 24 hours). The toxic stormwater was then subjected to different extractions to selectively remove various pollutants from the stormwater, after which additional toxicity tests were conducted. The first extractions were with activated carbon to remove oil and grease. The water was then split by filtering through 0.45- and 0.7-μm filters and further treated to remove metals (by chelation extraction) and organics (by solid-phase extraction). This procedure enabled the pollutant phase causing the toxicity to be identified: particulate bound pollutants, filterable metals, or filterable organics.

The EPA TIE protocols consist of three levels of confirmation (EPA 1991a). These methods were designed for analyzing wastewater effluents; however, they have been used for stormwaters, sediment pore waters, and whole sediments (e.g., EPA 1991d; Bailey et al. 2000; Werner et al. 2000; Vlaming et al. 2000; USGS 1999; Burgess et al. 1997; Ho et al. 1999; Kosian et al. 1999; Boucher and Watzin 1999). Usually, only Phase I is conducted due to the time and expense required. The TIE Phase I is a physical and chemical fractionation process that separates chemical groups by their properties. The principal groups of contaminants include pH-sensitive and volatile compounds (such as ammonia), metals, and nonpolar organics. This consists of exposing *Ceriodaphnia dubia* neonates and/or *P. promelas* larvae to water fractions for 48-hour periods. If toxicity is removed in any fraction, subsequent chemical analyses can be used to confirm the removal of compounds which may be contributing to toxicity. These methods are relatively complex and should be conducted by an experienced laboratory.

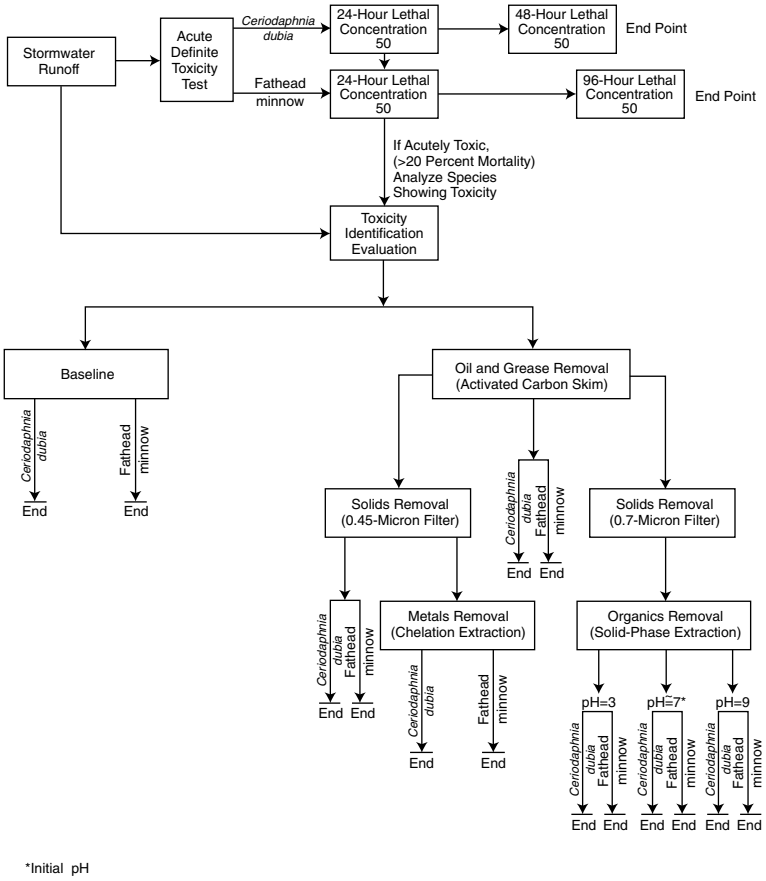

Figure 6.158 Toxicity identification evaluation (TIE) protocol. (From Lopes, T.J. et al. *Statistical Summary of Selected Physical, Chemical, and Microbial Characteristics, and Estimates of Constituent Loads in Urban Stormwater, Maricopa County, Arizona*. U.S. Geological Survey Water-Resources Investigations Report 94-4240. Tucson, AZ. 1995.)

Examples of Identifying Stressors

Diazinon was shown to be the primary toxicant in stormwater samples using *C. dubia* (Ohio EPA 1987). Anderson et al. (1991) compared numerous stormwater outfalls in the lower San Francisco Bay, CA. They found that nonpolar compounds in the most toxic stormwater (from a small, heavily industrialized drainage area) were the most important components of the toxicity, with lesser effects associated with suspended solids, metal chelates, and cationic metals. In another toxic stormwater study (from large parking areas surrounding an airport and industry), toxicity was most strongly influenced by cationic metals. Diazinon and chlorpyrifos in urban stormwater showed additive toxicity to *C. dubia* in a TIE (Bailey et al. 1997). TIE evaluations in the Sacramento–San Joaquin River basins confirmed that several organophosphate and carbamate pesticides were responsible for acute toxicity to *C. dubia* in water samples (Werner et al. 2000; Vlaming et al. 2000; Bailey et al. 2000). A TIE of pore water from a stormwater detention pond using *C. dubia* 48-hour exposures showed ammonia to be the primary toxicant, with some effects from metals (Zn, Fe, and Cu). The high level of ammonia may have obscured the metal toxicity (Wenholz and Crunkilton 1995).

Jirik et al. (1998) also used selected Phase 1 TIE studies to identify the toxicants most responsible for stormwater toxicity in the Santa Monica Bay area. Sea urchin fertilization tests

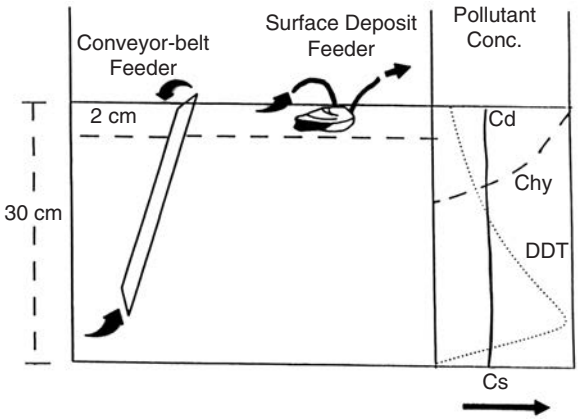

Figure 6.159 Different organism exposures should be matched with sampling of correct sediment depths. (From Lee, H., II. A clam's eye view of the bioavailability of sediment-associated pollutants, in *Organic Substances and Sediments in Water*, Vol. 3, *Biological*. Edited by R.A. Baker. Lewis Publishers, Boca Raton, FL. 1991. With permission.)

indicated EC50 values of stormwater of about 12 to 20%. Santa Monica Bay receiving waters were also found to be toxic, with the level of toxicity generally corresponding to the amount of stormwater in the receiving water. EDTA addition removed virtually all of the toxicity, implying that divalent metals were the likely toxicant component. Spiking studies showed that zinc, and sometimes copper, were the most likely metallic toxicants. Further studies, using EDTA vs. sodium thiosulfate for toxicity removal, also strongly implicated zinc as the likely cause of toxicity.

In situ tests also provide an excellent means for identifying the source and nature of the stressor by simply altering the exposure via chamber design and placement. It is essential to relate organism responses (e.g., mortality) with their correct, realistic exposure, such as overlying water, surficial sediment, or deeper sediments and pore waters (Figure 6.159). Useful *in situ* approaches to separating media effects and characterizing contaminant sources, pathways, and effects include characterization of benthic communities, *in situ* toxicity testing, and groundwater/surface water interactions (Greenberg et al. 2000; Figure 6.160). In a simplistic TIE approach, stressors can be partitioned out: overlying water, bulk sediment, interstitial water, light, suspended solids, flow velocity, and predator effects (Burton and Moore 1999) (see also Chapter 5). Strategic placement of chambers at reference and potentially impacted sites can identify both natural and anthropogenic stressors. Placement along known or suspected contamination gradients can provide an exposure–response relationship when combined with physicochemical measurements. For example, utilization of naturally occurring gradients (e.g., within and beyond a mixing zone) may facilitate an exposure–response characterization and regression analysis rather than a paired comparison (e.g., ANOVA) (Liber et al. 1992).

Useful *in situ* chambers for assessing stormwaters and surficial sediments are shown in Figures 6.161 through 6.164. Chambers are also buried in surficial sediments to assess sediment and groundwater associated contamination (Figures 6.165, 6.166, and 6.168) where chamber mesh

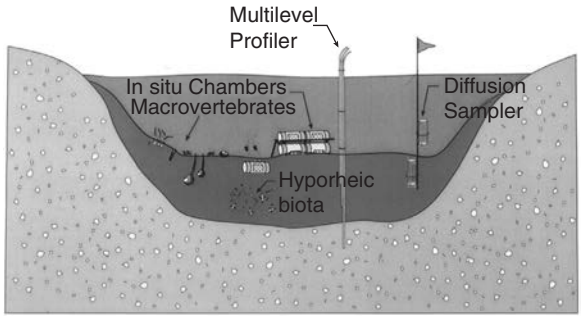

Figure 6.160 Integrated assessments of surface waters, sediments, and groundwater/pore waters.

Figure 6.161 *In situ* chambers optimized for surface water exposures on top of chambers optimized for surficial sediment exposures.

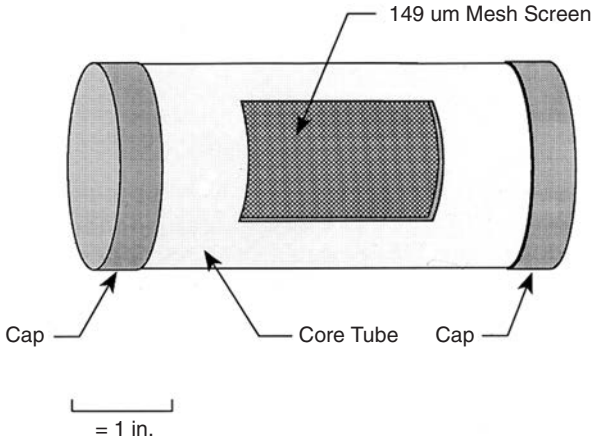

Figure 6.162 *In situ* chamber design components.

Figure 6.163 *In situ* chambers with water or sediment exposures with high-flow deflector.

Figure 6.164 *In situ* chambers with water or sediment exposures with high-flow deflector.

windows contact surficial sediments (bottom tray) or overlying water (top tray). Test organisms are placed within the chambers during low flow (Figure 6.169). Following organism addition, high flow guards (aluminum sheet metal) are attached to stakes to protect the chambers (Figures 6.163 and 6.164).

Assessments of PAH-contaminated sediments have demonstrated why both laboratory and field toxicity exposures were essential to adequately identify key stressors and characterize exposure dynamics (Ireland et al. 1996; Sasson-Brickson and Burton 1991; Stemmer et al. 1990). Sediment-associated toxicity increased in the laboratory exposure of *P. promelas, C. dubia, D. magna,* and *H. azteca* as compared to *in situ* exposures, whereas toxicity decreased in overlying waters (Figure 6.156). Photoinduced toxicity from PAH and UV interactions and sampling-induced artifacts accounted for these laboratory-to-field differences. Toxicity was also reduced significantly in the presence of UV light when the organic fraction of the stormwater was removed. Photoinduced toxicity occurred frequently during low flow conditions, but was reduced during high turbidity associated with high flow conditions. Toxicity was also higher in overlying waters near the contaminated sediment surface as opposed to waters several centimeters above the sediment–water interface.

An elevation in temperature of Des Plaines River water accentuated the toxicity of the water and of sediments, using both water column and benthic species (Brooker and Burton 1998; Burton and Rowland 1999; Lavoie and Burton 1998). Responses were replicated in laboratory, *in situ*, and

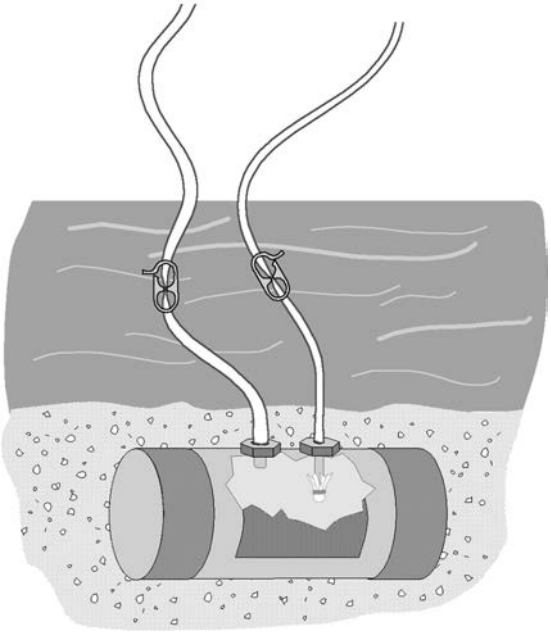

Figure 6.165 *In situ* chamber used as a "peeper" (buried for pore water exposure) or sediment–water interface (half-buried) exposure.

Figure 6.166 *In situ* sediment–water interface chambers buried.

Figure 6.167 *In situ* chambers optimized for surface water and photoinduced toxicity effects from PAHs and UV light.

Figure 6.168 Chambers for conducting sediment bioaccumulation studies.

Figure 6.169 Loading *in situ* chambers that are peepers for sediment–water interface exposures.

artificial, side-stream exposures. The laboratory exposures helped define exact threshold temperatures, critical exposure times, and interactions with ammonia (Figure 6.170). Field exposures, on the other hand, better defined real-world exposures and interactions with other stressors, such as suspended solids and fluctuating temperatures. Conclusions based on laboratory exposures would have underestimated stream effects.

An urban site receiving large loadings of residential, commercial, and industrial stormwater runoff was assessed using an integrated low and high flow assessment (Moore and Burton 1999). The effects of turbidity and flow were shown by reducing the mesh size in the *in situ* chambers (Figures 6.171 and 6.172). A survey of sediment quality during baseflow conditions found one depositional area where sediments were acutely toxic and contained elevated levels of contaminants. An *in situ* toxicity assessment found that low flow water was not toxic, but high flows were toxic, and suspended solids and flow contributed significantly to overall stress. However, indigenous communities appeared to be affected more strongly by contaminated sediments than high flow conditions.

Newer TIE methods include whole-sediment manipulations, exposure to UV (Kosian et al. 1998), or *in situ* exposures with various stressor partitioning methods and substrates (Burton et al. 1998; Greenberg et al. 1998; Moore and Burton 1999), and may reduce the likelihood of artifacts.

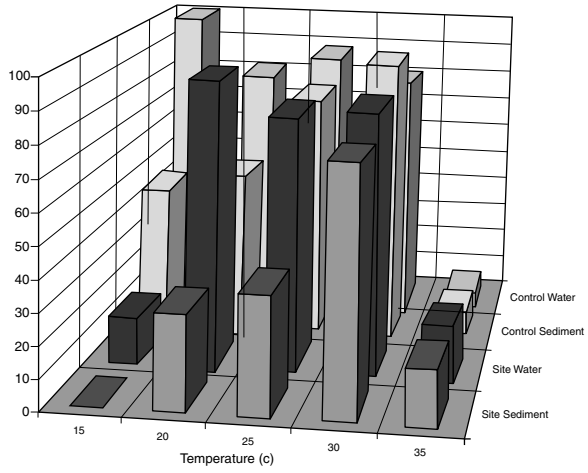

Figure 6.170 Temperature threshold determination in the presence of contaminated site water and sediment vs. control waters and sediment. Survival (%) of *Hyalella azteca*.

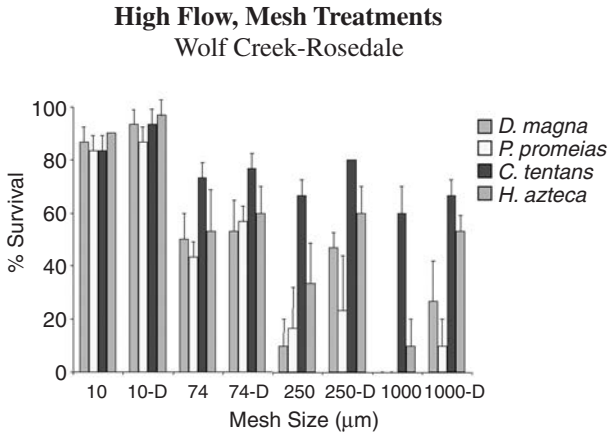

Figure 6.171 Relationship between toxicity and suspended solids/flow. *In situ* exposures in chambers with smaller mesh sizes decreased solids and flow and increased survival.

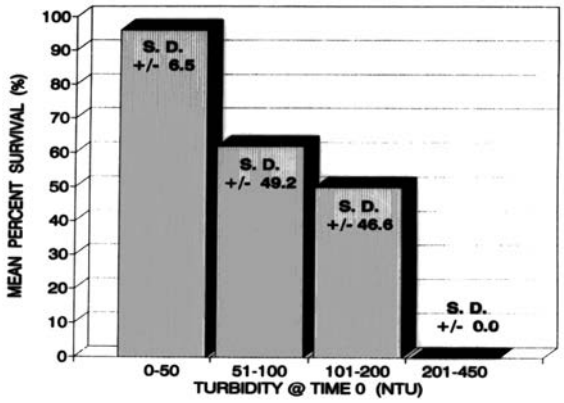

Figure 6.172 Relationship between turbidity and *Daphnia magna* toxicity in peeper exposures.

Toxicant Sampling and In-Stream Modeling Considerations

When sampling for, or predicting the fate of, toxicants, it is helpful to consider whether the likely contaminants tend to sorb to particulates, such as suspended solids or bedded sediments, or whether they tend to remain dissolved. Though metals will sorb to sediments in most waterways, if the water pH is acidic or if suspended colloids and solids concentrations are low, metals may remain in the water column. Dissolved metals do not necessarily equate with toxicity, as they may be complexed (e.g., carbonates, hydroxides) in less toxic forms. Many organics can be transported in dissolved forms at low suspended solid concentrations (EPA 1986). Adsorption can be predicted by knowing the octanol-water coefficient (K_{ow}), the organic carbon content of the suspended sediment, and then calculating the partition coefficient (K_p) (EPA 1986), as shown in Figure 6.173 (Novotny and Olem 1994). The K_p, however, is a site-specific value which varies at the site spatially and temporally during storm events and should thus be used with caution.

Sediment resuspension (scour) is an important mechanism affecting water column concentrations of many problematic constituents that tend to accumulate in stream sediments (especially pathogens, toxicants, and nutrients). Scouring of sediments can also be an important factor influencing water

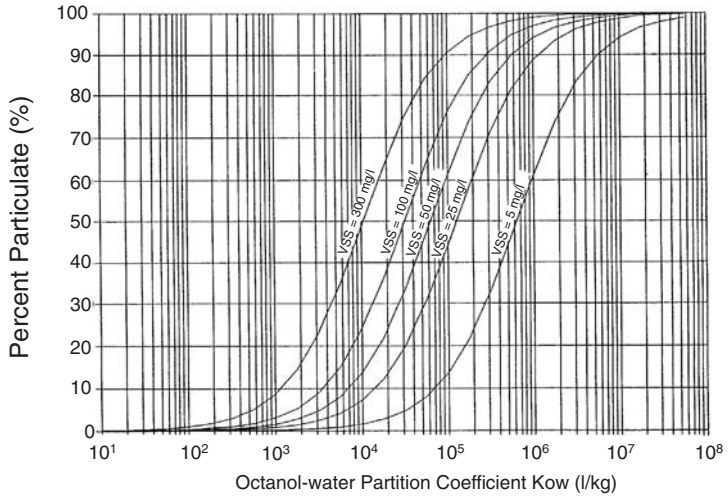

Figure 6.173 Relationship of dissolved and total concentrations of organic priority pollutant related to the octanol partioning number and volatile suspended solids content of runoff. (From Novotny, V. and H. Olem. *Water Quality: Prevention, Identification, and Management of Diffuse Pollution.* Van Nostrand Reinhold, New York. Copyright 1994. Used with permission of John Wiley & Sons.)

turbidity in some cases. Methods for measuring sediment scour were discussed previously in this chapter in the general habitat discussion. In that case, the significant role that scour has on habitat was stressed. The measurement methods described there (used in conjunction with sediment quality information) can also be used to measure the resuspension of contaminated sediments. Modeling of sediment resuspension can only be crudely predicted because site-specific details are rarely available in sufficient detail and local scour "hot spots" (small areas where the flowing water has excessive shear stress) are extremely difficult to predict. However, scour around bridge piers has been investigated for several thousand years, and there are methods to reduce sediment losses in those situations. In most cases, it is only possible to grossly predict average sediment resuspension based on average stream bed conditions. Therefore, careful scour measurements should be conducted to indicate likely sediment resuspension rates for different flows for specific streams.

Many organic toxicants move in and through an ecosystem being controlled primarily by one fate process. Volatilization controls the fate of compounds such as trichloroethylene, toluene, xylene, acetone, and benzene. Adsorption dominates the fate of polychlorinated biphenyls, dioxins, and furans. For many common contaminants, such as the metals, metalloids, polycyclic aromatic hydrocarbons, and nutrients, multiple processes (e.g., biodegradation, methylation, photolysis, hydrolysis) dominate at different stages in different microenvironments.

A number of stream models exist for predicting pollutant fates, ranging from simple to complex, which may in limited cases be useful tools for stormwater effect studies. A summary of screening approach data requirements for metals and organics are listed in Tables 6.61 and 6.62, respectively.

Contaminants may move from their source through the receiving system, (e.g., stream, lake, wetland), in a conservative or nonconservative manner depending on the fate processes that dominate in that system and are characteristics of that particular toxicant. Generalized toxicant concentration profiles shown in Figure 6.174a reflect stream dilution and toxicant decay. This profile is not representative of reactive (nonconservative) constituents, such as highly volatile compounds, nutrients, species, or dissolved oxygen concentrations. Effects from these stressors must always be considered when toxicant fate and effects are being assessed. As shown in Figure 6.174b, during high flow conditions, contaminated sediment scour may increase concentrations in some stream segments before dilution and first-order decay profiles return. By constructing suspended solids profiles at low and high flow conditions, both sources and erosion- and scour-related stressors (e.g., sorbed toxicants and nutrients, oxygen demand, solids-related filter/gill clogging, or suffocation) can be better defined (see Figure 6.175).

Table 6.61 Summary of Data Requirements for Screening Approach for Metals in Rivers

Data	Calculation Methodology Where Data Are Used*	Remarks
	Hydraulic Data	
1. Rivers:		
River flow rate, Q	D, R, S, L	An accurate estimation of flow rate is very important because of dilution considerations. Measure or obtain from USGS gauge.
Cross-sectional area, A	D, R, S	
Water depth, h	D, R, S, L	The average water depth is cross-sectional area divided by surface width.
Reach lengths, x	R, S	
Stream velocity, U	R, S	The required velocity is distance divided by travel time. It can be approximated by Q/A only when A is representative of the reach being studied.
2. Lakes:		
Hydraulic residence time, T	L	Hydraulic residence times of lakes can vary seasonally as the flow rates through the lakes change.
Mean depth, H	L	Lake residence times and depths are used to predict settling of absorbed metals in lakes.

Table 6.61 Summary of Data Requirements for Screening Approach for Metals in Rivers *(Continued)*

Data	Calculation Methodology Where Data Are Used*	Remarks
	Source Data	
1. Background		
Metal concentrations, C_T	D, R, S, L	Background concentrations should generally not be set to zero without justification.
Boundary flow rates, Q_U	D, R, S, L	
Boundary suspended solids, S_U	D, R, S, L	One important reason for determining suspended solids concentrations is to determine the dissolved concentration, C, of metals, based on C_T, S, and K_p. However, if C is known along with C_T and S, this information can be used to find K_p.
Silt, clay fraction of suspended solids	L	
Locations	D, R, S, L	
2. Point Sources		
Locations	D, R, S, L	
Flow rate, Q_w	D, R, S, L	
Metal concentration, C_{Tw}	D, R, S, L	
Suspended solids, S_w	D, R, S, L	
	Bed Data	
Depth of contamination		For the screening analysis, the depth of contamination is most useful during a period of prolonged scour when metal is being input into the water column from the bed.
Porosity of sediments, n		
Density of solids in sediments (e.g., 2.7 for sand), $:_s$		
Metal concentration in bed during prolonged scour period, C_{T2}		
	Derived Parameters	
Partition coefficient, Kp	All	The partition coefficient is a very important parameter. Site-specific determination is preferable.
Settling velocity, ws	S, L	This parameter is derived based on suspended solids vs. distance profile.
Resuspension velocity, Wrs	R	This parameter is derived based on suspended solids vs. distance profile.
	Equilibrium Modeling	
Water quality characterization of river:	E	Equilibrium modeling is required only if predominant metal species and estimated solubility controls are needed.
pH		
Suspended solids		
Conductivity		
Temperature		
Hardness		Water quality criteria for many metals are keyed to hardness, and allowable concentrations increase with increasing hardness.
Total organic carbon		
Other major cations and anions		

* D = dilution (includes total dissolved and adsorbed phase concentration predictions); R = dilution and resuspension; S = dilution and settling; L = lake; E = equilibrium modeling.

From EPA. *Handbook — Stream Sampling for Waste Load Allocation Applications.* U.S. Environmental Protection Agency. Office of Research and Development. Washington, D.C. EPA/625/6-86/013. 1986.

Table 6.62 Summary of Data Requirements for Screening Approach for Toxic Organics in Rivers

Data	Methodology Where Data Are Used	Remarks
River Hydraulic Data		
Flow rate, Q	D, DA, DAK	An accurate estimate of flow rate is very important because of dilution, which for many organics is the most important process that influences their fate. Measure or obtain from USGS gauge.
Cross-sectional area, A	D, DA, DAK	
Water depth, h	DAK	Water depth can influence rate processes such as volatilization and photolysis.
Reach lengths, x	DAK	
Stream velocity, U	DAK	U = Q/A should be used only where A is representative of the reach being analyzed. Otherwise dye tracers, measured from centroid to centroid of the dispersing dye, are a better method of finding velocity (indirectly as distance divided by travel time).
Source Data		
1. Background		
Toxicant concentrations	D, DA, DAK	Concentrations of organic toxicants may be negligible in areas not influenced by man.
Boundary flow rates	D, DA, DAK	
Boundary suspended solids	DA, DAK	Suspended solids are used to help determine the dissolved and adsorbed phase concentrations.
2. Point Source		
Locations	D, DA, DAK	
Flow rates, Q_w	D, DA, DAK	
Total toxicant concentration, C_T	D, DA, DAK	
Suspended solids, S_w	DA, DAK	
Partition Coefficient and Rate Constant Data		Difficult to calculate accurately.

From EPA. *Handbook — Stream Sampling for Waste Load Allocation Applications.* U.S. Environmental Protection Agency. Office of Research and Development. Washington, D.C. EPA/625/6-86/013. 1986.

Dye studies (as discussed earlier) are recommended in waste load allocation (WLA), or total maximum daily load (TMDL) studies to study point source mixing, movement of conservative pollutants, and to construct ambient toxicity profiles (EPA 1986; Figure 6.176). Multiple samples on a transect are necessary immediately downstream of sources or in wide streams (Figure 6.177). Samples of effluent from point sources (e.g., sewer overflow, culverts, tributaries [months], and stormwater) should be collected prior to dye studies, and both acute and chronic toxicity should be measured using EPA-recommended species (i.e., *Pimephales promelas*, *Ceriodaphnia dubia*), key surrogates (e.g., *Hyalella azteca*, *Selenastrum capricornutum*), and/or important resident species (e.g., trout). The dilution required to reach the no-observable-effects level (NOEL) in the toxicity tests should be the final sample points for constructing the dye isopleth (Figure 6.178; EPA 1986). These data may then be used to guide station location selection for ambient toxicity sample collection. In this manner, toxicity decay or persistence can be defined for various flow conditions.

SUMMARY

As indicated in many discussions in this book, multiple approaches are needed to effectively evaluate receiving water impacts in urban areas. This chapter presents details in collecting information pertaining to different ecosystem components and specific beneficial use impairments, including rainfall and flow monitoring; soil characteristics; aesthetics, litter, and safety; habitat

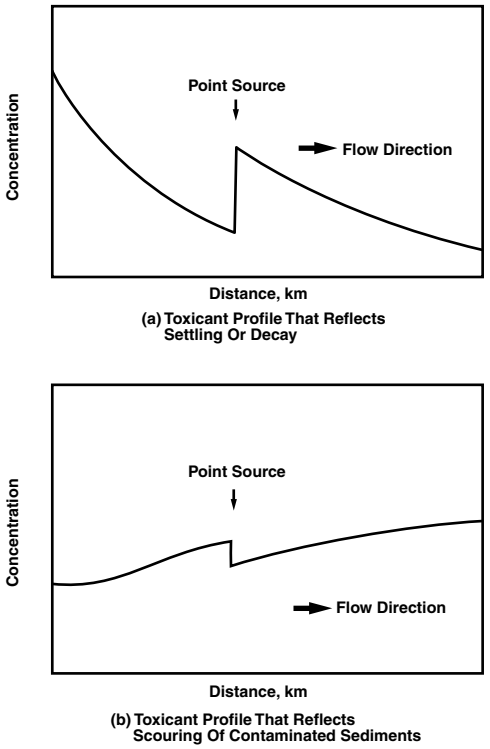

Figure 6.174 Typical concentration profiles of toxicants in rivers. (From EPA. *Handbook — Stream Sampling for Waste Load Allocation Applications.* U.S. Environmental Protection Agency. Office of Research and Development. Washington, D.C. EPA/625/6-96/013. 1986.)

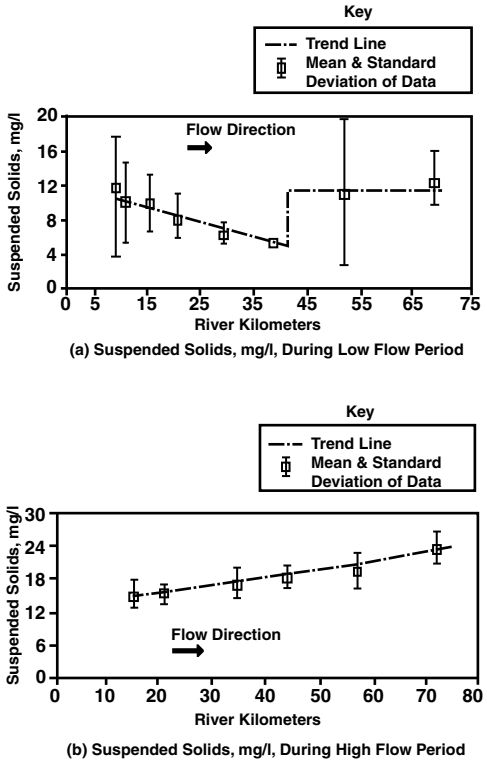

Figure 6.175 Typical suspended solids concentrations during (a) low flow and (b) high flow periods. (From EPA. *Handbook — Stream Sampling for Waste Load Allocation Applications.* U.S. Environmental Protection Agency. Office of Research and Development. Washington, D.C. EPA/625/6-96/013. 1986.)

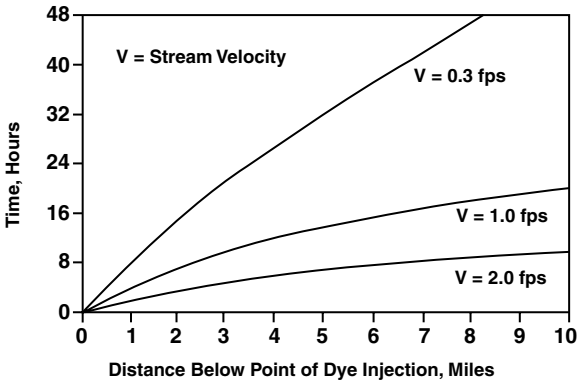

Figure 6.176 Time required for a continuous release of dye to reach steady-state concentrations at selected locations below the point of discharge. Note: the curves are based on a solution to the advection-dispersion equation which is used to predict when dye concentrations are 95% of steady-state levels. (From EPA. *Handbook — Stream Sampling for Waste Load Allocation Applications.* U.S. Environmental Protection Agency. Office of Research and Development. Washington, D.C. EPA/625/6-96/013. 1986.)

conditions; water and sediment chemical analyses; microorganism evaluations; benthos, zooplankton, and fish collecting; and tests for toxicity and bioaccumulation. This information supplements the information provided in Chapter 5 concerning collecting samples and selecting an experimental design. Chapter 7 briefly presents some statistical analyses tools, while Chapter 8 presents data interpretation for the complete study.

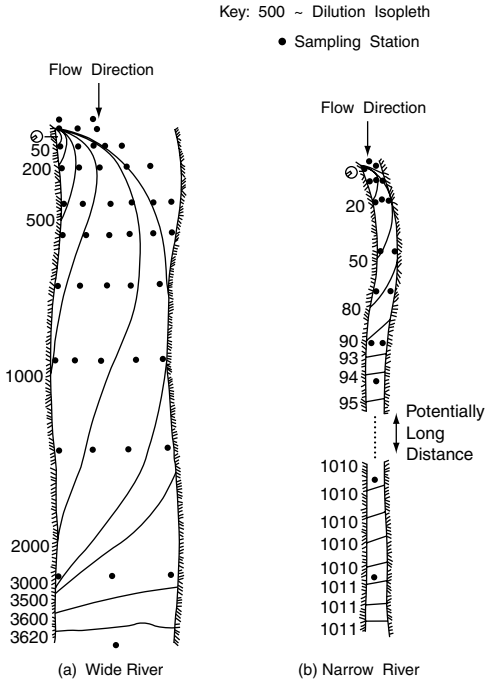

Figure 6.177 Example of sampling locations in wide and narrow rivers. (From EPA. *Handbook — Stream Sampling for Waste Load Allocation Applications.* U.S. Environmental Protection Agency. Office of Research and Development. Washington, D.C. EPA/625/6-96/013. 1986.)

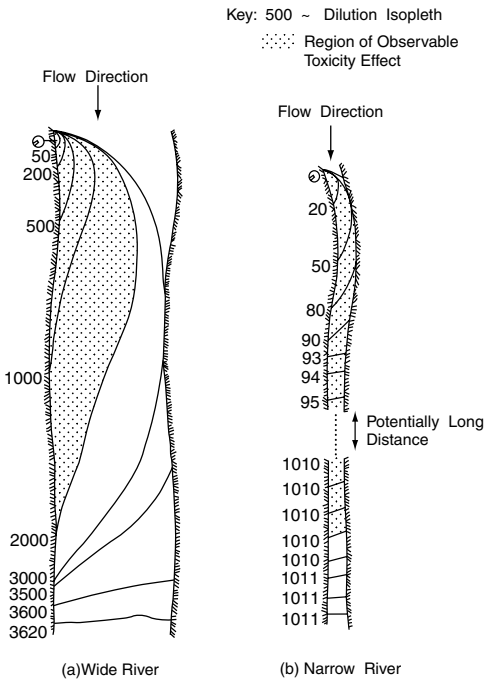

Figure 6.178 Regions of observable toxicity in wide and narrow rivers. (From EPA. *Handbook — Stream Sampling for Waste Load Allocation Applications.* U.S. Environmental Protection Agency. Office of Research and Development. Washington, D.C. EPA/625/6-96/013. 1986.)

It is essential that there be an accurate description of the system's hydrodynamics when assessing the effects of stormwater runoff on receiving waters. Flow represents the pollutant loading mechanism, and its power and frequency of occurrence can degrade the physical habitat. Instantaneous flow can be measured using traditional current meters, while long-term flow monitoring is usually conducted using stage recorders. Tracer methods are also useful, especially where the flows are quite shallow and the stream channel very rough. Tracers can also be used to effectively indicate diffusion and transport of pollutant discharges into small streams. Flow is also of primary consideration in supporting aquatic life, as minimum depths and velocities are needed for their survival. With urbanization, flow changes can be dramatic, with excessive flows occurring during wet periods and significantly reduced flows occurring during dry months.

The role that different rains have on wet weather-related receiving water effects is also important to understand through evaluation of local data. As an example, small rains (less than about 0.5 in in the upper Midwest) are important because they are associated with the majority of runoff events and they frequently exceed heavy metal and bacteria objectives, although they only account for a small fraction of the annual pollutant discharges. Intermediate-sized rains (from about 0.5 to 1.5 in in the upper Midwest) account for the majority of the pollutant discharges and subject the receiving waters to frequent high pollutant loads and moderate-to-high flow rates. Larger rains (from about 1.5 to 3 in in the upper Midwest) produce relatively small amounts of the annual pollutant discharges, but produce the most damaging flows from a habitat destruction standpoint. The largest rains are critical from a drainage aspect and must be controlled to provide safe conditions for inhabitants of the watershed. These rains must be controlled in the primary drainage systems, while excessive flows that exceed the capacities of these systems must be safely controlled in secondary

drainages (such as temporary flooding of some roads, parking areas, vacant fields, etc.). Therefore, the type of receiving water problem being addressed is likely associated with a specific set of rain conditions, typically much smaller than the rains used in the design of storm drainage.

Soils can play a significant role in watershed and receiving water assessments. Most of the particulates being transported in stormwater originate as local soil, and their texture can have dramatic effects on stream turbidity levels and the amounts of erosion from nonpaved areas. In addition, soils in urban areas undergo significant modifications and are generally greatly compacted compared to natural soil profiles. The compacted soils provide much less infiltration for the rain water, increasing the runoff flow rates. Soil surveys can describe the soil types, textures, depths, chemical quality, and amounts of compaction, which are all useful measures. Soil modifications to enhance infiltration, to capture pollutants during percolation above the groundwater, and improve the fertility of the soil to enhance plant growth with minimal fertilization can therefore be important stormwater control practices.

Aesthetics, litter, and safety are all critical receiving water attributes that need to be quantified to indicate if basic beneficial uses (such as noncontact recreation) are being met. Many municipalities currently suffer large litter accumulations along public streams that significantly detract from their use and respect. Habitat problems are probably some of the most important impairments to aquatic life beneficial uses. Unfortunately, "standards" for habitat goals are not likely to become possible, requiring local investigations to compare receiving waters to local reference conditions. The role that highly fluctuating flows have on habitat is beginning to be understood. The amount of large woody debris, and other channel-forming materials, can be directly measured in streams, along with the rate of channel enlargement. Stormwater controls can possibly be designed to overcome habitat problems if the role of the causative impairment factors in local waters is better understood.

Water quality measurements also need to be made in a comprehensive receiving water assessment. Historically, most studies overly relied on expensive water quality measurements, with little supportive information. Currently, many areas are almost totally eliminating water quality analyses in stream assessments and only examining several basic stream biological conditions. As noted in this book, it is important that a balanced set of parameters be included in an effective program, requiring a basic set of traditional, plus specialized water quality measurements. The specific water quality parameters to be monitored should be selected based on the beneficial uses of the stream, along with additional indicator parameters that can identify the presence of inappropriate discharges and other unusual conditions. This chapter describes different field monitoring options, along with modifications that may be needed for conventional laboratory methods to be most effective for stormwater samples. Needed detection limits, along with safety and complexity, are presented as the most important factors that determine the most appropriate analytical methods that should be used for the selected parameters.

Microorganism measurements are needed in most receiving water assessments, especially in areas having water-contact recreation and consumption of aquatic life beneficial uses. Newly available microorganism measurement methods and changes in guidance on target organisms require a reexamination of traditional approaches in the assessment of these important parameters in receiving waters.

Benthos sampling is one of the most important measurements in receiving water assessments (along with habitat evaluations). Much guidance is now available on obtaining and evaluating appropriate samples. Fish sampling, although more complex to conduct and evaluate, is an important assessment tool, especially when relating to beneficial uses that are easier for the interested public to understand. Currently accepted methods for benthos and fish sampling are described in detail in this chapter and in related appendices.

Toxicity and bioaccumulation measurements can be important tools, especially when trying to identify cause-and-effect relationships between different stressors and receiving water impacts. Recently developed *in situ* toxicity test methods are especially useful tools because they subject the test organisms to natural conditions, such as fluctuations in receiving water conditions, and to

the toxicity effects of in-place sediments. Traditional and newly developed methods for toxicity testing is presented in this chapter.

Chapter 6 presents a wide range of tools for characterizing many different components of ecosystems. Case studies also illustrate these procedures and show how they can be effectively utilized. Summaries of the advantages and disadvantages of the different methods are also frequently presented. Several appendices also present supportive information for the techniques given in this chapter.

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CHAPTER 7

Statistical Analyses of Receiving Water Data

"*Get your facts first, then you can distort them as much as you please.*"

Mark Twain

CONTENTS

SELECTION OF APPROPRIATE STATISTICAL ANALYSIS TOOLS AND PROCEDURES

The appropriate selection of statistical analyses must be an integral aspect of the experimental design activities for an effective data collection effort. Chapter 5 examined various sampling strategies and presented methods that can be used to estimate the sampling effort. This chapter reviews some typically used statistical analysis procedures that have been very effective in receiving water studies.

Statistical software packages have become an indispensable tool for research, but their selection and use can be frustrating. There are numerous comprehensive statistical software programs available that contain both conventional and specialized tools of interest to environmental researchers. The number of choices is almost overwhelming and covers a wide range of cost (from freeware to several thousand dollars). The selection process can therefore become difficult without some guidance. It is highly recommended that the selection of a software program be made based on consultation with a colleague who has experience with these tools, especially if that colleague can be relied on for assistance later.

When computerized statistical packages were first made available to typical users, it was very easy to rely too much on the wealth of available options, to produce copious piles of irrelevant printouts, and then to become dismayed at the prospect of sorting through the material to find what was important. This was exacerbated if there was also no appropriate experimental design developed at the onset of the data collection effort, or if one needed to rely solely on existing data that were not collected for the objectives at hand. However, examining existing data is an important initial step in experimental design activities, as described in Chapter 5. "Data mining" to identify trends and relationships hidden in large amounts of retrospective data that can be exploited has now become a common household term, even showing up in Dilbert® comic strips. Obviously, collecting haphazard data and relying on powerful computer programs to ferret out conclusions is not a very efficient experimental design for ongoing research. The need for carefully stated project objectives, an appropriate experimental design, and an understanding of the likely statistical tools that will be used for data analysis are all important initial steps in any research activity.

In addition to having access to appropriate software tools, it is imperative that the researcher have some knowledge of applied statistics. Most professionals involved in environmental research have been required to take some type of introductory statistical course for their degree. Few, however, have likely been exposed to the broad range of options that should be examined to select the few procedures that may be most efficient for the specific project objectives. Luckily, well-written articles are available in many technical journals that do an excellent job of describing the statistical tests that were used. In addition, statistical consultants are available through most university statistics and biostatistics departments, in addition to private statistical consultants and experienced colleagues, who are readily available to consult on environmental research projects examining receiving water impacts. Obviously, self-study by the researcher is also necessary, as the person involved in the specific study must take an active role and be ultimately responsible for the experimental design and data evaluation. This chapter therefore lists several applied statistics texts that the authors have found to be extremely valuable and that are well written and at a level understandable to those who are not experts in the statistics field.

Computer Software and Recommended Statistical References to Assist in Data Analysis

The following sections present brief information for useful print and software resources that the receiving water impact investigator may find useful.

Statistical Reference Books

The following books comprise a basic library in applied statistics and have proven very useful in environmental research. These are especially helpful in that they contain many example applications in the environmental sciences and engineering. The use of these books, along with consultation with statistical experts as needed, will enable more efficient experimental designs and data analyses.

- Exploratory data analysis

  Exploratory Data Analysis. John W. Tukey. Addison-Wesley Publishing Co. 1977. This is a basic book with many simple ways to examine data to find patterns and relationships.

  The Visual Display of Quantitative Information. Edward R. Tufte. Graphics Press, Box 430, Cheshire, CT 06410. 1983. This is a beautiful book with many examples of how to and how not to present graphical information. Tufte has two other books that are sequels: *Envisioning Information,* 1990, and *Visual Explanations: Images and Quantities, Evidence and Narrative*, 1997.

  Visualizing Data. William S. Cleveland. Hobart Press, P.O. Box 1473, Summit, NJ 07902, 1993, and *The Elements of Graphing Data*, 1994, are both continuations of the concept of beautiful and informative books on elements of style for elegant graphical presentations of data.

- Experimental design (and some basic methods)

  Statistics for Experimenters. George E. P. Box, William G. Hunter, and J. Stuart Hunter. John Wiley & Sons, 1978. This book contains detailed descriptions of basic statistical methods for comparing experimental conditions and model building.

  Statistical Methods for Environmental Pollution Monitoring. Richard O. Gilbert. Van Nostrand Company, 1987. This book contains a good summary of sampling designs and methods to identify trends, unusual conditions, etc.

- General statistics

  Statistics for Environmental Engineers. Paul Mac Berthouex and Linfield C. Brown. Lewis. 1994. This excellent book reviews the shortcomings and benefits of many common statistical procedures, enabling much more thoughtful evaluation of environmental data.

  Biostatistical Analysis. Jerrold H. Zar. Prentice-Hall. 1996. A highly recommended basic statistics text for the environmental sciences, especially with its many biological science examples.

  Primer on Biostatistics. Stanton A. Glantz. McGraw-Hill. 1992. This is one of the easiest to read and understand introductory texts on applied statistics available.

- Specialized statistical methods

  Nonparametrics: Statistical Methods Based on Ranks. E.L. Lehman and H.J.M. D'Abrera. Holden-Day and McGraw-Hill. 1975. This is a good discussion, with many examples of nonparametric methods for the analysis and planning of comparative studies.

  Applied Regression Analysis. Norman Draper and Harry Smith. John Wiley & Sons. 1981. Thorough treatment of one the most commonly used (and misused) statistical tools.

Statistical Software Programs

There are several tiers of software available for statistical analyses, although the distinctions in their capabilities are becoming blurred. Freeware and shareware (or otherwise inexpensive) software packages have traditionally been developed and made available by private individuals, or are "obsolete" versions of enhanced commercial products. The individually developed packages were typically created to solve a specific problem, or for cost-effective or straightforward use in classrooms. Many of these products are very good, but documentation is likely minimal.

Modern spreadsheet programs also contain many built-in statistical routines (at least regression analyses and simple comparison tests) and graphing options. Spreadsheets are now ubiquitous on all microcomputers, are familiar to users, and the available statistical capabilities should therefore be considered before purchasing additional software. Spreadsheets are extremely helpful for preliminary analyses and for concurrent data evaluation as the data observations are being collected and organized in the spreadsheet (especially critical for laboratory QA/QC control plots as described in Chapter 5). Relatively inexpensive spreadsheet add-ons are also available for decision analysis and Monte Carlo sampling routines, plus some contain rather complete sets of sophisticated statistical routines and graphing templates. Spreadsheets can also be programmed by the user with macros for "customized" statistical routines.

There are also many very elegant and easy-to-use commercial software packages that contain almost all that one would likely need. There is a wide range in price for these products, and some offer specialized capabilities. For comprehensive research, it is common for several different software products to be used for specific data evaluation objectives. Reviews of statistical software packages are commonly available in technical journals and should be consulted. In addition, much information is also available on the Internet. One outstanding example is the "Statistics on the Web" Internet page, created by Clay Helberg, which presents links to many statistical resources. The URL for this page is http://www.execpc.com/~helberg/statistics.html.

The following is a list of groupings of links available on Helberg's Web page:

- Professional organizations
- Institutes and consulting groups
- Educational resources (web courses and online textbooks)
- Publications and publishers
- Statistics book list
- Software-oriented pages
- Mailing lists and discussion groups
- Other lists of links
- Statisticians and other statistical people

Of special interest is his list of software-oriented pages where short reviews and descriptions are given for numerous freeware, shareware, and commercial statistical software. In addition, links are given to the sources of this software, enabling the user to download freeware and shareware packages, and in many cases, trial versions of the commercial packages. Another comprehensive listing of freeware, shareware, and commercial statistical software (for Windows, UNIX, DOS, and Macintosh computers) is available from *St@tServ* — Statistical Software, whose URL is http://www.statserv.com/.

The following programs are briefly mentioned here because of the authors' experience with them, and to indicate some of their major features. There are obviously many other suitable programs, including highly specialized programs emphasizing specific methods.

SYSTAT

SYSTAT (now available through SPSS, Inc., Chicago, IL) has been available to users of small computers for many years and is available for both Windows and Macintosh. It was one of the first comprehensive software packages that was competitive with the early mainframe statistical software packages. Not only did it offer a cost-effective alternative to other programs, but it was also noticeably easier to use and contained several areas of strength not readily available to many (especially nonlinear and multivariate analyses). Many of the examples in this book were prepared using various versions of SYSTAT. Recent versions of SYSTAT include many tools including cluster analysis, correlations and distance measures, factor analysis, multidimensional scaling, regression, analysis of variance, multivariate models, nonlinear models, nonparametric statistics, time series, and basic statistics. Numerous graphical options are also available, often integrated with the statistical methods. Three-dimensional graphing and multiminiatures are especially valuable. SYSTAT graphing is usually easy to use, such as when repeating many basic graphs for numerous parameters (e.g., automatically preparing probability plots for all constituents measured). However, fine-tuning the graphs was not straightforward in the earlier versions of SYSTAT. All data are entered (or imported) in spreadsheet-like tables, making large-scale analyses using very large data matrices easy. Numerous data transformations are also available. A very large number of options are usually available for each statistical tool, but basic setups are typically suitable for most analyses. The comprehensive documentation contains a great deal of information and some guidance, but the user should be reasonably knowledgeable about the techniques selected (as in all computer-based statistical programs).

SigmaStat and SigmaPlot

These programs were originally developed and distributed by Jandel Scientific, but are now owned and updated by SPSS, Inc., Chicago, IL. Probably the most important feature of these programs is their ease of use, especially the built-in guidance and evaluation of data pertaining to the selection of the most appropriate statistical procedure. In addition, it is easy to produce final publication-quality graphs. Exploratory data analysis is especially well covered by these programs.

Although not as comprehensive as some of the other available statistical programs (such as SYSTAT), SigmaStat and SigmaPlot offer complementary strengths. Recent versions include tests for comparing two or more groups (parametric and nonparametric tools), comparing reseated measures of the same individuals, comparing frequencies, rates, and proportions, prediction and correlation, computing power and sample size, and nonlinear regressions. Numerous data transformations are also available, and the data are also imported and managed in a spreadsheet-like table. SigmaPlot is a standalone program that can also be integrated with SigmaStat, offering a powerful data analysis package. The numerous graphing display options make for a very powerful and flexible tool, but also make it somewhat more difficult to prepare routine plots. However, they also offer several graphing templates for exploratory data analysis based on Tufte's excellent book (*The Visual Display of Quantitative Information*, Graphics Press, Cheshire, CT. 1983).

SAS

This statistical program is available from SAS Institute and covers a wide variety of statistical procedures. Although it is generally considered to emphasize business applications (such as database marketing, customer relationship management, clinical trials, quality improvement, fraud detection, etc.), it is also commonly used by researchers from all technical areas. This is one of the best supported statistical software packages, backed by many independent reference books covering SAS programming language for custom applications to specific statistical topics. It does require some training for most users. Because of its long history in academic computer centers, most users will be able to find assistance from experienced SAS users on most university campuses.

SPSS

SPSS, from SPSS, Inc., Chicago, IL, has also long been popular in the academic world and enjoys wide support. It also has numerous independently written books available for reference. The basic module contains the data management utilities, numerous basic statistical tools and graphs, and demographic analyses. Several add-on options are also available: advanced models for complex relationships, regression models, tables, and trends for forecasting. Again, a user should have little trouble finding assistance with this comprehensive program from university-based statistical consultants.

Statistica

Statistica, from StatSoft, of Tulsa, OK, is quickly gaining favor among environmental scientists for its ease of use, comprehensive set of tools, and intensive graphically based options. There are several levels of the program — student versions, a "quick" version, and the "full" version and associated optional add-on packages, including one for designs of experiments and another for quality control charts. Statistica's background was in basic statistical software written specifically for and by social science researchers using microcomputers. The ease of use and ease of interpretation objectives of the early programs are still very much evident in the current Windows- and Macintosh-based versions.

A great public service from StatSoft is the *Downloadable Electronic Statistics Textbook* (StatSoft, Inc. Tulsa, OK, 1999). This text can be used on-line through their web page, or downloaded to a local hard drive. According to StatSoft, this textbook offers training in the understanding and application of statistics. The material was developed at the StatSoft R&D department based on many years of teaching undergraduate and graduate statistics courses and covers a wide variety of applications, including laboratory research (biomedical, agricultural, etc.), business statistics and forecasting, social science statistics and survey research, data mining, engineering, and quality control applications. The *Electronic Textbook* begins with an overview of relevant elementary

concepts and continues with more in-depth explorations of specific areas of statistics representing classes of analytical techniques. A glossary of statistical terms and a list of references for further study are also included. The text requires about 30 min to download at 28.8 Kbps from http://www.statsoftinc.com/textbook/stathome.html. The textbook is lengthy and covers many subjects, and it is very well written for novice statistical software users. Surprisingly, however, it has few numeric examples, although it contains numerous graphical outputs from Statistica.

Selection of Statistical Procedures

Most of the objectives of receiving water studies can be examined through the use of relatively few statistical evaluation tools. The following briefly outlines some simple experimental objectives and a selected number of statistical tests (and their data requirements) that can be used for data evaluation.

Statistical Power

Type 1 and Type 2 errors (along with statistical confidence and power) are discussed in Chapter 5 in the experimental design section. Errors in decision making are usually divided into Type 1 (α: alpha) and Type 2 (β: beta) errors:

α (alpha) (Type 1 error) — a false positive, or assuming something is true when it is actually false. An example would be concluding that a tested water was adversely contaminated, when it was actually clean. The most common value of α is 0.05 (accepting a 5% risk of having a Type 1 error). Confidence is $1 - \alpha$, or the confidence of not having a false positive.

β (beta) (Type 2 error) — a false negative, or assuming something is false when it is actually true. An example would be concluding that a tested water was clean when it was actually contaminated. If the sample was an effluent, it would therefore be an illegal discharge with the possible imposition of severe penalties from a regulatory agency. In most statistical tests, β is usually ignored (if ignored, β is 0.5). If it is considered, a typical value is 0.2, implying accepting a 20% risk of having a Type 2 error. Power is $1 - \beta$, or the certainty of not having a false negative.

When evaluating data using a statistical test, power is the sensitivity of the test for rejecting the hypothesis. For an ANOVA test, it is the probability that the test will detect a difference among the groups if a difference really exists.

Comparison Tests

Probably the most common situation is to compare data collected from different locations, or seasons. Comparison of test with reference sites, of influent with effluent, of upstream with downstream locations, for different seasons of sample collection, of different methods of sample collection can all be made with comparison tests. If only two groups are to be compared (above/below; in/out; test/reference), then the two group tests can be used effectively, such as the simple Student's *t*-test or nonparametric equivalent. If the data are collected in "pairs," such as concurrent influent and effluent samples, or concurrent above and below samples, then the more powerful and preferred paired tests can be used. If the samples cannot be collected to represent similar conditions (such as large physical separation in sampling location, or different time frames), then the independent tests must be used.

If multiple groupings are used, such as from numerous locations along a stream, but with several observations from each location; or from one location, but for each season, then a one-way ANOVA is needed. If one has seasonal data from each of the several stream locations for multiple seasons, a two-way ANOVA can be used to investigate the effects of location, season, and the interaction of location and season together. Three-way ANOVA tests can be used to investigate another dimension of the data (such as contrasting sampling methods or weather for

the different seasons at each of the sampling locations), but that would obviously require substantially more data to represent each condition. (See the discussion on stratified random sampling in Chapter 5, for example.)

There are various data characteristics that influence which specific statistical test can be used for comparison tests. The parametric tests require the data to be normally distributed and that the different data groupings have the same variance, or standard deviation (checked with probability plots and appropriate test statistics for normality, such as the Kolmogorov–Smirnov one-sample test, the chi-square goodness of fit test, or the Lilliefors test). If the data do not meet the requirements for the parametric tests, the data may be transformed to better meet the test conditions (such as taking the $\log_{10}$ of each observation and conducting the test on the transformed values). The nonparametric tests are less restrictive, but are not free of certain requirements. Even though the parametric tests have more statistical power than the associated nonparametric tests, they lose any advantage if inappropriately applied. If uncertain, nonparametric tests should be used.

A few example statistical tests (as available in SigmaStat, SPSS, Inc.) are indicated below for different comparison test situations:

- Two groups
  - Paired observations
    - Parametric tests (data require normality and equal variance)
      - – Paired Student's *t*-test (more power than nonparametric tests)
    - Nonparametric tests
      - – Sign test (no data distribution requirements, some missing data accommodated)
      - – Friedman's test (can accommodate a moderate number of "nondetectable" values, but no missing values are allowed
      - – Wilcoxon signed rank test (more power than sign test, but requires symmetrical data distributions)
  - Independent observations
    - Parametric tests (data require normality and equal variance)
      - – Independent Student's *t*-test (more power than nonparametric tests)
    - Nonparametric tests
      - – Mann–Whitney rank sum test (probability distributions of the two data sets must be the same and have the same variances, but do not have to be symmetrical; a moderate number of "nondetectable" values can be accommodated)
- Many groups (use multiple comparison tests, such as the Bonferroni *t*-test, to identify which groups are different from the others if the group test results are significant).
  - Parametric tests (data require normality and equal variance)
    - One-way ANOVA for single factor, but for >2 "locations" (if 2 locations, use Student's *t*-test)
    - Two-way ANOVA for two factors simultaneously at multiple "locations"
    - Three-way ANOVA for three factors simultaneously at multiple "locations"
    - One factor repeated measures ANOVA (same as paired *t*-test, except that there can be multiple treatments on the same group)
    - Two factor repeated measures ANOVA (can be multiple treatments on two groups)
  - Nonparametric test
    - Kurskal–Wallis ANOVA on ranks (use when samples are from non-normal populations or the samples do not have equal variances)
    - Friedman repeated measures ANOVA on ranks (use when paired observations are available in many groups)
  - Nominal observations of frequencies (used when counts are recorded in contingency tables)
    - Chi-square (χ^2) test (use if more than two groups or categories, or if the number of observations per cell in a 2×2 table are > 5)
    - Fisher Exact test (use when the expected number of observations is <5 in any cell of a 2×2 table)
    - McNamar's test (use for a "paired" contingency table, such as when the same individual or site is examined both before and after treatment)

Data Associations and Model Building

These activities are an important component of the "weight-of-evidence" approach used to identify likely cause-and-effect relationships. The following list illustrates some of the statistical tools (as available in SigmaStat and/or SYSTAT, SPSS, Inc.) that can be used for evaluating data associations and subsequent model building:

- Data Associations
  - Simple
    - Pearson Correlation (residuals, the distances of the data points from the regression line, must be normally distributed. Calculates correlation coefficients between all possible data variables. Must be supplemented with scatterplots, or scatterplot matrix, to illustrate these correlations. Also identifies redundant independent variables for simplifying models)
    - Spearman Rank Order Correlation (a nonparametric equivalent to the Pearson test)
  - Complex (typically only available in advanced software packages)
    - Hierarchical Cluster Analyses (graphical presentation of simple and complex interrelationships. Data should be standardized to reduce scaling influence. Supplements simple correlation analyses)
    - Principal Component Analyses (identifies groupings of parameters by factors so that variables within each factor are more highly correlated with variables in that factor than with variables in other factors. Useful to identify similar sites or parameters).
- Model building/equation fitting (these are parametric tests and the data must satisfy various assumptions regarding behavior of the residuals)
  - Linear equation fitting (statistically-based models)
    - Simple linear regression ($y = b_0 + b_1x$, with a single independent variable, the slope term, and an intercept. It is possible to simplify even further if the intercept term is not significant)
    - Multiple linear regression ($y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + \ldots + b_kx_k$, having k independent variables. The equation is a multidimensional plane describing the data)
    - Stepwise regression (a method generally used with multiple linear regression to assist in identifying the significant terms to use in the model)
    - Polynomial regression ($y = b_0 + b_1x^1 + b_2x^2 + b_3x^3 + \ldots + b_kx^k$, having one independent variable describing a curve through the data)
  - Nonlinear equation fitting (generally developed from theoretical considerations)
    - Nonlinear regression (a nonlinear equation in the form: $y = b^x$, where x is the independent variable. Solved by iteration to minimize the residual sum of squares)
- Data trends
  - Graphical methods (simple plots of concentrations vs. time of data collection)
  - Regression methods (perform a least-squares linear regression on the above data plot and examine ANOVA for the regression to determine if the slope term is significant. Can be misleading due to cyclic data, correlated data, and data that are not normally distributed)
  - Mann–Kendall test (a nonparametric test that can handle missing data and trends at multiple stations. Short-term cycles and other data relationships affect this test and must be corrected)
  - Sen's estimator of slope (a nonparametric test based on ranks closely related to the Mann–Kendall test. It is not sensitive to extreme values and can tolerate missing data).
  - Seasonal Kendall test (preferred over regression methods if the data are skewed, serially correlated, or cyclic. Can be used for data sets having missing values, tied values, censored values, or single or multiple data observations in each time period. Data correlations and dependence also affect this test and must be considered in the analysis)

COMMENTS ON SELECTED STATISTICAL ANALYSES FREQUENTLY APPLIED TO RECEIVING WATER DATA

Determination of Outliers

Outliers in data collection can be recognized in the tails of the probability distributions. Observations that do not perfectly fit the probability distributions in the tails are commonly

considered outliers. They can be either very low or very high values. These values always attract considerable attention because they don't fit the mathematical probability distributions exactly and are usually assumed to be flawed and are then discarded. Certainly, these values (like any other suspect values) require additional evaluation to confirm that simple correctable errors (transcription, math, etc.) are not responsible. If no errors are found, then these values should be included in the data analyses because they represent rare conditions that may be very informative.

Analytical results less than the practical quantification limit (PQL) or the method detection limit (MDL) need to be flagged, but the result (if greater than the instrument detection limit, or IDL) should still be used in most of the statistical calculations. In some cases, the statistical test procedures can handle some undetected values with minimal modifications. In most cases, however, commonly used statistical procedures behave badly with undetected values. In these cases, results less than the IDL should be treated according to Berthouex and Brown (1994). Generally, the statistical procedures should be used twice, once with the less than detection values (LDV) equal to zero, and again with the LDV equal to the IDL. This procedure will determine if a significant difference in conclusions would occur with handling the data in a specific manner. In all cases of substituting a single value for LDV, the variability is artificially reduced, which can significantly affect comparison tests. It may therefore be best to use the actual instrument reported value for many statistical tests, even if it is below the IDL or MDL. This value may be considered a random value, but it is probably closer to the true value than a zero or other arbitrary value, plus it retains some aspects of the variability of the data sets. Of course, these values should not be "reported" in the project report, or to a regulatory agency, as they obviously do not meet the project QA/QC requirements.

Similarly, unusually high values need to be examined critically to identify possible errors. In most cases, the sample should be reanalyzed. This is a good reason to retain any "left over" sample until satisfied with the results. Of course, long-stored samples may not be very representative of actual conditions for many constituents, so care will have to be taken when using these reanalyzed values if they exceeded the recommended storage periods. It is difficult to reject wet-weather constituent observations solely because they are unusually high, as wet weather flows can easily have wide-ranging constituent observations. High values should not automatically be considered as outliers and therefore worthy of rejection, but as rare and unusual observations that may shed some light on the problem.

Exploratory Data Analyses

Exploratory data analysis (EDA) is an important tool to quickly review available data before a specific data collection effort is initiated. It is also an important first step in summarizing collected data to supplement the specific data analyses associated with the selected experimental designs. A summary of the data's variation is most important and can be presented using several simple graphical tools. *The Visual Display of Quantitative Information* (Tufte 1983) is a book with many examples of how to and how not to present graphical information. *Envisioning Information*, also by Tufte (1990), supplements his earlier book. Another important reference for basic analyses is *Exploratory Data Analysis* (Tukey 1977), which is the classic on this subject and presents many simple ways to examine data to find patterns and relationships. Cleveland (1993 and 1994) has also published two books related to exploratory data analyses: *Visualizing Data* and *The Elements of Graphing Data*. The basic plots described below can obviously be supplemented by many others presented in these books. Besides plotting the data, exploratory data analyses should always include corresponding statistical test results, if available.

Basic Data Plots

There are several basic data plots that need to be prepared as data are being collected and when all of the data are available. These plots are basically for QA/QC purposes and to demonstrate basic

data behavior. These basic plots include: time series plots (data observations as a function of time), control plots (generally the same as time series plots, but using control samples and with standard deviation bands, as described in Chapter 5), probability plots (described below), scatterplots (described below), and residual plots (needed for model building activity, especially for regression analyses, also described below).

Probability Plots

The most basic exploratory data analysis method is to prepare a probability plot of the available data. The plots indicate the possible range of the values expected, their likely probability distribution type, and the data variation. It is difficult to recommend another method that results in so much information using the available data. Histograms, for example, cannot accurately indicate the probability distribution type very accurately, but they more clearly indicate multimodal distributions. The observations are ranked in ascending order and probability values are calculated for each observation using the following formula:

$$P = (i - 0.5)/n$$

where i = the rank position
n = the total number of observations.

If 11 observations are available, the 6th ranked value would have a probability of 0.50 (50%), using the above formula. The values and corresponding probability positions are plotted on special normal-probability paper. This paper has a y-axis whose values are spread out for the extreme small and large probability values. When plotted on this paper, the values form a straight line if they are normally distributed (Gaussian). If the points do not form an acceptably straight line, they can then be plotted on log-normal probability paper (or the data observations can be log transformed and plotted on normal probability paper). If they form a straight line on the log-normal plot, then the data are log-normally distributed. Other data transformations are also possible for plotting on normal-probability paper, but these two (normal and log-normal) are usually sufficient for most receiving water analyses.

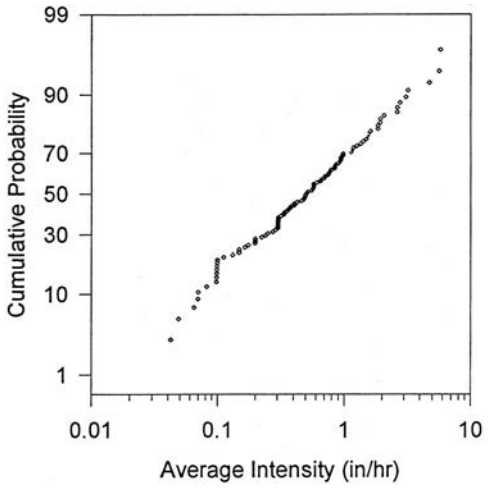

Figure 7.1 Log-normal probability distribution for 1976 Birmingham, AL, average rain intensities. (From Pitt, R. and S.R. Durrans. *Drainage of Water from Pavement Structures*. Alabama Department of Transportation. Research Project 930-275. Montgomery, AL. Sept. 1995.)

Figure 7.1 is an example of a probability plot of average rain intensity for Birmingham, AL, for 1976 (Pitt and Durrans 1995). This is a log-normal probability plot, as the rain intensity values are plotted on a log scale. These intensities plot along a reasonably straight line, indicating that they are generally log-normally distributed. Figure 7.2 shows three types of results that can be observed when plotting pollutant reduction observations on probability plots, using data collected at the Monroe St. wet detention pond in Madison, WI, by the USGS and the WI DNR. Figure 7.2a for suspended solids (particulate residue) shows that SS are effectively removed over a wide range of influent concentrations, ranging from 20 to over 1000 mg/L. A simple calculation of percentage reduction would not show this consistent removal over the wide range. In contrast, Figure 7.2b for total dissolved solids (filtered residue) shows poor removals of TDS for all concentration conditions, as expected for this wet detention pond. The average percentage

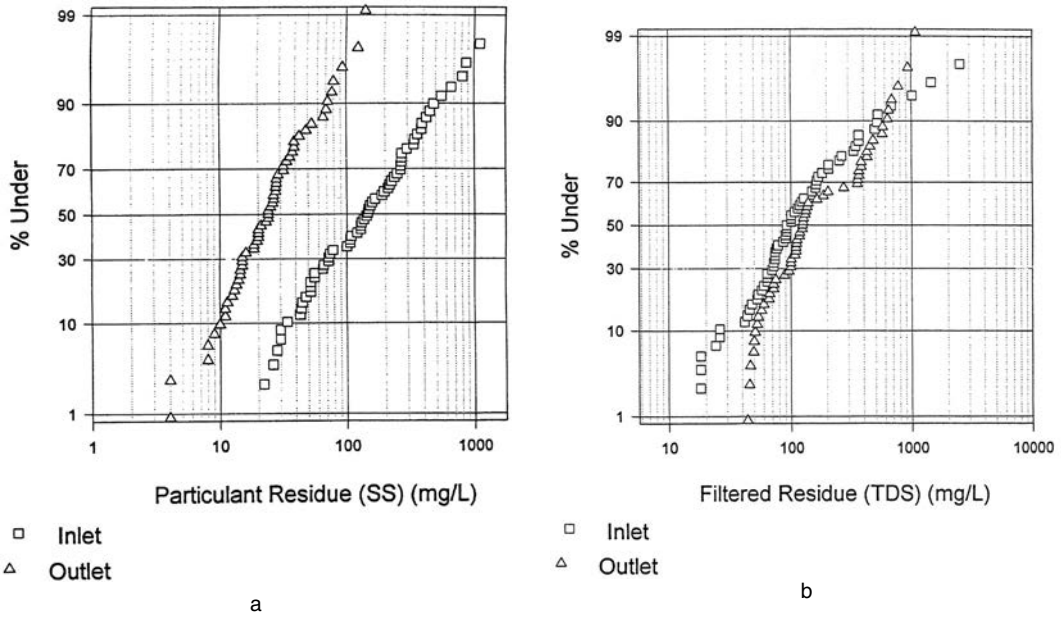

Figure 7.2 Influent and effluent observations for suspended solids, dissolved solids, and filtered COD at the Monroe St., Madison, WI, stormwater detention pond.

removal for TDS would be close to zero and no additional surprises are indicated on this plot. Figure 7.2c, however, shows a wealth of information that would not be available from simple numerical statistical summaries. In this plot, filtered COD is seen to be poorly removed for low concentrations (less than about 20 mg/L, but the removal increases substantially for higher concentrations. In addition, the COV of the effluent concentrations is much smaller than for the influent concentrations. Although not indicated on these plots, the rank order of concentrations was similar for both influent and effluent distributions for all three pollutants.

Generally, water quality observations do not form a straight line on normal probability paper, but do (at least from about the 10 to 90 percentile points) on log-normal probability paper. This indicates that the samples generally have a log-normal distribution, and many parametric statistical tests can probably be used, but only after the data are log-transformed. These plots indicate the central tendency (median) of the data, along with their possible distribution type and variance (the steeper the plot, the smaller the COV, and the flatter the slope of the plot, the larger the COV for the data). Multiple data sets can also be plotted on the same plot (such as for different sites, different seasons, different habitats, etc.) to indicate obvious similarities (or differences) in the data sets. Most statistical methods used to compare different data sets require that the sets have the same variances, and many require normal distributions. Similar variances would be indicated by generally parallel plots of the data on the probability paper, while normal distributions would be reflected by straight line plots on normal probability paper.

Probability plots should be supplemented with standard statistical tests that determine if the data are normally distributed. These tests (at least some are usually available in most software packages) include the Kolmogorov–Smirnov one-sample test, the chi-square goodness of fit test, and the Lilliefors variation of the Kolmogorov–Smirnov test. They basically are paired tests comparing data points from the best-fitted normal curve to the observed data. The statistical tests may be visualized by comparing the best-fitted normal curve data and the observed data plotted on normal probability paper. If the observed data cross the fitted curve data numerous times, it is much more likely to be normally distributed than if it only crossed the fitted curve a few times.

Digidot Plot

Berthouex and Brown (1994) point out that since the best way to display data is with a plot, it makes little sense to present the data in a table. They highly recommend a digidot plot, developed by Hunter (1988) based on Tukey (1977), as a basic presentation of characterization data. This plot indicates the basic distribution of the data, shows changes with time, and presents the actual values, all in one plot. A data table is therefore not needed in addition to the digidot plot. A stem and leaf plot of the data is presented as the y-axis, and the data are presented in a time series (in the order of collection) along the x-axis. Figure 7.3 is an example of a digidot plot, as presented by Berthouex and Brown (1994). The stem and leaf plot is constructed by placing the last digit of the value on the y-axis between the appropriate tic marks. In this example, the value 47 is represented with a 7 placed in the division between 45 and 50. Similarly, 33 is represented with a 3 placed in the division between 30 and 35. Values from 30 to 34 are placed between the 30 and 35 tic marks, while values from 35 to 39 are placed between the 35 and 40 tic marks. Simultaneously, the values are plotted in a time series in the order of collection. This plot can therefore be constructed in real time as the data are collected, and obvious trends can be noted with time. This plot also presents the actual numerical data that can also be used in later statistical analyses.

Scatterplots

According to Berthouex and Brown (1994), the majority of the graphs used in science are scatterplots. They stated that these plots should be made before any other analyses of the data are performed. Scatterplots are typically made by plotting the primary variable (such as a water quality constituent) against a factor that may influence its value (such as time, season, flow, another constituent like suspended solids, etc.). Figure 7.4 is a scatterplot showing COD values plotted against rain depth to investigate the possibility of a "first-flush," where higher concentrations are assumed to be associated with small runoff events (Pitt 1985). In this example, the smallest rains appear to have the highest COD concentrations associated with them, but the distribution of values is very wide. This may simply be a result of the much greater number of events having small rains and an increased likelihood of events having unusual observations when more observations are

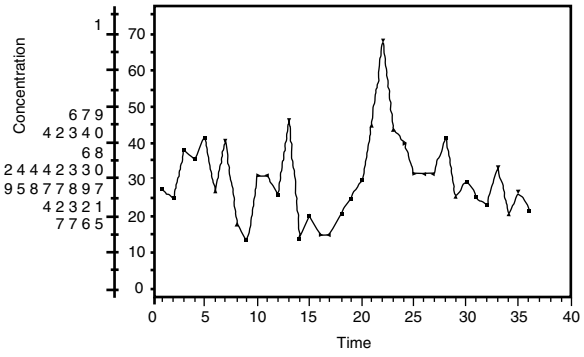

Figure 7.3 Digidot plot. (From Berthouex, P.M. and L.C. Brown. *Statistics for Environmental Engineers.* Lewis Publishers, Boca Raton, FL. 1994. With permission.)

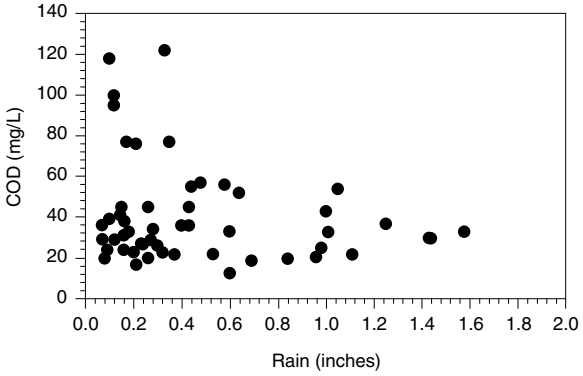

Figure 7.4 Scatterplot for Bellevue, WA, COD stormwater concentrations, by rain depth. (From Pitt, R. *Characterizing and Controlling Urban Runoff through Street and Sewerage Cleaning*. U.S. Environmental Protection Agency, Storm and Combined Sewer Program, Risk Reduction Engineering Laboratory. EPA/600/S2-85/038. PB 85-186500. Cincinnati, OH. 467 pp. June 1985.)

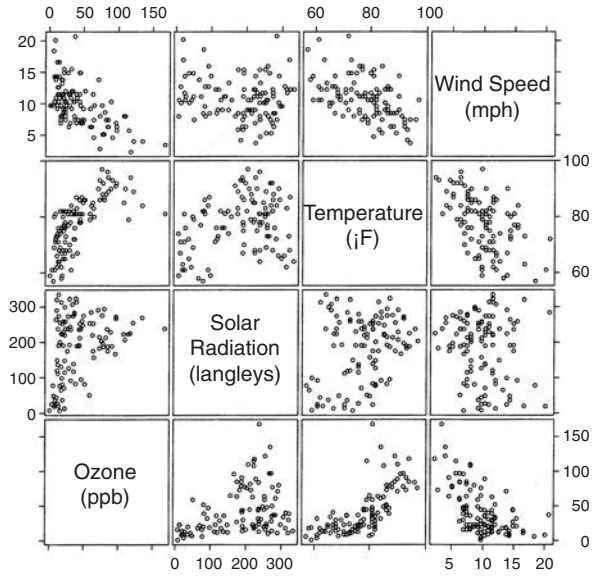

Figure 7.5 Grouped scatterplot for ozone, solar radiation, temperature, and wind speed. (From Cleveland, W.S. *The Elements of Graphing Data*. Hobart Press, Summit, NJ. 1994. With permission.)

made. When many data are observed for many sites, generally smaller rains do seem to be associated with the highest concentrations observed, but it is not a consistent pattern.

Grouped scatterplots (miniatures) of all possible combinations of constituents can be organized as in a correlation matrix (Figure 7.5; Cleveland 1994). This arrangement allows obvious relationships to be seen easily, and even indicates if the relationships are straight-lined or curvilinear. In this example, the highest ozone values occur on days having the highest temperatures, and the lowest ozone concentrations occur on days having brisk winds and low temperatures, for example.

Grouped Box and Whisker Plots

Another primary exploratory data analysis tool, especially when differences between sample groups are of interest, is the use of grouped box and whisker plots. Examples of their use include examining different sampling locations (such as above and below a discharge), influent and effluent of a treatment process, different seasons, etc. These plots indicate the range and major percentile locations of the data, as shown on Figure 7.6 (Pitt 1985). In this example, seasonal groupings of stormwater quality observations for COD (chemical oxygen demand) from Bellevue, WA, were plotted to indicate obvious differences in the values. If the 75 and 25 percentile lines of the boxes are higher or lower than the medians of other box and whisker plots, then the data groupings are likely significantly different (at least at the 95% level). When large numbers of data sets are plotted using box and whisker plots, the relative overlapping (or separation) of the plots can be used to

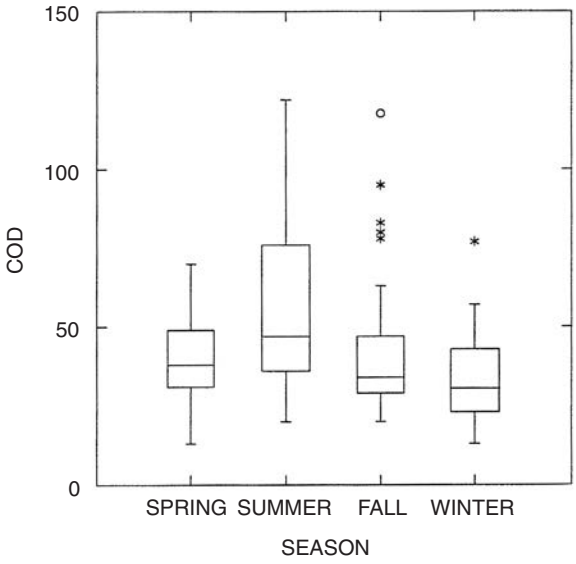

Figure 7.6 Grouped box and whisker plot for Bellevue, WA, COD stormwater concentrations, by season. (From Pitt, R. *Characterizing and Controlling Urban Runoff through Street and Sewerage Cleaning.* U.S. Environmental Protection Agency, Storm and Combined Sewer Program, Risk Reduction Engineering Laboratory. EPA/600/S2-85/038. PB 85-186500. Cincinnati, OH. 467 pp. June 1985.)

identify possible groupings of the separate sets. In this case, the winter has lower concentrations than the summer.

To supplement the visual presentation with the grouped box and whisker plots, a one-way ANOVA test (or the Kurskal–Wallis ANOVA on ranks test) should be conducted to determine if there are any statistically significant differences between the different boxes on the plot. ANOVA doesn't specifically identify which sets of data are different from others, however. A multiple comparison procedure (such as the Bonferroni *t*-test) can be used to identify significant differences between all cells if the ANOVA finds that a significant difference exists. Both of these tests (ANOVA and Bonferroni *t*-test) are parametric tests and require that the data be normally distributed. It may therefore be necessary to perform a log-transformation on the raw data. These tests will identify differences in sample groupings, but similarities (to combine data) are probably also important to know.

Comparing Multiple Sets of Data with Group Comparison Tests

Making comparisons of data sets is a fundamental objective of many receiving water investigations. Different habitats and seasons can produce significant affects on the observations. The presence of influencing factors, such as pollutant discharges or control practices, also affects the data observations. Berthouex and Brown (1994) and Gilbert (1987) present excellent summaries of the most common statistical tests that are used for these comparisons in environmental investigations. The significance of the test results (the α value, the confidence factor, along with the β value, the power factor, both discussed in Chapter 5) will indicate the level of confidence and power that the two sets of observations are the same. In most cases, an α level of less than 0.05 has been traditionally used to signify significant differences between two sets of observations, although this is an arbitrary criterion. In most cases, β is ignored (resulting in a default value of $1 - \beta$ of 0.5), although some use a $1 - \beta$ value of 0.8. An α value of 0.05 implies that the interpretation will be in error an average of 1 in 20 times. In some cases, this may be too conservative, while in others (such as where health and welfare implications are involved), it may be too liberal. The selection of the critical α value should be decided beforehand, while the calculated values for α should always be presented in the data evaluation (not simply stating that the results were significant or not significant at the 0.05 level, as is common). Even if the α level is significant, the magnitude of the difference, such as the pollutant reduction, may not be very important. The importance of the level of pollutant reductions should also be graphically presented using grouped box plots

indicating the range and variations of the concentrations at each of the sampling locations, as described previously.

Comparison tests are divided into simple tests between two groups (such as Student's *t*-test) and tests that examine larger numbers of groups and interactions (such as analysis of variance tests, or ANOVA).

Simple Comparison Tests with Two Groups

The main types of simple comparison tests are separated into independent and paired tests. These can be further separated into tests that require specific probability distribution characteristics (parametric tests) and tests that do not have as many restrictions based on probability distribution characteristics of the data (nonparametric data). If the parametric test requirements can be met, they should be used because they have more statistical power. However, if information concerning the probability distributions is not available, or if the distributions do not behave correctly, then the somewhat less powerful nonparametric tests should be used. Similarly, if the data gathering activity can allow for paired observations, they should be used preferentially over independent tests.

In many cases, observations cannot be related to each other, such as a series of observations at two locations during all of the rains during a season. Unless the sites are very close together, the rains are likely to vary considerably at the two locations, disallowing a paired analysis. However, if data can be collected simultaneously, such as at influent and effluent locations for a (rapid) treatment process, paired tests can be used to control all factors that may influence the outcome, resulting in a more efficient statistical analysis. Paired experimental designs ensure that uncontrolled factors basically influence both sets of data observations equally (Berthouex and Brown 1994).

The parametric tests used for comparisons are the Student's *t*-tests (both independent and paired *t*-tests). All statistical analysis software and most spreadsheet programs contain both of these basic tests. These tests require that the variances of the sample sets be the same and constant over the range of the values. These tests also require that the probability distributions be Gaussian (normal). Transformations can be used to modify the data sets to these conditions. Log-transformations can be used to produce Gaussian distributions of most water quality data. Square root transformations are also commonly used to make the variance constant over the data range, especially for biological observations (Sokal and Rohlf 1969). In all cases, it is necessary to confirm these requirements before the standard *t*-tests are used.

Nonparametrics: Statistical Methods Based on Ranks by Lehman and D'Abrera (1975) is a comprehensive general reference on nonparametric statistical analyses. Gilbert (1987) presents an excellent review of nonparametric alternatives to the Student's *t*-tests, especially for environmental investigations from which the following discussion is summarized. Even though the nonparametric tests remove many of the restrictions associated with the *t*-tests, the *t*-tests should be used if justifiable. Unfortunately, seldom are the Student's *t*-test requirements easily met with environmental data, and the slight loss of power associated with using the nonparametric tests is much more acceptable than misusing the Student's *t*-tests. Besides having few data distribution restrictions, many of the nonparametric tests can also accommodate a few missing data, or observations below the detection limits. The following paragraphs briefly describe the features of the nonparametric tests used to compare data sets.

Nonparametric Tests for Paired Data Observations

The sign test is the basic nonparametric test for paired data. It is simple to compute and has no requirements pertaining to data distributions. A few "not detected" observations can also be accommodated. Two sets of data are compared and the differences are used to assign a positive sign if the value in data set #1 is greater than the corresponding value in data set #2, or a negative sign is assigned if opposite. The number of positive signs are added and a statistical table (such as in Lehman

and D'Abrera 1975) is used to determine if the number of positive signs found is unusual for the number of data pairs examined.

The Mann–Whitney signed rank test has more power than the sign test, but it requires that the data distributions be symmetrical (but with no specific distribution type). Without transformations, this requirement may be difficult to justify for water quality data. This test requires that the differences between the data pairs in the two data sets be calculated and ranked before checking with a special statistical table (as in Lehman and D'Abrera 1975). In the simplest case for monitoring the effectiveness of treatment alternatives, comparisons can be made of inlet and outlet conditions to determine the level of pollutant removal and the statistical significance of the concentration differences. StatXact-Turbo (CYTEL, Cambridge, MA) is a microcomputer program that computes exact nonparametric levels of significance, without resorting to normal approximations. This is especially important for the relatively small data sets that will typically be evaluated during most environmental research activities.

Friedman's test is an extension of the sign test for several related data groups. There are no data distribution requirements and the test can accommodate a moderate number of "nondetectable" values, but no missing values are allowed.

Nonparametric Tests for Independent Data Observations

As for the *t*-tests, paired test experimental designs are superior to independent designs for nonparametric tests because of their ability to cancel out confusing properties. However, paired experiments are not always possible, requiring the use of independent tests. The Wilcoxon rank sum test is the basic nonparametric test for independent observations. The test statistic is also easy to compute and compare to the appropriate statistical table (as in Lehman and D'Abrera 1975). The Wilcoxon rank sum test requires that the probability distributions of the two data sets be the same (and therefore have the same variances). There are no other restrictions on the data distributions (they do not have to be symmetrical, for example). A moderate number of "nondetectable" values can be accommodated by treating them as ties.

The Kruskal–Wallis test is an extension of the Mann–Whitney rank sum test and allows evaluations of several independent data sets, instead of just two. Again, the distributions of the data sets must all be the same, but they can have any shape. A moderate number of ties and nondetectable values can also be accommodated.

Comparisons of Many Groups

If there are more than two groups of data to be compared (such as in-stream concentrations at several locations along a river, each with multiple observations), one of the analysis of variance, or ANOVA, tests should be used. The commonly available one-way, two-way, and three-way ANOVA tests are parametric tests and require that the data in each grouping be normally distributed and that the variances be the same in each group. This can be visually examined by preparing a probability plot for the data in each group displayed on the same chart. The probability plots would need to be parallel and straight. Obviously, log transformations of the data can be used if assumptions are met when the data is plotted using log-normal probability axes. In Figure 7.2a, the influent and effluent probability plots for suspended solids at the Monroe St. wet detention pond site in Madison, WI, the probability plots are reasonably parallel and straight when plotted as log-normal plots. However, Figure 7.2c, a similar plot for dissolved COD, indicates that the plots are not parallel. Of course, these figures contain only two groupings of data (influent and effluent), and one of the previous two-group tests would be more efficient for this data.

If data from multiple stations along a river were collected during different seasons, it would be possible to use the two-way ANOVA test to examine the effects of different seasons and different locations, along with the interaction of these parameters. Three-way ANOVA tests can be used to evaluate the results of similar field sampling data (different locations, different seasons) and another factor, such as natural vs. artificial substrate samplers for benthic macroinvertebrates (or seining

vs. electroshocking for fish sampling). These tests would then indicate if the results from these different sampling procedures varied significantly by season, or sampling location. These analyses are more flexible than the factorial tests described earlier in Chapter 5, as the factorial tests are most commonly only used for two levels (such as winter vs. summer; pools vs. riffles; and artificial substrate vs. natural substrate samplers). Factorial tests are more complicated when intermediate, or more than 2 levels, are being considered. However, the ANOVA tests are parametric tests and require multiple observations in each group, while the factorial tests are not and can be used with single observations per group (although that may not be a good idea considering the expected high variability in most environmental sampling).

A nonparametric test, usually included in statistical programs for comparing many groups, is the Kruskal–Wallis ANOVA on ranks test. This is only a one-way ANOVA test and is only suitable for comparing data from different sampling sites alone, for example. This would be a good test to supplement grouped box and whisker plots.

Grouped comparison tests indicate only that at least one of the groups is significantly different from at least one other, they do not indicate which ones. For that reason, some statistical programs also conduct multiple comparison tests. SigmaStat, for example, offers: the Tukey test, Student–Newman–Keuls test, Bonferroni *t*-test, Fisher's LDS, Dunner's test, and Duncan's multiple range test. These tests basically conduct comparisons of each group against each other group and identify which are different.

Data Associations

Identifying patterns and associations in data may be considered a part of exploratory data analyses, but many of the tools (especially cluster, principal component, and factor analyses) may require specialized procedures having multiple data handling options that are not available in all statistical software packages, while some (such as correlation matrices discussed here) are commonly available.

Identifying data associations, and possible subsequent model building, is another area of interest to many investigators examining receiving water conditions. This is a critical component of the "weight-of-evidence" approach for identifying possible cause and effect relationships. The following are possible steps for investigating data associations:

1. Reexamine the hypothesis of cause and effect (an original component of the experimental design previously conducted and the basis for the selected sampling activities).
2. Prepare preliminary examinations of the data, as described previously (most significantly, prepare scatterplots and grouped box/whisker plots).
3. Conduct comparison tests to identify significant groupings of data. As an example, if seasonal factors are significant, then cause and effect may vary for different times of the year.
4. Conduct correlation matrix analyses to identify simple relationships between parameters. Again, if significant groupings were identified, the data should be separated into these groupings for separate analyses, in addition to an overall analysis.
5. Further examine complex interrelationships between parameters by possibly using combinations of hierarchical cluster analyses, principal component analyses (PCA), and factor analyses.
6. Compare the apparent relationships observed with the hypothesized relationships and with information from the literature. Potential theoretical relationships should be emphasized.
7. Develop initial models containing the significant factors affecting the parameter outcomes. Simple apparent relationships between dependent and independent parameters should lead to reasonably simple models, while complex relationships will likely require further work and more complex models.

The following sections briefly describe these tools and present some interesting examples of their use.

Correlation Matrices

Knowledge of the correlations between data elements is very important in many environmental data analysis efforts. They are especially important when model building, such as with regression analysis. When constructing a model, it is important to include the important factors in the model, but the factors should be independent. Correlation analyses can assist by identifying the basic structure of the model.

Table 7.1 (Pitt 1987) is a standard correlation matrix that shows the relationships between measured rain and measured runoff parameters. This is a common Pearson correlation matrix, constructed using the microcomputer program SYSTAT (SPSS, Inc., Chicago, IL). It measures the strength of association between the variables. The Pearson correlation coefficients vary from –1 to +1. A coefficient of 0 indicates that neither of the two variables can be predicted from the other using a linear equation, while values of –1 or +1 indicate that perfect predictions can be made of one variable by only using the other variable. This example shows several very high correlations between pairs of parameters (>0.9). The paired parameters having high correlations are the same for both sites, possibly indicating the same basic processes for rainfall-runoff. High correlations are seen between total runoff depth (RUNTOT) and rain depth (RAINTOT) and between runoff duration (RUNDUR) and rain duration (RAINDUR).

It is very important not to confuse correlation with causation. Box et al. (1978) presents a historical example of a plot (Figure 7.7) of the population of Oldenburg, Germany, against the number of storks observed in each year. In this example, few would conclude that the high correlation between the increased number of storks observed and the simultaneous increase in population is a cause and effect relationship. The two variables observed are most likely related to another factor (such as time in this example, as both sets of populations increased over the years from 1930 to 1936). However, many investigators make similar improper assumptions of cause and effect from their observations, especially if high correlations are found. It is extremely important that theoretical knowledge of the system being modeled be considered. If this knowledge is meager, then specific tests to directly investigate cause and effect relationships must be conducted.

Hierarchical Cluster Analyses

Another method to examine correlations between measured parameters is by using hierarchical cluster analyses. Figure 7.8 (Pitt 1987) is a tree diagram (dendogram) produced by SYSTAT using the same data as presented in the correlation matrix. A tree diagram illustrates simple and complex correlations between parameters. Parameters with short branches linking them are more closely correlated than parameters linked by longer branches. In addition, the branches can encompass more than just two parameters. The length of the short branches linking only two parameters is indirectly comparable to the correlation coefficients (short branches signify correlation coefficients close to 1). The main advantage of a cluster analyses is the ability to identify complex correlations that cannot be observed using a simple correlation matrix. In this example, the rain total — runoff total and runoff duration — rain duration high correlation coefficients found previously are also seen to have simple relationships. In contrast, predicting peak runoff rates (PEAKDIS) requires more complex information. Therefore, the model used to predict peak runoff would have to be more complex, requiring additional information than required to merely predict total runoff.

Principal Component Analyses (PCA) and Factor Analyses

Another important tool to identify relationships and natural groupings of samples or locations is with principal component analyses (PCA). Normally, data are autoscaled before PCA in order to remove the artificially large influence of constituents having large values compared to constituents having small values. PCA is a sophisticated procedure where information is sorted to determine

Table 7.1 Pearson Correlation Matrix

	RAINTOT	RAINDUR	AVEINT	PEAKINT	DRYPER	RUNTOT	RUNDUR	AVEDIS	PEAKDIS	LAG
Emery (Industrial)										
RAINTOT	1.000									
RAINDUR	0.533	1.000								
AVEINT	0.138	–0.387	1.000							
PEAKINT	0.512	–0.039	0.675	1.000						
DRYPER	0.169	0.273	–0.096	–0.132	1.000					
RUNTOT	**0.906**	0.562	0.007	0.405	0.075	1.000				
RUNDUR	0.501	**0.965**	–0.348	0.035	0.184	0.556	1.000			
AVEDIS	0.709	–0.013	0.480	0.654	–0.095	0.680	–0.026	1.000		
PEAKDIS	0.729	0.129	0.372	**0.748**	0.041	0.699	0.150	**0.849**	1.000	
LAG	0.135	0.220	–0.217	–0.217	0.052	0.205	0.134	0.098	0.107	1.000
Thistledowns (Residential/Commercial)										
RAINTOT	1.000									
RAINDUR	0.553	1.000								
AVEINT	0.321	–0.295	1.000							
PEAKINT	0.564	–0.104	**0.827**	1.000						
DRYPER	0.281	0.308	–0.190	–0.122	1.000					
RUNTOT	**0.903**	0.448	0.187	0.551	0.283	1.000				
RUNDUR	0.508	**0.989**	–0.322	–0.148	0.337	0.402	1.000			
AVEDIS	0.398	–0.178	0.593	0.817	–0.037	0.585	–0.227	1.000		
PEAKDIS	0.600	–0.051	0.659	**0.917**	0.009	0.702	–0.106	**0.946**	1.00	
LAG	–0.192	–0.037	–0.114	–0.202	–0.122	–0.184	–0.094	–0.138	–0.173	1.000

From Pitt, R. *Small Storm Urban Flow and Particulate Washoff Contributions to Outfall Discharges*. Ph.D. dissertation submitted to the Department of Civil and Environmental Engineering, University of Wisconsin, Madison. 1987.

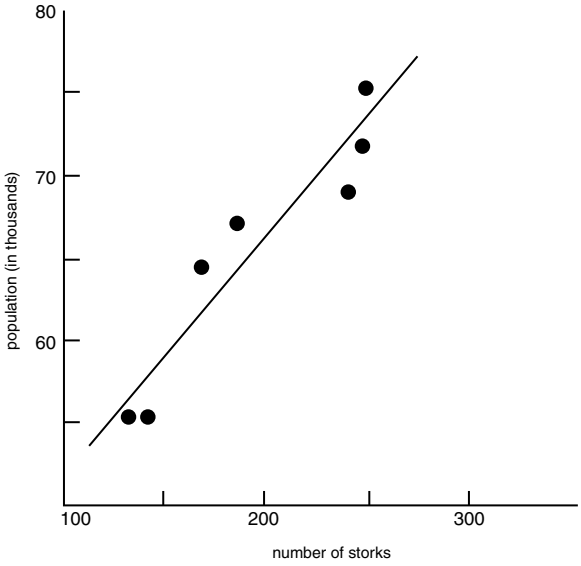

Figure 7.7 Possible cause and effect confusion from correlation tests. (From Box, G.E.P., W.G. Hunter, and J.S. Hunter. *Statistics for Experimenters*. John Wiley & Sons. New York. Copyright © 1978. John Wiley & Sons. This material is used by permission of John Wiley & Sons, Inc.)

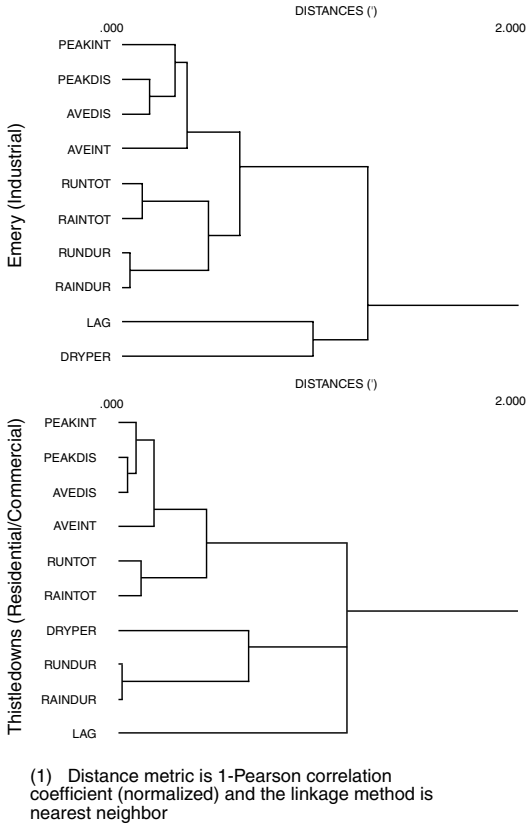

Figure 7.8 Tree diagram from cluster analyses of Toronto rainfall and runoff parameters. (From Pitt, R. *Small Storm Urban Flow and Particulate Washoff Contributions to Outfall Discharges*. Ph.D. dissertation submitted to the Department of Civil and Environmental Engineering, University of Wisconsin, Madison. 1987. With permission.)

	%	cum		%	cum
PC1	75.4	75.4	PC3	5.2	89.4
PC2	8.8	84.2	PC4	3.8	93.2

%, percent of variance; cum, cumulative variance.

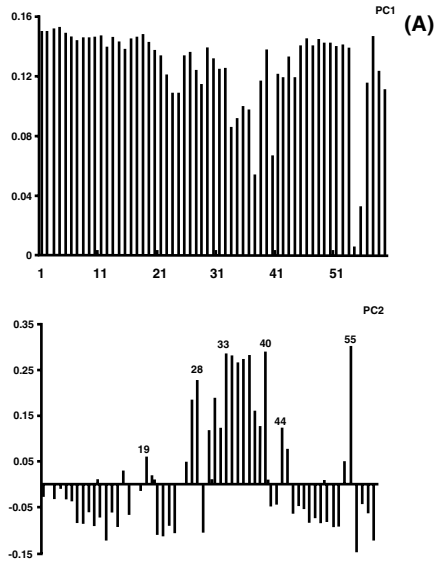

Figure 7.9A Loadings of principal components. (Reprinted with permission from Salau, J.S., R. Tauler, J.M. Bayona, and I. Tolosa. Input characterization of sedimentary organic contaminants and molecular markers in the northwestern Mediterranean Sea by exploratory data analysis. *Environ. Sci. Technol.*, Vol. 31, No. 12, pp. 3482. 1997. Copyright 1997. American Chemical Society.)

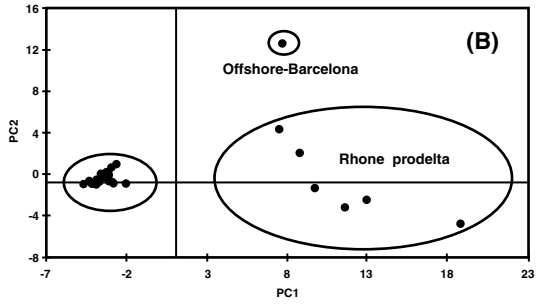

Figure 7.9B Score plots of principal components. (Reprinted with permission from Salau, J.S., R. Tauler, J.M. Bayona, and I. Tolosa. Input characterization of sedimentary organic contaminants and molecular markers in the northwestern Mediterranean Sea by exploratory data analysis. *Environ. Sci. Technol.*, Vol. 31, No. 12, pp. 3482. 1997. Copyright 1997. American Chemical Society.)

the components (usually constituents) needed to explain the variance of the data. Typically, very large numbers of constituents are available for PCA analyses and a relatively small number of sample groups are to be identified. Salau et al. (1997) used PCA (and then cluster analyses) to identify characteristics of sediment off Spain. Figure 7.9A shows the first two component loadings (collectively comprising most of the information) for 59 constituents. The first principal component (PC1) is seen to be a near reversed image of the second principal component (PC2) (if a constituent is very important in one PC, it should be much less important in the other). Figure 7.9B shows a scatterplot of PC1 vs. PC2 values for different sample locations, showing how there are three main groups of samples, which generally correspond to two sampling areas, plus a third group. The third

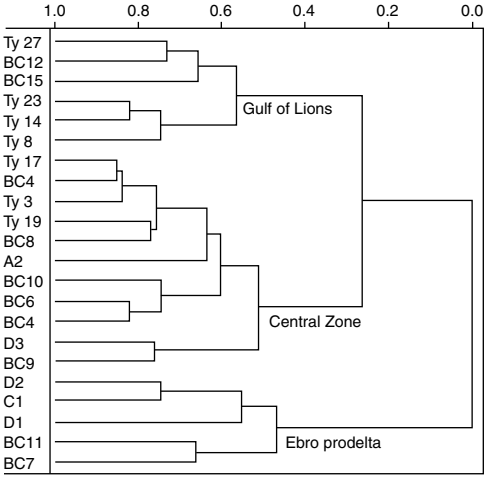

Figure 7.10 Dendogram of data, without two major groupings. (Reprinted with permission from Salau, J.S., R. Tauler, J.M. Bayona, and I. Tolosa. Input characterization of sedimentary organic contaminants and molecular markers in the northwestern Mediterranean Sea by exploratory data analysis. *Environ. Sci. Technol.*, Vol. 31, No. 12, pp. 3482. 1997. Copyright 1997. American Chemical Society.)

group was then further analyzed using cluster analysis to examine more complex groupings and sampling subareas, as shown in the dendogram of Figure 7.10.

Regression Analyses

Requirements for the Use of Regression Analyses

Regression analyses are a very popular, but commonly misused, statistical analysis tool. All statistical packages and most spreadsheets contain regression analysis routines. An excellent reference for regression analysis is *Applied Regression Analysis* by Draper and Smith (1981), while Berthouex and Brown (1994) have extensive discussions concerning misapplications and suggestions for proper use of regression analysis.

Regression analyses are best used to fit data to a theoretically derived equation that has some physical meaning. Theoretically derived equations often result in a nonlinear equation that cannot be evaluated using standard regression procedures, and many of the statistical programs available do not have any, or have only limited, nonlinear regression capabilities. Nonlinear regression analyses require assumptions and analyses steps similar to the more conventional regression analyses. Statistically based models (such as are common with stepwise regression or multiparameter polynomial regression equations) are very important and useful for many applications, but they are more limited in their transferability to other conditions and do not result in as useful understandings of the processes.

Regression models are most commonly misused when used to establish cause and effect, as illustrated in Figure 7.7, which showed an excellent correlation between stork and human populations. As described in Chapter 8, a weight-of-evidence approach (independent evaluations with a preponderance of supporting data) is typically needed to establish confidence in a proposed cause and effect relationship. Regression analyses are important components of most weight-of-evidence approaches, but they should not be overly relied upon. Besides these basic problems in objectives for conducting the test and in interpretation of regression analyses, many apply regression analyses improperly.

The following steps should be followed when conducting a regression (curve-fitting or model building) analysis:

1. Formulate the objectives of the curve-fitting exercise (a subset of the experimental design previously conducted).
2. Prepare preliminary examinations of the data, as described previously. (Most significantly, prepare scatterplots and probability plots of the data, plus correlation evaluations to examine independence between multiple parameters that may be included in the models.)

3. Identify alternative models from the literature that have been successfully applied for similar problems (part of the previously conducted experimental design activities in order to identify which parameters to measure, or to modify or control).
4. Evaluate the data to ensure that regression is applicable and make suitable data transformations.
5. Apply regression procedures to the selected alternative models.
6. Evaluate the regression results by examining the coefficient of determination (R^2) and the results of the analysis of variance of the model (standard error analyses and probability values for individual equation parameters and overall model).
7. Conduct an analysis of the residuals (as described below).
8. Evaluate the results and select the most appropriate model(s).
9. If not satisfied, it may be necessary to examine alternative models, especially those based on data patterns (through cluster analyses and principal component analyses) and to reexamine and modify the theoretical basis of existing models. Statistically based models can be developed using stepwise regression routines.

The following discussion presents the necessary assumptions and proper verification steps needed when using regression analyses. Draper and Smith (1981) list the following requirements for proper use of regression analyses:

- The residuals are independent
- The residuals have zero mean
- The residuals have a constant variance (σ^2)
- The residuals have a normal distribution (required for making F-tests)

Residuals are the unexplained variation of a model and are calculated as the differences between what is actually observed and what is predicted by the model (equation). Examination of the residuals should confirm if the fitted model is correct. The easiest method to confirm residual behavior is through graphical analyses, as described below. The examination of residuals applies to any model situation, not just regression models.

The Need for Graphical Analyses of Residuals

In all cases, graphical analysis of model residuals is necessary to confirm most of these requirements and to verify the use of the model. Berthouex and Brown (1994) list the following required residual graphical analyses for a regression model:

- Check for normality of the residuals (preferably by constructing a probability plot on normal probability paper and having the residuals form a straight line, or at least use an overall plot, as in Figure 7.11a).
- Plot the residuals against the predicted values (Figure 7.11b).
- Plot the residuals against the predictor variables (similar to Figure 7.11b).
- Plot the residuals against time in the order the measurements were made (Figure 7.11c).

Examples of these plots are shown in Figure 7.11 (Draper and Smith 1981) and in Figure 7.12 (Pitt 1987). The residuals need to be random and have the same variance for all these plots, as indicated in Figure 7.12a. If the residuals spread out (as in Figure 7.12b), then data transformations or a weighted least-squares analysis may be needed. If a trend is evident (as in Figure 7.12c), then a linear term should have been added to the model. If the residuals are curved (as in Figure 7.12d), then a higher level model (if a polynomial) may be needed.

Figure 7.13 shows a fitted regression model relating runoff volume to rain depth for 60 observations (Pitt 1987). Figure 7.13a shows the predicted and the observed runoff volumes, while Figure 7.13b is a probability plot of the model residuals. All of the 60 residuals fit the normal distribution, except for one low value and three high values. Figures 7.13c and 7.13d are plots of

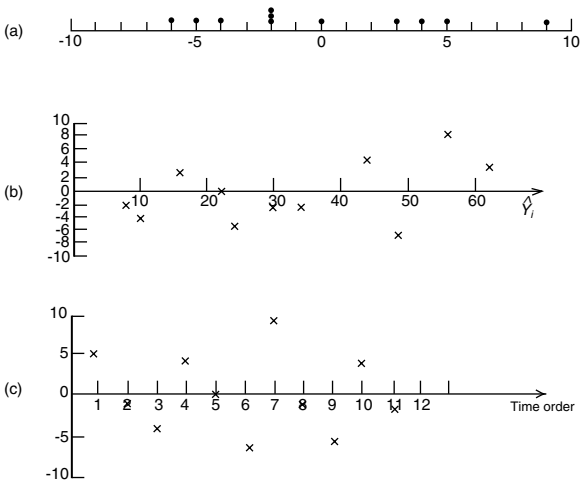

Figure 7.11 Graphical analyses of residuals: (a) overall plot, (b) by predicted value, and (c) in time order. (From Draper, N. and H. Smith. *Applied Regression Analysis*. John Wiley & Sons. New York. 1981. Copyright © 1981. John Wiley & Sons. This material is used by permission of John Wiley & Sons, Inc.)

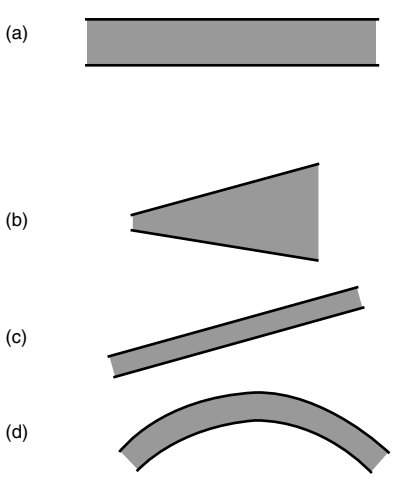

Figure 7.12 Interpretation of graphical analyses of residuals: (a) desired (even band), (b) transformations may be needed, (c) needs a linear term, and (d) higher-order model needed. (From Draper, N. and H. Smith. *Applied Regression Analysis*. John Wiley & Sons. New York. 1981. Copyright © 1981. John Wiley & Sons. This material is used by permission of John Wiley & Sons, Inc.)

the residuals with time and against the predicted runoff volume. All observations, except for 5, fall within one standard deviation of the mean residual (zero) (as expected, since ±1 standard deviation contains about $^2/_3$ of the data). The trends appear to be random, although there are many more observations associated with the smaller runoff volumes.

Simple lag plots should also be constructed to identify serial correlations of the residuals. Figure 7.14 (Draper and Smith 1981) shows two lag-1 serial correlation plots. To make lag-1 plots, the residuals are plotted against the preceding residual value. A lag-2 plot is prepared in a similar manner, by plotting a value against a preceding value skipping one. Different lag plots are normally prepared, although the lag-1 plot is usually the most informative. However, if daily samples are collected, sometimes lag-7 plots can be interesting by indicating some repeatable feature (such as associated with an industrial wastewater discharge), or if monthly samples are taken, lag-12 plots indicate seasonal changes. If these patterns are evident, then the model should be expanded to consider these possibly significant effects. If the resulting plot has a negative slope (as in Figure 7.14a), then the residuals are negatively serially correlated. If the resulting plot has a positive slope (as in Figure 7.14b), then the residuals are positively correlated. Both of these behaviors are undesirable for residuals because they indicate that the measurements are not independent. Serial correlation plots should be supplemented with a statistical procedure, such as the Durbin–Watson test for independence.

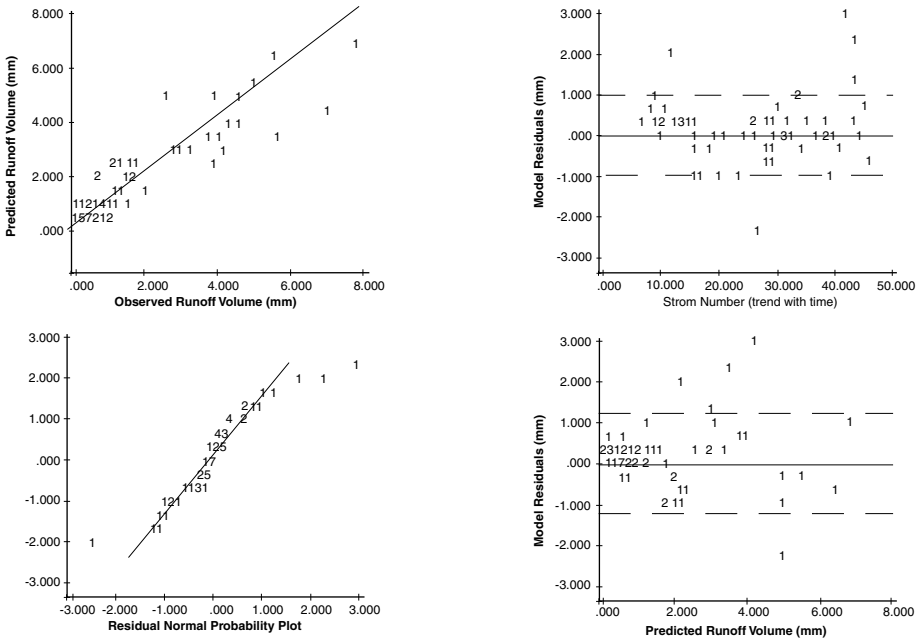

Figure 7.13 Example residual analysis for simple rainfall-runoff model. (From Pitt, R. *Small Storm Urban Flow and Particulate Washoff Contributions to Outfall Discharges*. Ph.D. dissertation submitted to the Department of Civil and Environmental Engineering, University of Wisconsin, Madison. 1987. With permission.)

Problems with Interpreting Regression Analysis Results

Berthouex and Brown (1994) present a fascinating discussion on the coefficient of determination (R^2) commonly used to "verify" a regression model. The following is a brief summary of that discussion. The coefficient of determination is the proportion of the total variability in the dependent variables that the regression equation accounts for. An R^2 of 1.0 indicates that the equation accounts for all of the variability of the dependent variables. Unfortunately, a high R^2 value, even if the model is statistically significant, doesn't guarantee that the model has any predictive value. Figure 7.15 shows plots of four data sets (from Anscombe 1973) having identical predicted regression equations with significant coefficients, the same R^2 values (0.67), and the same standard error values. However, the plots show that the relationships are vastly different from each other, stressing the need to always prepare basic scatterplots of the data and to perform residual analyses for the fitted equation (as described earlier).

Berthouex and Brown (1994) also show that having a low R^2 doesn't mean that the regression model is useless. The significance of the regression coefficients (presented in an ANOVA test of the regression equation) is highly dependent on the number of data observations. Highly significant equation coefficients are possible with a concurrent very low R^2 value if the number of data observations is large. The opposite is also true: a high R^2 value can occur with insignificant equation coefficients if only a few data observations are available. This leads to their comment that practical significance and statistical significance are not equivalent: a modest and unimportant true relationship may be established as statistically significant if a large number of observations are available. Conversely, a strong and important relationship may not be shown to be significant if only a few data are available. They therefore stress that great care needs to be exercised if a regression equation

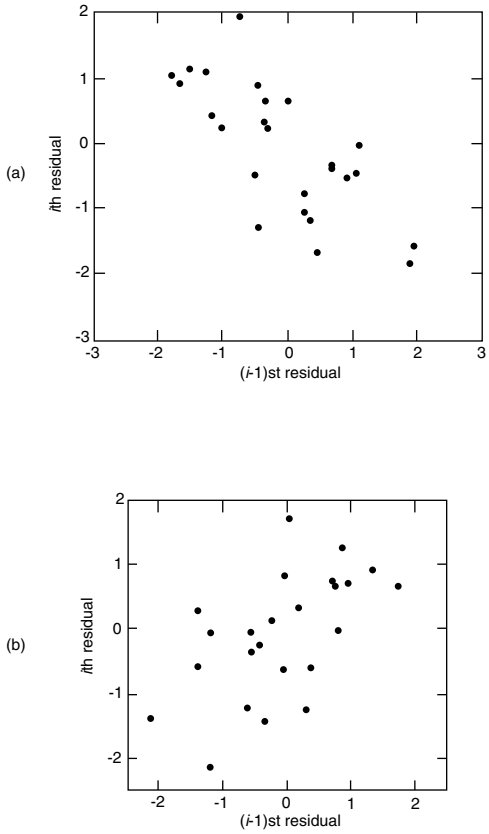

Figure 7.14 Example serial correlation analyses of residuals: (a) negative serial correlation, and (b) positive serial correlation. (From Draper, N. and H. Smith. *Applied Regression Analysis*. John Wiley & Sons. New York. 1981. Copyright © 1981. John Wiley & Sons. This material is used by permission of John Wiley & Sons, Inc.)

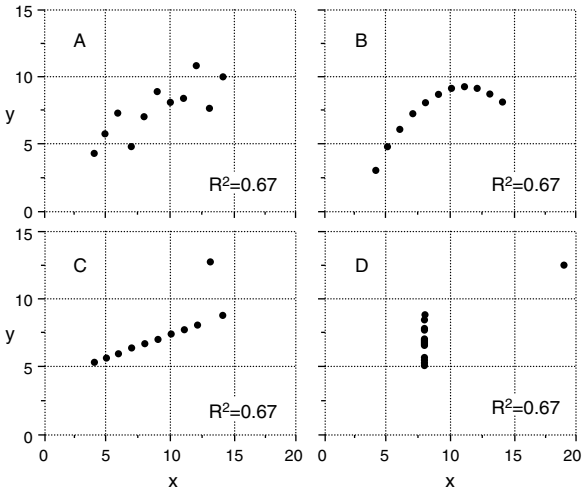

Figure 7.15 Problems when relying on coefficient of determination (R^2) to verify model. (From Anscombe, F.J. Graphs in statistical analysis. *Am. Stat.*, 27: 17–21. 1973. Reprinted with permission from *The American Statistician*. Copyright 1997 by the American Statistical Association. All rights reserved.)

is to be used for predictions because it is not possible to determine how accurate predictions will be based on the value of R^2. They strongly suggest that the model (such as a regression equation) be evaluated by: (1) examining the data and resultant model residuals graphically (as described previously), and (2) by using the standard error of the estimate (as in an ANOVA evaluation) as a more useful measure of the prediction capability of the model instead of relying only on R^2. The standard error of the estimate is computed from the variance of the predicted values using the model, so it is a more accurate indicator of the ability of the model to predict dependent variables.

Analysis of Trends in Receiving Water Investigations

The statistical identification of trends is very demanding. Several publications have excellent descriptions of statistical trend analyses for water quality data (as summarized by Pitt 1995). In addition to containing detailed descriptions and examples of experimental design methods to determine a required sampling effort, Gilbert (1987) devotes a large portion of his book to detecting trends in environmental data and includes the code for a comprehensive computer program for trend analysis. Reckhow and Stow (1990) present a comprehensive assessment of the effectiveness of different water quality monitoring programs in detecting water quality trends using EPA STORET data for several rivers and lakes in North Carolina. They found that most of the data (monthly phosphorus, nitrogen, and specific conductance values were examined) exhibited seasonal trends and inverse relations with flow. In many cases, large numbers of samples would be needed to detect changes of 25% or less (typical for stormwater retrofitting activities).

Spooner and Line (1993) present recommendations for monitoring requirements in order to detect trends in receiving water quality associated with nonpoint source pollution control programs, based on many years' experience with the Rural Clean Water Program. These recommendations, even though derived from rural experience, should also be applicable to urban receiving water trend analyses. The following is a general list of their recommended data needs for associating water quality trends with land use/treatment trends:

- Appropriate and sufficient control practices must be implemented. A high level of participation/control implementation is needed in the watershed to result in substantial and more easily observed water quality improvement. Controls need to be used in areas of greatest benefit (critical source areas, or in drainages below major sources), and most of the area must be treated.
- Control practice and land use monitoring is needed to separate and quantify the effects of changes in water quality due to the implemented controls by reducing the statistical confusion from other major factors. Monitor changes in land use and other activity on a frequent basis to observe temporal changes in the watershed. Seasonal variations in runoff quality can be great, along with seasonal variations in pollutant sources (monitor during all flow phases, such as during dry weather, wet weather, cold weather, warm weather, for example). Collect monitoring data and implement controls on a watershed basis.
- Monitor the pollutants affecting the beneficial uses of the receiving waters. Conduct the trend analyses for pollutants of concern, not just for easy, convenient parameters.
- Monitor for multiple years (at least 2 to 3 years for both pre- and post-control implementation) to account for year-to-year variability. Utilize a good experimental design, with preferable use of parallel watersheds (one must be a control and the other undergoing treatment).

Preliminary Evaluations before Trend Analyses Are Used

Gilbert (1987) illustrates several sequences of water quality data that can confuse trend analyses. It is obviously easiest to detect a trend when the trend is large and the random variation is very small. Cyclic data (such as seasonal changes) are often perceived as trends when no trends exist (Type 1 error), or they can mask trends that do exist (Type 2 error) (Reckhow and Stow 1990; Reckhow 1992). Three data characteristics need to be addressed before the data can be analyzed for trends because of confusing factors. These include:

- Measure data correlations, as most statistical tests require uncorrelated data. If data are taken close together (in time or in location), they are likely partially correlated. As an example, it is likely that a high value is closely surrounded by other relatively high values. Close data can therefore influence each other and do not provide unique information. This is especially important when determining confidence limits of predicted values or when determining the amount of data needed for a trend analyses (Reckhow and Stow 1990). Test statistics developed by Sen can use dependent data, but they may require several hundred data observations to be valid (Gilbert 1987).

- Remove any seasonal (or daily) effects or select a data analysis procedure that is unaffected by data cycles. The nonparametric Sen test can be used when no cycles are present or if cyclic effects are removed, while the seasonal Kendall test is not affected by cyclic data (Gilbert 1987).
- Identify any other likely predictable effects on concentrations and remove their influence. Normally occurring large variations in water quality data easily mask commonly occurring subtle trends. Typical relations between water quality and flow rate (for flowing water) can be detected by fitting a regression equation to a concentration vs. flow plot. The residuals from subtracting the regression from the data are then tested for trends using the seasonal Kendall test (Gilbert 1987).

Reckhow (1992) presents a chart listing specific steps that need to be taken to address the above problems. These steps are as follows:

1. Check the data for deterministic patterns of variability (such as concentration vs. flow by using graphical and statistical methods). If deterministic patterns exist, subtract the modeled pattern from the original data, leaving the residuals for subsequent seasonality analyses.
2. Examine the remaining residuals (or data, if no deterministic patterns exist) for seasonal (can be a short period, such as daily) variations. Again use graphical and statistical methods. If "seasonality" exists, subtract the modeled seasonality from the data (residuals from #1 above), leaving the remaining residuals for subsequent trend analyses.
3. Conduct the trend analysis on the residuals from #2 above, using the standard seasonal Kendall test. If a trend exists, subtract the trend, leaving the remaining residuals for subsequent autocorrelation analyses.
4. Test the remaining residuals from #3 above (or the raw data, if no deterministic or cyclic patterns or trends were found) for autocorrelation. If the autocorrelation is significant, reevaluate the trends using an autocorrelated-corrected version of the seasonal Kendall (or regular Kendall) test. If no autocorrelation was found, use the standard seasonal Kendall test if seasonality was identified, or the standard Kendall test if no seasonality was identified. The final residual variation is then used (after correcting for autocorrelation) in calculating the required number of samples needed to detect trends for similar situations.

Statistical Methods Available for Detecting Trends

Graphical Methods

Several sophisticated graphical methods are available for trend analyses that use special smoothing routines to reduce short-term variations so the long-term trends can be seen (Gilbert 1987). In all cases, simple plots of concentrations vs. time of data collection should be made. This will enable obvious data gaps, potential short-term variations, and distinct long-term trends to be possibly seen.

Regression Methods

A time-honored approach in trend analysis is to perform a least-squares linear regression on the quality vs. time plot and to conduct a *t*-test to determine if the true slope is not different from zero (Gilbert 1987). However, Gilbert (1987) points out that the *t*-test can be misleading due to cyclic data, correlated data, and data that are not normally distributed.

Mann–Kendall Test

This test is useful when missing data occur (due to gaps in monitoring, such as if waters freeze during the winters, equipment fails, or when data are reported as below the limit of detection). Besides missing data, this test can also consider multiple data observations per time period. This test also examines trends at multiple stations (such as surface waters and deep waters, etc.) and enables comparisons of any trends between the stations. This method also is not sensitive to the data distribution type. This test can be considered a nonparametric test for zero slope of water quality vs. time of sample collection (Gilbert 1987). Short-term (such as seasonal changes) cycles and other

data relationships (such as flow vs. concentration) affect this test and must be corrected. If data are highly correlated, then this test can be applied to median values in each discrete time grouping.

Sen's Nonparametric Estimator of Slope

Being a nonparametric test based on ranks, this method is not sensitive to extreme values (or gross data errors) when calculating slope (Gilbert 1987). This test can also be used when missing data occur in the set of observations. It is closely related to the Mann–Kendall test.

Seasonal Kendall Test

This method is preferred to most regression methods if the data are skewed, serially correlated, or cyclic (Gilbert 1987). This test can be used for data sets having missing values, tied values, censored values (less than detection limits), or single or multiple data observations in each time period. The testing of homogeneity of trend direction enables one to determine if the slopes at different locations are the same when seasonality is present. Data correlations (such as flow vs. concentration) and dependence also affect this test and must be considered in the analysis.

The code for the computer program contained in Gilbert (1987) computes Sen's estimator of slope for each station–season combination, along with the seasonal Kendall test, Sen's aligned test for trends, the seasonal Kendall slope estimator for each station, the equivalent slope estimator for each season, and confidence limits on the slope.

Chapter 4 contains a case study of receiving water improvements with time for a Swedish urban lake after the implementation of watershed controls. The above steps were used to identify and measure nutrient and transparency improvements after stormwater control to remove phosphates was installed.

Specific Methods Commonly Used for Evaluation of Biological Data

Many of the above examples reflect water quality data analyses. However, in many areas of science, specialized tests are often used to great advantage based on specific conditions that are commonly encountered. Biological data analysis is certainly one field where some of these specialized tests are worth noting. The following discussion specifically considers toxicity data and some of the unique statistical approaches that are useful.

Typically, there are a few differences between analyzing laboratory and field (*in situ*) toxicity data. Regardless of where an evaluation takes place, the focus of any toxicity test design is to determine if environmental stressors are affecting a biological system and to what degree they are doing so. Once a test design is chosen, relevant chemical (e.g., pH, conductivity, ammonia, and turbidity, etc.) and physical (e.g., temperature, flow rate, stage, rainfall, etc.) data should always be collected throughout testing. For *in situ* biomonitoring, physical and chemical characteristics should generally be monitored each day the exposure takes place. It is recommended that initial (i.e., Day 0 or exposure commencement) and final (i.e., the final day of exposure or end of the bioassay) measurements be made at a minimum. The same approach should be made for any laboratory testing. The field conditions at the time of environmental sample (e.g., sediments, effluents, or receiving waters) collection must be monitored. Once any effluent or receiving water bioassay commences in the laboratory, daily physical and chemical measurements should be compiled. Following an exposure, for either laboratory or *in situ* experiments, routine descriptive statistics are always calculated. At a minimum, means (e.g., survival, reproduction, or growth), standard deviations, and coefficients of variation should be calculated from resulting test data. In many situations, these descriptive statistics are sufficient for making an assessment of environmental impact, especially for a short-term, one-time-only exposure. However, in most cases further statistical analysis is needed to better explain the status of a biological community. These supplemental data and descriptive statistics are usually very useful in supporting statistical analysis or conclusions.

For most laboratory-derived toxicity data, it is recommended in the USEPA chronic (1993) and acute (1995) freshwater laboratory test methods that either hypothesis testing or point estimate approaches be used for analyzing resulting endpoints (e.g., survival, growth, and reproduction). Hypothesis testing is most frequently used to determine whether one or more biological responses resulting from exposure to a particular treatment differs as compared to the control response. These statistical tests can be done when effluents, receiving waters, or sediments are tested in the laboratory, and when field sites are evaluated *in situ*. Intuitively, the control response for any exposure should be representative of the condition being evaluated. Some hypothesis testing procedures require that the experiment yield a dose response or be conducted using a dilution series (e.g., effluent and receiving water tests). For experiments with a dilution series, hypothesis tests are used to yield specific effect levels, or concentrations at which either no effect or the first detection of an effect in the testing population appears. Therefore, the effect levels are either a No Observed Effect Concentration (NOEC) or a Lowest Observed Effect Concentration (LOEC). Prior to assigning NOEC and LOEC values, an analysis of variance (ANOVA) must be conducted on test data. An ANOVA allows the investigator to determine whether treatments differ from one another statistically; however, it does not identify which group(s) are different, only that there is at least one group that is statistically different from at least one other group. If statistical significance is detected after an ANOVA, the NOEC and LOEC values can be identified using a Student's *t*-test, or an equivalent nonparametric test. The NOEC is the highest concentration not significantly different from the control and the LOEC is the first concentration that is significantly different from the control. If the data are parametric (i.e., normally distributed and homogeneous) and test replicate numbers are equal, Dunnett's test is the appropriate choice. If test replicates are unequal, a *t*-test with Bonferroni adjustment is appropriate. Nonparametric data with an equal number of replicates require Steel's Many-One Rank test, and Wilcoxon Rank Sum test if they are not.

In situ toxicity tests may represent a natural, more "realistic," exposure period but never provide the luxury of the controlled laboratory bioassay. Dose–response restrictions are rarely possible during *in situ* evaluations, and toxicity (i.e., contaminant concentrations) at field sites usually varies greatly in no particular order. Currently, no EPA guidance exists for statistical analysis of *in situ* toxicity data, but hypothesis testing can be implemented quite easily. For most *in situ* biomonitoring studies, a weight-of-evidence approach utilizing a suite of established statistical tools and scientific judgment is the general process. In many cases, it is very useful to use ANOVAs in conjunction with various *post hoc* tests for a simple and useful means to detect significant differences between sample treatments. The *post hoc* multiple comparison tests are then required to differentiate those treatments. Opinion varies widely on which *post hoc* tests are best in certain situations. However, Tukey's honest significant difference (HSD) test or Duncan's multiple range test is sufficient in most cases for defining where significance lies in the data. Both Tukey's and Duncan's compare all treatments (i.e., control and contaminated treatments) against one another and can allow one to show all significant difference for all the data. Dunnett's can also be used again as a useful *post hoc* test to detect significant differences between all the treatments and only the control. Furthermore, it is sometimes recommended that to better meet the ANOVA assumptions of homogeneity of variance and normality, transforming binomial data (i.e., survival data) is sometimes needed. Typically, the square root, log, and arcsin-square root transformation are utilized most.

Almost all point estimate data analysis is conducted on data from laboratory effluent, receiving water, or reference toxicant testing. Data used to calculate point estimates are required to have a continuous, dose-response relationship, usually a function of a dilution series. Traditionally, they allow the investigator to describe the relationship between two variables (e.g., a sample concentration and biological response), in order to relate any adverse effects of known or suspected toxicants to a concentration or dose. Point estimation results are recorded as a lethal concentration (LC) for acute toxicity tests, and effective or inhibition concentration (EC or IC) for chronic tests. An LC is usually expressed as the concentration at which there is 50% mortality in the testing population

(i.e., an LC50 value). The EC and IC values are generally expressed as the concentration at which there is a 25% effect in a response, such as growth or reproduction (i.e., EC25 and IC25 values). Probit analysis is the only parametric, point estimate model where it is assumed the data are binomial (e.g., dead or alive, deformed or not) and normally distributed. For probit analysis, it is also required that there be at least two partial responses (i.e., no "all or nothing" responses). Probit effect levels are also reported as LC50 or EC50 values. A chi-square test (χ^2) for heterogeneity can be used to determine whether or not data will fit the probit model. The Spearman–Karber model is the preferred nonparametric model and yields an LC50 or EC50 value. However, no mathematical relationship for the concentration response is assumed for Spearman–Karber. A symmetrical distribution around the mean, including no response in the lowest concentration and 100% response in the highest concentration, is required for the untrimmed model, but the trimmed model is employed when the zero and/or 100% response is not met.

When a response variable or endpoint is dependent upon another variable(s), linear regression analysis may be useful. For example, for an *in situ* biomonitoring study where turbidity caused from suspended sediment is suspected of degrading water quality following storm events, numerous measurements must be taken to adequately assess impacts. After representative field sites are chosen, multiple measures of turbidity, flow, and particulate-associated contaminants throughout the exposure period would be needed. Trends can be detected by correlating the response of surrogate organisms (e.g., *Pimephales promelas*, *Hyalella azteca*, or *Chironomus tentans*) and physical or chemical measurements to strengthen a judgment of water quality and biological health of the waterway. A linear regression may be drawn between an endpoint and a single predictor variable (e.g., pH, temperature, or concentration of contaminant) in order to identify which independent variable is most closely related to the response. Multiple regression can be used to assess how an endpoint is related to multiple factors in a complex system. Linear regressions can be derived using many different functions (e.g., simple linear, exponential, hyperbolic). Least-squares estimates are used to determine the equation for the best fit line through the data, and this procedure is followed by computing the sum of squares (measures of the amount of variation in the response variable) and an ANOVA table. The ANOVA table partitions the variability of the responses and thus distinguishes what can be explained by regression and what remains unexplained (i.e., error). A large F value resulting from an ANOVA suggests that there is a significant linear relationship between the response (endpoint) and the predictor variable. However, a significant F value is not an indication that the regression equation used is the "best fit" model. Calculation of the Pearson's correlation (r), the coefficient of determination (R^2), and the coefficient of multiple correlation (in the case of multiple regression) indicate the fitness or strength of the regression. The SAS package offers a MAXR procedure for determining the best regression equation for a response variable and many predictor variables by optimizing R^2 while maintaining parsimony (i.e., yielding an equation with the fewest predictor variables). Further evaluation of the adequacy of the regression relationship is always needed through hypothesis testing (t-tests) of the equation constants (e.g., slope and intercept values), determination of confidence intervals for the response variables, and inspection of the plot of residuals. It should be noted that the above regression approach assumes only a single, or simple, interaction between expected causes and the observed effect. As described earlier, several tests that consider complex interactions (such as hierarchical cluster analyses or principal component analyses and factor analyses) may be necessary supplements to this traditional approach.

SUMMARY OF STATISTICAL ELEMENTS OF CONCERN WHEN CONDUCTING A RECEIVING WATER INVESTIGATION

This chapter briefly presented a number of tools available to the environmental researcher. These have been selected as having special utility when conducting experiments that are not easily controlled. The experimental design methods presented in Chapter 5 included simple and robust

experimental designs and stressed an adequate sampling effort to help ensure successful data analyses. Various exploratory data analyses procedures have been briefly presented in this chapter, along with several cautionary examples of common problems encountered when using popular statistical methods. In almost all cases, the researcher will need to rely on the methods as presented in the references, as this discussion has been mostly descriptive. The applied statistical reference books included in the reference list comprise a fundamental library to which the environmental researcher should have access.

Exploratory data analysis is a very useful tool for preliminary evaluations of historical data needed to help design data-gathering experiments, and, it should also be used as the first step in evaluating newly collected data. The comparison of data from multiple situations (upstream and downstream of an outfall, summer vs. winter observations, etc.) is a very common experimental objective. Similarly, the use of regression analyses is also a very common statistical tool for receiving water investigations. Trend investigations of water quality or biological conditions with time are also commonly conducted. The experimental design determines the location and conditions of the sampling for these statistical objectives, but several errors are commonly made when conducting the statistical evaluations of the collected data.

In all cases, statistical analyses should not be considered a last-minute thought. Even in the best of conditions, with carefully controlled experiments and simple project objectives, it is mandatory that a general outline of the proposed statistical analysis procedures be developed before the initial experimental design is developed. It is only possible to collect adequate and sufficient data if a comprehensive objective is available and if the most appropriate statistical methods are identified. Of course, it is likely that additional analyses, or even substitutions, will be used during the final data analysis activities, and some of these modifications may require the collection of additional data that was not anticipated at the beginning of the project.

A general strategy in data analysis should include several phases and layers of analyses. Graphical presentations of the data (using exploratory data analyses) should be conducted initially. Simple-to-complex relationships between variables may be more easily identified through visual data presentations for most people, compared to relying only on descriptive statistical summaries. Of course, graphical presentations should be supplemented with statistical test data to quantify the significance of any patterns observed.

This chapter outlined several basic approaches to data analysis divided into major categories (multiple data sets, data associations, regression analyses, and trends) that are generally of the most interest in receiving water assessments. There is a great number of statistical references, software products, and consultants available to assist the data analyst. Several are presented in this chapter for additional information.

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CHAPTER 8

Data Interpretation

"*If you get all the facts, your judgment can be right; if you don't get all the facts, it can't be right.*"

Bernard M. Baruch

CONTENTS

IS THERE A PROBLEM?

Unit 1 (Chapters 1 through 3) described problems associated with stormwater runoff. Unit 2 (Chapters 4 though 8) described the development of appropriate experimental designs that included selecting the components of the assessment process and determining an appropriate level of effort, plus specific sampling and monitoring activities to assess receiving water impacts. Unit 3 (the appendices) includes additional guidance on conducting specific field activities. There are numerous case study examples throughout these chapters showing how the recommended approach has functioned during previous successful projects. In this concluding chapter, these important issues are highlighted for the data interpretation process. Now that an assessment has been conducted, how does one determine whether or not the receiving waters are impaired and, if so, what is the source, or sources, of the impairment?

As indicated in Chapters 2 and 3, there is a variety of receiving water problems that may be associated with stormwater. The specific problems in any area are dependent on many site conditions and objectives. There are many documented cases, previously described, where stormwater has caused detrimental impairments on receiving water uses and goals. Probably the most common problem is associated with stormwater conveyance (flood prevention) caused by increased amounts of pavement in the drainage area. The increased flows, however, are also responsible for many habitat problems related to the increased stream power and associated unstable stream environment. Other common receiving water problems in urban waters are associated with noncontact recreation (linear parks, aesthetics, boating, etc.). The seemingly simple task of preventing floatable debris from being discharged can be very difficult to accomplish. Much of this book has addressed environmental health issues associated with biological uses (warm-water fishery, biological integrity, etc.). In addition to the habitat destruction problems associated with increased flows and increased stream power, contaminated sediment may be a significant causative agent affecting biological uses. Poor water quality obviously can also significantly affect most of the above uses, in addition to interfering with water contact recreation (swimming) and water supply uses. It is unlikely that these human health uses would be appropriate in any waterway located in a heavily urbanized watershed.

The study design is dependent on the expected problems likely to be encountered (see also Chapter 4). Without having that information at the beginning of a study, the initial list of parameters to be monitored has to be based on best judgment. The parameters to be monitored can be grouped into general categories, depending on expected beneficial use impairments, as follows:

- Flooding and drainage: debris and obstructions affecting conveyance are parameters of concern.
- Biological life/integrity: habitat destruction, high/low flows, taxonomic composition of existing aquatic life, inappropriate discharges, polluted sediment (texture, SOD, and toxicants), and wet weather quality (toxicity, bioaccumulation, toxicants, nutrients, DO) are key parameters.
- Noncontact recreation: odors, trash, high/low flows, aesthetics, and public access are the key parameters.
- Swimming and other contact recreation: pathogens, and above listed noncontact parameters, are key.
- Water supply: water quality standards (especially pathogens and toxicants) are key parameters.
- Shellfish harvesting and other consumptive fishing: pathogens, toxicants, and those listed under biological life/integrity, are key parameters.

Obviously, there are definite problems in receiving waters that will dictate many components of the sampling program and measures against which the data are to be compared. These problems may be minor if the watershed is relatively undeveloped, but they can be extreme for fully developed urban or agricultural areas. In addition, local use objectives also dramatically affect the definition of a "problem." In all cases, however, basic receiving water objectives should include safe drainage, noncontact recreation (acceptable aesthetics), and basic biological life objectives. It is unlikely that contact recreation or biological integrity, with the stream being able to support a full mixture of native organisms, would be reasonable receiving goals in a fully developed urban or agricultural watershed.

The information and guidance provided in this book should enable a researcher to investigate local conditions to identify local use impairments and to identify the most likely causes of these problems. Depending on the magnitude of the effort expended and the clarity of the problems in the local area, it may also be possible to quantify the magnitude of stream use improvements with different levels of reduction of the causative agent. Once the causes and sources of the problems are identified, choices pertaining to improvement, or prevention measures in other areas, can be examined.

The following sections outline the concept of "weight-of-evidence" as a tool to assemble a large amount of data to help in obtaining needed information pertaining to environmental health. An example risk assessment is also provided to show how risks associated with exposures to humans can be examined.

EVALUATING BIOLOGICAL STREAM IMPAIRMENTS USING THE WEIGHT-OF-EVIDENCE APPROACH

The Process

The term "weight-of-evidence" (WOE) has been used frequently during the past several years in the environmental assessment arena. However, there is no clear definition or approach accepted, and approaches have varied from those that are crude and qualitative to very complex and quantitative. As discussed in Chapter 4, no one assessment approach is adequate for drawing conclusions on the quality of a waterway because of the associated uncertainties and weaknesses of each approach. Therefore, there is now widespread acceptance that multiple approaches (lines of evidence) are essential in order to reach reliable conclusions of whether a problem exists. Using the WOE approach, however, does not ensure that accurate conclusions will be obtained. It is critical that a well-designed assessment design be used (see Chapter 4) and that the key ecosystem components (biological, chemical, and physical) be characterized correctly, noting their associated uncertainties. The following discussion presents useful approaches for WOE evaluations.

One of the first WOE approaches to gain widespread attention was the "sediment quality triad" (Chapman et al. 1987). In this approach, sediment toxicity, indigenous biota, and sediment chemistry were characterized at each test site and normalized as a percentage of the reference (background) site condition. Results were presented graphically in an X-Y-Z axis type format. Comparing test site conditions to reference sites has long been used, but the primary contribution of the triad was to promote the notion that components must be assessed together. In stormwater assessments, the triad approach should be expanded to include the physical conditions (i.e., habitat), water and sediment conditions, and the associated temporal dynamics of each assessment component (Table 8.1).

Unfortunately, it is difficult to make quantitative evaluations of significant differences from this original "triad" approach. The comparisons between sites are particularly difficult at intermediate levels of contamination or if significant variability exists in the monitoring data. This "weight-of-evidence" approach can be evaluated using both parametric and nonparametric procedures to address the following study objectives: which stations are significantly different (impacted) relative to other stations?; how do the stations relate to each other?; and which parameters (monitoring components)

Table 8.1 0Summary of Key Weight-of-Evidence Components for Assessing Stormwater Effects on Receiving Waters

Component	Media	Priority	Flow Level	Difficulty[a]
Benthic community	Sediment	High	Low	Low
Fish community	Water	Medium	Low	Medium
Toxicity				
Lab-based	Sediment	Medium	Low	Low–Med.
	Water	High	Low and High	Low–Med.
In situ-based	Sediment	High	Low	Low–Med.
	Water	High	Low and High	Low–Med.
Bioaccumulation				
Benthic species	Organism	Medium	Low	Med.–High
Fish species	Organism	Medium	Low	Med.–High
In situ passive	Water	Low	Low and High	Med.–High
Chemistry (metals, organics, conventional physicochemistry)	Sediment	High	Low	Med.–High
	Water	High	Low and High	Med.–High
Physical				
Flow	Water	High	Low and High	Low
Habitat	Whole stream	High	Low	Low

[a] Difficulty rating considers both level of effort and cost to measure by typical approaches described in Appendices.

are significantly different (impacted) relative to other stations? Initial exploratory data analyses should be used to identify relationships between variables, identify and rank important variables, and identify weighting factors or redundant variables (i.e., responses mimic each other). These analyses may include correlation analyses, scatterplots, and other ordination tests. Results can also be ranked, whereby endpoint measures are averaged at each station and stations are then ranked by performance. Sample average ranks can be compared to a critical value to determine if significant differences exist between stations. Ordination procedures can be used to determine distances among stations and endpoints (e.g., multidimensional scaling). Scatterplots will show similarity of ranked groups and the magnitude of relationships among measured endpoints.

The Massachusetts Department of Environmental Protection formalized the WOE approach (Menzie et al. 1996) for relating measurement endpoints to assessment endpoints in ecological risk assessments. They identified three major components:

1. Weight assigned to each measurement endpoint: measurement endpoints (e.g., mortality, growth) may vary in the degree they relate to the assessment endpoints, or their quality, and may therefore be assigned differing levels of weight (i.e., importance).
2. Magnitude of response in the measurement endpoint: a greater weight is assigned to strong responses.
3. Concurrence among measurement endpoints: there tends to be greater confidence in findings that agree with other lines of evidence. However, disagreement between components does not negate their validity or importance. For example, aquatic species have varying levels of sensitivity to different chemicals, or sampling may induce artifacts. Concordance of findings is more likely when very high levels of contamination are present, causing acute toxicity, as opposed to lower chronic toxicity exposures.

Numerical weighting values (e.g., 1 to 3 or 1 to 5) are assigned to elements of the process via professional judgment. This weighting of relative importance of the various tools has been done using Delphi techniques where a group of environmental professionals is surveyed. For example, each measurement endpoint (such as species population number) could be rated as high, medium, or low for three attributes (strength of relationship to an assessment endpoint, such as fish catch, data quality, and study design). These three attribute ratings may then be summed to get an overall measurement weight (of 1 to 3). The reliability of this best professional judgment approach is obviously related to the quality and comprehensive expertise of the survey group. After weighting values are assigned, measured responses are multiplied by their respective weights and summarized. The evidence showing the relationship between exposure to a stressor and a biological response (e.g., an assessment endpoint) is then assessed for risk. *This leads the assessor to the most critical point of the assessment where the question is asked: what is the relationship between exposure to the stressor of concern (e.g., suspended solids, zinc, pesticides, stormwater) and adverse biological effects?* The WOE process will help answer this question. While the WOE is the preferred approach, it is not without its shortcomings. Aside from not being a simple standardized protocol, the WOE is also not strictly quantitative, requiring best professional judgment. Statistically significant differences and relationships cannot readily be determined for the overall, integrated process. Certainty and accuracy are ensured via greater weight that is obtained through sound, comprehensive, integrated assessments. More importantly, as the WOE process is used in an area, it becomes "calibrated" through experience and observation and can become fine-tuned to better represent actual changes that may be occurring.

Benchmarks

In the process of interpreting exposure and effect relationships, there are a number of tools that can be used, ranging from "benchmarks," or deterministic approaches, to probabilistic methods. These are discussed in the following sections. Benchmarks refer to concentrations or levels of physical and chemical parameters above which adverse biological effects may occur. These are often derived from large scientific databases linking biological responses with exposures to

Table 8.2 Categories of Biological Impairment Benchmarks

Regional or National Water/Sediment Quality Criteria or Standards
State, Provincial, or Regional Water Quality Standards
Biological Criteria
Threshold (Toxicity) Effect Levels for Water, Sediment, or Tissues
Hazard Quotients (Threshold Level or Site Concentration vs. No Effect Level, Reference or Background Site)
Percentile Distributions
Statistical Significance of Test vs. Control or Reference

compounds. Examples of commonly used benchmarks are listed in Table 8.2. Specific benchmarks/criteria for water and sediment criteria and biota are also discussed in Appendices B, C, and G. For each of these benchmark categories, there exists chemical specific benchmarks calculated by a variety of methods. These methods vary in the amount of biological effect (toxicity) information they include, ranging from only acute toxicity information on one species, to acute and chronic toxicity on many species. In addition, the toxicity information generated in these benchmarks ranges from a site-specific nature to being applicable to large geographical areas (such as north America). As with any assessment tool, each has associated uncertainties that should be recognized and considered by the assessor. The optimal approach is to use multiple benchmarks to better ascertain whether impairment exists.

The most important issue to remember when using benchmarks is that they are simply "benchmarks" to use in the chemical-physical data interpretation process. They do not unequivocally determine whether adverse effects are occurring. Often, these benchmarks do not include site-specific biological effects data. In addition, the biological effect benchmarks may not be applicable to the conditions at your study site. For example, a suite of stressors may exist at your sites that interact to produce antagonistic or synergistic effects or conditions may alter the bioavailability of the chemical of concern. However, the use of multiple benchmarks that have been derived from large, scientifically valid, databases will assist in the weighting and data interpretation process.

The optimal method of establishing a relationship between biological effects and a site-specific parameter(s) is to thoroughly characterize exposure and effects. Benchmarks, unfortunately, only suggest that effects may be occurring if they are exceeded. If exceeded, they should at least be treated as "red flags," emphasizing areas where additional investigation is warranted. They do not address spatial and temporal variability or site-specific interactions. This requires carefully designed biological and toxicity studies during low and high flow conditions (Chapters 4 and 6). In the absence of site-specific effects data, use of probability modeling is preferred if adequate site data exist for determining spatial and temporal exposure-effects interactions (see Ecological Risk Assessment section below).

Perhaps the best recognized and accepted benchmarks are the U.S. EPA's National Ambient Water Quality Criteria (see Appendix G), which many states have adopted as their ambient water quality standards. The results of stormwater quality analyses have commonly been compared to water quality criteria in order to identify potentially toxic waters, and likely problematic pollutants. This has led to numerous problems with the interpretation of the data, especially concerning the "availability" of the toxicants to receiving water organisms and the exposure durations in receiving waters. The quality of stormwater, or of ambient waters immediately following high flow events, has been shown to be degraded in many studies with chemical concentrations that may exceed toxicity thresholds (e.g., Horner et al. 1994; Makepeace et al. 1995; Morrison et al. 1993; Waller et al. 1995). Stormwater toxicants are primarily associated with particulate fractions and are typically assumed to be "unavailable." Typically short and intermittent runoff events can also not be easily compared to the "long" duration criteria or standards. Chemical analyses, without biological analyses, would have underestimated the severity of the problems because the water column quality varied rapidly, while the major problems were associated with sediment quality and effects on macroinvertebrates (Lenat and Eagleson 1981; Lenat et al. 1981).

The contradictions noted between in-stream biological effects and water quality criteria should not be surprising, given the assumptions used by the EPA:

1. Single acute and chronic average exposure period that does not account for pulse or repeated exposures for short time periods
2. Single bioavailability normalization factors (such as hardness)
3. Laboratory-derived toxicity values for surrogate species are protective of indigenous species
4. Effects derived from single chemical exposures in clean solutions where the toxicant is in the dissolved form
5. Chemical exposures in the field based on limited grab sample analyses

To address magnitude and duration issues, the EPA developed the "Criterion Maximum Concentration" concept, with an exposure period assumption of 1 hour, and the "Criterion Continuous Concentration," with an average period assumption of 4 days. Yet, these assumptions do not accurately describe most wet weather runoff exposures. Tests with pentachloroethane (Erickson et al. 1989, 1991) showed that with intermittent exposures, higher pulse concentrations were needed to affect growth, and when averaged over the entire test, effects were elicited at concentrations lower than when under constant exposure. The simplest toxicity model (with first-order, single-compartment toxicokinetics and a fixed lethal threshold) could not completely describe the data. Erickson et al. (1989) concluded that kinetic models which predict mortality were reasonable; however, chronic toxicity effects were much more complicated, and no adequate models existed. Hickie et al. (1995) describe a one-compartment, first-order kinetics, pulse exposure model for residue-based toxicity of pentachlorophenol to *P. promelas.* Pulse exposures were of 2 min to 24 hours with durations of 2 to 24 hours, repeated 2 to 15 times. A comparison of three models (Cxt, Mancini, Breck 3 dimensional range repair) showed reasonable prediction of fish toxicity following 1 to 4 monochloramine pulses (2-h pulse, 22-h recovery). However, predictive capability decreased with greater than 4 pulses (Meyer et al. 1995). Beck et al. (1991) examined the transient nature of receiving water effects associated with stormwater, stressing the weaknesses associated with more typical steady-state approaches. They felt that there were still major misconceptions associated with modeling these effects.

Despite these limitations, water quality criteria and standards have been used effectively to identify potential stormwater problems and direct further assessment studies (see Chapter 6). Nonetheless, it is apparent that the use of water quality criteria to identify potential receiving water problems should be done with care. In many cases, the most direct comparison is made for concentrations of the soluble forms of the pollutants only and to use the short-term acute exposure criteria. This seems to be the most conservative approach, and if any measured pollutant exceeds this critical value, a problem pollutant is easily flagged. However, this approach is fraught with false negatives, as many chronic problems may still exist that are not recognized. As an example, numerous in-stream receiving water investigations (described in Chapter 3) have identified severe problems (indicated by lack of sensitive species) where the measured water quality met the criteria. Because the toxicants are strongly associated with particulates, secondary sediment contamination occurs that may be more important than water column conditions for aquatic life effects. In addition, habitat degradation caused by urbanization and agricultural activity (including highly fluctuating flows) are also likely responsible for many of the recognized receiving water problems. Finally, the irregular, but frequent, exposures of pollutant concentrations lower than the criteria may cause a greater problem than relatively constant, but higher, concentrations (see also Chapters 4 and 6). Therefore, direct comparisons of water quality criteria with monitored in-stream concentrations should be carefully conducted and used as adjuncts to direct in-stream biological use observations, plus evaluations of habitat and sediment quality. Human health criteria (such as pathogens for water-contact recreation and toxicants for drinking water supplies of fish/shellfish consumption) are more applicable to wet weather conditions and can be more directly used to flag potential problem pollutants.

Table 8.3 NURP Reported Median and 90th Percentile Event Mean Concentrations (EMC) (mg/L, unless otherwise noted) for Urban Runoff

Constituent	Median Urban Site EMC	Event to Event Variability in EMC (COV)	90th Percentile Urban Site EMC
Suspended solids	100	1–2	300
BOD_5	9	0.5–1.0	15
COD	65	0.5–1.0	140
Total P	0.33	0.5–1.0	0.70
Soluble P	0.12	0.5–1.0	0.21
TKN	1.5	0.5–1.0	3.3
NO_2 + NO_3 (as N)	0.68	0.5–1.0	1.8
Total copper (µg/L)	34	0.5–1.0	93
Total lead (µg/L)	144	0.5–1.0	350
Total zinc (µg/L)	160	0.5–1.0	500

From EPA (U.S. Environmental Protection Agency). *Final Report for the Nationwide Urban Runoff Program*. Water Planning Division, Washington, D.C. December 1983.

Appendix G presents a summary of the human health and aquatic health criteria for pollutants that commonly occur in urban runoff and receiving waters. Most of the criteria are expressed with a recommended exceedance frequency of 3 years. This is the EPA's best scientific judgment of the average amount of time it will take an unstressed system to recover from a detrimental event in which exposure to the pollutant exceeds the criterion. A stressed system, for example, one in which several outfalls occur in a limited area, would be expected to require more time for recovery. Obviously, if criteria are exceeded for most rain events (such as can occur for bacteria and total recoverable heavy metals), then 3 years are not available for recovery before the next runoff event.

The discussions on the effects of the pollutants on aquatic life and human health presented in Appendix G are summarized from the U.S. EPA's *Quality Criteria for Water, 1986* (EPA 1986). The criteria were also reviewed using the EPA's web page (http:/www.epa.gov) on the Internet for more recent changes. Some minor changes have been made since 1986 (chloride standards, for example, in 1988). Numeric criteria for heavy metals have been proposed as part of the states' Compliance for Toxic Pollutants (for the nine states subject to EPA's 1992 National Toxics Rule) as an interim rule. In most cases, only the short-term criteria are applicable for wet-weather receiving water conditions. Most runoff events last only a few hours; very few last for several days. However, degraded in-stream conditions can occur for several times the duration of the rain event itself. In addition, frequent exposures to concentrations less than the critical short-term criteria may result in significant problems that would not be predicted based on these criteria alone.

In some instances, acceptable stormwater concentration guidelines may be based on typical data as obtained during the Nationwide Urban Runoff Program (NURP) (EPA 1983). These data were almost solely represented by medium-density residential area runoff, with some data from other areas (such as shopping centers and light industrial areas). Useful benchmarks include the event mean concentrations, or EMC, (average of all observed concentrations) and the 90th percentile values of common parameters as measured during NURP (Table 8.3). The 90th percentile values are sometimes used as an upper limit for acceptable concentrations.

Ranking and Confirmatory Studies

If an adequate stormwater runoff study design is implemented (Chapter 4) and the weight-of-evidence process followed with the ensuing monitoring data, then sound decisions can be made. In reality, few comprehensive stormwater assessments look at all possible stressors and species of concern while characterizing the spatial and temporal dynamics of the system. Most environmental assessments are resource limited, requiring a tiered approach, where potential problem sites are identified, ranked, and then decisions made as to what further assessments are necessary. This is

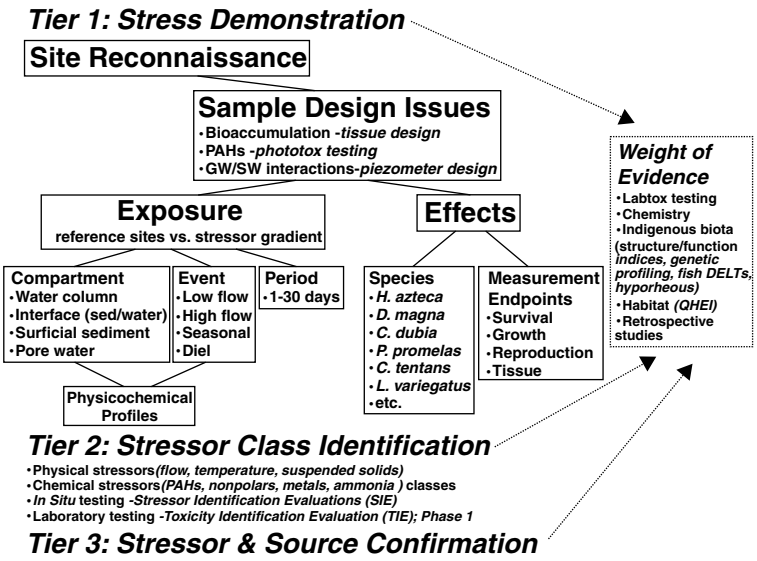

Figure 8.1 Example of a tiered weight-of-evidence approach used by Wright State University to evaluate stormwater runoff and aquatic ecosystem contamination.

particularly true for stormwater studies, where historical funding mechanisms do not exist and where the watershed-receiving water relationships are complex. It is important to be realistic in the expectations of initial screening studies. The goals should be to simply rank problem sites through the WOE approach (see above WOE discussion). Then, follow-up confirmatory (Tier 2) studies can focus on fewer sites, allowing for more quantitative characterization of the temporal dynamics and resulting effects of runoff events (Figure 8.1) (see also Chapter 4 example outline of a comprehensive runoff effect study).

For example, an approach used to identify stressors in aquatic ecosystems used by Wright State University is shown in Figure 8.1. During initial site reconnaissance, a determination is made as to whether three common sample design issues need to be incorporated: (1) Do pollutants (such as PCBs) that readily bioaccumulate likely occur? (2) Do PAHs likely occur? and/or, (3) Are there likely groundwater–surface water transition zones occurring in the area of contamination? If any of these three issues are present, then the typical Tier 1 sampling design may be modified to include: tissue residue or bioaccumulation testing, phototoxicity evaluations, and piezometer measures of groundwater movement (with concurrent chemical and toxicity testing of those compartments). The typical Tier 1 design will involve toxicity testing of two to four species which are exposed to three to four compartments (overlying water, sediment-water interface, surficial sediment, pore water) during low flow. At high flows, these same species are exposed to overlying waters. During their exposures, basic water quality measures are made, such as DO, conductivity, alkalinity, hardness, pH, temperature, turbidity (or TSS), and ammonia. If toxic effects are noted following these exposures, then Tier 2 testing may commence to better identify the type of stressor. Tier 2 testing may then require more in-depth chemical analyses and try to separate out stressors such as ammonia, metals, and nonpolar organics. Finally, in Tier 3, the focus can be to determine the significance of the dominant stressor(s) via a WOE approach.

The WOE process lends itself easily to ranking sites — particularly using broad categories such as high, medium, and low priority. For instance, this may separate sites that have acute toxicity and few pollution-sensitive benthic organisms from those with possible chronic toxicity and marginal benthic communities. The decision maker may then choose to immediately pursue installation of stormwater controls at the worst site, while conducting confirmatory studies at the marginal site to establish the extent and/or cause of the problem. Confirmatory studies are frequently necessary

to establish the: (1) dominant stressor(s); (2) exposure pathways/dynamics; (3) receptor organisms; (4) food web interactions; (5) environmental risk (human and ecological significance of effects); and (6) stressor sources. Confirmatory or Tier 2 studies are designed to answer very focused questions and use many of the same tools described for the more routine stormwater assessments. However, as the questions may be more focused, more specific and novel assessment techniques may be employed, such as DNA fingerprinting (RAPD PCR), toxicity identification evaluations (TIEs), or SPMDs (all described in Chapter 6). The environmental quality of many of our agricultural and urban waterways will also be less than pristine where anthropogenic influences are minimal. Therefore, the issue of whether significant ecological impairment exists will be more of a challenge in these human-dominated watersheds. The point of comparison for determinations of impairment should be an appropriate ecoregion reference or criteria, where manageable stressors have been removed (such as high temperature, erosion, pesticides, lack of riparian zone).

Comments Pertaining to Habitat Problems and Increases in Stream Flow

Habitat changes due to urbanization and agricultural activities are likely the cause for much of the degradation noted in biological conditions in streams. Appendix A outlines habitat evaluation schemes, while Chapter 6 also included descriptions on characterizing habitat. Understanding the effect that habitat has on stream biological uses is very important if these changes are to be minimized. This understanding needs to come from detailed local investigations, as our ability to predict habitat changes associated with stormwater discharges is rather poor. With site studies, some researchers have been able to recommend local guidelines to minimize habitat degradation.

MacRae (1997) found that stream bed and bank erosion is controlled by the frequency and duration of the mid-depth flows (generally occurring more often than once a year), not the bank-full condition (approximated by the 2-year event). During monitoring near Toronto, he found that the duration of the geomorphically significant predevelopment mid-bankfull flows increased by a factor of 4.2, after 34% of the basin had been urbanized, compared to before-development flow conditions. The channel had responded by increasing in cross-sectional area by as much as three times in some areas, and was still expanding. He also reported other studies that found channel cross-sectional areas began to enlarge after about 20 to 25% of the watershed was developed, corresponding to about a 5% impervious cover in the watershed. When the watersheds are completely developed, the channel enlargements were about five to seven times the original cross-sectional areas. Changes from stable stream bed conditions to unstable conditions appear to occur with basin imperviousness of about 10%, similar to the value reported previously for serious biological degradation. MacRae concluded that an effective criterion to protect stream stability (a major component of habitat protection) must address mid-bankfull events, especially by requiring similar durations and frequencies of stream power at these depths, compared to satisfactory reference conditions.

Much research on habitat changes and rehabilitation attempts in urban streams has occurred in the Seattle area of western Washington over the past 20 years. Sovern and Washington (1997) described the in-stream processes associated with urbanization in this area. The important factors that affect the direction and magnitude of changes in a steam's physical characteristics due to urbanization include:

- The depths and widths of the dominant discharge channel will increase directly proportional to the water discharge. The width is also directly proportional to the sediment discharge. The channel width divided by the depth (the channel shape) is also directly related to sediment discharge.
- The channel gradient is inversely proportional to the water discharge rate and is directly proportional to the sediment discharge rate and the sediment grain size.
- The sinuosity of the stream is directly proportional to the stream's valley gradient and is inversely proportional to the sediment discharge.
- Bedload transport is directly related to the stream power and the concentration of fine material and inversely proportional to the fall diameter of the bed material.

In their natural state, small streams in forested watersheds in western Washington have small low-flow channels (the aquatic habitat channel) with little meandering (Sovern and Washington 1997). The stream banks are nearly vertical because of clayey bank soils and heavy root structures, and the streams have numerous debris jams from fallen timber. The widths are also narrow, generally from 3 to 6 feet wide. Stable forested watersheds also support about 250 aquatic plant and animal species along the stream corridor. Pool/riffle habitat is dominant along streams having gradients less than about 2% slope, while pool/drop habitat is dominant along streams having gradients from 4 to 10%. The pools form behind large organic debris (LOD) or rocks. The salmon and trout in western Washington have evolved to take advantage of these stream characteristics. Sovern and Washington (1997) point out that less athletic fish species (such as chum and pink salmon) cannot utilize the steeper gradient, upper reaches of the streams. However, coho, steelhead, and cutthroat can use these upper areas.

Urbanization radically affects many of these natural stream characteristics. Pitt and Bissonnette (1984) reported that coho and cutthroat were affected by the increased nutrients and elevated temperatures of the urbanized streams in Bellevue, as studied by the University of Washington as part of the EPA's NURP project (EPA 1983). These conditions were probably responsible for accelerated growth of the fry, which were observed to migrate to Puget Sound and the Pacific Ocean sooner than their counterparts in the control forested watershed, which was also studied. However, the degradation of sediments, from decreased particle sizes, adversely affected their spawning areas in streams that had become urbanized.

Sovern and Washington (1997) reported that in western Washington, frequent high flow rates can be 10 to 100 times the predevelopment flows in urbanized areas, but that the low flows in the urban streams are commonly lower than the predevelopment low flows. They have concluded that the effects of urbanization on western Washington streams are dramatic, in most cases permanently changing the stream hydrologic balance by: increasing the annual water volume in the stream, increasing the volume and rate of storm flows, decreasing the low flows during dry periods, and increasing the sediment and pollutant discharges from the watershed. With urbanization, the streams increase in cross-sectional area to accommodate these increased flows, and headwater downcutting occurs to decrease the channel gradient. The gradients of stable urban streams are often only about 1 to 2%, compared to 2 to 10% gradients in natural areas. These changes in width and the downcutting result in very different and changing stream conditions. The common pool/drop habitats are generally replaced by pool/riffle habitats, and the stream bed material is comprised of much finer material, for example. Along urban streams, fewer than 50 aquatic plant and animal species are usually found. Researchers have concluded that once urbanization begins, the effects on stream shape are not completely reversible. Developing and maintaining quality aquatic life habitat is possible under urban conditions, but it requires human intervention and it will not be the same as for forested watersheds.

Other Seattle area researchers have specifically examined the role that large woody debris (LWD) has in stabilizing the habitat in urban streams. Booth and Jackson (1997) found that LWD performs key functions in undisturbed streams that drain lowland forested watersheds in western Washington. These important functions include dissipation of the flow energy, channel bank and bed stabilization, sediment trapping, and pool formation. Urbanization typically results in the almost complete removal of this material. They point out that logs and other debris have long been removed from channels in urban areas for many reasons, especially because of their potential for blocking culverts or forming jams at bridges. Also, they may increase bank scour, and many residents favor "neat" stream bank areas (a lack of woody debris in and near the water and even with mowed grass to the water's edge).

It is clear that stream hydraulics, sediment transport, and riparian vegetation dramatically affect habitat in streams. Water quality evaluations, by themselves, obviously do not consider these important factors. Evaluations of habitat conditions and effects of changing habitat on biological uses obviously require combinations of stream studies, modeling, and comparison with local

reference streams. The ability to predict habitat changes associated with urbanization, and the general success of habitat restoration efforts, is currently very poor. However, it is clear that detailed local investigations are critical and that habitat changes are likely one of the most important detrimental effects associated with urbanization.

EVALUATING HUMAN HEALTH IMPAIRMENTS USING A RISK ASSESSMENT APPROACH

The risk assessment paradigm is now well established in North America. The approach is basically the same for human health and ecological risk assessments (EPA 1989, 1998). The risk assessment paradigm is comprised of the following components: problem formulation, exposure and effects characterization followed by risk characterization, then the final risk management decisions. Risk assessment is a broad term which encompasses both risk characterization and risk management. The distinction between these two terms is an important one. The National Research Council's 1983 report on risk assessment in the federal government distinguished between risk assessment and risk management.

> Broader uses of the term [risk assessment] than ours also embrace analysis of perceived risks, comparisons of risks associated with different regulatory strategies, and occasionally analysis of the economic and social implications of regulatory decision functions that we assign to risk management. (EPA 1995)

The U.S. EPA has made the additional distinction of separating risk assessment from risk characterization. Risk characterization is the last step in risk assessment, is the starting point for risk managers, and is the foundation for regulatory decision making. The risk characterization identifies and highlights the noteworthy risk conclusions and related uncertainties (EPA 1995). The process described above is similar, but we have used different terminology. If the stormwater assessor is more comfortable using the EPA risk assessment approach, it can incorporate the guidelines of this handbook. The EPA guidance for conducting ecological risk assessments (ERAs) is quite general and does not provide specific methodologies and processes (EPA 1998). A number of good references (e.g., Suter 1993) exist that describe risk assessment approaches and considerations which are beyond the scope of this handbook. The two principal approaches for assessing adverse effects (hazard) in risk assessments are briefly described below.

Deterministic Approach

The simplest approach is the benchmark approach. This method (described above) basically ignores temporal exposure issues and focuses on point-in-time evaluations where threshold effect levels are compared to site contamination levels to ascertain risk. Many of the commonly used benchmarks (Appendix G) can be found in databases such the EPA's Ambient Water Quality Database, state water quality standards, ECOTOX, and the U.S. Department of Energy's Oak Ridge National Laboratory web site (http://www.hsrd.ornl.gov/ecorisk/ecorisk.html). This approach uses the quotient method for screening-level risk assessments. For compounds that bioaccumulate, it is easy to rearrange exposure equations involving uptake to back calculate ecotoxicity criteria for sediment, surface water, or soil (e.g., Pastorok et al. 1996).

Probabilistic Approach

A potentially more accurate and powerful assessment approach uses probability estimates to link likelihood of exposure with effects. This approach has been used frequently at Superfund sites,

looking at exposure pathway analysis and risk modeling to assess chemical risks to humans, and aquatic and terrestrial wildlife. Since food is a primary source of toxicants, food web models are important tools to describe potential ecosystem effects (Pastorok et al. 1996). The more advanced wildlife exposure models now contain three attributes: habitat spatial structure, food web complexity, and receptor behavior and physiology ranging from Tier 1 (steady-state, worst-case conservative) to Tier III (dynamic, stochastic). For assessments of aquatic stressor impacts, probabilistic assessments of pesticide effects have been conducted using the following steps (Solomon et al. 1996; World Wildlife Fund 1992):

1. Characterize sensitivity effects (select appropriate measurement endpoints and rank effect, e.g., EC50 or no observed effect levels, vs. concentration)
2. Characterize exposure (plot distribution of chemical concentrations vs. site vs. frequency of occurrence)
3. Risk characterization: compare exposure distribution with overlap of the sensitivity distribution, while considering uncertainty, confounding stressors, variables, and ecological relevance of the assessment

Example Risk Assessment for Human Exposure to Stormwater Pathogens

The following discussion, summarized from Meyland et al. (1998), describes waterborne pathogens in separate sewer overflow (SSO) discharges as an example of the risk assessment process applied to a wet-weather problem. SSOs are generally sanitary sewage discharges that occur at "relief" locations, resulting in untreated wastewater being discharged directly into receiving waters.

Hazard Identification

The first step in a risk assessment, hazard identification, can be examined by gathering information regarding waterborne disease outbreaks. The agent that causes disease could be chemical, physical, or biological. However, in this case we will focus on biological causes, or infectious agents, i.e., pathogenic microorganisms. Table 8.4 shows the agents that have caused waterborne disease outbreaks in the United States, from 1971 to 1990. Notice that the vast majority of known agents are microorganisms. Table 8.5 shows additional data compiled from waterborne disease outbreaks. This table shows the agent associated with the disease.

The Centers for Disease Control (CDC) keep detailed records regarding notifiable, or reportable, diseases. There are legal requirements for reporting cases of these diseases. This list of notifiable diseases includes cryptosporidiosis. The fact that this disease is notifiable means that it is recognized as being extremely hazardous. As of mid-April 1998, there were 520 cases of cryptosporidiosis (not notifiable in all 50 states) (CDC 1998).

Dose–Response

The concept of dose–response, the second step in a risk assessment, is critical. Briefly, dose–response describes a relationship between a given level of contaminant and the biological response induced. This relationship is usually incremental; i.e., increase in the dose causes an increase in the response. In this particular case, the dose is the number of pathogenic microorganisms that the human subject is exposed to (through ingestion, swimming, wading, etc.), and the response is the level of infection. Generally, there is a minimum infective dose threshold that must be reached in order to infect a given individual. Once an individual has been infected, there are increasing degrees of infection severity. A *subclinical* infection describes the case where the pathogen produces a detectable immune response or organisms may be found growing in the human host, but the subject exhibits no clinical signs or symptoms, e.g., diarrhea, vomiting, etc. A *clinical* infection

Table 8.4 Causative Agents of Waterborne Disease Outbreaks, 1971 to 1990

	Outbreaks		Illness	
	Number of Cases	Percentage of Total	Number of Cases	Percentage of Total
Gastroenteritis (unknown cause)	293	49.66	67,367	47.60
Giardiasis	110	18.64	26,531	18.75
Chemical poisoning	55	9.32	3877	2.74
Shigellosis	40	6.78	8806	6.22
Viral gastroenteritis	27	4.58	12,699	8.97
Hepatitis A	25	4.24	762	0.54
Salmonellosis	12	2.03	1370	0.97
Camplylobacterosis	12	2.03	5233	3.70
Typhoid fever	5	0.85	282	0.20
Yersiniosis	2	0.34	103	0.07
Cryptosporidiosis	2	0.34	13,117	9.27
Chronic gastroenteritis	1	0.17	72	0.05
Toxigenic *E. coli*	2	0.34	1243	0.88
Cholera	1	0.17	17	0.01
Dermatitis	1	0.17	31	0.02
Amebiasis	1	0.17	4	0.00
Cyanobacteria-like bodies	1	0.17	21	0.01
Total	590	100	141,535	100

Data from Committee on Groundwater Recharge, NRC (National Research Council), National Academy of Science. *Ground Water Recharge Using Waters of Impaired Quality.* National Academy Press, Washington, D.C. 284 pp. 1994.

Table 8.5 Waterborne Disease Outbreaks Due to Microorganisms[a]

Disease	Agent	Outbreaks[b] (%)	Cases[c] (%)
Bacteria			
Typhoid fever	*Salmonella typhi*	10	0.1
Shigellosis	*Shigella* spp.	9	2.6
Salmonellosis	*Salmonella paratyphi* and other *Salmonella* species	3	3.5
Gastroenteritis	*Escherichia coli*	0.3	0.7
	Campylobacter spp.	0.3	0.7
Viruses			
Infectious hepatitis	Hepatitis A virus	11	0.5
Diarrhea	Norwalk virus	1.5	0.6
Protozoa			
Giardiasis	*Giardia lamblia*	7	3.8
Cryptosporidiosis[d]	*Cryptosporidium parvum*	0.2	71
Unknown etiology			
Gastroenteritis		57	16.7

[a] Compiled from data provided by the Centers for Disease Control, Atlanta, GA.
[b] Of more than 650 outbreaks in recent decades.
[c] Of 520,000 cases over the same period.
[d] A single outbreak of cryptosporidiosis in 1993 caused illness in 370,000 individuals from Milwaukee, WI. This is the largest single recorded outbreak of a waterborne disease in history.

Data from Madigan, M.T., J.M. Martinko, and J. Parker. *Brock Biology of Microorganisms, 8th ed.* Prentice-Hall, Upper Saddle River, NJ. 1997.

refers to the condition in which there are clinical signs and symptoms present. In layman's terms, one would refer to a person with a clinical infection as being "ill." The most severe response to infection would be death, i.e., a *fatality*. Therefore, one usually refers to the MID50, that is, the minimum infective dose that will cause subclinical infection in 50% of people exposed to that number of pathogens. The minimal infective dose (MID) varies widely with the type of pathogen, as shown in Table 8.6 (Bitton 1994). Of those infected, a percentage will show clinical signs; this

Table 8.6 0Minimal Infective Doses for Some Pathogens and Parasites

Organism	Minimal infective Dose
Salmonella spp.	10^4 to 10^7
Shigella spp.	10^1 to 10^2
Escherichia coli	10^6 to 10^8
Vibrio cholerae	10^3
Giardia lamblia	10^1 to 10^2 cysts
Cryptosporidium	10^1 cysts
Entamoeba coli	10^1 cysts
Ascaris	1–10 eggs
Hepatitis A virus	1–10 PFU

Data from Bitton, G. *Wastewater Microbiology.* John Wiley & Sons, Inc. New York. 1994.

Table 8.7 0Values Used to Calculate Risks of Infection, Illness, and Mortality from Selected Enteric Microorganisms

	Probability of Infection from Exposure to One Organism (per million)	Ratio of Clinical Illness to Infection (%)	Mortality Rate (%)	Secondary Spread (%)
Campylobacter	7000			
Salmonella typhi	380			
Shigella	1000			
Vibrio cholerae	7			
Coxsackieviruses		5–96	0.12–0.94	76
Echoviruses	17,000	50	0.27–0.29	40
Hepatitis A virus		75	0.6	78
Norwalk virus			0.0001	30
Poliovirus 1	14,900	0.1–1	0.9	90
Poliovirus 3	31,000			
Rotavirus	310,000	28–60	0.01–0.12	
Giardia lamblia	19,800			

Data from Committee on Groundwater Recharge, NRC (National Research Council), National Academy of Science. *Ground Water Recharge Using Waters of Impaired Quality.* ISBN 0-309-05142-8. National Academy Press, Washington, D.C. 284 pp. 1994.

is referred to as the ratio of clinical illness to infection. In addition, a percentage of those infections will result in fatalities; this is referred to as the case fatality rate. Table 8.7 shows example values for these various levels of response to infection.

Notice that higher probabilities, rates, or percentages correspond to pathogens with higher virulence. For example, if 1 million people are exposed to one rotavirus each, then 310,000 may be infected. In contrast, if 1 million people are exposed to one *Vibrio cholerae* bacterium each, only seven may be infected. In general, viral pathogens are much more virulent than bacterial pathogens.

Table 8.8 shows another example of data that can be obtained from published studies. These data show, for instance, that once infected by *Salmonella* bacteria, approximately 41% will exhibit clinical infection. In addition, *Cryptosporidium* infection results in a 71% clinical infection frequency.

Another study (DuPont et al. 1995) published results pertaining to infection rates from the oral introduction of *Cryptosporidium* oocysts into healthy volunteers. Various doses of oocysts, from 30 to 1 million, were given to volunteers in gelatin capsules, and these subjects were followed up to record the incidence of infection. Table 8.9 gives these results. A linear regression analysis of the data yielded a correlation coefficient of 0.983 and an infectious dose of 50 of 132 oocysts. This is an excellent example of the dose–response relationship, as increasing doses of oocysts caused increasing rates of infection.

Table 8.8 0Ratio of Clinical to Subclinical Infections and Case Fatality Rates for Enteric Microorganisms

Microorganism	Frequency of Clinical Illness (%)	Case:Fatality Rate (%)
Viruses		
Hepatitis (adults)	75	0.6
Rotavirus	25–60	0.01
Astrovirus (adults)	12.5	0.12
Coxsackie A16	50	0.59–0.94
Coxsackie B	5–96	
Bacteria		
Salmonella	41	0.1
Shigella	46	0.2
Protozoan parasites		
Giardia	50–67	
Cryptosporidium	71	

Data from Gerba, C.P., J.B. Rose, C.N. Hass, and K.D. Crabtree. Waterborne rotavirus: a risk assessment. *Water Research*, Vol. 30, No 12, pp. 2929–2940. Dec. 1996.

Table 8.9 0Rate of Infection, Enteric Symptoms, and Clinical Cryptosporidiosis, According to the Intended Dose of Oocysts

Intended Dose of Oocysts	No. of Subjects	Infection	Enteric Symptoms	Cryptosporidiosis
		Number (percent)		
30	5	1 (20)	0	0
100	8	3 (37.5)	3 (37.5)	3 (37.5)
300	3	2 (66.7)	0	0
500	6	5 (83.3)	3 (50)	2 (33.3)
>1000	7	7 (100)	5 (71.4)	2 (28.6)
Total	29	18	11	7

Data from DuPont, H.L., C.L. Chappell, C.R. Sterling, P.C. Okhuysen, J.B. Rose, and W. Jakubowski. The infectivity of *Cryptosporidium parvum* in healthy volunteers. *N. End. J. Med.*, Vol. 332, No. 13, pp. 855–859. 1995.

Exposure Assessment

The exposure assessment is the third step in a risk assessment. Several factors contribute to whether or not contact with a particular pathogen may cause disease. Among these factors are virulence, mode of transmission, portal of entry, and host susceptibility. Virulence is defined as a particular organism's ability to cause disease in humans and is related to the dose of infectious agent necessary for host infection and causing disease (Bitton 1994). The mode of transmission is the particular method in which the organism is transported from the reservoir of pathogens (such as a contaminated outfall) to the host, i.e., person-to-person, waterborne, or foodborne.

The research conducted by Meyland et al. (1998) concentrated on the waterborne transmission route of SSOs to exposed individuals, but exposure assessment can also be evaluated based on portal of entry. The portal of entry is dictated by the mechanism of contact; examples or entry portals are access through the gastrointestinal tract, respiratory tract, or skin. Host susceptibility is dependent upon resistance to infectious agents, which consists of the roles of the immune system and nonspecific factors (Bitton 1994). Immunity can be both natural (genetic) and acquired from previous contact with the pathogen.

There are many documented examples of waterborne transmission of pathogenic microorganisms. Recently, in the United States, there has been widespread concern about *Cryptosporidium* contamination of water supplies. This is an example of waterborne transmission via a contaminated drinking water supply. Table 8.10 summarizes the available information regarding *Cryptosporidium* outbreaks in the United States. Outbreaks caused by this organism are a

Table 8.10 0*Cryptosporidium* Outbreaks: Affected Populations and Characteristics of the Raw Water Supply

County, State (City)	Date	Estimated No. of People Affected (Confirmed Cases)[a]	Raw Water Source	Suspected Sources of Contamination
Bexar County, TX (Braun Station)	May–Jul 1984	2,000 (47)	Well	Raw sewage[b]
Bernalillo County, NM (Albuquerque)	Jul–Oct 1986	(78)	Surface water	Surface runoff from livestock grazing areas
Carroll County, GA (Carrollton)	Jan–Feb 1987	13,000	River	Raw sewage and runoff from cattle grazing areas
Berks County, PA	Aug 1991	551	Well	Septic tank effluent, nearby creek
Jackson County, OR (Talent and Medford)	Jan–Jun 1992	15,000	Spring/river	Surface water, treated wastewater,[b] or runoff from agricultural areas
Milwaukee County, WI (Milwaukee)	Jan–Apr 1993	403,000	Lake	Cattle wastes, slaughterhouse wastes, and sewage carried by tributary rivers
Yakima County, WA	Apr 1993	7 (3)	Well	Infiltration of runoff from cattle, sheep, or elk grazing areas
Cook County, MN (Grand Marais)	Aug 1993	27 (5)	Lake	Backflow of sewage or septic tank effluent into distribution, raw water inlet lines, or both
Clark County, NV (Las Vegas)	Jan–Apr 1994	(78)[c]	Lake	Treated wastewater, sewage from boats
Walla Walla County, WA	Aug–Oct 1994	86 (15)	Well	Treated wastewater[b]
Alachua County, FL	Jul 1995	(72)	N/A	Backflow of contaminated water

[a] Estimates are based on epidemiologic studies; confirmed cases correspond to patients whose stool samples tested positive for *Cryptosporidium*.
[b] Strong evidence to support effect of wastewater.
[c] 103 laboratory-confirmed cases were associated with the outbreak; 78 of these were documented during the epidemiologic study period.

Data from Solo-Gabriele, H. and S. Neumeister. US outbreaks of cryptosporidiosis. *Journal American Water Works Association*, Vol. 88, No. 9, pp. 76–86. Sept. 1996.

significant health threat (more than 400,000 people were infected during the 1993 Milwaukee outbreak). Moreover, notice that the suspected source of contamination is likely to be sewage. In fact, municipal wastewater was implicated as the source in roughly half of the outbreaks (Solo-Gabriele and Neumeister 1996). The remaining outbreaks were likely caused by contaminated agricultural runoff.

Another important mode of transmission is water-contact recreation. This type of transmission is usually associated with swimming beach exposures. Many studies have shown an association between illness and swimming near stormwater, SSO, or CSO (combined sewer overflow) outfalls. In general, most of these studies found an increased risk of illness resulting from swimming in waters that contained fecal contamination indicators or pathogenic microorganisms. The SMBRP (Santa Monica Bay Restoration Project) Study is unique in that it found a distance-dependent association between contamination sources and health effects. In this study, there was a higher rate of enteric illness in swimmers who swam within 400 ft of a stormwater outfall than in those who swam more than 400 ft away. In many urban receiving waters, children frequently play in and near potentially contaminated small streams and creeks, well away from designated swimming beaches. In most cases, this exposure route is not considered, because this is not a designated use of the water. The most important pathogens contained in stormwater are likely from sewage contamination.

Table 8.11 Sensitive Populations in the United States

Population	Individuals	Year
Pregnancies	5,657,900	1989
Neonates	4,002,000	1989
Elderly people (over 65)	29,400,000	1989
Residences in nursing homes or related care facilities	1,553,000	1986
Cancer patients (non-hospitalized)	2,411,000	1986
Transplant organ patients (1981–1989)	110,270	1981–1989
AIDS patients	142,000	1981–1990

Data from Gerba, C.P., J.B. Rose, C.N. Hass, and K.D. Crabtree. Waterborne rotavirus: a risk assessment. *Water Research*, Vol. 30, No 12, pp. 2929–2940. Dec. 1996.

Pitt et al. (1993) conducted a study of inappropriate pollutant entries into storm drainage systems in which many illegal sanitary sewer connections to storm drain systems were commonly found.

Another possible exposure route is through the consumption of contaminated fish or shellfish. One example of this type of outbreak occurred in Louisiana in 1993 (Kohn 1995). This outbreak was caused by contaminated oysters that were consumed raw. The agent implicated in this outbreak was Norwalk virus, which causes gastroenteritis. Seventy of the 84 people (83% infection rate) who ate the raw oysters became ill. The epidemiologic investigation found that this outbreak was probably caused by overboard sewage disposal by harvesters near the oyster bed.

An additional consideration that one must account for when assessing the adverse health effects of contact with pathogenic microorganisms is that certain individuals within the population are at higher risk for serious infections. Individuals who are at higher risk are the very young, elderly people, pregnant individuals, and immunocompromised (organ transplants, cancer, and AIDS) patients (Gerba et al. 1996). This collective group represents almost 20% of the current U.S. population (Table 8.11). In addition, elderly and immunocompromised people are an increasing segment of the population.

Calculation of Risk

The fourth step in a risk assessment is the calculation of risk for a specific situation. As indicated in the first three steps, it is possible to identify which components pose a risk for a specific activity, to estimate the doses necessary to cause different responses, and to conduct the exposure assessment. For an urban receiving water study, it is possible to consider an important, but commonly overlooked, scenario: exposure to pathogens by children who are wading in an urban stream. The list of potential pathogens may be lengthy, depending on the likelihood of sanitary sewage contamination. Even with separate stormwater, numerous pathogens are still commonly present (such as *Pseudomonas aeruginosa* and *Shigella*). Sanitary sewage contamination lengthens the list considerably, as shown on the above lists. The dose–response curves can be examined for each likely pathogen and route of exposure (such as water contact for *P. aeruginosa*, or ingestion for *Giardia* and *Cryptosporidium*). The difficulty is estimating the magnitude of discharge for each pathogen and its fate after the discharge to the likely point of contact. Exposure duration, or ingestion quantity, of the water also needs to be estimated, along with likely time between discharge and exposure. This is especially important for wading exposures, for example, because water-contact recreation is unlikely during a runoff event but can certainly occur soon after a rain has ended. However, exposure to pathogens when wading is likely to be made more severe through stirring up contaminated sediments. Bacteria have been shown to survive for long periods in stream sediments in areas of deposition (pools, backwaters, or behind small dams), many of which attract children because of the deeper water (Burton et al. 1987; Burton 1982). Therefore, it would be necessary to predict areas where the particulates, with which pathogens are associated, are likely to accumulate.

The local monitoring program must therefore consider characterization studies of the pathogens in the discharge, along with the hydraulic characteristics of the receiving water that would affect transport and fate of the pathogens. This information is used in a receiving water model to predict the conditions (concentrations in the water) at the time and location of exposure. Appendix H summarizes representative receiving water models that may be applicable for certain fate and route pathways. HSPF, one of the more complex urban stream models, contains many options, but requires a great deal of monitoring data for calibration and verification. Other, simpler models, such as WASP5, may be suitable for this task. The predicted conditions at the likely sites of exposure can be used, along with assumptions pertaining to exposure duration. This information is then compared to the dose–response information to estimate the likelihood of contacting disease.

IDENTIFYING AND PRIORITIZING CRITICAL STORMWATER SOURCES

An important goal of a receiving water study is to learn enough about local problems to be able to reduce them in the future, and to prevent new problems from occurring. The tools and techniques presented in this book enable local watershed managers to obtain a good understanding of their local problems and their likely causes.

After receiving water problems (beneficial use impairments) are described, the likely causes for these problems must be identified. Many of the problems are directly associated with measured parameters in excessive quantities and are therefore inherently obvious (such as high bacteria concentrations interfering with water-contact recreation and fish consumption; high flows and debris blockages affecting drainage; trash or odors affecting noncontact recreation; and high toxicant concentrations affecting water supplies). The most difficult task is identifying the possible causes for declining aquatic life conditions, which can be associated with numerous causes, including habitat destruction, high/low flows, inappropriate discharges, polluted sediment, and water quality. The weight-of-evidence approach, described above, has therefore become a useful framework to understand possible cause-and-effect relationships for biological resources.

Once the critical pollutants (or flow conditions) are identified, it is more straightforward to identify the likely sources in the watershed contributing these problem constituents. Classical approaches include watershed modeling to develop mass balances for targeted pollutants. The following discussion describes this approach, along with a case study describing how the Wisconsin Nonpoint Source Program has integrated field monitoring, data analysis, watershed modeling, and the development of stormwater controls to reduce future receiving water problems. It is also important to recognize additional potential sources of contaminants in a watershed that are not easily addressed by most watershed models. The most important include snowmelt and inappropriate sources. These two sources can be much greater contributors of some critical pollutants than warm-weather runoff alone. Field investigations to locate and quantify inappropriate sources (sanitary sewage and discharges from small industries and commercial establishments are the most important) were described in Chapter 6 and must be conducted along with conventional watershed modeling activities. Snowmelt contributions should also be quantified for comparison with the more traditional sources.

Sources of Urban Stormwater Contaminants

Urban runoff is comprised of many separate source area flow components that are combined within the drainage area and at the outfall before entering the receiving water. It may be adequate to consider the combined outfall conditions alone when evaluating the long-term, areawide effects of many separate outfall discharges to a receiving water. However, if better predictions of outfall characteristics (or the effects of source area controls) are needed, the separate source area components

must be characterized. The discharge at the outfall is made up of a mixture of contributions from different source areas. The "mix" depends on the characteristics of the drainage area and the specific rain event. The effectiveness of source area controls is therefore highly site and storm specific.

Various urban source areas contribute different quantities of runoff and pollutants, depending on their specific characteristics. Impervious source areas may contribute most of the runoff during small rain events. Examples of these source areas include paved parking lots, streets, driveways, roofs, and sidewalks. Pervious source areas become important contributors for larger rain events. These pervious source areas include gardens, lawns, bare ground, unpaved parking areas and driveways, and undeveloped areas. The relative importance of each individual source is a function of their areas, their pollutant washoff potentials, and the rain characteristics.

The washoff of debris and soil during a rain is dependent on the energy of the rain and the properties of the material. Pollutants are also removed from source areas by winds, litter pickup, or other cleanup activities. The runoff and pollutants from the source areas flow directly into the drainage system, onto impervious areas that are directly connected to the drainage system, or onto pervious areas that will attenuate some of the flows and pollutants before they discharge to the drainage system.

Sources of pollutants on paved areas include on-site particulate storage that cannot be removed by the usual processes e.g., rain, wind, street cleaning, etc. Atmospheric deposition, deposition from activities on these paved surfaces (auto traffic, material storage, etc.), and the erosion of material from upland areas that discharge flows directly onto these areas are the major sources of pollutants to the paved areas. Pervious areas contribute pollutants mainly through erosion processes where the rain energy dislodges soil from between plants. The runoff from these source areas enters the storm drainage system where sedimentation in catchbasins or in the sewerage may affect their ultimate discharge to the outfall. In-stream physical, biological, and chemical processes affect the pollutants after they are discharged to the ultimate receiving water.

It is important to know when the different source areas become "active" (when runoff initiates from the area, carrying pollutants to the drainage system). If pervious source areas are not contributing runoff or pollutants, the prediction of urban runoff quality is much simplified. The mechanisms of washoff, and delivery yields of runoff and pollutants from paved areas, are much better known than from pervious urban areas (Novotny and Chesters 1981). In many cases, pervious areas are not active runoff contributors except during rain events greater than at least 5 or 10 mm. For smaller rain depths, almost all of the runoff and pollutants originate from impervious surfaces (Pitt 1987). However, in many urban areas, pervious areas may contribute the majority of the runoff, and some pollutants, when rain depths are greater than about 20 mm. The actual importance of the different source areas is highly dependent on the specific land use and rainfall patterns. Obviously, in areas having relatively low-density development, especially where moderate- and large-sized rains occur frequently (such as in the Southeast), pervious areas typically dominate outfall discharges. In contrast, in areas having significant paved areas, especially where most rains are relatively small (such as in the arid West), the impervious areas dominate outfall discharges. The effectiveness of different source controls is therefore quite different for different land uses and climatic patterns.

If the number of events exceeding a water quality objective is important, then the small rain events are of most concern. Stormwater runoff typically exceeds some water quality standards for practically every rain event (especially for bacteria and some heavy metals). In the upper Midwest, the median rain depth is about 6 mm, while in the Southeast, the median rain depth is about twice this depth. In most urban areas, no runoff is observed until the rain depth exceeds 2 or 3 mm. For these small rain depths and for most urban land uses, directly connected paved areas usually contribute most of the runoff and pollutants. However, if annual mass discharges are more important, e.g., for long-term effects, then the moderate rains are more important. Rains from about 10 to 50 mm produce most of the annual runoff volume in many areas of the United States. Runoff from both impervious and pervious areas can be very important for these rains. The largest rains (greater than about 100 mm) are relatively rare and do not contribute significant amounts of runoff pollutants

during normal years, but are very important for drainage design. The specific source areas that are most important (and controllable) for these different conditions vary widely.

Case Study: Wisconsin Nonpoint Source Program in Urban Areas

The urban stormwater evaluation methodology used by the Nonpoint Source Program at the Wisconsin Department of Natural Resources was developed to supplement the extensive ongoing rural aspects of the statewide watershed planning program (Pitt 1986). This comprehensive urban methodology includes:

- Evaluation of receiving water goals, based on established water use objectives and through meetings of citizen groups and technical experts
- Identification of current problems and sources of problem pollutants and flows through monitoring and modeling
- Identification and evaluation of suitable source area and outfall treatment options, including the development of model ordinances for construction site erosion control and stormwater management, plus the development of design manuals for constructing controls
- Demonstration projects evaluating alternative controls
- Receiving water evaluations to confirm or to modify recommendations

An important element of this methodology was to extensively modify SLAMM, the Source Loading and Management Model (Pitt and Voorhees 1995; www.winslamm.com). This model was developed to identify sources of pollutants in urban areas and to evaluate many alternative stormwater control programs. This methodology is generally used in the development of the watershed plans and to determine suitable cost-sharing aspects of the Wisconsin Nonpoint Source Program. The Milwaukee area was the first urban watershed planning effort to use this methodology. Milwaukee has a great deal of stormwater quality information that was used in this planning and implementation effort.

Stormwater quality management in the Milwaukee area was initiated as part of the Wisconsin Priority Watershed Program. This program was developed in 1978 to help combat both urban and rural nonpoint sources of pollution (EPA 1990a, 1991). This program is one of the oldest in the nation funding nonpoint pollution abatement. An important element of this program is retrofitting control practices in both rural and urban areas. The program was initially heavily involved in rural areas, with technical assistance from the NRCS. A unique aspect of the program is that it is implemented on a watershed, and not on a political jurisdiction basis. Of the state's 330 watersheds, 130 (mostly located in the southern part of Wisconsin) will likely require comprehensive management activities to control nonpoint pollutants. A 25-year plan was developed in 1982 which would require the start-up of about eight or nine new watershed abatement efforts per year. The watershed plans are prepared by the state with cooperation and reviews by local government agencies. They contain detailed analyses of the water resources objectives (existing and desired beneficial uses, including the problems and threats to these uses), the critical sources of problem pollutants, and the control practices that can be applied within each watershed. The plans also include implementation schedules and budgets to meet the pollution reduction objectives.

Each plan requires 1 year to prepare, including the necessary fieldwork. Various field inventory activities are needed to prepare the plans, including aquatic biology and habitat surveys to identify existing and potential fishery uses, stream bank surveys to identify the nature and magnitude of stream bank erosion problems and to help design needed controls, field and barnyard surveys to supply information needed to estimate and rank their pollution potentials and to design farm control practices, and urban surveys needed to evaluate urban runoff pollution potential and its control.

Urban planning was initiated in 1983 in the Milwaukee and Madison areas, with other urban areas of the state following. The urban practices eligible for cost sharing identified in these plans have included stream bank protection, detention basins, and infiltration devices for existing urbanized areas. Construction site erosion controls are also usually required as a condition for a grant

agreement in an urban area, but they are not eligible for state cost sharing. About $3 to $5 million per year will be used by the nonpoint source program over a 20-year period in controlling urban runoff. An outcome of the Milwaukee River South Watershed plan included goals for reducing urban stormwater discharges (D'Antuono 1998). These goals were 50% reductions for suspended solids and heavy metals, and 50 to 70% reductions for phosphorus.

Detailed studies on toxicant sources, effects, and controls have also been conducted in Milwaukee, including a study conducted in the heavily urbanized Lincoln Creek (having a 19-mi^2 watershed and being 9 mi long). A seven-tiered indicator program, incorporating many physical, chemical, and biological tests, was simultaneously conducted which identified long-term toxicity problems, likely associated with resuspended contaminated sediments having high levels of organic compounds (Claytor 1996). It was found that discharges of these fine sediments could be significantly reduced through the use of well-designed and maintained wet detention basins. The in-stream toxicity monitoring methods developed and used during the Lincoln Creek study can be used by other municipalities to answer the following basic questions:

- Are toxic conditions present?
- What is causing the toxicity?
- How much is too much urbanization?
- Can stormwater controls reduce these problems?

The benefits of stormwater controls have also been evaluated in Milwaukee, especially grass swales, wet detention ponds, and underground devices for critical source areas. The Southeastern Wisconsin Regional Planning Commission also prepared a comprehensive report documenting costs associated with construction site erosion and stormwater control.

Use of SLAMM to Identify Pollutant Sources and to Evaluate Control Programs

A logical approach to stormwater management requires knowledge of the problems to be solved, the sources of the problem pollutants, and the effectiveness of stormwater management practices that can control the problem pollutants at their sources and at outfalls. The Source Loading and Management Model (SLAMM) is designed to provide information on these last two aspects of this approach. The first versions of SLAMM were developed by Pitt in the mid-1970s to help evaluate the results from early EPA stormwater projects (Pitt and Voorhees 1995). Further information on SLAMM, especially its integration with GIS systems, is included in Appendix H and at www.winslamm.com.

The development of SLAMM began in the mid-1970s, primarily as a data reduction tool for use in early street cleaning and pollutant source identification projects sponsored by the EPA's Storm and Combined Sewer Pollution Control Program (Pitt 1979, 1984; Pitt and Bozeman 1982). Additional information contained in SLAMM was obtained during the EPA's Nationwide Urban Runoff Program (NURP) (EPA 1983), especially the early Alameda County, CA (Pitt and Shawley 1982), and the Bellevue, WA (Pitt and Bissonnette 1984) projects. The completion of the model was made possible by the remainder of the NURP projects and additional field studies and programming support sponsored by the Ontario Ministry of the Environment (Pitt and McLean 1986), the Wisconsin Department of Natural Resources (Pitt 1986), and Region V of the U.S. Environmental Protection Agency. Early users of SLAMM included the Ontario Ministry of the Environment's Toronto Area Watershed Management Strategy (TAWMS) study (Pitt and McLean 1986) and the Wisconsin Department of Natural Resources' Priority Watershed Program (Pitt 1986). SLAMM can now be effectively used as a tool to enable watershed planners to obtain a better understanding of the effectiveness of different control practice programs.

Some of the major users of SLAMM have been associated with the Nonpoint Source Pollution Control Program of the Wisconsin Department of Natural Resources, where SLAMM has been used for a number of years to support their extensive urban stormwater planning and cost-sharing program (Thum et al. 1990; Kim et al. 1993a,b; Ventura and Kim 1993; Bachhuber 1996; Banner-

man et al. 1996; Haubner and Joeres 1996; Legg et al. 1996). Many of these applications have included the integrated use of SLAMM with GIS models, as illustrated in Appendix H.

SLAMM was developed primarily as a planning-level tool to generate information needed for planning-level decisions, while not generating or requiring superfluous information. Its primary capabilities include predicting flow and pollutant discharges that reflect a broad variety of development conditions and the use of many combinations of common urban runoff control practices. Control practices evaluated by SLAMM include detention ponds, infiltration devices, porous pavements, grass swales, catchbasin cleaning, and street cleaning. These controls can be evaluated in many combinations and at many source areas as well as the outfall location. SLAMM also predicts the relative contributions of different source areas (roofs, streets, parking areas, landscaped areas, undeveloped areas, etc.) for each land use investigated. As an aid in designing urban drainage systems, SLAMM also calculates correct NRCS curve numbers that reflect specific development and control characteristics. These curve numbers can then be used in conjunction with available urban drainage procedures to reflect the water quantity reduction benefits of stormwater quality controls.

SLAMM is normally used to predict source area contributions and outfall discharges. However, it has been used in conjunction with a receiving water model (HSPF) to examine the ultimate receiving water effects of urban runoff (Ontario 1986), and has been recently been modified to be integrated with SWMM (Pitt et al. 1999) to more accurately consider the joint benefits of source area controls on drainage design. The following example illustrates how SLAMM is used to identify the most important source areas and how to select the most appropriate control programs.

The areas of the different surfaces in each land use are very important for SLAMM evaluations. Figure 8.2 is an example showing the areas of different surfaces for a medium-density residential area in Milwaukee. As shown in this example, streets make up between 10 and 20% of the total area, while landscaped areas can make up about half of the drainage area. The variation of these different surfaces can be very large within a designated area. The analysis of many candidate areas might therefore be necessary to understand how effective or how consistent the model results might be for a general land use classification.

One of the first problems in evaluating an urban area for stormwater controls is the need to understand where the pollutants of concern are originating under different rain conditions.

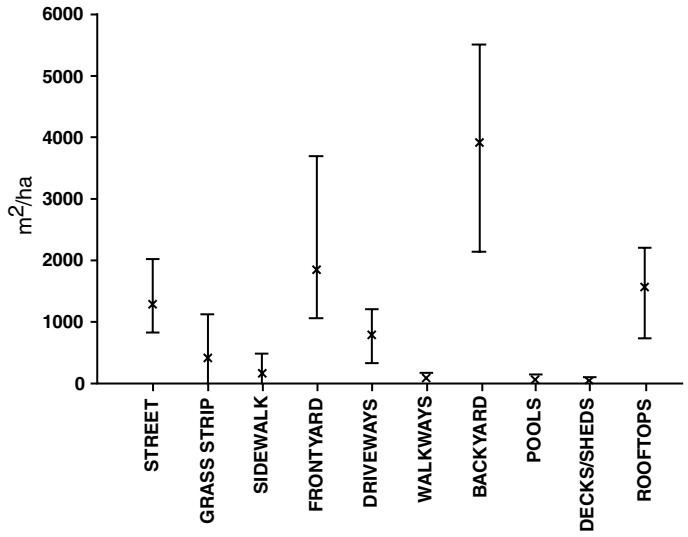

Figure 8.2 Source areas — Milwaukee medium-density residential areas (without alleys). (From Pitt, R. *Small Storm Flow and Particulate Washoff Contributions to Outfall Discharges*. Ph.D. dissertation, Department of Civil and Environmental Engineering, the University of Wisconsin, Madison, November 1987. With permission.)

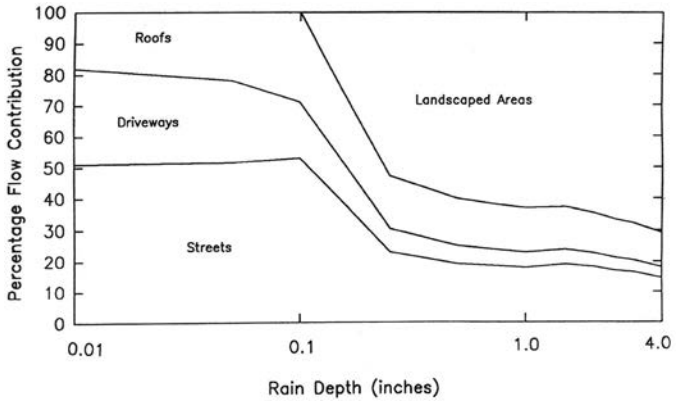

Figure 8.3 Flow sources for example medium-density residential area having clayey soils. (From Pitt, R. and J. Voorhees. Source loading and management model (SLAMM). *Seminar Publication: National Conference on Urban Runoff Management: Enhancing Urban Watershed Management at the Local, County, and State Levels*. March 30–April 2, 1993. Center for Environmental Research Information, U.S. Environmental Protection Agency. EPA/625/R-95/003. Cincinnati. OH. pp. 225–243. April 1995.)

Figure 8.3 is an example of a typical medium-density residential area, showing the percentage of runoff originating from different major sources, as a function of rain depth. For storms of up to about 0.1 in in depth, street surfaces contribute about one half of the total runoff to the outfall. This contribution decreased to about 20% for storms greater than about 0.25 in in depth. This decrease in the significance of streets as a source of water is associated with an increase of water contributions from landscaped areas (which make up more than 75% of the area and have clayey soils). Similarly, the significance of runoff from driveways and roofs also starts off relatively high and then decreases with increasing storm depth. Obviously, this is just an example plot, and the source contributions would vary greatly for different land uses/development conditions, rainfall patterns, and the use of different source area controls.

A major use of SLAMM is to better understand the role of different sources of pollutants and the suitability of controls that can be applied at the sources and at outfalls. As an example, to control suspended solids, street cleaning (or any other method to reduce the washoff of particulates from streets) may be very effective for the smallest storms, but would have very little benefit for storms greater than about 0.25 in in depth. However, erosion control from landscaped surfaces may be effective over a wider range of storms. The following list shows the different control programs that were investigated in this hypothetical medium-density residential area:

- Base level (as built in 1961–1980 with no additional controls)
- Catchbasin cleaning
- Street cleaning
- Grass swales
- Roof disconnections
- Wet detention pond
- Catchbasin and street cleaning combined
- Roof disconnections and grass swales combined
- All of the controls combined

This residential area, which was based upon actual Birmingham, AL, field observations for homes built between 1961 and 1980, has no controls, including no street cleaning or catchbasin cleaning. The use of catchbasin cleaning in the area, in addition to street cleaning, was evaluated. Grass swale use was also evaluated, but swales are an unlikely retrofit option and would only be appropriate for newly developing areas. However, it is possible to disconnect some of the roof drainages and divert the roof runoff away from the drainage system and onto grass surfaces for

infiltration in existing developments. In addition, wet detention ponds can be retrofitted in different areas and at outfalls. Besides those controls examined individually, catch basin and street cleaning controls combined were also evaluated, in addition to the combination of disconnecting some of the rooftops and the use of grass swales. Finally, all of the controls were also examined together.

The following list shows a general description of this hypothetical area:

- All curb and gutter drainage (in fair condition)
- 70% of roofs drain to landscaped areas
- 50% of driveways drain to lawns
- 90% of streets are intermediate texture (remaining are rough)
- No street cleaning
- No catchbasins

About one half of the driveways currently drain to landscaped areas, while the other half drain directly to the pavement or the drainage system. Almost all of the streets are of intermediate texture, and about 10% are rough textured. As noted earlier, there currently is no street cleaning or catchbasin cleaning.

The level of catchbasin use that was investigated for this site included 950 ft^3 of total sump volume per 100 acres (typical for this land use), with a cost of about $50 per catchbasin cleaning. Typically, catchbasins in this area could be cleaned about twice a year, for a total annual cost of about $85 per acre of the watershed. Street cleaning could also be used, with a monthly cleaning effort for about $30 per year per watershed acre. Grass swale drainage was also investigated. Assuming that swales could be used throughout the area, there could be 350 ft of swales per acre (typical for this land use), and the swales could be 3.5 ft wide. Because of the clayey soil conditions, an average infiltration rate of about 0.5 in per hour was used in this analysis, based on many different double ring infiltrometer tests of typical soil conditions. Swales cost much less than conventional curb and gutter systems, but have an increased maintenance frequency. Again, the use of grass swales is appropriate for new development, but not for retrofitting in this area.

Roof disconnections could also be utilized as a control measure by directing all roof drains to landscaped areas. The objective would be to direct *all* the roof drains to landscaped areas. Since 70% of the roofs already drain to the landscaped areas, only 30% would be further disconnected, at a cost of about $125 per household. The estimated total annual cost for roof disconnections would be about $10 per watershed acre. An outfall wet detention pond suitable for 100 acres of this medium-density residential area would have a wet pond surface of 0.5% of drainage area to provide about 90% suspended solids control. It would need 3 ft of dead storage and live storage equal to runoff from 1.25 in of rain. The total annual cost for wet detention ponds was estimated to be about $130 per watershed acre.

Table 8.12 summarizes the SLAMM results for runoff volume, suspended solids, filterable phosphate, and total lead for 100 acres of this medium-density residential area. The only control practices evaluated that would reduce runoff volume are the grass swales and roof disconnections. All of the other control practices evaluated do not infiltrate stormwater. Table 8.12 also shows the total annual average volumetric runoff coefficient (Rv) for these different options. The base level of control has an annual flow-weighted Rv of about 0.3, while the use of swales would reduce the Rv to about 0.1. Only a small reduction of Rv (less than 10%) would be associated with complete roof disconnections, compared to the existing situation, because of the large number of roof disconnections that already exist. The suspended solids analyses show that catchbasin cleaning alone could result in about 14% suspended solids reductions. Street cleaning would have very little benefit, while the use of grass swales would reduce the suspended solids discharges by about 60%. Grass swales would have minimal effect on the reduction of suspended solids concentrations at the outfall, but provide about 60% reductions in annual pollutant mass discharges (they are primarily an infiltration device, having very little filtering benefit). Wet detention ponds would remove about 90% of the mass and concentrations of suspended solids. Similar observations can be made for filterable phosphates and total lead.

Table 8.12 SLAMM Predicted Runoff and Pollutant Discharge Conditions for Example[a]

Birmingham 1976 rains: (112 rains, 55 in. total 0.01–3.84 in. each)	Runoff Volume			Suspended Solids		Filterable Phosphate		Total Lead	
	Annual ft³/acre	Flow-wtg. Rv	CN Range	Flow–wtg. mg/L	Annual lbs/acre	Flow-wtg. µg/L	Annual lbs/acre	Flow-wtg. µg/L	Annual lbs/acre
Base (no controls)	59800	0.3	77–100	385	1430	157	0.58	543	2.0
Catchbasin cleaning		0.3	77–100	331	1230	157	0.58	468	1.7
reduction (lbs or ft³)	59800			14	200	0	0	14	0.29
reduction (%)	0				14		0		14
cost ($/lb or $/ft³)	0				0.43		N/A		293
($85/acre/yr)	N/A								
Street cleaning		0.3	77–100	385	1430	157	0.58	543	2.0
reduction (lbs or ft³)	59800			0	0	0	0	0	0.01
reduction (%)	0				0		0		0.49
cost ($/lb or $/ft³)	0				N/A		N/A		3000
($30/acre/yr)	N/A								
Grass swales		0.12	63–100	380	554	151	0.22	513	0.75
reduction (lbs or ft³)	23300			1	876	1	0.36	6	1.28
reduction (%)	36500				61		62		63
cost ($/lb or $/ft³)	61				minimal		minimal		minimal
($minimal/acre/yr)	minimal								
Roof disconnections		0.28	76–100	410	1430	156	0.55	443	1.6
reduction (lbs or ft³)	56000			–6	0	1	0.03	18	0.48
reduction (%)	3800				0		5		24
cost ($/lb ir $/ft³)	6				N/A		333		21
(10/acre/yr)	0								
Wet detention pond		0.3	77–100	49	185	157	0.58	69	0.26
reduction (lbs or ft³)	59800			87	1250	0	0	87	1.8
reduction (%)	0				87		0		87
cost ($/lb or $/ft³)	0				0.10		N/A		73
($130/acre/yr)	N/A								
CB and street cleaning		0.3	77–100	331	1230	157	0.58	468	1.7
reduction (lbs. or $/ft³)	59800			14	200	0	0	14	0.29
reduction (%)	0				14		0		14
cost ($/lb or $/ft³)	0				0.58		N/A		397
($115/acre/yr)	N/A								
Roof dis. and swales		0.1	63–100	403	526	139	0.18	352	0.46
reduction (lbs or ft³)	20900			–5	904	11	0.4069	35	1.6
reduction (%)	38900				63		25		77
cost ($/lb or $/ft³)	65				0.01				6.4
($10/acre/yr)	0.00026								

Table 8.12 SLAMM Predicted Runoff and Pollutant Discharge Conditions for Example[a] (continued)

Birmingham 1976 rains: (112 rains, 55 in. total 0.01–3.84 in. each)	Runoff Volume			Suspended Solids		Filterable Phosphate		Total Lead	
	Annual ft^3/acre	Flow-wtg. Rv	CN Range	Flow–wtg. mg/L	Annual lbs/acre	Flow-wtg. µg/L	Annual lbs/acre	Flow-wtg. µg/L	Annual lbs/acre
All above controls		0.1	63–100	42	55	139	0.18	36	0.05
reduction (lbs or ft^3)	20900			89	1375	11	0.40	93	1.98
reduction (%)	38900				96		69		97
cost ($/lb or $/$ft^3$)	65				0.19		638		129
($255/acre/yr)	0.0066								

[a] Medium-density residential area, developed in 1961–1980, with clayey soils (curbs and gutters); new development controls (not retrofit).

From Pitt, R. and J. Voorhees. Source loading and management model (SLAMM). *Seminar Publication: National Conference on Urban Runoff Management: Enhancing Urban Watershed Management at the Local, County, and State Levels*. March 30–April 2, 1993. Center for Environmental Research Information, U.S. Environmental Protection Agency. EPA/625/R-95/003. Cincinnati. OH. pp. 225–243. April 1995.

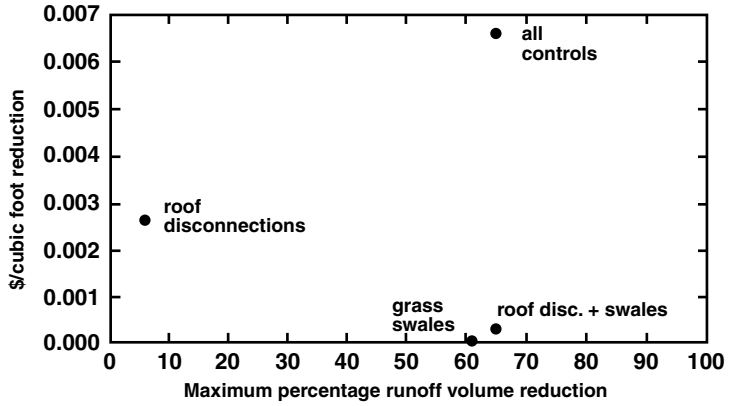

Figure 8.4 0 Cost-effectiveness data for runoff volume reduction benefits. (From Pitt, R. and J. Voorhees. Source loading and management model (SLAMM). *Seminar Publication: National Conference on Urban Runoff Management: Enhancing Urban Watershed Management at the Local, County, and State Levels*. March 30–April 2, 1993. Center for Environmental Research Information, U.S. Environmental Protection Agency. EPA/625/R-95/003. Cincinnati. OH. pp. 225–243. April 1995.)

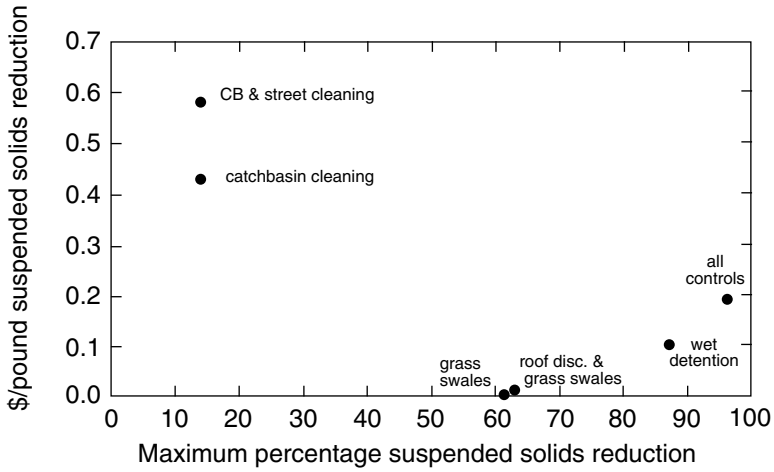

Figure 8.5 0 Cost-effectiveness data for suspended solids reduction benefits. (From Pitt, R. and J. Voorhees. Source loading and management model (SLAMM). *Seminar Publication: National Conference on Urban Runoff Management: Enhancing Urban Watershed Management at the Local, County, and State Levels*. March 30–April 2, 1993. Center for Environmental Research Information, U.S. Environmental Protection Agency. EPA/625/R-95/003. Cincinnati. OH. pp. 225–243. April 1995.)

Figures 8.4 through 8.7 show the maximum percentage reductions in runoff volume and pollutants, along with associated unit removal costs. As an example, Figure 8.4 shows that roof disconnections would have a very small potential maximum benefit for runoff volume reduction and at a very high unit cost compared to the other practices. The use of grass swales could have about a 60% reduction at minimal cost. The use of roof disconnections plus swales would slightly increase the maximum benefit to about 65%, at a small unit cost. Obviously, the use of roof disconnections alone, or all control practices combined, is very inefficient for this example. For suspended solids control, catchbasin cleaning and street cleaning would have minimal benefit at high cost, while the use of grass swales would produce a substantial benefit at very small cost. However, if additional control is necessary, the use of wet detention ponds may be necessary at a higher cost. If close to 95% reduction of suspended solids was required, then all of the controls investigated could be used together, but at substantial cost.

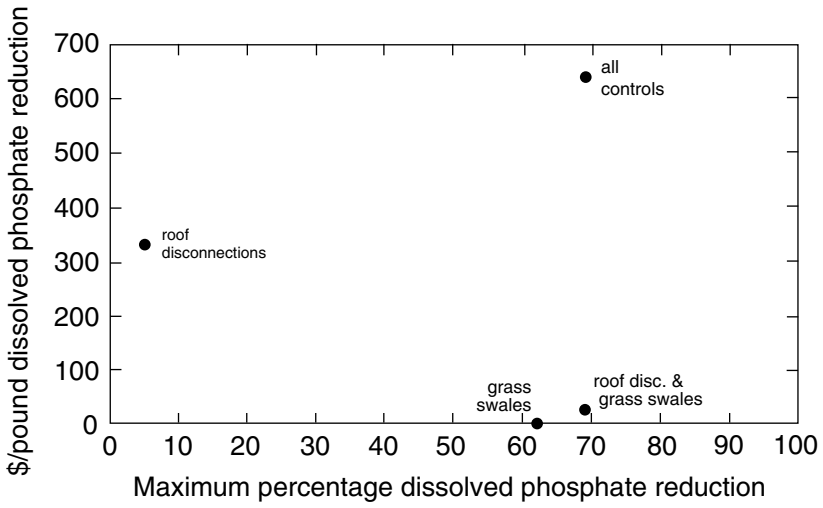

Figure 8.6 0 Cost-effectiveness data for dissolved phosphate reduction benefits. (From Pitt, R. and J. Voorhees. Source loading and management model (SLAMM). *Seminar Publication: National Conference on Urban Runoff Management: Enhancing Urban Watershed Management at the Local, County, and State Levels*. March 30–April 2, 1993. Center for Environmental Research Information, U.S. Environmental Protection Agency. EPA/625/R-95/003. Cincinnati. OH. pp. 225–243. April 1995.)

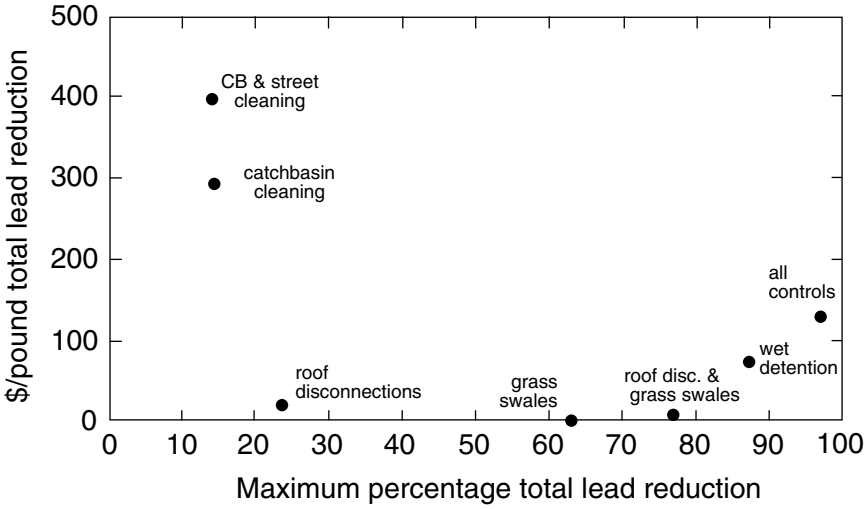

Figure 8.7 0 Cost-effectiveness data for total lead reduction benefits. (From Pitt, R. and J. Voorhees. Source loading and management model (SLAMM). *Seminar Publication: National Conference on Urban Runoff Management: Enhancing Urban Watershed Management at the Local, County, and State Levels*. March 30–April 2, 1993. Center for Environmental Research Information, U.S. Environmental Protection Agency. EPA/625/R-95/003. Cincinnati. OH. pp. 225–243. April 1995.)

SUMMARY

This chapter has provided some, but limited, insight into how an investigator of urban receiving waters can interpret collected data and develop appropriate conclusions. This is obviously not an easy task. The main chapters of the book include many case studies and a large number of references to illustrate how prior investigators have accomplished this difficult task. The investigator should

consult selected references that are similar in location, scope, and/or objectives. Some major theses of this book are summarized below.

It is critical that the investigator have a good idea of what is to be accomplished and develop a suitable experimental design with an appropriate tiered approach. Shortcomings of many investigations can be traced to a lack of initial thought and suitable study hypotheses. In addition, while it is critical to retain flexibility and increase attention given to newly uncovered interesting phenomena, it is important not to keep changing direction based on preliminary conclusions. Of course, the reality of limited resources also precludes continued increases in project scope. Most receiving water investigations are probably only initial investigations, with little prior specific data for the location being studied, and it is natural for many new questions to develop during the studies. A tiered, weight-of-evidence approach enables the most significant objectives to be adequately addressed, with resources available for more detailed investigations to clarify issues.

It is difficult to examine the collected data and clearly identify some beneficial use impairments, especially considering the dynamic and seasonal nature of watersheds and receiving waters. It is easy to miss important short-term events and to misjudge the significance of other seemingly obvious events. Careful and complete sampling, especially if conducted using a stratified random sampling strategy, can help reduce these problems. Well-calibrated and verified models are also important because they allow a long-term perspective of the discretely collected data.

Cause-and-effect relationships tying together stressors and biological impairments are especially difficult to identify and quantify, requiring specialized tests and the weight-of-evidence approach. The use of adequate and appropriate reference sites is very important for biological evaluations, as comparisons to water quality criteria are uncertain for stormwater-related problems, habitat guidance is in its infancy, and contaminated stream sediment guidelines are unclear. The use of available criteria is needed, obviously, but criteria exceedances should be considered red flags to focus site-specific investigations. Human risks should be much more closely related to water quality criteria for water contact, drinking water supplies, and fish consumption, but there is still a potential for error when predicting actual exposure associated with stormwater sources.

The implications of receiving water investigations can be extremely important, especially if remedial action is warranted or if problems are not to be worsened in the future. It is therefore necessary that the whole watershed and associated urban infrastructure be considered. It would be very unusual to find an urban or agricultural receiving water in a completely developed watershed that has significant acceptable uses in the absence of dramatic stormwater controls. In most cases, these streams are managed, it at all, solely for flood control and drainage, with no acknowledgment of other reasonably acceptable uses of noncontact recreation and biological life. Unfortunately, many urban and agricultural streams attract children who play around and in them. Obviously, water-contact recreation and fish consumption are not appropriate uses of most urban and agricultural streams, but these uses do occur, often by members of the community most at risk. These concerns and challenges can be effectively addressed by linking assessments of stormwater effects with progressive watershed management.

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UNIT 3

Toolbox of Assessment Methods

APPENDIX A

Habitat Characterization

CONTENTS

While more advanced habitat quantification methods are available (see Chapters 5 and 6), rapid methods have proven to be quite useful. Two of the more popular and similar approaches for assessing the habitat of streams and rivers are described below. These methods are not particularly useful for large rivers, lakes, or coastal areas. Habitat effects may be qualitatively evaluated using methods of the Ohio EPA (OEPA 1989) or the USEPA Rapid Bioassessment Protocol (EPA 1999), both of which are described in this appendix. The Qualitative Habitat Evaluation Index (QHEI) was issued by the OEPA for fish sampling, but it may be also used in any type of stream survey.

THE QUALITATIVE HABITAT EVALUATION INDEX (QHEI) (OEPA 1989)

A general evaluation of macrohabitat is made while sampling each location using the Ohio EPA Site Description Sheet — Fish (Figures A.1 and A.2). This form is used to tabulate data and information for calculating the Qualitative Habitat Evaluation Index (QHEI). The following guidance should be used when completing the site evaluation form.

Geographical Information

1. *Stream, River Mile (RM), Date*
   The official stream name may be found in the *State Gazetteer* of streams or on USGS 7.5 minute topographic maps.

Ohio EPA Site Description Sheet - **QHEI SCORE:** ☐

Stream_____ FM_____ Date_____ River Code_____

Location_____ Crew_____

1) SUBSTRATE (Check *Only* Two Substrate TYPE BOXES. Check all types present): **SUBSTRATE SCORE:** ☐

TYPE	Pool	Ruffle	Pool	Ruffle	**SUBSTRATE QUALITY**		
[][]-Bldr /Slabs(10)	____	____	[][]-Gravel(7)	____	____	**Substrate Origin**(Check all)	**Silt Cover** (Check One)
[][] -Boulder (9)	____	____	[][]-Sand(6)	____	____	[]-Limestone(1) []-Riptrap (0)	[]-Silt Heavy(-2)
[][]-Cobble(8)	____	____	[][]-Bedrock(5)	____	____	[]-Tells(1) []-Hardpan(0)	[]-Silt Moderate(-1)
[][]-Hardpan(4)	____	____	[][]-Detritus(3)	____	____	[]-Sandstone(0)	[]-Silt Normal (0) []-Silt Free(1)
[][]-Muck(2)	____	____	[][]-Artific(0)	____	____	[]-Shale(-1)	**Extent of Embeddness** (Check One)

Total Number of Substrate Types: []>4 (2), []<=4 (0) []-Coal Fines(-2) []-Extensive (-2) []Moderate(-1)

NOTE: (Ignore Sludge that originates from point-sources; score based on natural substances) []-Low(0) []-None(1)

Comments:_____

COVER SCORE: ☐

2) INSTREAM COVER **Amount:** (Check *ONLY* One or check 2 and AVERAGE)

TYPE (Check *All* That Apply)

[]-Undercut Banks(1)	[]-Deep Pools(2)	[]-Oxbows(1)	[]-Extensive > 75% (11)
[]-Overhanging Vegetation(1)	[]-Rootwads(1)	[]-Aquatic Macrophytes(1)	[]-Moderate 25-75% (7)
[]-Shallows (In Slow Water)(1)	[]-Boulders(1)	[]-Logs or Woody Debris(1)	[]-Sparse 5-25% (3)
			[]-Nearly Absent < 5% (1)

Comments:_____

3) CHANNEL MORPHOLOGY: (Check *ONLY* One PER Category OR check 2 and AVERAGE) **CHANNEL:** ☐

SINUOSITY	**DEVELOPMENT**	**CHANNELIZATION**	**STABILITY**	**MODIFICATIONS/OTHER**	
[]-High (4)	[]-Excellent (7)	[]-None (6)	[]-High (3)	[]-Snagging	[]-Impound
[]-Moderate(3)	[]-Good (5)	[]-Recovered (4)	[]-Moderate(2)	[]-Relocation	[]-Islands
[]-Low (2)	[]-Fair (3)	[]-Recovering (3)	[]-Low (1)	[]-Canopy Removal	[]-Leveed
[]-None (1)	[]-Poor (1)	[]-Recent or No Recovery(1)		[]-Dredging	[]-Bank Shaping
				[]-One Side Channel Modifications	

Comments:_____

RIPARIAN: ☐

4) RIPARIAN ZONE AND BANK EROSION: (Check *ONE* box per bank or check 2 and AVERAGE per bank)

River Right Looking Downstream

RIPARIAN WIDTH	**EROSION/RUNOFF - FLOOD PLAIN QUALITY**		**BANK EROSION**
L R (Per Bank)	L R (Most Predominant Per Bank)	L R (Per Bank)	
[][]-Wide>50m (4)	[][]-Forest, Swamp (3)	[][]-Urban or Industrial(0)	[][]-None or Little (3)
[][]-Moderate10-50m (3)	[][]-Open Pasture/Rowcrop (0)	[][]-Shrub or Old Field(2)	[][]-Moderate (2)
[][]-Narrow 1-5m (2)	[][]-Resid. Park, New Field (1)	[][]-Conserv. Tillage (1)	[][]-Heavy or Severe(1)
[][]-Very Narrow1-5m(1)	[][]-Fenced Pasture (1)	[][]-Mining/construction(0)	
[][]-None (0)			

Comments:_____

5) POOL/GLIDE AND RIFFLE/RUN QUALITY **POOL:** ☐

MAX DEPTH (Check 1)	**MORPHOLOGY** (Check 1)	**POOL/RUN/RIFFLE CURRENT VELOCITY** (Check *All* That Apply)	
[]- >1m (6)		[]-Torrential (-1)	[]-Eddies (1)
[]- 0.7-1m (4)	[]-Pool Width>Riffle Width(2)	[]-Fast (1)	[]-Interstitial (-1)
[]- 0.4-0.7m (2)	[]-Pool Width=Riffle Width(1)	[]-Moderate (1)	[]-Intermittent (-2)
[]- <0.4m (1)	[]-Pool Width<Riffle Width(0)	[]-Slow (1)	[]-No Pool (0)
[]- <0.2m (Pool=0)			

Comments:_____

RIFFLE: ☐

RIFFLE/RUN DEPTH	**RIFFLE/RUN SUBSTRATE**	**RIFFLE/RUN EMBEDDEDNESS**	
[]-Generally>10cm,Max>50(4)	[]-Stable (e.g. Cobble, Boulder) (2)	[]-Extensive (-1)	
[]-Generally>10cm,Max<50(3)	[]-Mod. Stable (e.g. Pea Gravel)(1)	[]-Moderate (0)	
[]-Generally5-10cm (1)	[]-Unstable (Gravel, Sand) (0)	[]-Low (1)	
[]Generally <5cm (Riffle=0)		[]-None (2)	[]-No Ruffle (0)

Comments:_____

6) Gradient (feet/mile):_____ %Pool:_____ %Ruffle:_____ %Run:_____ **GRADIENT:** ☐

Figure A.1 Front side of the Ohio EPA Site Description Sheet for the Qualitative Habitat Evaluation Index (QHEI). (From Ohio Environmental Protection Agency. *The Qualitative Habitat Evaluation Index (QHEI): Rationale, Methods, and Application*. Ecological Assessment Section, Ohio Environmental Protection Agency, Columbus, OH. 1989.)

2. *Specific Location*
   A brief description of the sampling location should include proximity to a local landmark such as a bridge, road, discharge outfall, railroad crossing, park, tributary, dam, etc.
3. *Field Sampling Crew*
   The field crew involved with the sampling is noted on the sheet, with the person who filled out the sheet listed first.

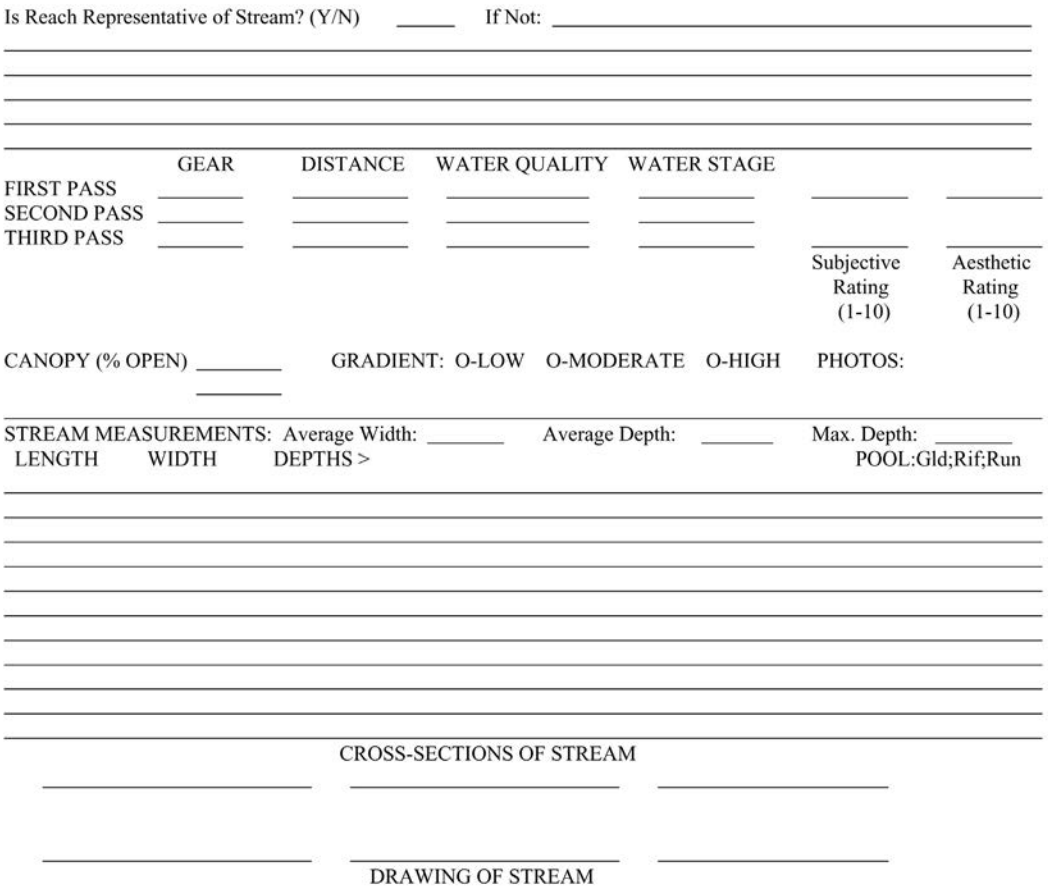

Figure A.2 Reverse side of the Ohio EPA Site Description Sheet for evaluating the geographical and physical characteristics of fish sampling locations. This is used to record additional information about the sampling site and adjacent area. (From Ohio Environmental Protection Agency). *The Qualitative Habitat Evaluation Index (QHEI): Rationale, Methods, and Application*. Ecological Assessment Section, Ohio Environmental Protection Agency, Columbus, OH. 1989.)

4. *Habitat Characteristics: QHEI Metrics*
   The Qualitative Habitat Evaluation Index (QHEI) is a physical habitat index designed to provide an empirical, quantified evaluation of the general lotic *macrohabitat* characteristics that are important to fish communities. The QHEI is composed of six principal metrics each of which is described below. The maximum possible QHEI site score is 100. Each of the metrics is scored individually and then summed to provide the total QHEI site score. This is completed at least once for each sampling site during each year of sampling. An exception to this convention is when substantial changes to the macrohabitat have occurred between sampling passes. Standardized definitions for pool, run, and riffle habitats, for which a variety of existing definitions and perceptions exist, are essential for using the QHEI accurately. It is recommended that this reference also be consulted prior to scoring individual sites.

Riffle and Run Habitats

Riffle — areas of the stream with fast current and shallow depth; the water surface is visibly broken.

Run — areas of the stream that have a rapid, nonturbulent flow; runs are deeper than riffles, with a faster current velocity than pools, and are generally located downstream from riffles where the stream narrows; the stream bed is often flat beneath a run and the water surface is not visibly broken.

Pool and Glide Habitats

Pool — an area of the stream with slow current velocity and a depth greater than riffle and run areas; the stream bed is often concave and stream width frequently is the greatest; the water surface slope is nearly zero. If a pool or glide has a maximum depth of less than 20 cm, it is deemed to have lost its functionality and the metric is scored a 0.

Glide — an area common to most modified stream channels that does not have distinguishable pool, run, and riffle habitats; the current and flow are similar to that of a canal; the water surface gradient is nearly zero.

The following is a description of each of the six QHEI metrics and the individual metric components. Guidelines on how to score each are presented. Generally, metrics are scored by checking boxes. In certain cases, the biologist completing the QHEI sheet may interpret a habitat characteristic as being intermediate between the possible choices; in cases where this is allowed (denoted by the term "Double-Checking"), two boxes may be checked and their scores averaged.

Metric 1: Substrate

This metric includes two components, *substrate type* and *substrate quality.*

Substrate Type — Check the two most common substrate types in the stream reach. If one substrate type predominates (greater than approximately 75 to 80%) of the bottom area *or* is clearly the most *functionally* predominant substrate), then this substrate type should be checked twice. **DO NOT CHECK MORE THAN TWO BOXES**. *Note the category for artificial substrates.* Spaces are provided to note the presence (by check marks, or estimates of %, if time allows) of *all* substrate types present in pools and riffles that each comprise at least 5% of the site (i.e., they occur in sufficient quantity to support species that may commonly be associated with the habitat type). This section must be filled out completely to permit future analyses of this metric. If there are more than four substrate types in the zone that are present in greater than approximately 5% of the sampling area, check the appropriate box.

Substrate Quality — Substrate *origin* refers to the "parent" material that the stream substrate is derived from. Check **ONE** box under the substrate origin column *unless* the parent material is from multiple sources (*e.g.*, limestone and tills). **Embeddedness** is the degree to which cobble, gravel, and boulder substrates are surrounded, impacted in, or covered by fine materials (sand and silt). Substrates should be considered embedded if >50% of surface of the substrates is embedded in fine material. Embedded substrates cannot be easily dislodged. This also includes substrates that are concreted or "armor-plated." Naturally sandy streams are not considered embedded; however, a sand-predominated stream that is the result of anthropogenic activities that have buried the natural coarse substrates is considered embedded. Boxes are checked for *extensiveness* (area of sampling zone) of the embedded substrates as follows: **Extensive**: >75% of site area, **Moderate**: 50 to 75%, **Sparse**: 25 to 50%, **Low**: <25%.

Silt Cover — the extent to which substrates are covered by a silt layer (i.e., more than 1 inch thick). **Silt Heavy** means that nearly all of the stream bottom is layered with a deep covering of silt. **Moderate** includes extensive coverings of silts, but with some areas of cleaner substrate (e.g., riffles). **Normal** silt cover includes areas where silt is deposited in small amounts along the stream margin *or* is present as a "dusting" that appears to have little functional significance. If substrates are exceptionally clean, the **Silt Free** box should be checked.

Substrate types are defined as:

a. *Bedrock* — solid rock forming a continuous surface.
b. *Boulder* — rounded stones over 250 mm in diameter (10 in) or large "slabs" more than 256 mm in length (*boulder slabs*).
c. *Cobble* — stones from 64 to 256 mm (2½ to 10 in) in diameter.
d. *Gravel* — mixture of rounded coarse material from 2 to 64 mm (0.8 to 2½ in) in diameter.
e. *Sand* — materials 0.06 to 2.0 mm in diameter, gritty texture when rubbed between fingers.
f. *Silt* — 0.004 to 0.06 mm in diameter; generally this is fine material which feels "greasy" when rubbed between fingers.
g. *Hardpan* — particles less than 0.004 mm in diameter, usually clay, which form a dense, gummy surface that is difficult to penetrate.
h. *Marl* — calcium carbonate; usually grayish-white; often contains fragments of mollusc shells.
i. *Detritus* — dead, unconsolidated organic material covering the bottom, which could include sticks, wood, and other partially or undecayed coarse plant material.
j. *Muck* — black, fine, flocculent, completely decomposed organic matter (*does not include* sewage sludge).
k. *Artificial* — substrates such as rock baskets, gabions, bricks, trash, concrete, etc., placed in the stream for reasons *OTHER* than habitat mitigation.

Sludge is defined as thick layers of organic matter, that is decidedly of human or animal origin. **NOTE: SLUDGE THAT ORIGINATES FROM POINT SOURCES IS NOT INCLUDED; THE SUBSTRATE SCORE IS BASED ON THE UNDERLYING MATERIAL.**

Substrate Metric Score — Although the theoretical maximum metric score is > 20, the maximum score allowed for the QHEI is limited to 20 points.

Metric 2: In-Stream Cover

This metric consists of *in-stream cover type* and *in-stream cover amount*. All of the cover types that are present in amounts greater than approximately 5% of the sampling area (i.e., they occur in sufficient quantity to support species that may commonly be associated with the habitat type) should be checked. Cover should not be counted when it is in areas of the stream with insufficient depth (usually <20 cm) to make it useful. For example, a logjam in 5 cm of water contributes very little if any cover and may be dry at low flow. Other cover types with limited utility in shallow water include *undercut banks and overhanging vegetation, boulders, and rootwads.* Under *amount*, one or two boxes may be checked. *Extensive* cover is that which is present throughout the sampling area, generally greater than about 75% of the stream reach. Cover is *moderate* when it occurs over 25 to 75% of the sampling area. Cover is *sparse* when it is present in less than 25% of the stream margins (sparse cover usually exists in one or more isolated patches). Cover is *nearly absent* when no large patch of any type of cover exists anywhere in the sampling area. This situation is usually found in recently channelized streams or other highly modified reaches (e.g., ship channels). If cover is thought to be intermediate in amount between two categories, *check two boxes and* ***average*** *their scores*. Cover types include: (1) undercut banks, (2) overhanging vegetation, (3) shallows (in slow water), (4) logs or woody debris, (5) deep pools (>70 cm), (6) oxbows, (7) boulders, (8) aquatic macrophytes, and (9) rootwads (tree roots that extend into stream). Do not check undercut banks AND rootwads unless undercut banks exist *along with* rootwads as a major component.

Cover Metric Score — Although the theoretical maximum score is >20, the maximum score assigned for the QHEI for the in-stream cover metric is limited to 20 points.

Metric 3: Channel Morphology

This metric emphasizes the quality of the stream channel that relates to the creation and stability of macrohabitat. It includes channel sinuosity (i.e., the degree to which the stream meanders), channel development, channelization, and channel stability. One box under each should be checked unless conditions are considered to be intermediate between two categories; in these cases *check two boxes and **average** their scores*.

a. *Sinuosity* — **No** sinuosity is a straight channel. **Low** sinuosity is a channel with only one or two poorly defined outside bends in a sampling reach, or perhaps slight meandering within modified banks. **Moderate** sinuosity is more than two outside bends, with at least one bend well defined. **High** sinuosity is more than two or three well-defined outside bends with deep areas outside and shallow areas inside. Sinuosity may be more conceptually described by the ratio of the stream distance between these same two points, taken from a topographic map. Check *only* one box.
b. *Development* — This refers to the development of riffle/pool complexes. **Poor** means *riffles* are absent, or if present, shallow with sand and fine gravel substrates; *pools*, if present, are shallow. Glide habitats, if predominant, receive a **Poor** rating. **Fair** means riffles are poorly developed or absent; however, pools are more developed with greater variation in depth. **Good** means better defined riffles present with larger substrates (gravel, rubble, or boulder); pools vary in depth and there is a distinct transition between pools and riffles. **Excellent** means development is similar to the Good category except the following characteristics must be present: pools must have a maximum depth of >1 m and deep riffles and runs (>0.5 m) must also be present. In streams sampled with wading methods, a sequence of riffles, runs, and pools must occur more than once in a sampling zone. Check *one* box.
c. *Channelization* — This refers to anthropogenic channel modifications. **Recovered** refers to streams that have been channelized in the past, but which have recovered most of their natural channel characteristics. **Recovering** refers to channelized streams which are still in the process of regaining their former, natural characteristics; however, these habitats are still degraded. This category also applies to those streams, especially in the Huron/Erie Lake Plain ecoregion (NW Ohio), that were channelized long ago and have a riparian border of mature trees, but still have **Poor** channel characteristics. **Recent** or **No Recovery** refers to streams that were recently channelized or those that show no significant recovery of habitats (e.g., drainage ditches, grass lined or rock riprap banks, etc.). The specific type of habitat modification is also checked in the two columns, but not scored.
d. *Stability* — This refers to channel stability. Artificially stable (concrete) stream channels receive a **High** score. Even though they are generally a negative influence on fish, the negative effects are related to features other than their stability. Channels with **Low** stability are usually characterized by fine substrates in riffles that often change location, have unstable and severely eroding banks, and a high bedload that slowly creeps downstream. Channels with **Moderate** stability are those that appear to maintain stable riffle/pool and channel characteristics, but which exhibit some symptoms of instability, e.g., high bedload, eroding or false banks, or show the effects of wide fluctuations in water level. Channels with **High** stability have stable banks and substrates, and little or no erosion and bedload.
e. *Modifications/Other* — Check the appropriate box if impounded, islands present, or leveed (these are not included in the QHEI scoring) as well as the appropriate source of habitat modifications.

The maximum QHEI metric score for Channel Morphology is **20 points**.

Metric 4: Riparian Zone and Bank Erosion

This metric emphasizes the quality of the riparian buffer zone and quality of the floodplain vegetation. This includes riparian zone width, floodplain quality, and extent of bank erosion. Each of the three components requires scoring the left *and* right banks (looking downstream). The *average* of the left and right banks is taken to derive the component value. One box per bank should be

checked unless conditions are considered to be intermediate between two categories; in these cases *check two boxes and **average** their scores*.

a. *Width of Floodplain Vegetation* — This is the width of the riparian (stream side) vegetation. Width estimates are only done for forest, shrub, swamp, and old field vegetation. Old field refers to a fairly mature successional field that has stable, woody plant growth; this generally does not include weedy urban or industrial lots that often still have high runoff potential. Two boxes, one each for the left and right bank (looking downstream), should be checked and then averaged.
b. *Floodplain Quality* — The two most predominant floodplain quality types should be checked, one each for the left and right banks (includes urban, residential, etc.), and then averaged. By floodplain we mean the areas *immediately outside* the riparian zone *or greater than 100 ft from the stream,* whichever is wider on each side of the stream. These are areas adjacent to the stream that can have direct runoff and erosional effects during normal wet weather. We do not limit it to the riparian zone, and it is much less encompassing than the stream basin.
c. *Bank Erosion* — The following Streambank Soil Alteration Ratings should be used; check one box for each side of the stream and average the scores. *False banks* mean banks that are no longer adjacent to the normal flow of the channel but have been moved back into the floodplain, most commonly as a result of livestock trampling.

    1. **None** — stream banks are stable and not being altered by water flows or animals (e.g., livestock) — Score **3**.
    2. **Little** — stream banks are stable, but are being lightly altered along the transect line; less than 25% of the stream bank is receiving any kind of stress, and if stress is being received it is very light; less than 25% of the stream bank is false, broken down, or eroding — Score **3**.
    3. **Moderate** — stream banks are receiving moderate alteration along the transect line; at least 50% of the stream bank is in a natural stable condition; less than 50% of the stream bank is false, broken down, or eroding; false banks are rated as altered — Score **2**.
    4. **Heavy** — stream banks have received major alterations along the transect line; less than 50% of the stream bank is in a stable condition; over 50% of the stream bank is false, broken down, or eroding — Score **1**.
    5. **Severe** — stream banks along the transect line are severely altered; less than 25% of the stream bank is in a stable condition; over 75% of the stream bank is false, broken down, or eroding — Score **1**.

The maximum score for Riparian Zone and Erosion metric is **10 points**.

Metric 5: Pool/Glide and Riffle-Run Quality

This metric emphasizes the quality of the pool, glide, and/or riffle-run habitats. This includes pool depth, overall diversity of current velocities (in pools *and* riffles), pool morphology, riffle-run depth, riffle-run substrate, and riffle-run substrate quality.

A. POOL/GLIDE QUALITY

1. *Maximum depth of pool or glide* — check one box only (Score **0** to **6**). Pools or glides with maximum depths of less than 20 cm are considered to have lost their function and the total *metric* is scored a **0**. No other characteristics need be scored in this case.
2. *Current Types* — check each current type that is present in the stream (including riffles and runs; score — **2** to **4**), definitions are:

    Torrential — extremely turbulent and fast flow with large standing waves; water surface is very broken with no definable, connected surface; usually limited to gorges and dam spillway tail-waters.

    Fast — mostly nonturbulent flow with small standing waves in riffle-run areas; water surface may be partially broken, but there is a visibly connected surface.

    Moderate — nonturbulent flow that is detectable and visible (i.e., floating objects are readily transported downstream); water surface is visibly connected.

Slow — water flow is perceptible, but very sluggish.
Eddies — small areas of circular current motion usually formed in pools immediately downstream from riffle-run areas.
Interstitial — water flow that is perceptible only in the interstitial spaces between substrate particles in riffle-run areas.
Intermittent — no flow is evident anywhere leaving standing pools that are separated by dry areas.

3. *Morphology* — Check *Wide* if pools are wider than riffles, *Equal* if pools and riffles are the same width, and *Narrow* if the riffles are wider than the pools (Score **0** to **2**). If the morphology varies throughout the site, *average* the types. If the entire stream area (including areas outside of the sampling zone) is pool or riffle, then check riffle = pool.

Although the theoretical maximum score is >12, the maximum score assigned for the QHEI for the Pool Quality metric is limited to **12 points**.

B. RIFFLE-RUN QUALITY
(score **0** for this metric if no riffles are present)

1. *Riffle/Run Depth* — Select one box that most closely describes the depth characteristics of the riffle (Score **0** to **4**). If the riffle is generally less than 5 cm in depth, riffles are considered to have lost their function and the entire riffle metric is scored a 0.
2. *Riffle/Run Substrate Stability* — Select one box from each that best describes the substrate type and stability of the riffle habitats (Score **0** to **2**).
3. *Riffle/Run Embeddedness* — **Embeddedness** is the degree that cobble, gravel, and boulder substrates are surrounded or covered by fine material (sand, silt). We consider substrates embedded if >50% of the surface of the substrates is embedded in fine material, as these substrates cannot be easily dislodged. This also includes substrates that are concreted. Boxes are checked for *extensiveness* (riffle area of sampling zone) with embedded substrates: **Extensive**: >75% of stream area, **Moderate**: 50 to 75%, **Sparse**: 25 to 50%, **Low**: <25%.

The maximum score assigned for the QHEI for the Riffle/Run Quality metric is **8 points**.

Metric 6: Map Gradient

Local or map gradient is calculated from USGS 7.5 minute topographic maps by measuring the elevation drop through the sampling area. This is done by measuring the stream length between the first contour line upstream and the first contour line downstream of the sampling site and dividing the distance by the contour interval. If the contour lines are closely "packed," a minimum distance of at least 1 mile should be used. Some judgment may need to be exercised in certain anomalous areas (e.g., in the vicinity of waterfalls, impounded areas, etc.), and this can be compared to an in-field, visual estimate, which is recorded on the back of the habitat sheet.

Scoring for ranges of stream gradient takes into account the varying influence of gradient with stream size (measured as drainage area in square miles or stream width). Gradient classifications (Table A.1) were modified from Trautman (1981), and scores were assigned, by stream size category, after examining scatterplots of IBI vs. natural log of gradient in ft/mile. Scores are listed in Table A.1.

The maximum QHEI metric score for Gradient is **10 points**.

Computing the Total QHEI Score

To compute the total **QHEI** score, add the components of each metric to obtain the metric scores and then sum the metric scores to obtain the total **QHEI** score. The **QHEI** metric scores cannot exceed the Metric Maximum Score indicated below:

Additional Information

Additional information is recorded on the reverse side of the Site Description Sheet (Figure A.2) and is described as follows:

Table A.1 Classification of Stream Gradients for Ohio, Corrected for Stream Size. Scores Were Derived from Plots of IBI vs. the Natural Log of Gradient for Each Stream Size Category

Stream Width (m)	Drainage Area (mi^2)	Very Low	Low	Low–Moderate	Moderate	Moderate–High	High	Very High[a]
0.3–4.7	0–9.2	0–1.0	1.1–5.0	5.1–10.0	10.1–15.0	15.1–20	20.1–30	30.1–40
		2	**4**	**6**	**8**	**10**	**10**	**8**
4.8–9.2	9.2–41.6	0–1.0	1.1–3.0	3.1–6.0	6.1–12.0	12.1–18.0	18.0–30	30.1–40
		2	**4**	**6**	**10**	**10**	**8**	**6**
9.2–13.8	41.6–103.7	0–1.0	1.1–2.5	2.6–5.0	5.1–7.5	7.6–12.0	12.1–20	20.1–30
		2	**4**	**6**	**8**	**10**	**8**	**6**
3.9–30.6	103.7–622.9 15.1–25		0–1.0	1.1–2.0	2.1–4.0	4.1–6.0	6.1–10.0	10.1–15
		4	**6**	**8**	**10**	**10**	**8**	**6**
>30.6	> 622.9	—	0–0.5	0.6–1.0	1.1–2.5	2.6–4.0	4.1–9.0	> 9.0
			6	**8**	**10**	**10**	**10**	**8**

[a] Any site with a gradient greater than the upper bound of the "very high" gradient classification is assigned a score of 4.

1. *Additional Comments/Pollution Impacts* — Different types of pollution sources (e.g., wastewater treatment plant, feedlot, industrial discharge, nonpoint source inputs) are noted with their proximity (in 0.1-mile increments) to the sampling site; any evidence of litter, either in-stream or on the stream bank, is also noted.
2. *Sampling Gear/Distance Sampled* — The type of fish sampling gear used during each pass is specified, and any variation in sampling procedures is noted (e.g., sampler type A specifies sampling along one shoreline of 0.5 km, but due to local restriction, sampling may be performed on both shorelines to accumulate 0.5 km); the total sampling distance in kilometers for each sampling site for each pass is recorded.
3. *Water Clarity* — The following descriptions can be used as a guide:
   a. Clear — bottom is clearly visible (if shallow enough), and the water contains no apparent color or staining.
   b. Stained — usually a brownish (or other) color to the water; the bottom may be visible in shallow areas.
   c. Turbid — bottom seldom visible at more than a few inches; caused by suspended sediment particles. The apparent source of stained (e.g., tannic acid, leaf decay, etc.) and turbid (e.g., runoff [clay/silt], algae/diatoms, sewage, etc.) water may be specified under additional comments.
4. *Water Stage* — This is the general water level of the stream during each pass; suggested descriptors are: a) flood, b) high, c) elevated, d) normal, e) low, and f) interstitial. (Note: sampling should not be conducted during flood or high flows.)
5. *Canopy* — This is the percentage of the sampling site that is not covered or shaded by woody bank vegetation. In wide streams and rivers, this determination should be made along both sides of the river or stream (i.e., the percent of the sampling path that is open).
6. *Gradient* — Check the box that best describes the gradient at the site. This will be used to check the accuracy of gradients taken from topographic maps.
7. *Field Crew* — The names of all individuals involved with the sampling/site description at each site are included.
8. *Photographs* — The number of each photograph taken is recorded; the subject of the photograph is briefly described.
9. *Stream Measurements (optional)* — When measuring the individual sampling sites, length, width, and average and maximum depth information should be recorded; each measurement should be recorded as either a riffle, run, or pool or glide by placing an X in the correct box to the right of where measurements are recorded (Figure A.2); see the introduction for definitions of riffles, runs, etc.

   The number of width measurements is left to the discretion of the field crew leader. Short riffles may require only one or two width measurements, while long pools will probably require more,

depending on the degree of variation that exists in the stream's width. Depth measurements should be made in association with individual width measurements. Depths should be taken at the stream margins and various points across the stream. Up to nine depth measurements may be taken, depending on the variability in the stream bottom. Maximum depth is the deepest spot in the stream section sampled. One purpose of this information is to calculate pool volume.

10. *Stream Diagram — Cross sections*: Two or three cross sections of the stream are drawn to provide information on features of the stream bank, stream bottom, stream channel, and floodplain.

    Channel — The cross section containing the stream that is distinct from the surrounding area due to breaks in the general slope of the land, lack of terrestrial vegetation, and changes in the composition of the substrate materials. The channel is made up of stream banks and stream bottoms.

    Banks — The portion of the channel cross section that tends to restrict lateral movement of water. The banks often have a slope steeper than 45° and exhibit a distinct break in slope from the stream bottom. Also, an obvious change in substrate materials is a reliable delineation of the bank.

    Stream Bottom — The portion of the channel cross section not classified as bank. The bottom is usually composed of stream sediments or water transported debris and may be covered by rooted or clinging aquatic vegetation. In some geologic formations, the stream bottom may consist of bedrock rather than sediments.

    Floodplain — The area adjacent to the channel that is seasonally submerged under water. Usually the floodplain is a low area covered by various types of riparian vegetation.

Stream Map

The entire sampling zone is sketched in the area provided. Important physical features are noted on the map with standard symbols used where possible. The sampling path taken is described, along with any other pertinent information.

THE USEPA HABITAT ASSESSMENT FOR THE RAPID BIOASSESSMENT PROTOCOLS (EPA 1999)

Rosgen (1985, 1994, 1996) presented a stream and river classification system that is founded on the premise that dynamically stable stream channels have a morphology that provides appropriate distribution of flow energy during storm events. Further, he identifies eight major variables that affect the stability of channel morphology, but are not mutually independent: channel width, channel depth, flow velocity, discharge, channel slope, roughness of channel materials, sediment load, and sediment particle size distribution. When streams have one of these characteristics altered, some of their capability to dissipate energy properly is lost (Leopold et al. 1964; Rosgen 1985) and will result in accelerated rates of channel erosion. Some of the habitat structural components that function to dissipate flow energy are sinuosity, roughness of bed and bank materials, presence of point bars (slope is an important characteristic), vegetative conditions of stream banks and the riparian zone, condition of the floodplain (accessibility from bank, overflow, and size are important characteristics).

Measurement of these parameters or characteristics serves to stratify and place streams into distinct classifications. However, none of these habitat classification techniques attempts to differentiate the quality of the habitat and the ability of the habitat to support the optimal biological condition of the region. Much of our understanding of habitat relationships in streams has emerged from comparative studies that describe statistical relationships between habitat variables and abundance of biota (Hawkins et al. 1993). A rapid and qualitative habitat assessment approach has been developed to describe the overall quality of the physical habitat (Ball 1982; Ohio EPA 1987; Plafkin

et al. 1989; Barbour and Stribling 1991, 1994; Rankin 1991, 1995). For a more detailed guidance, please refer to the original document (USEPA 1999, www.epa.gov/owow/monitoring/rbp/).

The habitat assessment matrix developed for the Rapid Bioassessment Protocols (RBPs) in Plafkin et al. (1989) were originally based on the Stream Classification Guidelines for Wisconsin developed by Ball (1982) and "Methods of Evaluating Stream, Riparian, and Biotic Conditions" developed by Platts et al. (1983). Barbour and Stribling (1991, 1994) modified the habitat assessment approach originally developed for the RBPs to include additional assessment parameters for high-gradient streams and a more appropriate parameter set for low-gradient streams. All parameters are evaluated and rated on a numerical scale of 0 to 20 (highest) for each sampling reach. The ratings are then totaled and compared to a reference condition to provide a final habitat ranking. Scores increase as habitat quality increases. To ensure consistency in the evaluation procedure, descriptions of the physical parameters and relative criteria are included in the rating form (Figures A.3 through A.8).

A biologist who is well versed in the ecology and zoogeography of the region can generally recognize optimal habitat structure as it relates to the biological community. The ability to accurately assess the quality of the physical habitat structure using a visual-based approach depends on several factors: the parameters selected to represent the various features of habitat structure need to be relevant and clearly defined; a continuum of conditions for each parameter must exist that can be characterized from the optimum for the region or stream type under study to the poorest situation reflecting substantial alteration due to anthropogenic activities; the judgment criteria for the attributes of each parameter should minimize subjectivity through either quantitative measurements or specific categorical choices, in which the investigators are experienced or adequately trained, for stream assessments in the region under study (Hannaford et al. 1997); adequate documentation and ongoing training must be maintained to evaluate and correct errors resulting in outliers and aberrant assessments.

Habitat evaluations are first made on in-stream habitat, followed by channel morphology, bank structural features, and riparian vegetation. Generally, a single, comprehensive assessment is made that incorporates features of the entire sampling reach as well as selected features of the catchment. Additional assessments may be made on neighboring reaches to provide a broader evaluation of habitat quality for the stream ecosystem. The actual habitat assessment process involves rating the 10 parameters as optimal, suboptimal, marginal, or poor, based on the criteria included on the Habitat Assessment Field Data Sheets. Some state programs, such as Florida Department of Environmental Protection (DEP) (1996) and Mid-Atlantic Coastal Streams Workgroup (MACS) (1996) have adapted this approach using somewhat fewer and different parameters.

Reference conditions are used to scale the assessment to the "best attainable" situation. This approach is critical to the assessment because stream characteristics will vary dramatically across different regions (Barbour and Stribling 1991). The ratio between the score for the test station and the score for the reference condition provides a percent comparability measure for each station. The station of interest is then classified on the basis of its similarity to expected conditions (reference condition), and its apparent potential to support an acceptable level of biological health. Use of a percent comparability evaluation allows for regional and stream-size differences which affect flow or velocity, substrate, and channel morphology. Some regions are characterized by streams having a low channel gradient, such as coastal plains or prairie regions.

Other habitat assessment approaches or a more rigorously quantitative approach to measuring the habitat parameters may be used (see Klemm and Lazorchak 1994; Kaufmann and Robison 1994; Meador et al. 1993). However, holistic and rapid assessment of a wide variety of habitat attributes along with other types of data is critical if physical measurements are to be used to best advantage in interpreting biological data.

A generic habitat assessment approach based on visual observation can be separated into two basic approaches — one designed for high-gradient streams, and one designed for low-gradient streams. High-gradient or riffle/run prevalent streams are those in moderate- to high-gradient landscapes. Natural high-gradient streams have substrates primarily composed of coarse sediment particles

PHYSICAL CHARACTERIZATION/WATER QUALITY FIELD DATA SHEET (FRONT)

STREAM NAME	LOCATION	
STATION #_____ RIVERMILE_____	STREAM CLASS	
LAT _____ LONG _____	RIVER BASIN	
STORET #	AGENCY	
INVESTIGATORS		
FORM COMPLETED BY	DATE _____ TIME _____ AM PM	REASON FOR SURVEY

WEATHER CONDITIONS	**Now** ❑ storm (heavy rain) ❑ rain (steady rain) ❑ showers (intermittent) ____%❑ %cloud cover ❑ clear/sunny	**Past 24 hours** ❑ ❑ ❑ ❑____% ❑
	Has there been a heavy rain in the last 7 days? ❑ Yes ❑ No **Air Temperature**_____° C **Other**_____	
SITE LOCATION/MAP	**Draw a map of the site and indicate the areas sampled (or attach a photograph)**	
STREAM CHARACTERIZATION	**Stream Subsystem** ❑ Perennial ❑ Intermittent ❑ Tidal **Stream Origin** ❑ Glacial ❑ Non-glacial montane ❑ Swamp and bog ❑ Spring-fed ❑ Mixture of origins ❑ Other_____	**Stream Type** ❑ Coldwater ❑ Warmwater **Catchment Area**_____km²

Figure A.3 For use in Rapid Bioassessment Protocols. (From EPA. *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates and Fish.* Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 841/B-99/002. 1999.)

(i.e., gravel or larger) or frequent coarse particulate aggregations along stream reaches. Low-gradient or glide/pool prevalent streams are those in low- to moderate-gradient landscapes. Natural low-gradient streams have substrates of fine sediment or infrequent aggregations of more coarse (gravel or larger) sediment particles along stream reaches. The entire sampling reach is evaluated for each parameter. A brief set of decision criteria is given for each parameter corresponding to each of the four categories, reflecting a continuum of conditions on the field sheet (optimal, suboptimal, marginal, and poor).

PHYSICAL CHARACTERIZATION/WATER QUALITY FIELD DATA SHEET (BACK)

WATERSHED FEATURES

Predominant Surrounding Landuse
❑ Forest ❑ Commercial
❑ Field/Pasture ❑ Industrial
❑ Agricultural ❑ Other _____
❑ Residential

Local Watershed NPS Pollution
❑ No evidence ❑ Some potential sources
❑ Obvious sources

Local Watershed Erosion
❑ None ❑ Moderate ❑ Heavy

RIPARIAN VEGETATION (18 meter buffer)

Indicate the dominant type and record the dominant species present
❑ Trees ❑ Shrubs ❑ Grasses ❑ Herbaceous

dominant species present _____

INSTREAM FEATURES

Estimated Reach Length _____m

Estimated Stream Width _____m

Sampling Reach Area _____m^2

Area in km^2 (m^2x1000) _____km^2

Estimated Stream Depth _____m

Surface Velocity (at thalweg) _____m/sec

Canopy Cover
❑ Partly open ❑ Partly shaded ❑ Shaded

High Water Mark _____m

Proportion of Reach Represented by Stream Morphology Types
❑ Riffle_____% ❑ Run_____%
❑ Pool_____%

Channelized ❑ Yes ❑ No

Dam Present ❑ Yes ❑ No

LARGE WOODY DEBRIS

LWD _____m^2

Density of LWD _____m^2/km^2 **(LWD/ reach area)**

AQUATIC VEGETATION

Indicate the dominant type and record the dominant species present
❑ Rooted emergent ❑ Rooted submergent ❑ Rooted floating ❑ Free floating
❑ Floating Algae ❑ Attached Algae

dominant species present _____

Portion of the reach with aquatic vegetation _____%

WATER QUALITY

Temperature_____° C

Specific Conductance_____

Dissolved Oxygen _____

pH _____

Turbidity _____

WQ Instrument Used _____

Water Odors
❑ Normal/None ❑ Sewage
❑ Petroleum ❑ Chemical
❑ Fishy ❑ Other_____

Water Surface Oils
❑ Slick ❑ Sheen ❑ Globs ❑ Flecks
❑ None ❑ Other_____

Turbidity (if not measured)
❑ Clear ❑ Slightly turbid ❑ Turbid
❑ Opaque ❑ Stained ❑ Other_____

SEDIMENT/ SUBSTRATE

Odors
❑ Normal ❑ Sewage ❑ Petroleum
❑ Chemical ❑ Anaerobic ❑ None
❑ Other_____

Oils
❑ Absent ❑ Slight ❑ Moderate ❑ Profuse

Deposits
❑ Sludge ❑ Sawdust ❑ Paper fiber ❑ Sand
❑ Relict shells ❑ Other_____

Looking at stones which are not deeply embedded, are the undersides black in color?
❑ Yes ❑ No

INORGANIC SUBSTRATE COMPONENTS (should add up to 100%)			ORGANIC SUBSTRATE COMPONENTS (does not necessarily add up to 100%)		
Substrate Type	**Diameter**	**% Composition in Sampling Reach**	**Substrate Type**	**Characteristic**	**% Composition in Sampling Area**
Bedrock			Detritus	sticks, wood, coarse plant materials (CPOM)	
Boulder	> 256 mm (10")				
Cobble	64-256 mm (2.5"-10")		Muck-Mud	black, very fine organic (FPOM) .	
Gravel	2-64 mm (0.1"-2.5")				
Sand	0.06-2mm (gritty)		Marl	grey, shell fragments	
Silt	0.004-0.06 mm				
Clay	< 0.004 mm (slick)				

Figure A.4 For use in Rapid Bioassessment Protocols. (From EPA. *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates and Fish.* Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 841/B-99/002. 1999.)

Use of a percent comparability evaluation allows for regional and stream-size differences that affect flow or velocity, substrate, and channel morphology. Some regions are characterized by streams having a low channel gradient. Such streams are typically shallower, have a greater pool/riffle or run/bend ratio, and have a less stable substrate than streams with a steep channel gradient. Although some low-gradient streams do not provide the diversity of habitat or fauna afforded by steeper-

HABITAT ASSESSMENT FIELD DATA SHEET—HIGH GRADIENT STREAMS (FRONT)

STREAM NAME	LOCATION	
STATION #_____ RIVERMILE_____	STREAM CLASS	
LAT _____ LONG _____	RIVER BASIN	
STORET #	AGENCY	
INVESTIGATORS		
FORM COMPLETED BY	DATE _____ TIME _____ AM PM	REASON FOR SURVEY

Parameters to be evaluated in sampling reach

Habitat Parameter	Condition Category			
	Optimal	Suboptimal	Marginal	Poor
1. Epifaunal Substrate/ Available Cover	Greater than 70% of substrate favorable for epifaunal colonization and fish cover; mix of snags, submerged logs, undercut banks, cobble or other stable habitat and at stage to allow full colonization potential (i.e., logs/snags that are not new fall and not transient).	40-70% mix of stable habitat; well-suited for full colonization potential; adequate habitat for maintenance of populations; presence of additional substrate in the form of newfall, but not yet prepared for colonization (may rate at high end of scale).	20-40% mix of stable habitat; habitat availability less than desirable; substrate frequently disturbed or removed.	Less than 20% stable habitat; lack of habitat is obvious; substrate unstable or lacking.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
2. Embeddedness	Gravel, cobble, and boulder particles are 0-25% surrounded by fine sediment. Layering of cobble provides diversity of niche space.	Gravel, cobble, and boulder particles are 25-50% surrounded by fine sediment.	Gravel, cobble, and boulder particles are 50-75% surrounded by fine sediment.	Gravel, cobble, and boulder particles are more than 75% surrounded by fine sediment.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
3. Velocity/Depth Regime	All four velocity/depth regimes present (slow-deep, slow-shallow, fast-deep, fast-shallow). (Slow is < 0.3 m/s, deep is > 0.5 m.)	Only 3 of the 4 regimes present (if fast-shallow is missing, score lower than if missing other regimes).	Only 2 of the 4 habitat regimes present (if fast-shallow or slow-shallow are missing, score low).	Dominated by 1 velocity/ depth regime (usually slow-deep).
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
4. Sediment Deposition	Little or no enlargement of islands or point bars and less than 5% of the bottom affected by sediment deposition.	Some new increase in bar formation, mostly from gravel, sand or fine sediment; 5-30% of the bottom affected; slight deposition in pools.	Moderate deposition of new gravel, sand or fine sediment on old and new bars; 30-50% of the bottom affected; sediment deposits at obstructions, constrictions, and bends; moderate deposition of pools prevalent.	Heavy deposits of fine material, increased bar development; more than 50% of the bottom changing frequently; pools almost absent due to substantial sediment deposition.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
5. Channel Flow Status	Water reaches base of both lower banks, and minimal amount of channel substrate is exposed.	Water fills >75% of the available channel; or <25% of channel substrate is exposed.	Water fills 25-75% of the available channel, and/or riffle substrates are mostly exposed.	Very little water in channel and mostly present as standing pools.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0

Figure A.5 For use with Rapid Bioassessment Protocols. (From EPA. *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates and Fish.* Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 841/B-99/002. 1999.)

gradient streams, they are characteristic of certain regions. Using the approach presented here, these streams may be evaluated relative to other low-gradient streams (USEPA 1989).

Assessment Category	Percent of Comparability
Comparable to reference	≥90%
Supporting	75–88%
Partially supporting	60–73%
Nonsupporting	≤58%

Water Quality

Information requested in this section is standard to many aquatic studies and allows for some comparison between sites. Additionally, conditions that may significantly affect aquatic biota are

HABITAT ASSESSMENT FIELD DATA SHEET—HIGH GRADIENT STREAMS (BACK)

Parameters to be evaluated broader than sampling reach

Habitat Parameter	Condition Category			
	Optimal	Suboptimal	Marginal	Poor
6. Channel Alteration	Channelization or dredging absent or minimal; stream with normal pattern.	Some channelization present, usually in areas of bridge abutments; evidence of past channelization, i.e., dredging, (greater than past 20 yr) may be present, but recent channelization is not present.	Channelization may be extensive; embankments or shoring structures present on both banks; and 40 to 80% of stream reach channelized and disrupted.	Banks shored with gabion or cement; over 80% of the stream reach channelized and disrupted. Instream habitat greatly altered or removed entirely.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
7. Frequency of Riffles (or bends)	Occurrence of riffles relatively frequent; ratio of distance between riffles divided by width of the stream <7:1 (generally 5 to 7); variety of habitat is key. In streams where riffles are continuous, placement of boulders or other large, natural obstruction is important.	Occurrence of riffles infrequent; distance between riffles divided by the width of the stream is between 7 to 15.	Occasional riffle or bend; bottom contours provide some habitat; distance between riffles divided by the width of the stream is between 15 to 25.	Generally all flat water or shallow riffles; poor habitat; distance between riffles divided by the width of the stream is a ratio of >25.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
8. Bank Stability (score each bank) Note: determine left or right side by facing downstream.	Banks stable; evidence of erosion or bank failure absent or minimal; little potential for future problems. <5% of bank affected.	Moderately stable; infrequent, small areas of erosion mostly healed over. 5-30% of bank in reach has areas of erosion.	Moderately unstable; 30-60% of bank in reach has areas of erosion; high erosion potential during floods.	Unstable; many eroded areas; "raw" areas frequent along straight sections and bends; obvious bank sloughing; 60-100% of bank has erosional scars.
SCORE ___ (LB)	Left Bank 10 9	8 7 6	5 4 3	2 1 0
SCORE ___ (RB)	Right Bank 10 9	8 7 6	5 4 3	2 1 0
9. Vegetative Protection (score each bank)	More than 90% of the streambank surfaces and immediate riparian zone covered by native vegetation, including trees, understory shrubs, or nonwoody macrophytes; vegetative disruption through grazing or mowing minimal or not evident; almost all plants allowed to grow naturally.	70-90% of the streambank surfaces covered by native vegetation, but one class of plants is not well-represented; disruption evident but not affecting full plant growth potential to any great extent; more than one-half of the potential plant stubble height remaining.	50-70% of the streambank surfaces covered by vegetation; disruption obvious; patches of bare soil or closely cropped vegetation common; less than one-half of the potential plant stubble height remaining.	Less than 50% of the streambank surfaces covered by vegetation; disruption of streambank vegetation is very high; vegetation has been removed to 5 centimeters or less in average stubble height.
SCORE ___ (LB)	Left Bank 10 9	8 7 6	5 4 3	2 1 0
SCORE ___ (RB)	Right Bank 10 9	8 7 6	5 4 3	2 1 0
10. Riparian Vegetative Zone Width (score each bank riparian zone)	Width of riparian zone >18 meters; human activities (i.e., parking lots, roadbeds, clear-cuts, lawns, or crops) have not impacted zone.	Width of riparian zone 12-18 meters; human activities have impacted zone only minimally.	Width of riparian zone 6-12 meters; human activities have impacted zone a great deal.	Width of riparian zone <6 meters: little or no riparian vegetation due to human activities.
SCORE ___ (LB)	Left Bank 10 9	8 7 6	5 4 3	2 1 0
SCORE ___ (RB)	Right Bank 10 9	8 7 6	5 4 3	2 1 0

Total Score _____

Figure A.6 For use with Rapid Bioassessment Protocols. (From EPA. *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates and Fish.* Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 841/B-99/002. 1999.)

documented. Documentation of recent and current weather conditions is important because of the potential impact that weather may have on water quality. To complete this phase of the bioassessment, a photograph may be helpful in identifying station location and documenting habitat conditions. Any observations or data not requested but deemed important by the field observer should be recorded. This section is identical for all protocols, and the specific data requested are described below:

Temperature (C), Dissolved Oxygen, pH, Conductivity — Measure and record values for each of the water quality parameters indicated, using the appropriate calibrated water quality instrument(s). Note the type of instrument and unit number used.

HABITAT ASSESSMENT FIELD DATA SHEET—LOW GRADIENT STREAMS (FRONT)

STREAM NAME	LOCATION	
STATION #_____ RIVERMILE_____	STREAM CLASS	
LAT _____ LONG _____	RIVER BASIN	
STORET #	AGENCY	
INVESTIGATORS		
FORM COMPLETED BY	DATE _____ TIME _____ AM PM	REASON FOR SURVEY

Parameters to be evaluated in sampling reach

Habitat Parameter	Condition Category			
	Optimal	Suboptimal	Marginal	Poor
1. Epifaunal Substrate/ Available Cover	Greater than 50% of substrate favorable for epifaunal colonization and fish cover; mix of snags, submerged logs, undercut banks, cobble or other stable habitat and at stage to allow full colonization potential (i.e., logs/snags that are not new fall and not transient).	30-50% mix of stable habitat; well-suited for full colonization potential; adequate habitat for maintenance of populations; presence of additional substrate in the form of newfall, but not yet prepared for colonization (may rate at high end of scale).	10-30% mix of stable habitat; habitat availability less than desirable; substrate frequently disturbed or removed.	Less than 10% stable habitat; lack of habitat is obvious; substrate unstable or lacking.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
2. Pool Substrate Characterization	Mixture of substrate materials, with gravel and firm sand prevalent; root mats and submerged vegetation common.	Mixture of soft sand, mud, or clay; mud may be dominant; some root mats and submerged vegetation present.	All mud or clay or sand bottom; little or no root mat; no submerged vegetation.	Hard-pan clay or bedrock; no root mat or vegetation.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
3. Pool Variability	Even mix of large-shallow, large-deep, small-shallow, small-deep pools present.	Majority of pools large-deep; very few shallow.	Shallow pools much more prevalent than deep pools.	Majority of pools small-shallow or pools absent.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
4. Sediment Deposition	Little or no enlargement of islands or point bars and less than <20% of the bottom affected by sediment deposition.	Some new increase in bar formation, mostly from gravel, sand or fine sediment; 20-50% of the bottom affected; slight deposition in pools.	Moderate deposition of new gravel, sand or fine sediment on old and new bars; 50-80% of the bottom affected; sediment deposits at obstructions, constrictions, and bends; moderate deposition of pools prevalent.	Heavy deposits of fine material, increased bar development; more than 80% of the bottom changing frequently; pools almost absent due to substantial sediment deposition.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
5. Channel Flow Status	Water reaches base of both lower banks, and minimal amount of channel substrate is exposed.	Water fills >75% of the available channel; or <25% of channel substrate is exposed.	Water fills 25-75% of the available channel, and/or riffle substrates are mostly exposed.	Very little water in channel and mostly present as standing pools.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0

Figure A.7 For use with Rapid Bioassessment Protocols. (From EPA. *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates and Fish.* Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 841/B-99/002. 1999.)

Stream Type — Note the appropriate stream designation according to state water quality standards.

Water Odors — Note those odors described (or include any other odors not listed) that are associated with the water in the sampling area.

Water Surface Oils — Note the term that best describes the relative amount of any oils present on the water surface.

Turbidity — Note the term which, based upon visual observation, best describes the amount of material suspended in the water column.

Physical Characterization

Physical characterization parameters include estimations of general land use and physical stream characteristics such as width, depth, flow, and substrate. The evaluation begins with the riparian zone (stream bank and drainage area) and proceeds in-stream to sediment/substrate descriptions. Such information will provide insight as to what organisms may be present or are expected to be

HABITAT ASSESSMENT FIELD DATA SHEET—LOW GRADIENT STREAMS (BACK)

Parameters to be evaluated broader than sampling reach

Habitat Parameter	Condition Category			
	Optimal	Suboptimal	Marginal	Poor
6. Channel Alteration	Channelization or dredging absent or minimal; stream with normal pattern.	Some channelization present, usually in areas of bridge abutments; evidence of past channelization, i.e., dredging, (greater than past 20 yr) may be present, but recent channelization is not present.	Channelization may be extensive; embankments or shoring structures present on both banks; and 40 to 80% of stream reach channelized and disrupted.	Banks shored with gabion or cement; over 80% of the stream reach channelized and disrupted. Instream habitat greatly altered or removed entirely.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
7. Channel Sinuosity	The bends in the stream increase the stream length 3 to 4 times longer than if it was in a straight line. (Note - channel braiding is considered normal in coastal plains and other low-lying areas. This parameter is not easily rated in these areas.)	The bends in the stream increase the stream length 1 to 2 times longer than if it was in a straight line.	The bends in the stream increase the stream length 1 to 2 times longer than if it was in a straight line.	Channel straight; waterway has been channelized for a long distance.
SCORE	20 19 18 17 16	15 14 13 12 11	10 9 8 7 6	5 4 3 2 1 0
8. Bank Stability (score each bank)	Banks stable; evidence of erosion or bank failure absent or minimal; little potential for future problems. <5% of bank affected.	Moderately stable; infrequent, small areas of erosion mostly healed over. 5-30% of bank in reach has areas of erosion.	Moderately unstable; 30-60% of bank in reach has areas of erosion; high erosion potential during floods.	Unstable; many eroded areas; "raw" areas frequent along straight sections and bends; obvious bank sloughing; 60-100% of bank has erosional scars.
SCORE ___ (LB)	Left Bank 10 9	8 7 6	5 4 3	2 1 0
SCORE ___ (RB)	Right Bank 10 9	8 7 6	5 4 3	2 1 0
9. Vegetative Protection (score each bank) Note: determine left or right side by facing downstream.	More than 90% of the streambank surfaces and immediate riparian zone covered by native vegetation, including trees, understory shrubs, or nonwoody macrophytes; vegetative disruption through grazing or mowing minimal or not evident; almost all plants allowed to grow naturally.	70-90% of the streambank surfaces covered by native vegetation, but one class of plants is not well-represented; disruption evident but not affecting full plant growth potential to any great extent; more than one-half of the potential plant stubble height remaining.	50-70% of the streambank surfaces covered by vegetation; disruption obvious; patches of bare soil or closely cropped vegetation common; less than one-half of the potential plant stubble height remaining.	Less than 50% of the streambank surfaces covered by vegetation; disruption of streambank vegetation is very high; vegetation has been removed to 5 centimeters or less in average stubble height.
SCORE ___ (LB)	Left Bank 10 9	8 7 6	5 4 3	2 1 0
SCORE ___ (RB)	Right Bank 10 9	8 7 6	5 4 3	2 1 0
10. Riparian Vegetative Zone Width (score each bank riparian zone)	Width of riparian zone >18 meters; human activities (i.e., parking lots, roadbeds, clear-cuts, lawns, or crops) have not impacted zone.	Width of riparian zone 12-18 meters; human activities have impacted zone only minimally.	Width of riparian zone 6-12 meters; human activities have impacted zone a great deal.	Width of riparian zone <6 meters: little or no riparian vegetation due to human activities.
SCORE ___ (LB)	Left Bank 10 9	8 7 6	5 4 3	2 1 0
SCORE ___ (RB)	Right Bank 10 9	8 7 6	5 4 3	2 1 0

Total Score _____

Figure A.8 For use with Rapid Bioassessment Protocols. (From EPA. *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates and Fish.* Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 841/B-99/002. 1999.)

present, and to presence of stream impacts. The information requested in the Physical Characterization section of the Field Data Sheet is briefly discussed below:

Predominant Surrounding Land Use — Observe the prevalent land-use type in the vicinity (noting any other land uses in the area which, although not predominant, may potentially affect water quality).

Local Watershed Erosion — The existing or potential detachment of soil within the local watershed (the portion of the watershed that drains directly into the stream) and its movement into a stream are noted. Erosion can be rated through visual observation of watershed and stream characteristics. (Note any turbidity observed during water quality assessment below.)

Local Watershed Nonpoint Source Pollution — This item refers to problems and potential problems other than siltation. Nonpoint source pollution is defined as diffuse agricultural and urban runoff. Other compromising factors in a watershed that may affect water quality are feedlots, wetlands, septic systems, dams and impoundments, and/or mine seepage.

Estimated Stream Width (m) — Estimate the distance from shore to shore at a transect representative of the stream width in the area.

Estimated Stream Depth (m) — Riffle, run, and pool. Estimate the vertical distance from water surface to stream bottom at a representative depth at each of the three habitat types.

High Water Mark (m) — Estimate the vertical distance from the stream bank to the peak overflow level, as indicated by debris hanging in bank or floodplain vegetation, and deposition of silt or soil. In instances where bank overflow is rare, a high water mark may not be evident.

Velocity — Record an estimate of stream velocity in a representative run area.

Dam Present — Indicate the presence or absence of a dam upstream or downstream of the sampling station. If a dam is present, include specific information relating to alteration of flow.

Channelized — Indicate whether or not the area around the sampling station is channelized.

Canopy Cover — Note the general proportion of open to shaded area which best describes the amount of cover at the sampling station.

Sediment Odors — Disturb sediment and note any odors described (or include any other odors not listed) which are associated with sediment in the area of the sampling station.

Sediment Oils — Note the term which best describes the relative amount of sediment oils observed in the sampling area.

Sediment Deposits — Note those deposits described (or include any other deposits not listed) which are present in the sampling area. Also indicate whether the undersides of rocks not deeply embedded are black (which generally indicates low dissolved oxygen or anaerobic conditions).

Inorganic Substrate Components — Visually estimate the relative proportion of each of the several substrate/particle types listed that are present in the sampling area.

Organic Substrate Components — Indicate relative abundance of each of the three substrate types listed.

Listed below is a general explanation of some major habitat parameters to be evaluated.

Substrate and In-Stream Cover

The in-stream habitat characteristics directly pertinent to the support of aquatic communities consist of substrate type and stability, availability of refugia, and migration/passage potential.

Bottom Substrate — This refers to the availability of habitat for support of aquatic organisms. A variety of substrate materials and habitat types is desirable. The presence of rock and gravel in flowing streams is generally considered the most desirable habitat. However, other forms of habitat may provide the niches required for community support. For example, logs, tree roots, submerged or emergent vegetation, undercut banks, etc., will provide excellent habitat for a variety of organisms, particularly fish. Bottom substrate is evaluated and rated by observation.

Embeddedness — The degree to which boulders, rubble, or gravel are surrounded by fine sediment indicates suitability of the stream substrate as habitat for benthic macroinvertebrates and for fish spawning and egg incubation. Embeddedness is evaluated by visual observation of the degree to which larger particles are surrounded by sediment. In some western areas of the United States, embeddedness is regarded as the stability of cobble substrate by measuring the depth of burial of large particles (cobble, boulders).

Stream Discharge and/or Stream Velocity — Stream discharge relates to the ability of a stream to provide and maintain a stable aquatic environment. Stream discharge (and water quality) is most critical to the support of aquatic communities when the representative low flow is ≤0.15 cms (5 cfs). In these small streams, discharge should be estimated in a straight stretch of run area where banks are parallel and bottom contour is relatively flat. Even where a few stations may have discharges in excess of 0.15 cms, discharge may still be the predominating constraint. Therefore, the evaluation is based on discharge rate rather than velocity.

In larger streams and rivers (>0.15 cms), velocity, in conjunction with depth, has a more direct influence than the discharge rate on the structure of benthic communities (Osborne and Hendricks 1983) and fish communities (Oswood and Barber 1982). The quality of the aquatic habitat can therefore be evaluated in terms of a velocity and depth relationship. As patterned after Oswood and Barber (1982), four general categories of velocity and depth are optimal for benthic and fish communities: (1) slow (<0.3 m/s), shallow (<0.5 m); (2) slow (<0.3 m/l), deep (>0.5 m); (3) fast (>0.3 m/s), deep (0.5 m); and (4) fast (>0.3 m/s), shallow (<0.5 m). Habitat quality is reduced in the absence of one or more of these four categories.

Channel Morphology

Channel morphology is determined by the flow regime of the stream, local geology, land surface form, soil, and human activities (Platts et al. 1983). The sediment movement along the channel, as influenced by the tractive forces of flowing water and the sinuosity of the channel, also affects habitat conditions.

Channel Alteration — The character of sediment deposits from upstream is an indication of the severity of watershed and bank erosion and stability of the stream system. The growth, or appearance, of sediment bars tends to increase with continued watershed disturbance. Channel alteration also results in deposition, which may occur on the inside of bends, below channel constrictions, and where stream gradient flattens out. Channelization (e.g., straightening, construction of concrete embankments) decreases stream sinuosity, thereby increasing stream velocity and the potential for scouring.

Bottom Scouring and Deposition — These parameters relate to the destruction of in-stream habitat resulting from the problems described above. Characteristics to observe are scoured substrate and degree of siltation in pools and riffles. Scouring results from high-velocity flows. The potential for scouring is increased by channelization. Deposition and scouring result from the transport of sediment or other particulates and may be an indication of large-scale watershed erosion. Deposition and scouring are rated by estimating the percentage of an evaluated reach that is scoured or silted (i.e., 50-ft silted in a 100-ft stream length equals 50%).

Pool/Riffle or Run/Bend Ratio — These parameters assume that a stream with riffles or bends provides more diverse habitat than a straight (run) or uniform depth stream. Bends are included because low-gradient streams may not have riffle areas, but excellent habitat can be provided by the cutting action of water at bends. The ratio is calculated by dividing the average distance between riffles or bends by the average stream width. If a stream contains riffles and bends, the dominant feature with the best habitat should be used.

Riparian and Bank Structure

Well-vegetated banks are usually stable regardless of bank undercutting; undercutting actually provides excellent cover for fish (Platts et al. 1983). The ability of vegetation and other materials on the stream banks to prevent or inhibit erosion is an important determinant of the stability of the stream channel and in-stream habitat for indigenous organisms. Because riparian and bank structure indirectly affect the in-stream habitat features, they are weighted less than the primary or secondary parameters.

The upper bank is the land area from the break in the general slope of the surrounding land to the normal high water line. The upper bank is normally vegetated and covered by water only during extreme high water conditions. Land forms vary from wide, flat floodplains to narrow, steep slopes. The lower bank is the intermittently submerged portion of the stream cross section from the normal high water line to the lower water line. The lower channel defines the stream width.

Bank Stability — Bank stability is rated by observing existing or potential detachment of soil from the upper and lower stream bank and its potential movement into the stream. Steeper banks are generally more subject to erosion and failure, and may not support stable vegetation. Streams with

poor banks will often have poor in-stream habitat. Adjustments should be made in areas with clay banks where steep, raw areas may not be as susceptible to erosion as other soil types.

Bank Vegetative Stability — Bank soil is generally held in place by plant root systems. Erosional protection may also be provided by boulder, cobble, or gravel material. An estimate of the density of bank vegetation (or proportion of boulder, cobble, or gravel material) covering the bank provides an indication of bank stability and potential in-stream sedimentation.

Streamside Cover — Streamside cover vegetation is evaluated in terms of stream-shading and escape cover or refuge for fish. A rating is obtained by visually determining the dominant vegetation type covering the exposed stream bottom, bank, and top of bank. Platts (1974) found that streamside cover consisting primarily of shrub had a higher fish standing crop than similar-size streams having tree or grass streamside cover. Riparian vegetation dominated by shrubs and trees provides the CPOM source in allochthonous systems.

Procedure for Performing the Habitat Assessment

1. Select the reach to be assessed. The habitat assessment is performed on the same 100-m reach (or other reach designation [e.g., 40 × stream wetted width]) from which the biological sampling is conducted. Some parameters require an observation of a broader section of the catchment than just the sampling reach.
2. Complete the station identification section of each field data sheet and habitat assessment form (Figures A.2 through A.7).
3. It is best for the investigators to obtain a close look at the habitat features to make an adequate assessment. If the physical and water quality characterization and habitat assessment are done before the biological sampling, care must be taken to avoid disturbing the sampling habitat.
4. Complete the Physical Characterization and Water Quality Field Data Sheet. Sketch a map of the sampling reach on the back of the form.
5. Complete the Habitat Assessment Field Data Sheet, in a team of two or more biologists, if possible, to come to a consensus on determination of quality. Those parameters to be evaluated on a scale greater than a sampling reach require traversing the stream corridor to the extent deemed necessary to assess the habitat feature. As a general rule-of-thumb, use two lengths of the sampling reach to assess these parameters.

Quality Assurance Procedures

1. Each biologist is to be trained in the visual-based habitat assessment technique for the applicable region or state.
2. The judgment criteria for each habitat parameter are calibrated for the stream classes under study. Some text modifications may be needed on a regional basis.
3. Periodic checks of assessment results are completed using pictures of the sampling reach and discussions among the biologists in the agency.

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APPENDIX B

Benthic Community Assessment

CONTENTS

RAPID BIOASSESSMENT PROTOCOL: BENTHIC MACROINVERTEBRATES (EPA 1989, 1999)

As with the habitat assessments, there are more advanced and complex methods for characterizing benthic communities than what is presented below. However, the Rapid Bioassessment Protocols (RBP) outlined by the U.S. EPA (EPA 1989, 1999) have been proven to be efficient and effective in small streams and rivers. The EPA is currently developing guidance for benthic characterization in lakes, large rivers, and coastal areas. States such as Ohio, Maine, and North Carolina use approaches that are also very useful, and similar in many ways. The following are direct excerpts from EPA (1989, 1999; www.epa.gov/owow/monitoring/rbp) and Ohio EPA (1989) guidance manuals. For more extensive information, the reader should refer directly to those manuals. In addition to the references given in the following text, other useful information for identifying benthic macroinvertebrates is found in Barbour et al. 1999; Beck 1977; Harris and Lawrence 1978; Hubbard and Peters 1978; Surdick and Gaufin 1978; USDA 1985.

Rapid Bioassessment Protocol (RBP) utilizes the systematic field collection and analysis of major benthic taxa. The data are compiled into various metrics. The optimal metrics will vary across (and even within) ecoregions, so a qualified benthic ecologist should be used to select the most appropriate metrics. The protocol can be used to prioritize sites for more intensive evaluation (i.e., replicate sampling, ambient toxicity testing, chemical characterization). The EPA 1989 guid-

ance described three levels of RBPs, each with more accurate taxonomic resolution. This approach also recommended sampling a single habitat type. The 1999 guidance describes methods for multi-habitat assessments, which are more appropriate in low-gradient streams and rivers where there is little cobble and riffle area. The description below focuses on single habitat characterization.

Sample Collection

The collection procedure provides representative samples of the macroinvertebrate fauna from comparable habitat types at all stations constituting a site evaluation, and is supplemented with separate coarse particulate organic matter (CPOM) samples (e.g., leaves, decaying vegetation). This RBP single habitat approach focuses on the riffle/run habitat because it is the most productive habitat available in stream systems and includes many pollution-sensitive taxa of the scraper and filtering collector functional feeding groups. The CPOM sample provides a measure of effects (particularly toxicity effects) on a third trophic component of the benthic community, the shredders.

In sampling situations where a riffle/run habitat with a rock substrate is not available, any submerged fixed structure will provide a substrate for the scraper and filtering collector functional groups emphasized here. This allows for the same approach to be used in non-wadable streams and large rivers and wadable streams and rivers with unstable substrates.

Riffle/Run Sample

Riffle areas with relatively fast currents and cobble and gravel substrates generally provide the most diverse community. Riffles should be sampled using a kick net to collect from an approximately 1-m^2 area. A minimum of two 1-m^2 riffle samples should be collected at each station: one from an area of fast current and one from an area of slower current. The samples are composited for processing. In streams lacking riffles, run areas with cobble or gravel substrate are also appropriate for kick net sampling.

Where riffle/run communities with a rock substrate are not available, other submerged fixed structures (e.g., submerged boulders, logs, bridge abutments, pier pilings) should be sampled by hand picking. These structures provide suitable habitat for the scrapers and filtering collectors and will allow use of the RBP in a wider range of regions and stream orders.

CPOM Sample

In addition to the riffle/run sample collected for evaluation of the scraper and filtering collector functional feeding groups, a CPOM sample should also be collected to provide data on the abundance of shredders at the site. Large particulate shredders are important in forested areas of stream ecosystems ranging from stream orders 1 through 4 (Minshall et al. 1985). The absence of shredders of large particulate material is characteristic of unstable, poorly retentive headwater streams in disturbed watersheds or in dry areas where leaf material processing is accomplished by terrestrial detritivores (Minshall et al. 1985). McArthur et al. (1988) reported that very few shredders were found in summer leaf packs in South Carolina because processing was so rapid.

The CPOM sample is processed separately from the riffle/run sample and used only for characterizing the functional feeding group representation. Sampling the CPOM component requires a composite collection of various plant parts such as leaves, needles, twigs, bark, or their fragments. Potential sample sources include leaf packs, shore zones, and other depositional areas where CPOM may accumulate. Only the upper surface of litter accumulation in depositional areas should be sampled to ensure that it is from the aerobic zone. For the shredder community analysis, several handfuls of material should be adequate. A variety of CPOM forms should be collected if available. CPOM collected may be washed in a dip net or a sieve bucket.

Shredder abundance is maximum when the CPOM is partially decomposed (Cummins et al. 1989). Care must be taken to *avoid* collecting recent or fully decomposed leaf litter to optimize collection of the shredder community. For this CPOM collection technique, seasonality may have an important influence on shredder abundance data. For instance, fast-processing litter (e.g., basswood, alder, maples, birch) would have the highest shredder representation in the winter (Cummins et al. 1989). The slow-processing litter (e.g., oaks, rhododendrons, beech, conifers) would have the highest shredder representation in the summer.

Sample Sorting and Identification

Riffle/Run Sample

Sorting and enumeration in the field to obtain a 100 (or higher) -count organism subsample is recommended for the riffle/run sample. After processing in the field, the organisms and sample residue should be preserved for archiving. Thus, a reanalysis (for quality control) or more thorough processing (e.g., larger counts, more detailed taxonomy) would be possible. The subsampling method described in this protocol is based on Hilsenhoff's Improved Biotic Index (Hilsenhoff 1987) and is similar to that used by the New York Department of Environmental Conservation (Bode 1988). This subsampling technique provides for a consistent unit of effort and a representative estimate of the benthic fauna (modified from Hilsenhoff 1987):

1. Thoroughly rinse sample in a (500-µm) screen or the sampling net to remove fine sediments. Any large organic material (whole leaves, twigs, algal or macrophyte mats) should be rinsed, visually inspected, and discarded.
2. Place sample contents in a large, flat pan with a light-colored (preferably white) bottom. The bottom of the pan should be marked with a numbered grid pattern, each block in the grid measuring 5×5 cm. (Sorting using a gridded pan is only feasible if the organism movement in the sample can be slowed by the addition of club soda or tobacco to the sample. If the organisms are not anesthetized, 100 organisms should be removed from the pan as randomly as possible.) A 30×45 cm pan is generally adequate, although pan size ultimately depends on sample size. Larger pans allow debris to be spread more thinly, but they are unwieldy. Samples too large to be effectively sorted in a single pan may be thoroughly mixed in a container with some water, and half of the homogenized sample placed in each of two gridded pans. Each half of the sample must be composed of the same kinds and quantity of debris, and an equal number of grids must be sorted from each pan to ensure a representative subsample.
3. Add just enough water to allow complete dispersion of the sample within the pan; excessive water will allow sample material to shift within the grid during sorting. Distribute sample material evenly within the grid.
4. Use a random numbers table to select a number corresponding to a square within the gridded pan. Remove all organisms from within that square and proceed with the process of selecting squares and removing organisms until the total number sorted from the sample is within 10% of 100. Any organism that is lying over a line separating two squares is considered to be in the square containing its head. In those instances where it is not possible to determine the location of the head (worms for instance), the organism is considered to be in the square containing the largest portion of its body. Any square sorted must be sorted in its entirety, even after the 100 count has been reached. In order to lessen sampling bias, the investigator should attempt to pick smaller, cryptic organisms as well as the larger, more obvious ones.

An alternative method of subsampling live samples in the field is to simply sort 100 organisms in a random manner. Narcotization to slow the organisms is less important with this subsampling technique. To lessen sampling bias, the investigator should pick smaller, cryptic organisms, as well as the larger, more obvious organisms.

BENTHIC MACROINVERTEBRATE FIELD DATA SHEET

STREAM NAME	LOCATION	
STATION #_____ RIVERMILE_____	STREAM CLASS	
LAT _____ LONG _____	RIVER BASIN	
STORET #	AGENCY	
INVESTIGATORS		LOT NUMBER
FORM COMPLETED BY	DATE _____ TIME _____ AM PM	REASON FOR SURVEY

HABITAT TYPES	**Indicate the percentage of each habitat type present** ❑ Cobble____% ❑ Snags____% ❑ Vegetated Banks____% ❑ Sand____% ❑ Submerged Macrophytes____% ❑ Other ( )____%
SAMPLE COLLECTION	**Gear used** ❑ D-frame ❑ kick-net ❑ Other _____ **How were the samples collected?** ❑ wading ❑ from bank ❑ from boat **Indicate the number of jabs/kicks taken in each habitat type.** ❑ Cobble____ ❑ Snags____ ❑ Vegetated Banks____ ❑ Sand____ ❑ Submerged Macrophytes____ ❑ Other ( )____
GENERAL COMMENTS	

QUALITATIVE LISTING OF AQUATIC BIOTA
Indicate estimated abundance: 0 = Absent/Not Observed, 1 = Rare, 2 = Common, 3= Abundant, 4 = Dominant

Periphyton	0	1	2	3	4	Slimes	0	1	2	3	4
Filamentous Algae	0	1	2	3	4	Macroinvertebrates	0	1	2	3	4
Macrophytes	0	1	2	3	4	Fish	0	1	2	3	4

FIELD OBSERVATIONS OF MACROBENTHOS
Indicate estimated abundance: 0 = Absent/Not Observed, 1 = Rare (1-3 organisms), 2 = Common (3-9 organisms), 3= Abundant (>10 organisms), 4 = Dominant (>50 organisms)

Porifera	0	1	2	3	4	Anisoptera	0	1	2	3	4	Chironomidae	0	1	2	3	4
Hydrozoa	0	1	2	3	4	Zygoptera	0	1	2	3	4	Ephemeroptera	0	1	2	3	4
Platyhelminthes	0	1	2	3	4	Hemiptera	0	1	2	3	4	Trichoptera	0	1	2	3	4
Turbellaria	0	1	2	3	4	Coleoptera	0	1	2	3	4	Other	0	1	2	3	4
Hirudinea	0	1	2	3	4	Lepidoptera	0	1	2	3	4						
Oligochaeta	0	1	2	3	4	Sialidae	0	1	2	3	4						
Isopoda	0	1	2	3	4	Corydalidae	0	1	2	3	4						
Amphipoda	0	1	2	3	4	Tipulidae	0	1	2	3	4						
Decapoda	0	1	2	3	4	Empididae	0	1	2	3	4						
Gastropoda	0	1	2	3	4	Simuliidae	0	1	2	3	4						
Bivalvia	0	1	2	3	4	Tabinidae	0	1	2	3	4						
						Culcidae	0	1	2	3	4						

Figure B.1 Benthic macroinvertebrate field data sheet. (From EPA. *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 841/B-99/002. 1999.)

All organisms in the subsample should be classified according to functional feeding group. Field classification is important because many families comprise genera and species representing a variety of functional groups. Knowing the family-level identification of the organisms will generally be insufficient for categorization by functional feeding group. Functional feeding group classification can be done in the field, on the basis of morphological and behavioral features, using Cummins and Wilzbach (1985). Care should be taken in noting early instars, which may constitute different functional feeding groups from the later instars. Recommended forms for recording benthic data are presented in Figures B.1 through B.4 (EPA 1999).

The scraper and filtering collector functional groups are the most important indicators in the riffle/run community. Numbers of individuals representing each of these two groups are recorded on the Benthic Macroinvertebrate Field Data Sheet (Figure B.1) (EPA 1999). The Benthic

page __ of __

BENTHIC MACROINVERTEBRATE SAMPLE LOG-IN SHEET

Date Collected	Collected By	Number of Containers	Preservation	Station #	Stream Name and Location	Date Received by Lab	Lot Number	Date of Completion		
								sorting	mounting	identification

Serial Code Example: B0754001(1)
B = Benthos (F = Fish; P = Periphyton) ■ 0754 = project number ■ 001 = sample number ■ (1) = lot number (e.g., winter 1996 =1; summer 1996 = 2)

Figure B.2 Benthic macroinvertebrate sample log-in sheet. (From EPA. *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 841/B-99/002. 1999.)

Macroinvertebrate Sample Log-In Sheet (Figure B.2) (EPA 1999) is used to record all collections and is an important part of the QA/QC and sample tracking activities.

All organisms in the subsample should be identified to family or order, enumerated, and recorded, along with any observations on abundance of other aquatic biota, on this data sheet. A summary of all benthic data to be used in the final analysis will be recorded on the Benthic Macroinvertebrate Laboratory Bench Sheet (Figures B.3 and B.4) (EPA 1999) upon return to the laboratory. The use of family-level identification in this protocol is based on Hilsenhoff's Family Biotic Index, which uses higher taxonomic levels of identification (Hilsenhoff 1988).

CPOM Sample

Organisms collected in the supplemental CPOM sample are classified as shredders or non-shredders. Taxonomic identification is not necessary for this component. The composited CPOM sample may be field sorted in a small pan with a light-colored bottom or in the net or sieve through which it was rinsed. (If a large number of benthic macroinvertebrates have been collected, a representative subsampling of 20 to 60 organisms may be removed for functional feeding group classification.) Numbers of individuals representing the shredder functional group, as well as total number of macroinvertebrates collected in this sample, should be recorded for later analysis. The shredder/nonshredder metric may be deemed optional in rivers or in some regions where shredder abundance is naturally low. However, the potential utility of such a metric for assessing toxicant effects warrants serious consideration in this bioassessment approach.

Data Analysis Techniques

Biological impairment of the benthic community may be indicated by the absence of generally pollution-sensitive macroinvertebrate taxa such as Ephemeroptera, Plecoptera, and Trichoptera (EPT); excess dominance by any particular taxon, especially pollutant-tolerant forms such as some

BENTHIC MACROINVERTEBRATE LABORATORY BENCH SHEET (FRONT)

page _____ of _____

STREAM NAME	LOCATION
STATION #_____ RIVERMILE_____	STREAM CLASS
LAT _____ LONG _____	RIVER BASIN
STORET #	AGENCY
COLLECTED BY DATE_____	LOT #
TAXONOMIST DATE_____	SUBSAMPLE TARGET ❑ 100 ❑ 200 ❑ 300 ❑ Other ____

Enter Family and/or Genus and Species name on blank line.

Organisms		No.	LS	TI	TCR	Organisms		No.	LS	TI	TCR
Oligochaeta						Megaloptera					
Hirudinea						Coleoptera					
Isopoda											
Amphipoda						Diptera					
Decapoda											
Ephemeroptera											
						Gastropoda					
						Pelecypoda					
Plecoptera											
						Other					
Trichoptera											
Hemiptera											

Taxonomic certainty rating (TCR) 1-5:1=most certain, 5=least certain. If rating is 3-5, give reason (e.g., missing gills). LS= life stage: I = immature; P = pupa; A = adult TI = Taxonomists initials

Total No. Organisms _____ **Total No. Taxa** _____

Figure B.3 Benthic macroinvertebrate laboratory bench sheet (front). (From EPA. *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 841/B-99/002. 1999.)

BENTHIC MACROINVERTEBRATE LABORATORY BENCH SHEET (BACK)

SUBSAMPLING/SORTING INFORMATION

Sorter _____

Date _____

Number of grids picked: _____

Time expenditure _____ No. of organisms _____

Indicate the presence of large or obviously abundant organisms:

QC: ❑ YES ❑ NO **QC Checker** _____

organisms originally sorted ÷ (# organisms recovered by checker + # organisms originally sorted) = % sorting efficiency

≥90%, sample passes _____

<90%, sample fails, action taken _____

TAXONOMY

ID _____

Date _____

Explain TCR ratings of 3-5:

Other Comments (e.g. condition of specimens):

QC: ❑ YES ❑ NO **QC Checker** _____

Organism recognition ❑ pass ❑ fail

Verification complete ❑ YES ❑ NO

General Comments (use this space to add additional comments):

Figure B.4 Benthic macroinvertebrate laboratory bench sheet (back). (From EPA. *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 841/B-99/002. 1999.)

Chironomidae and Oligochaeta taxa; low overall taxa richness; or appreciable shifts in community composition relative to the reference condition. Impairment may also be indicated by an overabundance of fungal slimes or filamentous algae, or an absence of expected populations of fish. All of these indicators can be evaluated using the sampling data generated. A number of useful metrics exist (Tables B.2 and B.3), while Figure B.5 (EPA 1999) is a preliminary assessment score sheet.

On the basis of observations made in the assessment of habitat, water quality, physical characteristics, and the qualitative biosurvey, the investigator concludes whether impairment is detected. If impairment is detected, an estimation of the probable cause and source should be made. The aquatic biota that indicated an impairment, are noted along with observed indications of potential

PRELIMINARY ASSESSMENT SCORE SHEET
(PASS)

page ____ of ____

STREAM NAME	LOCATION
STATION # _____ RIVERMILE _____	STREAM CLASS
LAT _____ LONG _____	RIVER BASIN
STORET #	AGENCY
COLLECTED BY DATE _____	LOT # _____ NUMBER OF SWEEPS _____
HABITATS: ❑ COBBLE ❑ SHOREZONE ❑ SNAGS ❑ VEGETATION	

Enter Family and/or Genus and Species name on blank line.

Organisms	No.	LS	TI	TCR	Organisms	No.	LS	TI	TCR
Oligochaeta					Megaloptera				
Hirudinea					Coleoptera				
Isopoda									
Amphipoda					Diptera				
Decapoda									
Ephemeroptera					Gastropoda				
					Pelecypoda				
Plecoptera					Other				
Trichoptera									
Hemiptera					Taxonomic certainty rating (TCR) 1-5:1=most certain, 5=least certain. If rating is 3-5, give reason (e.g., missing gills). LS= life stage: I = immature; P = pupa; A = adult TI = Taxonomists initials				

	Site Value	Target Threshold	If 2 or more metrics are ≥ target threshold, site is
Total No. Taxa			**HEALTHY**
EPT Taxa			If less than 2 metrics are within target range, site is
Tolerance Index			**SUSPECTED IMPAIRED**

Figure B.5 Preliminary assessment score sheet. (From EPA. *Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers: Periphyton, Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 841/B-99/002. 1999.)

problem sources. The downstream extent of impact is estimated and multiplied by appropriate stream width to provide an estimate of the areal extent of the problem.

The data analysis scheme used in this RBP integrates several community, population, and functional parameters into a single evaluation of biotic integrity. Each parameter, or metric, measures a different component of community structure and has a different range of sensitivity to pollution stress (Figure B.6). This integrated approach provides more assurance of a valid assessment because a variety of parameters are evaluated. Deficiency of any one metric in a particular situation should not invalidate the entire approach.

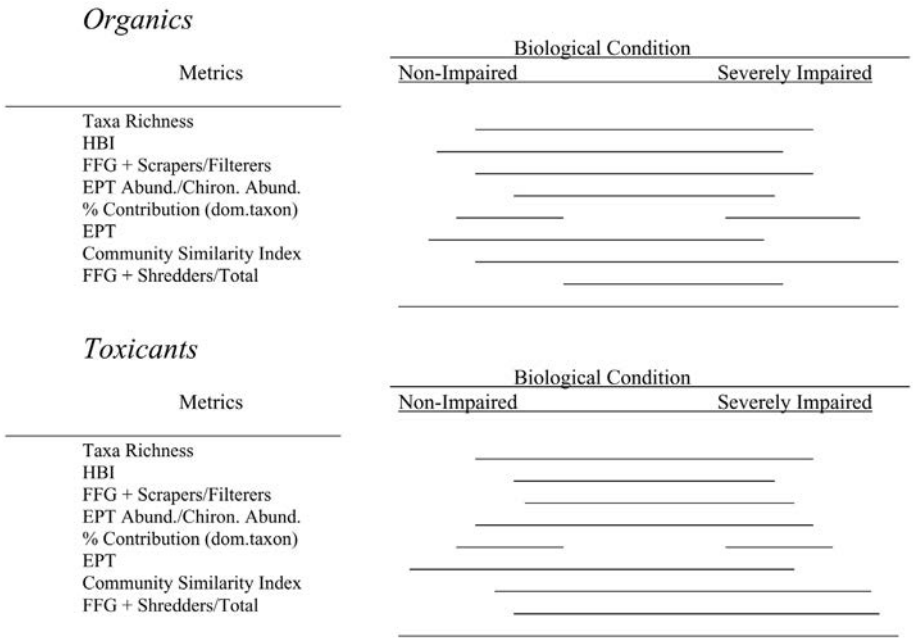

Figure B.6 Range of sensitivities of Rapid Bioassessment Protocol II and III benthic metrics in assessing biological condition in response to organics and toxicants.

The integrated data analysis (Figure B.7) is performed as follows. Using the raw benthic data, a numerical value is calculated for each metric. Calculated values are then compared to values derived from either a reference site within the same region, a reference database applicable to the region, or a suitable control station on the same stream. Each metric is then assigned a score according to the comparability (percent similarity) of calculated and reference values. Scores for the eight metrics are then totaled and compared to the total metric score for the reference station. The percent comparison between the total scores provides a final evaluation of biological condition. The criteria to be used for scoring the eight metrics *may need to be adjusted for use in particular regions.*

Inherent variability in each metric was considered in establishing percent comparability criteria (Figure B.6). The metrics based on taxa richness, FBI, and EPT Indices have low variability (Resh 1988). This variability is accounted for in the criteria for characterization of biological condition, based on existing data. For metrics based on standard taxa richness and FBI and EPT Indices, differences of 10 to 20% relative to the reference condition would be considered nominal, and the station being assessed would receive the maximum metric score. Because increasing FBI values denote worsening biological condition, percent difference for this metric is calculated by dividing the reference value by the value for the station of comparison.

Metrics that utilize ratios fluctuate more widely, however, and comparing percent differences between ratios (ratios of ratios) will compound the variability. Scoring increments are therefore set at broad intervals of 25% or greater. For metrics based on functional feeding group ratios, Cummins (1987, personal communication) contends that differences as great as 50% from the reference may be acceptable, but differences in the range of 50 to 100% are not only important, but discriminate degrees of impact more clearly.

The contribution of the dominant taxon to total abundance is a simple estimator of evenness. Scoring criteria are based on theoretical considerations rather than direct comparison with a reference.

The Community Loss Index (a representative similarity index) already incorporates a comparison with a reference. Therefore, actual index values are used in scoring.

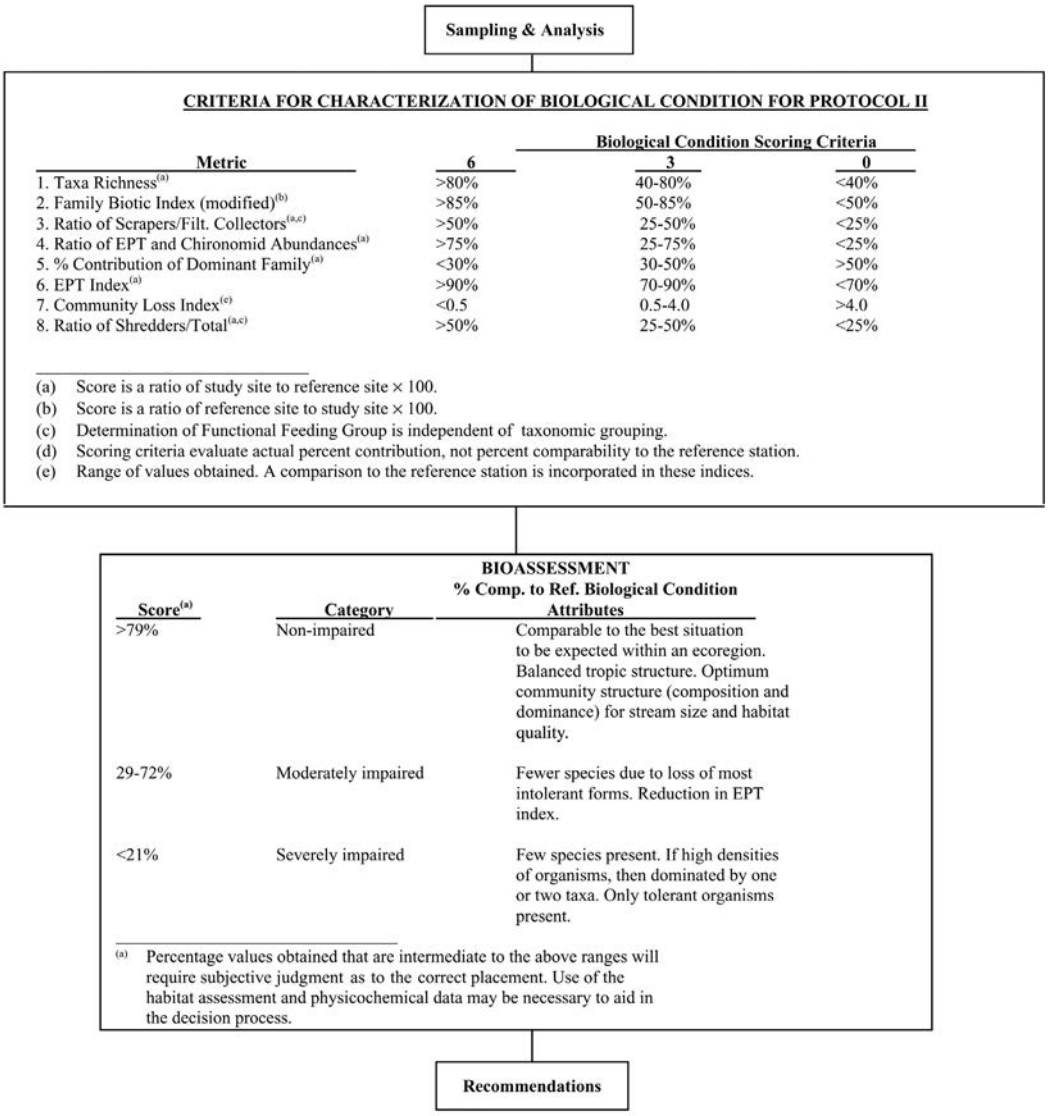

Figure B.7 Flowchart of bioassessment approach advocated for a Rapid Bioassessment Protocol.

The metrics used to evaluate the benthic data and their significance are explained below and in Tables B.1 and B.2.

Riffle/Run Sample

Metric 1. Taxa Richness

Reflects health of the community through a measurement of the variety of taxa (total number of families) present. Generally increases with increasing water quality, habitat diversity, and habitat suitability. Sampling of highly similar habitats will reduce the variability in this metric attributable to factors such as current speed and substrate type. Some pristine headwater streams may be naturally unproductive, supporting only a very limited number of taxa. In these situations, organic enrichment may result in an increased number of taxa (including EPT taxa).

Table B.1 Definitions of Best Candidate Benthic Metrics and Predicted Direction of Metric Response to Increasing Perturbation

Category	Metric	Definition	Predicted Response to Increasing Perturbation
Richness measures	Total No. taxa	Measures the overall variety of the macroinvertebrate assemblage	Decrease
	No. EPT taxa	Number of taxa in the insect orders Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies)	Decrease
	No. Ephemeroptera taxa	Number of mayfly taxa (usually genus or species level)	Decrease
	No. Plecoptera taxa	Number of stonefly taxa (usually genus of species level)	Decrease
	No. Trichoptera taxa	Number of caddisfly taxa (usually genus or species level)	Decrease
Composition measures	% EPT	Percent of the composite of mayfly, stonefly, and caddisfly larvae	Decrease
	% Ephemeroptera	Percent of mayfly nymphs	Decrease
Tolerance/intolerance measures	No. intolerant taxa	Taxa richness of those organisms considered to be sensitive to perturbation	Decrease
	% tolerant organisms	Percent of macrobenthos considered to be tolerant of various types of perturbation	Increase
	% dominant taxon	Measures the dominance of the single most abundant taxon. Can be calculated as dominant 2, 3, 4, or 5 taxa.	Increase
Feeding measures	% filterers	Percent of the macrobenthos that filter FPOM from either the water column or sediment	Variable
	% grazers and scrapers	Percent of the macrobenthos that scrape or graze upon periphyton	Decrease
Habit measures	No. clinger taxa	Number of taxa of insects	Decrease
	% clingers	Percent of insects having fixed retreats or adaptations for attachment to surfaces in flowing water.	Decrease

Data from DeShon 1995; Barbour et al. 1996b; Fore et al. 1996; Smith and Voshell 1997.

Metric 2. Modified Family Biotic Index

Tolerance values range from 0 to 10 for families and increase as water quality decreases. The index was developed by Hilsenhoff (1988) to summarize the various tolerances of the benthic arthropod community with a single value. The Modified Family Biotic Index was developed to detect organic pollution and is based on the original species-level index (Hilsenhoff 1982). Tolerance values for each family were developed by weighting species according to their relative abundance in the State of Wisconsin.

The family-level index has been modified for this document to include organisms other than just arthropods using the genus and species-level biotic index developed by the State of New York (Bode 1988). The formula for calculating the Family Biotic Index is:

$$HBI = \frac{\sum x_i t_j}{n}$$

where x_i = number of individuals within a taxon
t_j = tolerance value of a taxon
n = total number of organisms in the sample

Table B.2 Definitions of Additional Potential Benthic Metrics and Predicted Direction of Metric Response to Increasing Perturbation

Category	Metric	Definition	Predicted Response to Increasing Perturbation	References
Richness measures	No. *Pteronarcys* species	The presence or absence of a long-lived stonefly genus (2–3 year life cycle)	Decrease	Fore et al. 1996
	No. Diptera taxa	Number of "true" fly taxa, which includes midges	Decrease	DeShon 1995
	No. Chironomidae taxa	Number of taxa of chironomid (midge) larvae	Decrease	Hayslip 1993; Barbour et al. 1996b
Composition measures	% Plecoptera	Percent of stonefly nymphs	Decrease	Barbour et al. 1994
	% Trichoptera	Percent of caddisfly larvae	Decrease	DeShon 1995
	% Diptera	Percent of all "true" fly larvae	Increase	Barbour et al. 1996b
	% Chironomidae	Percent of midge larvae	Increase	Barbour et al. 1994
	% Tribe Tanytarsini	Percent of Tanytarisinid midges to total fauna	Decrease	DeShon 1995
	% Other Diptera and noninsects	Composite of those organisms generally considered to be tolerant to a wide range of environmental conditions	Increase	DeShon 1995
	% *Corbicula*	Percent of Asiatic clam in the benthic assemblage	Increase	Kerans and Karr 1994
	% Oligochaeta	Percent of aquatic worms	Variable	Kerans and Karr 1994
Tolerance/ intolerance measures	No. intol. snail and mussel species	Number of species of molluscs generally thought to be pollution intolerant	Decrease	Kerans and Karr 1994
	% sediment tolerant organisms	Percent of infaunal macrobenthos tolerant of perturbation	Increase	Fore et al. 1996
	Hilsenhoff Biotic Index	Uses tolerance values to weight abundance in an estimate of overall pollution; originally designed to evaluate organic pollution	Increase	Barbour et al. 1992; Hayslip 1993; Kerans and Karr 1994
	Florida Index	Weighted sum of intolerant taxa, which are classed as 1 (least tolerant) or 2 (intolerant); Florida Index = 2 × Class 1 taxa + Class 2 taxa	Decrease	Barbour et al. 1996b
	% Hydropsychidae to Trichoptera	Relative abundance of pollution tolerant caddisflies (metric could also be regarded as a composition measure)	Increase	Barbour et al. 1992; Hayslip 1993
Feeding measures	% omnivores and scavengers	Percent of generalists in feeding strategies	Increase	Kerans and Karr 1994
	% ind. gatherers and filterers	Percent of collector feeders of CPOM and FPOM	Variable	
	% gatherers	Percent of the macrobenthos that "gather"	Variable	Barbour et al. 1996b
	% predators	Percent of the predator functional feeding group; can be made restrictive to exclude omnivores	Variable	Kerans and Karr 1994
	% shredders	Percent of the macrobenthos that "shreds" leaf litter	Decrease	Barbour et al. 1992; Hayslip 1993
Life cycle measures	% multivoltine	Percent of organisms having short (several per year) life cycle	Increase	Barbour et al. 1994
	% univoltine	Percent of organisms relatively long-lived (life cycles of 1 or more years)	Decrease	Barbour et al. 1994

Hilsenhoff's family-level tolerance values may require modification for some regions. Alternative tolerance classifications and biotic indices have been developed by some state agencies. Additional biotic indices are listed in EPA (1983).

Although the FBI may be applicable for toxic pollutants, it has only been evaluated for organic pollutants. The State of Wisconsin is conducting a study to evaluate the ability of Hilsenhoff's index to detect nonorganic effects.

Metric 3. Ratio of Scraper and Filtering Collector Functional Feeding Groups

The scraper and filtering collector metric reflects the riffle/run community foodbase. When compared to a reference site, shifts in the dominance of a particular feeding type indicate a community responding to an overabundance of a particular food source. The predominant feeding strategy reflects the type of impact detected. Assignment of individuals to functional feeding groups is independent of taxonomy, with some families representing several functional groups.

A description of the functional feeding group concept can be found in Cummins (1973) and Merritt and Cummins (1984). Functional feeding group designations for most aquatic insect families may be found in Merritt and Cummins (1984). Most aquatic insects can also be classified to functional feeding group in the field, on the basis of morphological and behavioral features, using Cummins and Wilzbach (1985).

The relative abundance of scrapers and filtering collectors in the riffle/run habitat is an indication of the periphyton community composition, availability of suspended fine particulate organic material (FPOM), and availability of attachment sites for filtering. Scrapers increase with increased diatom abundance and decrease as filamentous algae and aquatic mosses (which scrapers cannot efficiently harvest) increase. However, filamentous algae and aquatic mosses provide good attachment sites for filtering collectors, and the organic enrichment often responsible for overabundance of filamentous algae can also provide FPOM that is utilized by the filterers.

Filtering collectors are also sensitive to toxicants bound to fine particles and should be the first group to decrease when exposed to steady sources of such bound toxicants. This situation is often associated with point-source discharges where certain toxicants adsorb readily to dissolved organic matter (DOM), forming FPOM during flocculation. Toxicants thus become available to filterers via FPOM. The scraper to filtering collector ratio may not be a good indicator of organic enrichment if adsorbing toxicants are present. In these instances the FBI and EPT Index may provide additional insight. Qualitative field observations on periphyton abundance may also be helpful in interpreting results.

Metric 4. Ratio of EPT and Chironomidae Abundances

The EPT and Chironomidae abundance ratio uses relative abundance of these indicator groups (Ephemeroptera, Plecoptera, Trichoptera, and Chironomidae) as a measure of community balance. Good biotic condition is reflected in communities with an even distribution among all four major groups and with substantial representation in the sensitive groups Ephemeroptera, Plecoptera, and Trichoptera. Skewed populations having a disproportionate number of the Chironomidae relative to the more sensitive insect groups may indicate environmental stress (Ferrington 1987; Shackleford 1988). Certain species of some genera such as *Cricotopus* are highly tolerant (Lenat 1983; Mount et al. 1984), and as opportunists may become numerically dominant in habitats exposed to metal discharges where EPT taxa are not abundant, thereby providing a good indicator of toxicant stress (Winner et al. 1980). Clements et al. (1988) found that mayflies were more sensitive than chironomids to exposure levels of 15 to 32:g/L of copper. Chironomids tend to become increasingly dominant in terms of percent taxonomic composition and relative abundance along a gradient of increasing enrichment or heavy metals concentration (Ferrington 1987).

An alternative to the ratio of EPT and Chironomidae abundance metric is the Indicator Assemblage Index (IAI) developed by Shackleford (1988). The IAI integrates the relative abundances of the EPT taxonomic groups and the relative abundances of chironomids and annelids upstream and downstream of a pollutant source to evaluate impairment. The IAI may be a valuable metric in areas where the annelid community may fluctuate substantially in response to pollutant stress.

Metric 5. Percent Contribution of Dominant Family

The percent contribution of the dominant family to the total number of organisms uses abundance of the numerically dominant taxon relative to the rest of the population as an indication of community balance at the family level. A community dominated by relatively few families would indicate environmental stress. This metric may be redundant if the Pinkham and Pearson Similarity Index is used as a community similarity index for metric number 7.

Metric 6. EPT Index

The EPT Index generally increases with increasing water quality. The EPT Index value is the total number of distinct taxa within the groups Ephemeroptera, Plecoptera, and Trichoptera. The EPT Index value summarizes the taxa richness within the insect groups that are generally considered pollution sensitive. This was developed for species-level identifications; however, the concept is valid for use at family-level identifications.

Headwater streams which are naturally unproductive may experience an increase in taxa (including EPT taxa) in response to organic enrichment.

Metric 7. Community Similarity Indices

Community Similarity Indices are used in situations where a reference community exists, either through sampling or through prediction for a region. Data sources or ecological data files may be available to predict a reference community to be used for comparison. The combined information provided through a regional analysis and EPA's ERAPT ecological database (Dawson and Hellenthal 1986) may be useful for this analysis. These indices are designed to be used with either species level identifications or higher taxonomic levels. Three of the many community similarity indices available are discussed below:

- Community Loss Index. Measures the loss of benthic taxa between a reference station and the station of comparison. The Community Loss Index was developed by Courtemanch and Davies (1987) and is an index of compositional dissimilarity, with values increasing as the degree of dissimilarity with the reference station increases. Values range from 0 to "infinity." Based on preliminary data analysis, this index provides greater discrimination than either of the following two community similarity indices.
- Jaccard Coefficient of Community Similarity. Measures the degree of similarity in taxonomic composition between two stations in terms of taxon presence or absence. The Jaccard Coefficient discriminates between highly similar collections. Coefficient values, ranging from 0 to 1.0, increase as the degree of similarity with the reference station increases. See Jaccard (1912), Boesch (1977), and EPA (1983) for more detail. The formulae for the Community Loss Index and the Jaccard Coefficient are

$$\text{Community Loss} = \frac{d - a}{e}$$

$$\text{Jaccard Coefficient} = \frac{a}{a + b + c}$$

where a = number of taxa common to both samples
b = number of taxa present in Sample B but not A
c = number of taxa present in Sample A but not B
d = total number of taxa present in Sample A
e = total number of taxa present in Sample B
Sample A = reference station (or mean of reference database)
Sample B = station of comparison

- Pinkham and Pearson Community Similarity Index Incorporates abundance and compositional information and can be calculated with either percentages or numbers. A weighting factor can be added that assigns more significance to dominant taxa. See Pinkham and Pearson (1976) and EPA (1983) for more detail. The formula is

$$S.I._{ab} = \sum \frac{\min(x_{ia}, x_{ib})}{\max(x_{ia}, x_{ib})} \left[\frac{\frac{x_{ia}}{x_a} \bullet \frac{x_{ib}}{x_b}}{2} \right]$$

where x_{ia}, x_{ib} = number of individuals in the ith taxon in Sample A or B

Other community similarity indices include Spearman's Rank Correlation (Snedecor and Cochran 1967), Morisita's Index (Morisita 1959), Biotic Condition Index (Winget and Mangum 1979), and Bray-Curtis Index (Bray and Curtis 1957; Whittaker 1952). Calculation of a chi-square "goodness of fit" (Cochran 1952) may also be appropriate.

CPOM Sample

Metric 8. Ratio of Shredder Functional Feeding Group and Total Number of Individuals Collected

Also based on the Functional Feeding Group concept, the abundance of the shredder functional group relative to the abundance of all other functional groups allows evaluation of potential impairment as indicated by the CPOM-based shredder community. Shredders are sensitive to riparian zone impacts and are particularly good indicators of toxic effects when the toxicants involved are readily adsorbed to the CPOM and either affect microbial communities colonizing the CPOM or the shredders directly (Cummins 1987, personal communication).

The degree of toxicant effects on shredders vs. filterers depends on the nature of the toxicants and the organic particle adsorption efficiency. Generally, as the size of the particle decreases, the adsorption efficiency increases as a function of the increased surface to volume ratio (Hargrove 1972). Because waterborne toxicants are readily adsorbed to FPOM, toxicants of a terrestrial source (e.g., pesticides, herbicides) accumulate on CPOM prior to leaf fall, thus having a substantial effect on shredders (Swift et al. 1988a,b). The focus on this approach is on a comparison to the reference community which should have a reasonable representation of shredders as dictated by seasonality, region, and climate. This allows for an examination of shredder or collector "relative" abundance as indicators of toxicity.

The data collected in the 100-organism riffle/run subsample and the CPOM sample are summarized according to the information required for each metric and entered on the Data Summary Sheet.

Each metric result is given a score based on percent comparability to a reference station. Scores are totaled and compared to the total metric score for the reference station. The percent comparison between the total scores provides a final evaluation of biological condition. Values obtained may sometimes be intermediate to established ranges and require some judgment as to assessment of

biological condition. In these instances, habitat assessment, physical characterization, and water quality data may aid in the evaluation process.

Guidance for Data Summary Sheets for Benthic RBP

Station Number: Indicate station number for each data set recorded.

Station Location: Record brief description of sampling site relative to established landmarks (i.e., roads, bridges).

Taxa Richness: Record total number of families (or higher taxa) collected in the 100-organism riffle subsample.

FBI (modified): Record the Family Biotic Index value (Hilsenhoff 1988) calculated for the 100-organism riffle subsample using the formula presented in RBP II. Tolerance classification values can be entered into the computer database to simplify calculation.

Functional Feeding Group: Functional feeding group classifications may be entered into the computer database to simplify calculations.

Riffle Community: Scrapers/filtering collectors: enter the value obtained by dividing the number of individuals in the riffle subsample representing the scraper functional group, by the number representing the filtering collector functional group.

CPOM Community: Shredders/total: enter the value obtained by dividing the number of individuals in the CPOM sample (or subsample) representing the shredder functional group, by the total number of organisms in the sample (or subsample).

EPT/Chironomidae: Enter the value obtained by dividing the number of individuals in the 100-organism riffle subsample in the family Chironomidae, by the total number of individuals in the orders Ephemeroptera, Plecoptera, and Trichoptera.

Percent Contribution (Dominant Family): Record the value obtained by dividing the number of individuals in the family that is most abundant in the 100-organism riffle subsample, by the total number of individuals in the sample.

EPT Index: Record the total number of taxa in the 100-organism riffle subsample representing the orders Ephemeroptera, Plecoptera, and Trichoptera.

Community Similarity Index: Enter the value calculated for the appropriate community similarity index, using data from the 100-organism riffle subsample.

Values obtained for each metric should be assigned a score based on percent comparability to the control or reference station data. Scores are summed for both the impaired and reference station. The percent comparison between the total scores provides the final evaluation of biological condition.

Family-Level Tolerance Classification

The original RBP II (EPA 1989) is based on family-level identifications. The adequate assessment of biological condition for RBP II requires the use of a tolerance classification for differentiating among responses of the benthic community to pollutants. Hilsenhoff's Family Biotic Index (FBI) is used as a basis for the family-level tolerance classification.

The biotic index (BI) of organic pollution is adapted (Hilsenhoff 1987) for rapid evaluation by providing tolerance values for families (Tables B.3 and B.4) to allow a family-level biotic index (FBI) to be calculated in the field. The FBI is an average of tolerance values of all arthropod families in a sample. It is not intended as a replacement for the BI and can be effectively used in the field only by biologists who are familiar enough with arthropods to be able to identify families without using keys.

Using the same method and more than 2000 stream samples from throughout Wisconsin that were used to revise tolerance values for species and genera (Hilsenhoff 1987) family-level tolerance values were established by comparing occurrence of each family with the average BI of streams in which they occurred in the greatest numbers. Thus, family-level tolerance values tend to be a weighted average of tolerance values of species and genera within each family based on their relative abundance in Wisconsin.

Table B.3 Tolerance Values for Families of Stream Arthropods in the Western Great Lakes Region

Plecoptera	Capniidae 1, Chloroperlidae 1, Leuctridae 0, Nemouridae 2, Perlidae 1, Perlodidae 2, Pteronarcyidae 0, Taeniopterygidae 2
Ephemeroptera	Baetidae 4, Baetiscidae 3, Caenidae 7, Ephemerellidae 1, Ephemeridae 4, Heptageniidae 4, Leptophlebiidae 2, Metretopodidae 2, Oligoneuriidae 2, Polymitarcyidae 2, Potomanthidae 4, Siphlonuridae 7, Tricorythidae 4
Odonata	Aeshnidae 3, Calopterygidae 5, Coenagrionidae 9, Cordulegastridae 3, Corduliidae 5, Gomphidae 1, Lestidae 9, Libellulidae 9, Macromiidae 3
Trichoptera	Brachycentridae 1, Glossosomatidae 0, Helicopsychidae 3, Hydropsychidae 4, Hydroptilidae 4, Lepidostomatidae 1, Leptoceridae 4, Limnephilidae 4, Molannidae 6, Odontoceridae 0, Philopotamidae 3, Phryganeidae 4, Polycentropodidae 6, Psychomyiidae 2, Rhyacophilidae 0, Sericostomatidae 3
Megaloptera	Corydalidae 0, Sialidae 4
Lepidoptera	Pyralidae 5
Coleoptera	Dryopidae 5, Elmidae 4, Psephenidae 4
Diptera	Athericidae 2, Blephariceridae 0, Ceratopogonidae 6, Blood-red Chironomidae (Chironomini) 8, other (including pink) Chironomidae 6, Dolochopodidae 4, Empididae 6, Ephyridae 6, Psychodidae 10, Simuliidae 6, Muscidae 6, Syrphidae 10, Tabanidae 6, Tipulidae 3
Amphipoda	Gammaridae 4, Talitridae 8
Isopoda	Asellidae 8

Data from Hilsenhoff, W.L. Rapid field assessment of organic pollution with a family-level biotic index. *J. North Am. Benthol. Soc.*, 7: 65–68. 1988; EPA. *Rapid Bioassessment Protocols for Use in Streams and Rivers: Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 444/4-89/001. 1989.

THE OHIO EPA INVERTEBRATE COMMUNITY INDEX APPROACH (OEPA 1989)

Field Methods — Quantitative Sampling

The primary sampling equipment used for the collection of benthic macroinvertebrates is the modified Hester–Dendy multiple-plate artificial substrate sampler. The sampler is constructed of $^1/_8$-in tempered hardboard cut into 3-in square (or circular) plates and 1-in square spaces. A total of eight plates and 12 spacers are used for each sampler. The plates and spacers are placed on a $^1/_4$-in stainless steel eyebolt so that there are three single spaces, three double spaces, and one triple space between the plates. The total surface area of the sampler, excluding the eyebolt, is 145.6 in^2.

Samplers placed in streams are tied to a concrete construction block, which anchors them in place and prevents the multiple-plates from coming into contact with the natural substrates. In water deeper than 4 ft, a float (1 quart cubitainer) is attached to the samplers to keep them within 4 ft of the surface. Whenever possible, the samplers are placed in runs rather than pools or riffles and an attempt is made to establish stations in as similar an ecological situation as possible. All samplers are exposed for a 6-week period. A set of samplers consists of three multiple-plate samplers (about 3 ft^2 of surface area) at National Ambient Water Quality Monitoring Network (NAWQMN) stations and five multiple-plate samplers at all other sampling locations. All NAWQMN stations and most routine monitoring stations are sampled from June 15 to September 30.

Retrieval of the sampler is accomplished by cutting them from the block and placing them in 1-quart, wide-mouth plastic containers while still submersed. Care is taken to avoid disturbing the samplers and thereby dislodging any organisms. Enough formalin is added to each container to equal an approximate 10% solution.

Qualitative samples of macroinvertebrates inhabiting the natural substrates are also collected at the time of sampler retrieval. In shallow water, samples are taken in a stream segment covering all available habitats near where the samplers were placed. Samples are collected using triangular ring frame 30-mesh dip nets and hand picking with forceps. Grab samplers (i.e., Ekman, Peterson, or Ponar) can also be used in deep water. The qualitative sampling continues until, by gross examination, no new taxa are being taken. A station description sheet is filled out by the collector at the time

Table B.4 . Tolerance Values for Some Macroinvertebrates Not Included in Hilsenhoff (1982, 1987)

Acariformes	4
Decapoda	6
Gastropoda	
Amnicola	8
Bithynia	8
Ferrissia	6
Gyraulus	8
Helisoma	6
Lymnaea	6
Physa	8
Sphaeriidae	8
Oligochaeta	
Chaetogaster	6
Dero	10
Nais barbata	8
Nais behningi	6
Nais bretscheri	6
Nais communis	8
Nais elinguis	10
Nais pardalis	8
Nais simplex	6
Nais variabilis	10
Pristina	8
Stylaria	8
Tubificidae	
Aulodrilus	8
Limnodrilus	10
Hirudinea	
Helobdella	10
Turbellaria	4

From Bode, R.W. *Quality Assurance Workplan for Biological Stream Monitoring in New York State*. New York State Department of Environmental Conservation, Albany. 1988; EPA. *Rapid Bioassessment Protocols for Use in Streams and Rivers: Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 444/4-89/001. 1989.

of sampler retrieval. The substrate is described using the categories for substrate characterization indicated in the U.S. EPA biological field manual (Weber 1973).

In situations where quantitative biological samples are collected from the natural substrates using a Surber square foot sampler (30-mesh netting), the collector stands on the downstream side of the sampler and works the substrate using a hand cultivator with 2-in tines. Large rocks are gently scrubbed with a brush. The material collected is placed in sealed containers, preserved in 10% formalin, and transported to the laboratory. Three to five Surber samples are taken at each site.

In situations where Ekman, Peterson, or Ponar grab samples are used for quantitative purposes, three to five samples are collected and then treated in essentially the same manner as the Surber samples. The material collected with the grab is washed through a bucket with a 30-mesh screen bottom, placed in sealed containers, preserved in 10% formalin, and returned to the laboratory.

Laboratory Methods — Quantitative Sampling

Samples are coded and sample numbers are immediately entered into a log book upon arrival at the laboratory. Samples are given a log number derived from the date, e.g., 871108-10, where 87 represents the year, 11 represents the month, and 08 the day. The number following this six-digit date, i.e., the number 10 in the previous example, indicates that this was the 10th sample logged that day. Other information in the log book includes the name(s) of field personnel who collected the sample, date, stream or lake name, basin name, entity (where applicable), general location, sample type, sampling method(s) used, the person who conducted the analyses, and any other comments considered pertinent to the collection and analysis of the sample.

Macroinvertebrate Counts and Identifications

Composite samples consisting of five multiple-plate samplers are used in station evaluations for routine monitoring. However, replicate samples (three multiple-plate samplers) are reported to the EPA for NAWQMN stations. Replicate sets of five multiple-plate samplers can be used if deemed necessary in cases where sampling is for litigation purposes. In all cases, the multiple-plate samplers are disassembled in a bucket of water and cleaned of organisms and debris. The organism/debris mixture is then passed through U.S. Standard Testing Sieves number 30 (0.589-mm openings) and number 40 (0.425-mm openings). The material retained in each sieve is preserved in properly labeled and coded jars containing 70% alcohol.

The following procedures are used during the course of analyzing an artificial substrate, Surber, or grab sample:

1. Sorting the sample is done in a white enamel pan followed by scanning under the dissecting microscope (10× magnification). Subsamples are produced using the following guidelines:
   a. A Folsom sample splitter is used for all subsampling. In an effort to determine the accuracy of the Folsom sample splitter, a sample composed of 200 individuals of five frequently collected organisms was prepared and repeatedly split. Statistical analysis of the data yielded a chi-square value of 2.56, df = 4, indicating that the subsamples were not significant at the 95% probability level.
   b. After an entire sample has been sorted, subsampling within families containing unmanageable numbers is acceptable.
   c. Very large samples may be subsampled prior to sorting, but only after examination in a white enamel pan to remove obvious rare taxa, e.g., hellgramites, non-hydropsychid caddisflies.
   d. A minimum of 250 organisms are identified, with at least 50 to 100 midges, 70 caddisflies, 70 mayflies.
2. Dipterans of the family Chironomidae are prepared for identification by clearing the larvae in hot 10% KOH for 30 min and then mounting in water on microscope slides. Permanent slides for the voucher collection are mounted in Euparol mounting medium.
3. Material retained in the #40 screen is counted and identified or counted and extrapolated when identification is impossible or impractical. (Artificial substrate sample only.)
4. Organisms determined to be dead before the time of collection are discarded.
5. When only one sex or life stage can be identified, it is assumed that the other sex or stage is the same species.
6. Sections of bryozoan colonies are removed from the plates and saved for identification. Only colonies, not individuals, are counted. (Artificial substrate sample only.)
7. Early instars that cannot be identified are extrapolated where possible.
8. Species-level identifications are made where possible and practical. Generic or higher level classifications are made if specimens are damaged beyond identification, in those cases where taxonomy is incomplete or laborious and time-consuming, or where the specimen is an unidentifiable early instar.
9. Organisms are listed in tables following the laboratory table format.
10. Two end fragments of an oligochaete are counted as one individual. Fragments without ends are not counted.
11. Any taxonomic key in the laboratory may be used as an aid in the identification of an organism. Also indicated is the level of taxonomy attainable with the keys listed.

Macroinvertebrate Data Analysis

Invertebrate Community Index

The principal measure of overall macroinvertebrate community condition used by the Biological Field Evaluations Group is the Invertebrate Community Index (ICI), a measurement derived in-house from information collected over many years. The ICI is a modification of the Index of Biotic Integrity (IBI) for fish developed by Karr (1981). The ICI consists of 10 structural community metrics, each with four scoring categories of 6, 4, 2, and 0 points (Table B.5). The point system evaluates a sample against a database of 247 relatively undisturbed reference sites throughout Ohio. Six points will be scored if a given metric has a value comparable to those of exceptional stream communities, 4 points for those metric values characteristic of more typical good communities, 2 points for metric values slightly deviating from the expected range of good values, and 0 points for metric values strongly deviating from the expected range of good values. The summation of the individual metric scores (determined by the relevant attributes of an invertebrate sample with some consideration given to stream drainage area) results in the ICI value. Metrics 1 through 9 are all generated from the artificial substrate sample data, while Metric 10 is based solely on the

Table B.5 . Invertebrate Community Index (ICI) Metrics and Scoring Criteria Based on Macroinvertebrate Community Data from 247 Reference Sites throughout Ohio

Metric		Scoring Criteria			
		0	2	4	6
1.	Total number of taxa	Scoring of each metric varies with drainage area; see Ohio EPA (1987)			
2.	Total number of mayfly taxa				
3.	Total number of caddisfly taxa				
4.	Total number of dipteran taxa				
5.	Percent mayflies				
6.	Percent caddisflies				
7.	Percent tribe tanytarsini midges				
8.	Percent other dipterans and non-insects				
9.	Percent tolerant organisms				
10.	Total number of qualitative Ephemeroptera, Plecoptera, and Trichoptera (EPT) taxa				

qualitative sample data from natural substrates. More discussion of the derivation of the ICI including descriptions of each metric and the data plots and other information used to score each metric can be found in Ohio EPA (1987).

Community Similarity Index

A coefficient of similarity between two stations can be calculated using Van Horn's (1950) equation modified from the general formula described by Gleason (1920):

$$c = \frac{2w}{a + b}$$

The variables in this expression can be based either on the number of taxa present or absent at each station or on actual numerical data collected at each site. If the presence/absence method is being used:

a = the number of taxa collected at one station
b = the number of taxa collected at the other station
w = the number of taxa common to both stations

When actual numerical data are being used, each taxon is assigned a prominence value calculated by multiplying the density of the taxon by the square root of its frequency of occurrence (Beals 1961; Burlington 1962). In this case:

a = the sum of the prominence values of all of the taxa at one station
b = the sum of the prominence values of all of the taxa at the other station
c = the sum of the prominence values of all of the taxa of one station which it has in common with the other station. The lower of the two resulting values of w is used in the equation.

Rank Correlation Coefficient

A rank correlation coefficient between measured biological, chemical, or other physical data can be calculated using the formula defined by Spearman (1904):

$$r_s = 1 - \frac{6\sum_{i-1}^{n} D_i^2}{n(n^2 - 1)}$$

where n = the number of paired observations ($x_i y_i$) and D_i = the rank of x_i minus the rank of y_i.

Table B.6 Benthic Macroinvertebrate Equipment and Supplies

Item	Unit	Source[a]
Boat, flat bottom, 14–16 ft, snatch-block meter, wheel and trailer, 18 hp outboard motor. Life jackets, other accessories	1	(7,15)
Boat crane kit and winch	1	(3,15)
Boat, inflatable with oar set	1	(1,15)
Cable fastening tools		(4,15)
Cable clamps, 1/8"	25	
Nicro-press clamps, 1/8"	100	
Nicro-press tool, 1/8"	1	
Wire cutter, Felco	1	
Wire thimbles, 1/8"	25	
Cable, 1/8", galvanized steel	1000 ft	(3,15)
Large capacity metal wash tub	1	
Sample wash bucket (sieve)	1	(8,14)
Core sampler, hand held	1	(3,8,14)
Box corer	1	(14)
K-B corer	1	(8)
Wide-barrel gravity corer	1	(14)
Phleger corer	1	(8,14)
Ballchek single or multiple corer	1	(8,14)
Ewing portable piston corer	1	(14)
Hardboard multiplate sampler	10	(3,8)
Ceramic multiplate sampler	10	(14)
Trawl net	1	(8)
Dredge	1	(3,8,14)
Rectangular box sediment sampler	1	(14)
Drift net, stream	6	(8,14)
Triple-net drift sampler	2	(14)
Stream bottom sampler, Surber type	2	(3,8,14)
Portable invertebrate box sampler	2	(13)
Stream-bed fauna sampler, Hess type	2	(14)
Hess stream bottom sampler	2	(8)
Grab sampler, Ponar	1	(3,8,14)
Wildco box corer	1	(8)
Grab sampler, Ekman	1	(3,8,14)
Grab sampler, Petersen	1	(3,8,14)
Grab sampler, Smith-McIntyre	1	(14)
Grab sampler, Van Veen	1	(14)
Grab sampler, Orange Peel	1	(14)
Grab sediment sampler, Shipek	1	(8)
Basket, bar B-Q, tumbler (#740-0035)	12	(9,11)
Sieves, US Standard No. 30	2	(5)
Flowmeter, mechanical	1	(3)
Mounting media, CMCP-9/9AF with stain	4 oz	No longer available
Mounting medium, CMCP-9	4 oz	(6)
Mounting medium, CMCP-10	4 oz	(6)
Fuchsin basic, C.I. dye	25 g	(6)
Mounting medium, Aquamount	4 oz	(12)
Refrigerated circulator	1	(5)
Water pump, epoxy-coated	2	(1)
Holding tank, constant temp	1	(10)
Balance, top-loading	1	(5)
Counter, 12-unit, 2 × 6	1	(3)
Counter, hand tally	2	(3)
Waders, with suspenders	1 pr	(1,15)
Boots, hip	1 pr	(1,15)
Raincoat	1	(3,15)

Table B.6 Benthic Macroinvertebrate Equipment and Supplies (continued)

Item	Unit	Source[a]
Magni-focuser, 2×	1	(5)
Microscope, field	1	(3)
Magnifier, illuminated + base	1	(3)
Magnifier, pocket, 5×, 10×, and 15×	1	(3)
Microscope, compound, with phase and bright-field, trinocular, 10× and 15× eyepieces, 4×, 10×, 20×, 45× and 100× objectives	1	(5)
Microscope, stereoscopic, with stand	1	(2)
Microscope slide dispenser	1	(1)
Microscope slides and cover slips, 12 and 15 mm circles	10 gross	(1)
Photographic system, photostar	1	(5)
Camera, photomicrographic, with 50 mm lens	1	(1,15)
Stirrer, magnetic	1	(5)
Aquarium, 10 gal., with cover, air pump and filter	1	(1,15)
Aquatic dip net, Model 412D	2	(3)
Jars, screw cap, specimen	5 dz	(1)
Bottles, wide mouth, 32 oz	1 case	(1)
Specimen jars, wide mouth, 4 oz	48	(1)
Specimen jars, wide mouth, 6 oz	48	(1)
Vials, specimen, 1 oz	10 gross	(1)
Petri dish, ruled grid	4	(1)
Petri dish, compartmented	1 case	(1)
Watch glasses	10	(1)
Vacuum oven	1	(5)
Sounding lead and calibrated line	1	(3)
Forceps, watchman's, stainless	1 pr	(1)
Forceps, microdissection	2 pr	(1)
Dissecting set, basic	1	(1)
Water test kit, limnology	1	(1)
Thermometer, digital	1	(1)
Wash bottle, wide mouth, 500 mL	4	(1)
Wash bottle, polyethylene, 4 oz	2	(1)
Dropper bottle, polystop, 30 mL	2	(2)
Desiccator, polypropylene	1	(1)
Clipboard with cover	2	(3,15)
Calculator, scientific	1	(3,15)
Marker, permanent, black	2	(3,15)
Pen set, slim pack, Koh-i-noor	1	(3,15)
Heavy paper tags with string	1000	(1,15)
Ice chest, insulated, 48 qt	2	(3,15)
Blue ice, soft pack	10	(3,15)
Plastic bags	100	(3,15)
Formalin, 10%	4 L	(2)
Ethyl alcohol	20 L	(2)
Trays, polypropylene, sorting	6	(5)

Note: Listed above are equipment and supplies needed for the collection and analysis of macroinvertebrate samples. The data quality objectives and sampling and analysis methods should determine the type of equipment and supplies needed. The source numbers refer to the companies that are listed at the end of the table. Mention of these sources or products does not constitute endorsement by the U.S. Environmental Protection Agency.

Table B.6 Benthic Macroinvertebrate Equipment and Supplies (continued)

[a] Sources of equipment and supplies:

1. Carolina Biological Supply Co.
   2700 York Road
   Burlington, NC 27215
2. Fisher Scientific
   50 Fadem Road
   Springfield, NJ 07081
3. Forestry Suppliers, Inc.
   205 West Rankin Street
   Jackson, MS 39284-8397
4. Industrial Rope Supply
   5250 River Road
   Cincinnati, OH 45233
5. Curtin Matheson Scientific, Inc.
   9999 Veterans Memorial Drive
   Houston, TX 77038-2499
6. Polyscience
   400 Valley Road
   Warrington, PA 18976
7. MonArk Boat Company
   Monticello, AK 71655
8. Wildlife Supply Company
   301 Case Street
   Saginaw, MI 48602
9. Tenaco
   2007 NE 27th Avenue
   Gainesville, FL 32609
10. Frigid Units, Inc.
    3214 Sylvania Avenue
    Toledo, OH 43613
11. W.C. Bradley Enterprises, Inc.
    P.O. Box 1240
    Columbus, GA 31993
12. Gallard-Schlesinger Chemical Mfg. Corp.
    584 Mineola Avenue
    Carle Place, NY 11514
13. Ellis-Rutter Associates
    P.O. Box 401
    Punta Gorda, FL 33950
14. Kahl Scientific Instrument Corp.
    P.O. Box 1166
    El Cajon, CA 92022-1166
15. Locally

From EPA. *Biological Criteria: Guide to Technical Literature*. U.S. Environmental Protection Agency, Washington, D.C. EPA-440-5-91-004. 1991.

Coefficient of Variation

In cases where replicate analyses are conducted (e.g., litigation purposes of NAWQMN stations), a coefficient of variation (CV or COV) between replicates is determined following the procedures outlined by Li (1964) using the formula:

$$CV = \frac{s}{x} \bullet 100\%$$

where s = the sample standard deviation
x = the sample mean.

A PARTIAL LISTING OF AGENCIES THAT HAVE DEVELOPED TOLERANCE CLASSIFICATIONS AND/OR BIOTIC INDICES

Florida Department of Environmental Regulation
Illinois EPA
New York Department of Environmental Conservation
North Carolina Department of Environmental Management
Ohio EPA
U.S. Department of Agriculture, Forest Service, Intermountain Region
U.S. EPA Region V
Vermont Department of Environmental Conservation

Table B.7. Phylogenetic Order for Macroinvertebrate Listing Including Level of Taxonomy Generally Used

Porifera:	Species	Plecoptera	
Coelenterata:	Genus	Pteronarcyidae:	Genus
Platyhelminthes:	Class	Peltoperfidae:	Genus
Nematomorpha:	Genus	Taeniopterygidae:	Genus
Bryozoa:	Species	Nemounidae:	Species
Entoprocta:	Species	Leuctridae:	Genus
Annelida		Capniidae:	Genus
Oligochaeta:	Class	Perfidae:	Species
Hirudinea:	Species	Perlodidae:	Species
Arthropoda		Chloroperfidae:	Genus
Crustacea		Hemiptera	
Isopoda:	Genus	Belostomatidae:	Genus
Amphipoda:	Genus/Species	Nepidae:	Genus
Decapoda:	Species	Pleidae:	Genus
Arachnoidea		Naucoridae:	Genus
Hydracarina:	Class	Corixidae:	Genus
Insecta		Notonectidae:	Genus
Ephemeroptera		Megaloptera	
Siphlonuridae:	Genus	Sialidae:	Genus
Baetidae:	Genus	Corydalidae:	Species
Oligoneuriidae:	Genus	Neuroptera:	Genus
Heptageniidae:	Genus/Species	Trichoptera	
Leptophlebiidae	Genus	Philopotamidae:	Genus/Species
Ephemerelidae:	Species	Psychomyiidae:	Species
Tricorythidae:	Genus	Polycentropodidae:	Genus
Caenidae:	Genus	Hydropsychidae:	Genus/Species
Baetiscidae:	Species	Rhyacophilidae:	Genus/Species
Potamanthidae:	Genus	Glossosomatidae:	Genus
Ephemeridae:	Genus	Hydroptidae:	Genus/Species
Polymitarchidae:	Species	Phryganeidae:	Genus
Odonata		Brachycentridae:	Genus
Zygoptera		Limnophilidae:	Genus
Calopterygidae:	Genus	Lepidostomatidae:	Genus
Lestidae:	Species	Beraeidae:	Genus
Coenagrionidae:	Family/Genus	Sericostomatidae:	Genus
Anisoptera		Odontocaridae:	Genus
Aeshnidae:	Species	Molannidae:	Genus
Gomphidae:	Species	Helicopsychidae:	Species
Cordulegastridae:	Species	Calamoceratidae:	Genus
Macromiidae:	Species	Leptocaridae:	Genus/Species
Corduliidae:	Species	Lepidoptera:	Genus
Libellulidae:	Species		

Table B.8. Level of Macroinvertebrate Taxonomy Attainable Using Keys

Coleoptera	
Gynnidae:	Genus
Haliplidae:	Genus
Dytiscidae:	Genus
Noteridae:	Genus
Hydrophilidae:	Genus
Hydraenidae:	Genus
Psepheriidae:	Species
Dryopidae:	Genus
Scirtidae:	Family
Elmidae:	Genus/Species
Limnichidae:	Genus
Heteroceridae:	Family
Ptilodactylidae:	Family
Chrysomelidae:	Family
Curculionidae:	Family
Lampyridae:	Family
Diptera	
Tipulidae:	Genus
Psychodidae:	Genus
Ptychopteridae:	Genus
Dixidae:	Genus
Chaoboridae:	Genus
Culicidae:	Genus
Thaumaleidae:	Genus
Simuliidae:	Genus
Certopogonidae:	Family/Genus/Species
Chironomidae	
Tanypodinae:	Genus/Species
Diamesinae:	Genus/Species
Prodiamesinae:	Genus/Species
Orthocladinae:	Genus/Species
Chironominae	
Chironomini:	Genus/Species
Pseudochironomini:	Genus/Species
Tanytarsini:	Genus/Species
Tabanidae:	Genus/Species
Athericidae:	Species
Stratiomyidae:	Genus
Empididae:	Family
Dolichopodidae:	Family
Syrphidae:	Family/Genus
Sciomyzidae:	Family/Genus
Ephydridae:	Family/Genus
Muscidae:	Species
Mollusca	
Gastropoda:	Family/Genus/Species
Pelecypoda:	Family/Genus/Species

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APPENDIX C

Fish Community Assessment

CONTENTS

RAPID BIOASSESSMENT PROTOCOL V — FISH (EPA 1989, 1999)

The following are excerpts from U.S. EPA (1989, 1999; www.epa.gov/owow/monitoring/rbp) guidance manuals. For more extensive information, the reader should refer directly to those manuals.

Rapid Bioassessment Protocol V (RBP V) is a rigorous approach similar to species-level identification with the macroinvertebrate RBP in accuracy and effort, but focuses on fish. Electrofishing, the most common technique used by agencies that monitor fish communities, and the most widely applicable approach for stream habitats, is the sampling technique recommended for use with RBP V.

The fish community biosurvey data are designed to be representative of the fish community at all station habitats, similar to the "representative qualitative sample" proposed by Hocutt (1981). The sampling station should be representative of the reach, incorporating at least one (preferably two) riffle(s), run(s), and pool(s) if these habitats are typical of the stream in question. Sampling of most species is most effective near shore and cover (macrophytes, boulders, snags, brush). Sampling procedures effective for large rivers are described in Gammon (1980), Hughes and Gammon (1987), and Ohio EPA (1987).

Typical sampling station lengths range from 100 to 200 m for small streams and 500 to 1000 m in rivers, but are best determined by pilot studies. The size of the reference station should be sufficient to produce 100 to 1000 individuals and 80% of the species expected from a 50% increase in sampling distance. Sample collection is usually done during the day, but night sampling can be more effective if the water is especially clear and there is little cover (Reynolds 1983). Use of block nets set (with as little wading as possible) at both ends of the reach increases sampling efficiency for large, mobile species sampled in small streams.

The RBP V fish community assessment requires that all fish species (not just gamefish) be collected. Small fish that require special gear for their effective collection may be excluded. Exclusion of young-of-the-year fish during collection has only a minor effect on IBI scores (Angermeier and Karr 1986), but lowers sampling costs and reduces the need for laboratory identification. Karr et al. (1986) recommended exclusion of fish less than 20 mm in length. However, this may prevent detection of species-specific effects, or early life stage effects from recent pollution incidents. This

recommendation should be considered on a regional basis and is also applicable to large fish requiring special gear for collection (e.g., sturgeon). The intent of the sample (as with the entire protocol) is to obtain a representative estimate of the species present, and their abundances, for a reasonable amount of effort. However, if threatened or endangered species are present, special attention should be given to documenting their presence and numbers.

Sampling effort among stations is standardized as much as possible. Regardless of the gear used, the collection method, site length (or area), and work hours expended must be comparable to allow comparison of fish community status among sites. Major habitat types (riffle, run, and pool) sampled at each site and the proportion of each habitat sampled should also be comparable. Generally 1 to 2 hours of actual sampling time are required, but this varies considerably with the gear used and the size and complexity of the site.

Atypical conditions, such as high flow, excessive turbidity or turbulence, heavy rain, drifting leaves, or other unusual conditions that affect sampling efficiency, are best avoided. Glare, a frequent problem, is reduced by wearing polarized glasses during sample collection.

Sample Processing

A field collection data sheet (Figure C.1) is completed for each sample. Sampling duration and area or distance sampled are recorded in order to determine level of effort. Species may be separated into adults and juveniles by size and coloration; then total numbers and weights and the incidence of external anomalies are recorded for each group. Reference specimens of each species from each site are preserved in 10% formaldehyde, the jar labeled, and the collection placed with the state ichthyological museum to confirm identifications and to constitute a biological record. This is especially important for uncommon species, for species requiring laboratory identification, and for documenting new distribution records. If retained in a live well, most fish can be identified, counted, and weighed in the field by trained personnel and returned to the stream alive. In warmwater sites, where handling mortality is highly probable, each fish is identified and counted, but for abundant species, subsampling (weigh, measure, observe for abnormalities, and return) may be considered. When subsampling is employed, the subsample is extrapolated to obtain a final value. Subsampling for weight is a simple, straightforward procedure, but failure to examine all fish to determine frequency of anomalies (which may occur in about 1% of all specimens) can bias results. The trade-off between handling mortality and data bias must be considered on a case-by-case basis. If a site is to be sampled repeatedly over several months (i.e., monitoring), the effect of sampling mortality might outweigh data bias. Holding fish in live-boxes in shaded, circulating water will substantially reduce handling mortality. More information on field methods is presented in Karr et al. (1986) and Ohio EPA (1987).

Data Analysis Techniques

Based on observations made in the assessment of habitat, water quality, physical characteristics, and the fish biosurvey, the investigator concludes whether impairment is detected. If impairment is detected, the probable cause and source are estimated and recorded on an Impairment Assessment Sheet (Figure C.2).

Data can be analyzed using the Index of Biotic Integrity (IBI) (or individual IBI metrics), the Index of Well Being (IWB) (Gammon 1980), or modified IWB (OEPA 1989; Gammon 1989), and multivariate statistical techniques to determine community similarities. Detrended correspondence analysis (DCA) is a useful multivariate analysis technique for revealing regional community patterns and patterns among multiple sites. It also demonstrates assemblages with compositions differing from others in the region or reach. See Gauch (1982) and Hill (1979) for descriptions of, and software for, DCA. Data analyses and reporting, including parts of the IBI, can be computer generated. Computerization reduces the time needed to produce a report and increases staff capability to examine

Page ____ of _____

FISH FIELD COLLECTION DATA SHEET

Drainage _____ Date_____
Sampling Duration (min)_____

Sampling Distance (m)_____ Sampling Area (m2)_____ Crew_____

Habitat Complexity/Quality (excellent good fair poor very poor)

Weather_____ Flow (flood bankfull moderate low)

Gear Used_____ Gear/Crew Performance_____

Comments_____

Fish (preserved) Number of Individuals_____ Number of Anomalies_____

Genus/Species	Adults		Juveniles		Anomalies(*)	
	No.	Wt.	No.	Wt.	No.	Wt.

(*) Discoloration, deformities, eroded fins, excessive mucus, excessive external parasites, fungus, poor condition, reddening, tumors, and ulcers

Figure C.1 Fish field collection data sheet for use with Rapid Bioassessment Protocol V. (From EPA. *Rapid Bioassessment Protocols for Use in Streams and Rivers: Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA 444/4-89/001. 1989.)

data patterns and implications. Illinois EPA has developed software to assist professional aquatic biologists in calculating IBI values in Illinois streams (Bickers et al. 1988). (Use of this software outside Illinois without modification is not recommended.) However, hand calculation in the initial use of the IBI promotes understanding of the approach and provides insight into local inconsistencies. Metrics should be optimized for specific ecoregions. See EPA (1999) for a range of alternative IBI metrics.

Each metric is scored against criteria based on expectations developed from appropriate regional reference sites. Metric values approximating, deviating slightly from, or deviating greatly from values occurring at the reference sites are scored as 5, 3, or 1, respectively. The scores of the 12 metrics are added for each station to give an IBI of 60 (excellent) to 12 (very poor). Trophic and tolerance classifications of many species are listed below. Additional classifications can be derived from information in state and regional fish texts or by objectively assessing a large statewide database. Use of the IBI in the southeastern and southwestern United States and its widespread use by water resource agencies may result in further modifications. Past modifications have occurred (Miller et al. 1988) without changing the IBI's basic theoretical foundations.

IMPAIRMENT ASSESSMENT SHEET

1. Detection of impairment: Impairment detected (Complete Item 2-6) / No impairment detected (Stop here)

2. Biological impairment indicator:

Fish

_____ sensitive species reduced/absent
_____ dominance of tolerant species
_____ skewed trophic structure
_____ abundance reduced/unusually high
_____ biomass reduced/unusually high
_____ hybrid or exotic abundance unusually high
_____ poor size class representation
_____ high incidence of anomalies

Other aquatic communities

_____ Macroinvertebrates
_____ Periphyton
_____ Macrophytes

3. Brief description of problem: _____
Year and date of previous surveys: _
Survey data available in: _____

4. Cause (indicate potential cause): organic enrichment toxicants flow sediment temperature poor habitat
other _____

5. Estimated areal extent of problem (m2) and length of stream reach affected (m) where applicable: _____

6. Suspected source(s) of problem

_____ point source
_____ urban runoff
_____ agricultural runoff
_____ silvicultural runoff
_____ livestock
_____ landfill
_____ mine
_____ dam or diversion
_____ channelization or snagging
_____ natural
_____ other
_____ unknown

Comments: _____

Figure C.2 Impairment assessment sheet for use with Fish Rapid Bioassessment Protocol V. (From EPA. *Rapid Bioassessment Protocols for Use in Streams and Rivers: Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency. Washington, D.C. EPA 444/4-89/001. 1989.)

The steps in calculating the IBI are explained below:

1. Assign species to trophic guilds; identify and assign species tolerances. Where published data are lacking, assignments are made based on knowledge of closely related species and morphology.
2. Develop scoring criteria for each IBI metric. Maximum species richness (or density) lines are developed from a reference database.
3. Conduct field study and identify fish; note anomalies, eroded fins, poor condition, excessive mucus, fungus, external parasites, reddening, lesions, and tumors. Complete field data sheets.
4. Enumerate and tabulate number of fish species and relative abundances.
5. Summarize site information for each IBI metric.

6. Rate each IBI score to one of the five integrity classes.
7. Translate total IBI score to one of the five integrity classes.
8. Interpret data in the context of the habitat assessment. Individual metric analysis may be necessary to ascertain specific trends.

Species Richness and Diversity

These metrics assess the species richness component of diversity and the health of the major taxonomic groups and habitat guilds of fishes. Two of the metrics assess community composition in terms of tolerant or intolerant species. Scoring for the first five of these metrics and their substitutes requires development of species–water body size relationships for different zoogeographic regions. Development of this relationship requires data sufficient to plot the number of species collected from regional reference sites of various stream sizes against a measure of stream size (watershed area, stream order) of those sites. A line is then drawn with slope fit by eye to include 95% of the points. Finally the area under the line is trisected into areas that are scored as 5, 3, or 1. A detailed description of these methods can be found in Fausch et al. (1984), Ohio EPA (1987), and Karr et al. (1986).

Metric 1. Total number of fish species — Substitute metrics: total number of native fish species, and salmonid age classes.

This number decreases with increased degradation; hybrids and introduced species are not included. In cold-water streams supporting few fish species, the age classes of the species found represent the suitability of the system for spawning and rearing. The number of species is strongly affected by stream size at small stream sites, but not at large river sites (Karr et al. 1986; Ohio EPA 1987). Thus, scoring depends on developing species–waterbody size relationships.

Metric 2. Number and identity of darter species — Substitute metrics: number and identity of sculpin species, benthic insectivore species, salmonid yearlings (individuals); number of sculpins (individuals); percent round-bodied suckers, sculpin and darter species.

These species are sensitive to degradation resulting from siltation and benthic oxygen depletion because they feed or reproduce in benthic habitats (Kuehne and Barbour 1983; Ohio EPA 1987). Many smaller species live within the rubble interstices, are weak swimmers, and spend their entire lives in an area of 100 to 400 m^2 (Hill and Grossman 1987; Matthews 1986). Darters are appropriate in most Mississippi basin streams; sculpins and yearling trout occupy the same niche in western streams. Benthic insectivores and sculpins or darters are used in small Atlantic slope streams that have few sculpins or darters, and round-bodied suckers are suitable in large midwestern rivers. Scoring requires development or species–water body size relationships.

Metric 3. Number and identity of sunfish species — Substitutes: number and identity of cyprinid species, water column species, salmonid species, headwater species, and sunfish and trout species.

These pool species decrease with increased degradation of pools and in-stream cover (Gammon et al. 1981; Angermeier 1983; Platts et al. 1983). Most of these fishes feed on drifting and surface invertebrates and are active swimmers. The sunfishes and salmonids are important sport species. The sunfish metric works for most Mississippi basin streams, but where sunfish are absent or rare, other groups are used. Cyprinid species are used in cool-water western streams; water column species occupy the same niche in northeastern streams; salmonids are suitable in cold-water streams; headwater species serve for midwestern headwater streams; and trout and sunfish species are used in southern Ontario streams. Karr et al. (1986) and Ohio EPA (1987) found the number of sunfish species to be dependent on stream size in small streams, but Ohio EPA (1987) found no relationship between stream size and sunfish species in medium to large streams, nor between stream size and headwater species in small streams. Scoring of this metric requires development of species–water body size relationships.

Metric 4. Number and identity of sucker species — Substitutes: number of adult trout species, number of minnow species, and number of suckers and catfish.

These species are sensitive to physical and chemical habitat degradation and commonly comprise most of the fish biomass in streams. All but the minnows are long-lived species and provide a multiyear integration of physicochemical conditions. Suckers are common in medium and large streams; minnows dominate small streams in the Mississippi basin; and trout occupy the same niche in cold-water streams. The richness of these species is a function of stream size in small and medium-sized streams but not in large rivers. Scoring of this metric requires development of species–water body size relationships.

Metric 5. Number and identity of intolerant species — Substitutes: number and identity of sensitive species (5), amphibian species, and presence of brook trout.

This metric distinguishes high- and moderate-quality sites using species that are intolerant of various chemical and physical perturbations. Intolerant species are typically the first to disappear following a disturbance. Species classified as intolerant or sensitive should only represent the 5 to 10% most susceptible species; otherwise this becomes a less discriminating metric. Candidate species are determined by examining regional ichthyological books for species that were once widespread but have become restricted to only the highest quality streams. Ohio EPA (1987) uses number of sensitive species (which includes highly intolerant and moderately intolerant species) for headwater sites because highly intolerant species are generally not expected in such habitats. Moyle (1976) suggested using amphibians in northern California streams because of their sensitivity to silvicultural impacts. This also may be a promising metric in Appalachian streams which may naturally support few fish species. Steedman (1988) found that the presence of brook trout had the greatest correlation with IBI score in Ontario streams. The number of sensitive and intolerant species increases with stream size in small and medium-sized streams but is unaffected by size of large rivers. Scoring this metric requires development of species–water body size relationships.

Metric 6. Proportion of individuals as green sunfish — Substitutes: proportion of individuals as common carp, white sucker, tolerant species, creek chub, and dace.

This metric is the reverse of Metric 5. It distinguishes low- from moderate-quality waters. These species show increased distribution or abundance despite the historical degradation of surface waters, and they shift from incidental to dominant in disturbed sites. Green sunfish are appropriate in small midwestern streams; creek chubs were suggested for central Appalachian streams; common carp were suitable for a cool-water Oregon river; white suckers were selected in the Northeast and Colorado where green sunfish are rare to absent; and dace (*Rhinichthys* species) were used in southern Ontario. To avoid weighting the metric on a single species, Karr et al. (1986) and Ohio EPA (1987) suggest using a small number of highly tolerant species. Scoring of this metric may require development of expectations based on water body size.

Trophic Composition Metrics

These three metrics assess the quality of the energy base and trophic dynamics of the community. Traditional process studies, such as community production and respiration, are time-consuming, and the results are equivocal; distinctly different situations can yield similar results. The trophic composition metrics offer a means to evaluate the shift toward more generalized foraging that typically occurs with increased degradation of the physicochemical habitat.

Metric 7. Proportion of individuals as omnivores — Substitutes: proportion of individuals as yearlings.

The percent of omnivores in the community increases as the physical and chemical habitat deteriorates. Omnivores are defined as species that consistently feed on substantial proportions of

plant and animal material. Ohio EPA (1987) excludes sensitive filter-feeding species such as paddlefish and lamprey ammocoetes and opportunistic feeders like channel catfish. Where omnivorous species are nonexistent, such as in trout streams, the proportion of the community composed of yearlings, which initially feed omnivorously, may be substituted.

Metric 8. Proportion of individuals as insectivorous cyprinids — Substitutes: proportion of individuals as insectivores, specialized insectivores, and insectivorous species; and number of juvenile trout.

Insectivores or invertivores are the dominant trophic guild of most North American surface waters. As the invertebrate food source decreases in abundance and diversity due to physicochemical habitat deterioration, there is a shift from insectivorous to omnivorous fish species. Generalized insectivores and opportunistic species, such as blacknose dace and creek chub, were excluded from this metric by the Ohio EPA (1987). This metric evaluates the midrange of biotic integrity.

Metric 9. Proportion of individuals as top carnivores — Substitutes: proportion of individuals as catchable salmonids, catchable wild trout, and pioneering species.

The top carnivore metric discriminates between systems with high and moderate integrity. Top carnivores are species that, as adults, feed predominantly on fish, other vertebrates, or crayfish. Occasional piscivores, such as creek chub and channel catfish, are not included. In trout streams, where true piscivores are uncommon, the percent of large salmonids is substituted for percent piscivores. These species often represent popular sport fish such as bass, pike, walleye, and trout. Pioneering species are used by Ohio EPA (1987) in headwater streams typically lacking piscivores.

Fish Abundance and Condition Metrics

The last three metrics (plus the final optional matrix) indirectly evaluate population recruitment mortality, condition, and abundance. Typically, these parameters vary continuously and are time-consuming to estimate accurately. Instead of such direct estimates, the final results of the population parameters are evaluated. Indirect estimation is less variable and much more rapidly determined.

Metric 10. Number of individuals in sample — Substitutes: density of individuals.

This metric evaluates population abundance and varies with region and stream size for small streams. It is expressed as catch per unit effort, either by area, distance, or time sampled. Generally sites with lower integrity support fewer individuals, but in some nutrient-poor regions, enrichment increases the number of individuals. Steedman (1988) addressed this situation by scoring catch per minute of sampling greater than 25 as a three, and less than 4 as a one. Unusually low numbers generally indicate toxicity, making this metric most useful at the low end of the biological integrity scale. Hughes and Gammon (1987) suggest that in larger streams, where sizes of fish may vary in orders of magnitude, total fish biomass may be an appropriate substitute or additional metric.

Metric 11. Proportion of individuals as hybrids — Substitutes: proportion of individuals as introduced species, simple lithophils, and number of simple lithophilic species.

This metric is an estimate of reproductive isolation or the suitability of the habitat for reproduction. Generally, as environmental degradation increases, the percent of hybrids and introduced species also increases, but the proportion of simple lithophils decreases. However, minnow hybrids are found in some high-quality streams; hybrids are often absent from highly impacted sites; and hybridization is rare and difficult for many to detect. Thus, Ohio EPA (1987) substitutes simple lithophils for hybrids. Simple lithophils spawn where their eggs can develop in the interstices of sand, gravel, and cobble substrates without parental care. Hughes and Gammon (1987) and Miller et al. (1988) proposed using percent introduced individuals. This metric is a direct measure of the loss of species segregation between midwestern and western fishes that existed before the introduction of midwestern species to western rivers.

Metric 12. Proportion of individuals with disease, tumors, fin damage, and skeletal anomalies — this metric depicts the health and condition of individual fish. These conditions occur infrequently or are absent from minimally impacted reference sites but occur frequently below major pollutant sources. They are excellent measures of the subacute effects of chemical pollution and the aesthetic value of game and nongame fish.

Metric 13. Total fish biomass (optional) — Hughes and Gammon (1987) suggest that in larger areas, where sizes of fish may vary in orders of magnitude, this additional metric may be appropriate.

Because the IBI is an adaptable index, the choice of metrics and scoring criteria is best developed on a regional basis through use of available publications (Karr et al. 1986; Ohio EPA 1987; Miller et al. 1988). Several steps are common to all regions. The fish species must be listed and assigned to trophic and tolerance guilds. Scoring criteria are developed through use of high-quality historical data and data from minimally impacted regional reference sites. This has been done for much of the country, but continued refinements are expected as more fish community ecology data become available. Once scoring criteria have been established, a fish sample is evaluated by listing the species and their abundances, calculating values for each metric, and comparing these values with the scoring criteria. Individual metric scores are added to calculate the total IBI score (Figure C.3).

Station No. _____

Site _____

Metrics[a]	Scoring Criteria[b] 5 %	3 %	1 %	Metric Value	Metric Source
1. Number of Native Fish Species	>67	33-67	<33		
2. Number of Darter or Benthic Species	>67	33-67	<33		
3. Number of Sunfish or Pool Species	>67	33-67	<33		
4. Number of Sucker or Long-Lived Species	>67	33-67	<33		
5. Number of Intolerant Species	>67	33-67	<33		
6. % Green Sunfish or Tolerant Individuals	<10	10-25	>25		
7. % Omnivores	<20	20-45	>45		
8. % Insectivores or Invertivores	>45	20-45	<20		
9. % Top Carnivores	>5	1 - 5	<33		
10. Total Number of Individuals	>67	33-67	<33		
11. % Hybrids or Exotics	0	0 - 1	>1		
12. % Anomalies	<1	1 - 5	>5		

Scorer _____ IBI Score _____

Comments: _____

[a] Karr's original metrics or commonly used substitutes. See text for other possibilities.

[b] Karr's original scoring criteria or commonly used substitutes. These may require refinement in other ecoregions.

Figure C.3 Data summary sheet for Rapid Bioassessment Protocol V. (From EPA. *Rapid Bioassessment Protocols for Use in Streams and Rivers: Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency. Washington, D.C. EPA 444/4-89/001. 1989.)

Hughes and Gammon (1987) and Miller et al. (1988) suggest that scores lying at the extremes of scoring criteria can be modified by a plus or minus; a combination of three pluses or three minuses results in a two-point increase or decrease in IBI. Ohio EPA (1987) scores proportional metrics as 1 when the number of species and individuals in samples are fewer than 6 and 75, respectively, when their expectations are of higher numbers.

Table C.1 Tolerance, Trophic Guilds, and Origins of Selected Fish Species

	Trophic Level	Tolerance	Origin
Willamette Species			
Salmonidae			
Chinook salmon	piscivore	intolerant	native
Cutthroat trout	insectivore	intolerant	native
Mountain whitefish	insectivore	intolerant	native
Rainbow trout	insectivore	intolerant	native
Cyprinidae			
Chiselmouth	herbivore	intermediate	native
Common carp	omnivore	tolerant	exotic
Goldfish	omnivore	tolerant	exotic
Leopard dace	insectivore	intermediate	native
Longnose dace	insectivore	intermediate	native
Northern squawfish	piscivore	tolerant	native
Peamouth	insectivore	intermediate	native
Redside shiner	insectivore	intermediate	native
Speckled dace	insectivore	intermediate	native
Catostomidae			
Largescale sucker	omnivore	tolerant	native
Mountain sucker	herbivore	intermediate	native
Ictaluridae			
Brown bullhead	insectivore	tolerant	exotic
Yellow bullhead	insectivore	tolerant	exotic
Percopsidae			
Sand roller	insectivore	intermediate	native
Gasterosteidae			
Threespine stickleback	insectivore	intermediate	native
Centrarchidae			
Bluegill	insectivore	tolerant	exotic
Largemouth bass	piscivore	tolerant	exotic
Smallmouth bass	piscivore	intermediate	exotic
White crappie	insectivore	tolerant	exotic
Percidae			
Yellow perch	insectivore	intermediate	exotic
Cottidae			
Paiute sculpin	insectivore	intolerant	native
Prickly sculpin	insectivore	intermediate	native
Reticulate sculpin	insectivore	tolerant	native
Torrent sculpin	insectivore	intolerant	native
Midwest Species			
Petromyzontidae			
Silver lamprey	piscivore	intermediate	native
Northern brook lamprey	filterer	intolerant	native
Mountain brook lamprey	filterer	intolerant	native
Ohio lamprey	piscivore	intolerant	native
Least brook lamprey	filterer	intermediate	native
Sea lamprey	piscivore	intermediate	exotic
Polyodontidae			
Paddlefish	filterer	intolerant	native

Table C.1 Tolerance, Trophic Guilds, and Origins of Selected Fish Species (continued)

	Trophic Level	Tolerance	Origin
Acipenseridae			
Lake sturgeon	invertivore	intermediate	native
Shovelnose sturgeon	insectivore	intermediate	native
Lepisosteidae			
Alligator gar	piscivore	intermediate	native
Shortnose gar	piscivore	intermediate	native
Spotted gar	piscivore	intermediate	native
Longnose gar	piscivore	intermediate	native
Amiidae			
Bowfin	piscivore	intermediate	native
Hiodontidae			
Goldeye	insectivore	intolerant	native
Mooneye	insectivore	intolerant	native
Clupeidae			
Skipjack herring	piscivore	intermediate	native
Alewife	invertivore	intermediate	exotic
Gizzard shad	omnivore	intermediate	native
Threadfin shad	omnivore	intermediate	native
Salmonidae			
Brown trout	insectivore	intermediate	exotic
Rainbow trout	insectivore	intermediate	exotic
Brook trout	insectivore	intermediate	native
Lake trout	piscivore	intermediate	native
Coho salmon	piscivore	intermediate	exotic
Chinook salmon	piscivore	intermediate	exotic
Lake herring	piscivore	intermediate	native
Lake whitefish	piscivore	intermediate	native
Osmeridae			
Rainbow smelt	invertivore	intermediate	exotic
Umbridae			
Central mudminnow	insectivore	tolerant	native
Esocidae			
Grass pickerel	piscivore	intermediate	native
Chain pickerel	piscivore	intermediate	native
Northern pike	piscivore	intermediate	native
Muskellunge	piscivore	intermediate	native
Cyprinidae			
Common carp	omnivore	tolerant	exotic
Goldfish	omnivore	tolerant	exotic
Golden shiner	omnivore	tolerant	native
Horneyhead chub	insectivore	intolerant	native
River chub	insectivore	intolerant	native
Silver chub	insectivore	intermediate	native
Bigeye chub	insectivore	intolerant	native
Streamline chub	insectivore	intolerant	native
Gravel chub	insectivore	intermediate	native
Speckled chub	insectivore	intolerant	native
Blacknose dace	generalist	tolerant	native
Longnose dace	insectivore	intolerant	native
Creek chub	generalist	tolerant	native
Tonguetied minnow	insectivore	intolerant	native
Suckermouth minnow	insectivore	intermediate	native
Southern redbelly dace	herbivore	intermediate	native
Redside dace	insectivore	intolerant	native
Pugnose minnow	insectivore	intolerant	native
Emerald shiner	insectivore	intermediate	native
Silver shiner	insectivore	intolerant	native

Table C.1 Tolerance, Trophic Guilds, and Origins of Selected Fish Species (continued)

	Trophic Level	Tolerance	Origin
Cyprinidae			
Rosyface shiner	insectivore	intolerant	native
Redfin shiner	insectivore	intermediate	native
Rosefin shiner	insectivore	intermediate	native
Striped shiner	insectivore	intermediate	native
Common shiner	insectivore	intermediate	native
River shiner	insectivore	intermediate	native
Spottail shiner	insectivore	intermediate	native
Blackchin shiner	insectivore	intolerant	native
Bigeye shiner	insectivore	intolerant	native
Steelcolor shiner	insectivore	intermediate	native
Spotfin shiner	insectivore	intermediate	native
Bigmouth shiner	insectivore	intermediate	native
Sand shiner	insectivore	intermediate	native
Mimic shiner	insectivore	intolerant	native
Ghost shiner	insectivore	intermediate	native
Blacknose shiner	insectivore	intolerant	native
Pugnose shiner	insectivore	intolerant	native
Silverjaw minnow	insectivore	intermediate	native
Mississippi silvery minnow	herbivore	intermediate	native
Bullhead minnow	omnivore	intermediate	native
Bluntnose minnow	omnivore	tolerant	native
Fathead minnow	omnivore	tolerant	native
Central stoneroller	herbivore	intermediate	native
Popeye shiner	insectivore	intolerant	native
Grass carp	herbivore	intermediate	exotic
Red shiner	omnivore	intermediate	native
Brassy minnow	omnivore	intermediate	native
Central silvery minnow	herbivore	intolerant	native
Catostomidae			
Blue sucker	insectivore	intolerant	native
Bigmouth buffalo	insectivore	intermediate	native
Black buffalo	insectivore	intermediate	native
Smallmouth buffalo	insectivore	intermediate	native
Quillback	omnivore	intermediate	native
River carpsucker	omnivore	intermediate	native
Highfin carpsucker	omnivore	intermediate	native
Silver redhorse	insectivore	intermediate	native
Black redhorse	insectivore	intolerant	native
Golden redhorse	insectivore	intermediate	native
Shorthead redhorse	insectivore	intermediate	native
Greater redhorse	insectivore	intolerant	native
River redhorse	insectivore	intolerant	native
Harelip sucker	invertivore	intolerant	native
Northern hog sucker	insectivore	intolerant	native
White sucker	omnivore	tolerant	native
Longnose sucker	insectivore	intermediate	native
Spotted sucker	insectivore	intermediate	native
Lake chubsucker	insectivore	intermediate	native
Creek chubsucker	insectivore	intermediate	native
Ictaluridae			
Blue catfish	piscivore	intermediate	native
Channel catfish	generalist	intermediate	native
White catfish	insectivore	intermediate	native
Yellow bullhead	insectivore	intolerant	native
Brown bullhead	insectivore	intolerant	native
Black bullhead	insectivore	tolerant	native

Table C.1 Tolerance, Trophic Guilds, and Origins of Selected Fish Species (continued)

	Trophic Level	Tolerance	Origin
Ictaluridae			
Flathead catfish	piscivore	intermediate	native
Stonecat	insectivore	intolerant	native
Mountain madtom	insectivore	intolerant	native
Slender madtom	insectivore	intolerant	native
Freckled madtom	insectivore	intermediate	native
Northern madtom	insectivore	intolerant	native
Scioto madtom	insectivore	intolerant	native
Brindled madtom	insectivore	intolerant	native
Tadpole madtom	insectivore	intermediate	native
Anguillidae			
American eel	piscivore	intolerant	native
Cyprinodontidae			
Western banded killifish	insectivore	intolerant	native
Eastern banded killifish	insectivore	tolerant	native
Blackstripe topminnow	insectivore	intermediate	native
Poeciliidae			
Mosquitofish	insectivore	intermediate	exotic
Gadidae			
Burbot	piscivore	intermediate	native
Percopsidae			
Trout-perch	insectivore	intermediate	native
Aphredoderidae			
Pirate perch	insectivore	intermediate	native
Atherinidae			
Brook silverside	insectivore	intermediate	native
Percichthyidae			
White bass	insectivore	intermediate	native
Striped bass	insectivore	intermediate	exotic
White perch	insectivore	intermediate	exotic
Yellow bass	insectivore	intermediate	native
Centrarchidae			
White crappie	invertivore	intermediate	native
Black crappie	invertivore	intermediate	native
Rock bass	piscivore	intermediate	native
Smallmouth bass	piscivore	intermediate	native
Spotted bass	piscivore	intermediate	native
Largemouth bass	piscivore	intermediate	native
Warmouth	invertivore	intermediate	native
Green sunfish	invertivore	tolerant	native
Bluegill	insectivore	intermediate	native
Orangespotted sunfish	insectivore	intermediate	native
Longear sunfish	insectivore	intolerant	native
Redear sunfish	insectivore	intermediate	native
Pumpkinseed	insectivore	intermediate	native
Percidae			
Sauger	piscivore	intermediate	native
Walleye	piscivore	intermediate	native
Yellow perch	piscivore	intermediate	native
Dusky darter	insectivore	intermediate	native
Blackside darter	insectivore	intermediate	native
Longhead darter	insectivore	intolerant	native
Slenderhead darter	insectivore	intolerant	native
River darter	insectivore	intermediate	native
Channel darter	insectivore	intolerant	native
Gilt darter	insectivore	intolerant	native
Logperch	insectivore	intermediate	native

Table C.1 Tolerance, Trophic Guilds, and Origins of Selected Fish Species (continued)

	Trophic Level	Tolerance	Origin
Percidae			
Crystal darter	insectivore	intolerant	native
Eastern sand darter	insectivore	intolerant	native
Western sand darter	insectivore	intolerant	native
Johnny darter	insectivore	intermediate	native
Greenside darter	insectivore	intermediate	native
Banded darter	insectivore	intolerant	native
Variegate darter	insectivore	intolerant	native
Spotted darter	insectivore	intolerant	native
Bluebreast darter	insectivore	intolerant	native
Tippecanoe darter	insectivore	intolerant	native
Iowa darter	insectivore	intermediate	native
Rainbow darter	insectivore	intermediate	native
Orangethroat darter	insectivore	intermediate	native
Fantail darter	insectivore	intermediate	native
Least darter	insectivore	intermediate	native
Slough darter	insectivore	intermediate	native
Sciaenidae			
Freshwater drum	invertivore	intermediate	native
Cottidae			
Spoonhead sculpin	insectivore	intermediate	native
Mottled sculpin	insectivore	intermediate	native
Slimy sculpin	insectivore	intermediate	native
Deepwater sculpin	insectivore	intermediate	native
Gasterosteidae			
Brook stickleback	insectivore	intermediate	native

From EPA. *Rapid Bioassessment Protocols for Use in Streams and Rivers: Benthic Macroinvertebrates and Fish*. Office of Water, U.S. Environmental Protection Agency. Washington, D.C. EPA 444/4-89/001. 1989.

Table C.2 National List of Intolerant Fish Species[a]

Common Name	Latin Name
Cisco	*Coregonus artedii .*
Arctic cisco	*Coregonus autumnalis .*
Lake whitefish	*Coregonus clupeaformis .*
Bloater	*Coregonus hoyi .*
Kiyi	*Coregonus kiyi .*
Bering cisco	*Coregonus laurettae .*
Broad whitefish	*Coregonus nasus .*
Humpback whitefish	*Coregonus pidschian .*
Hortnose cisco	*Coregonus reighardi .*
Least cisco	*Coregonus sardinella .*
Shortjaw cisco	*Coregonus zenithicus .*
Pink salmon	*Oncorhynchus gorbuscha .*
Chum salmon	*Oncorhynchus keta .*
Coho salmon	*Oncorhynchus kisutch .*
Sockeye salmon	*Oncorhynchus nerka .*
Chinook salmon	*Oncorhynchus tshawytscha .*
Pygmy whitefish	*Prosopium coulteri .*
Round whitefish	*Prosopium cylindraceum .*
Mountain whitefish	*Prosopium williamsoni .*
Golden trout	*Salmo aguabonita .*
Arizona trout	*Salmo apache .*
Cutthroat trout	*Salmo clarki .*

Table C.2 National List of Intolerant Fish Species[a] (continued)

Common Name	Latin Name
Rainbow trout	*Salmo gairdneri/O. mykiss .*
Atlantic salmon	*Salmo salar .*
Brown trout	*Salmo trutta .*
Arctic char	*Salvelinus alpinus .*
Bull trout	*Salvelinus confluentus .*
Brook trout	*Salvelinus fontinalis .*
Dolly varden	*Salvelinus malma .*
Lake trout	*Salvelinus namaycush .*
Inconnu	*Stenodus leucichthys .*
Arctic grayling	*Thymallus arcticus .*
Largescale stoneroller	*Campostoma oligolepis .*
Redside dace	*Clinostomus elongatus .*
Cutlips minnow	*Exoglossum maxillingua .*
Bigeye chub	*Hybobsis amblops .*
River chub	*Nocomis micropogon .*
Pallid shiner	*Notropis amnis .*
Pugnose shiner	*Notropis anogenus .*
Rosefin shiner	*Notropis ardens .*
Bigeye shiner	*Notropis boops .*
Pugnose minnow	*Notropis emiliae .*
Whitetail shiner	*Notropis galacturus .*
Blackchin shiner	*Notropis heterodon .*
Blacknose shiner	*Notropis heterloepis .*
Spottail shiner	*Notropis hudsonius .*
Sailfin shiner	*Notropis hypselopterus .*
Tennessee shiner	*Notropis leuciodus .*
Yellowfin shiner	*Notropis lutipinnis .*
Ozark minnow	*Notropis nubilus .*
Ozark shiner	*Notropis ozarcanus .*
Silver shiner	*Notropis photogenis .*
Duskystripe shiner	*Notropis pilsbryi .*
Rosyface shiner	*Notropis rubellus .*
Safron shiner	*Notropis rubricroceus .*
Flagfin shiner	*Notropis signipinnis .*
Telescope shiner	*Notropis telescopus .*
Topeka shiner	*Notropis topeka .*
Mimic shiner	*Notropis volucellus .*
Steelcolor shiner	*Notropis whipplei .*
Coosa shiner	*Notropis zaenocephalus .*
Bleeding shiner	*Notropis zonatus .*
Bandfin shiner	*Notropis zonistius .*
Blackside dace	*Phoxinus cumberlandensis .*
Northern redbelly dace	*Phoxinus eos .*
Southern redbelly dace	*Phoxinus erythrogaster .*
Blacknose dace	*Rhinichthys atratulus .*
Pearl dace	*Semotilus margarita .*
Alabama hog sucker	*Hypentelium etowanum .*
Northern hog sucker	*Hypentelium nigricans .*
Roanoke hog sucker	*Hypentelium roanokense .*
Spotted sucker	*Minytrema melanops .*
Silver redhorse	*Moxostoma anisurum .*
River redhorse	*Moxostoma carinatum .*
Black jumprock	*Moxostoma cervinum .*
Gray redhorse	*Moxostoma congestum .*
Black redhorse	*Moxostoma duquesnei .*
Rustyside sucker	*Moxostoma hamiltoni .*
Greater jumprock	*Moxostoma lachneri .*
Blacktail redhorse	*Moxostoma poecilurum .*

Table C.2 National List of Intolerant Fish Species[a] (continued)

Common Name	Latin Name
Torrent sucker	*Moxostoma rhothoecum* .
Striped jumprock	*Moxostoma rupiscartes* .
Greater redhorse	*Moxostoma valenciennesi* .
Ozark madtom	*Noturus albater* .
Elegant madtom	*Noturus elegans* .
Mountain madtom	*Noturus eleutherus* .
Slender madtom	*Noturus exilis* .
Stonecat	*Noturus flavus* .
Black madtom	*Noturus funebris* .
Least madtom	*Noturus hildebrandi* .
Margined madtom	*Noturus insignis* .
Speckled madtom	*Noturus leptacanthus* .
Brindled madtom	*Noturus miurus* .
Frecklebelly madtom	*Noturus minitus* .
Brown madtom	*Noturus phaeus* .
Roanoke bass	*Ambloplites cavifrons* .
Ozark rockbass	*Ambloplites constellatus* .
Rock bass	*Ambloplites rupestris* .
Longear sunfish	*Lepomis megalotis* .
Darters[a]	*Ammocrypta* sp.
Darters[a]	*Etheostoma* sp.
Darters[a]	*Percina* sp.
Sculpins[a]	*Cottus* sp.
O'opu alamoo (goby)	*Lentipes concolor* .
O'opu nopili (goby)	*Sicydium stimpsoni* .
O'opu nakea (goby)	*Awaous stamineus* .
Johnny darter	*Etheostoma nigrum* .
Bluntnose darter	*E. chlorosomum* .
Slough darter	*E. gracile* .
Cypress darter	*E. proeliare* .
Orangethroat darter	*E. spectabile* .
Swamp darter	*E. fusiforme* .
River darter	*Percina shumardi* .

[a] Reader note that there are inconsistencies between some tolerance rankings with Table C.1 (UEPA 1989).
[b] The United States has 150 species of darters and sculpins, the great majority of which are intolerant species. Possible exceptions include:

From EPA. *Methods for Chemical Analysis of Water and Wastes*. EPA-600/4-79-020, U.S. Environmental Protection Agency, Cincinnati, Ohio. 1983b.

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APPENDIX D

Toxicity and Bioaccumulation Testing

CONTENTS

GENERAL TOXICITY TESTING METHODS

There are a large number of toxicity and bioaccumulation test methods that can be used in laboratory or field (*in situ*) settings. The strengths and weaknesses of the two settings were discussed in Chapter 6. The toxicity test methods most commonly used in North America are those required by the EPA and state environmental protection agencies, such as *Pimephales promelas* and *Ceriodaphnia dubia* for wastewater effluent testing. While these tests have been used successfully to evaluate stormwaters, there are also other options that may be acceptable to the regulatory authorities, since they have been found useful in the scientific peer-reviewed literature. In addition, there are many standardized test methods approved by Environment Canada (Table D.1) and ASTM (Table D.2) that are often quite similar to U.S. EPA procedures. Only a few examples are listed below to help familiarize the user with the procedures and associated quality assurance issues. The project manager should verify that the appropriate test methods are being used to meet any regulatory requirements. These tests should only be conducted by laboratories with documented experience in aquatic toxicology. Given the potential for sampling and method-related artifacts (Chapters 5 and 6), it is important that the project manager ensure that proper study design, sample collection, and testing protocols are adhered to. The categories of assessment tools that are useful in receiving water assessments are shown in Table D.3. The methods recommended for screening are listed on Tables D.4 through D.14.

METHODS FOR CONDUCTING LONG-TERM SEDIMENT TOXICITY TESTS WITH *HYALELLA AZTECA*

The EPA recently finalized methods for long-term chronic toxicity testing of sediments (EPA 2000). These methods have not been widely used but have been found to be more sensitive to sediment contaminants than the 10-day assays. In addition, they were found to have acceptable levels of variability based on interlaboratory variance studies. Since these assays require 42 days and longer to run, they are somewhat costly to perform. Conditions for evaluating sublethal endpoints in a sediment toxicity test with *H. azteca* are summarized in Table D.15. A general activity schedule is outlined in Table D.16.

The 42-day sediment toxicity test with *H. azteca* is conducted at 23°C with a 16L:8D photoperiod at an illuminance of about 500 to 1000 lux. Test chambers are 300-mL high-form lipless beakers containing 100 mL of sediment and 175 mL of overlying water. Amphipods in each test chamber are fed 1.0 mL of YCT daily. Each test chamber receives two volume additions/day of overlying water.

A total of 12 replicates, each containing ten 7- to 8-d-old amphipods are tested for each sample. For the total of 12 replicates, the assignment of beakers is as follows: 12 replicates are set up on Day –1, of which 4 replicates are used for 28-day growth and survival endpoints and 8 replicates for measurement of survival and reproduction on Day 35, and survival, reproduction, or growth on Day 42.

Placement of Sediment into Test Chambers

The day before the sediment test is started (Day –1), each sediment is thoroughly homogenized and added to the test chambers. Sediment is visually inspected to judge the degree of homogeneity.

Each test chamber will contain the same amount of sediment, determined by volume. Overlying water is added to the chambers on Day –1 in a manner that minimizes suspension of sediment. Renewal of overlying water is started on Day –1. A test begins when the organisms are added to the test chambers (Day 0). Hardness, alkalinity, and ammonia concentrations in the water above the sediment within a treatment should not vary by more than 50% during the test.

Table D.1 Status Report — Environment Canada Biological Test Method Development Program[a] (Revised December 1999)

Test Method / Supporting Guidance Documents		Status	Publication Date	Report Number
	Universal Test Methods			
1.	Acute Lethality Test Using Rainbow Trout	Published	July 1990	EPS 1/RM/9
2.	Acute Lethality Test Using Threespine Stickleback	Published	July 1990	EPS 1/RM/10
3.	Acute Lethality Test Using *Daphnia* spp.	Published	July 1990	EPS 1/RM/11
4.	Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*	Published	February 1992	EPS 1/RM/21
5.	Test of Larval Growth and Survival Using Fathead Minnows	Published	February 1992	EPS 1/RM/22
6.	Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*)	Published	October 1992	EPS 1/RM/24
7.	Growth Inhibition Test Using the Freshwater Alga (*Selenastrum capricornutum*)	Published	November 1992	EPS 1/RM/25
8.	Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods	Published	December 1992	EPS 1/RM/26
9.	Fertilization Assay with Echinoids (Sea Urchins and Sand Dollars)	Published	December 1992	EPS 1/RM/27
10.	Early Life-Stage Toxicity Tests Using Salmonid Fish (Rainbow Trout) – Second Edition	Published	July 1998	EPS 1/RM/28
11.	Survival and Growth in Sediment Using Freshwater Midge Larvae *Chironomus tentans* or *riparius*	Published	December 1997	EPS 1/RM/32
12.	Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*	Published	December 1997	EPS 1/RM/33
13.	Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte *Lemna minor*	Published	March 1999	EPS 1/RM/37
14.	Survival and Growth in Sediment Using Estuarine or Marine Polychaete Worms	Final draft in preparation	Early 2001	—
	Reference Methods			
1.	Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	Published	July 1990	EPS 1/RM/13
2.	Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	Published	July 1990	EPS 1/RM/14
3.	Reference Method for Determining Acute Lethality of Sediment to Estuarine or Marine Amphipods	Published	1999	EPS 1/RM/35
	Supporting Guidance Documents			
1.	Control of Toxicity Test Precision Using Reference Toxicants	Published	August 1990	EPS 1/RM/12
2.	Collection and Preparation of Sediment for Physicochemical Characterization and Biological Testing	Published	December 1994	EPS 1/RM/29
3.	Measurement of Toxicity Test Precision Using Control Sediments Spiked with a Reference Toxicant	Published	September 1995	EPS 1/RM/30
4.	Application and Interpretation of Single-Species Test Data in Environmental Toxicology	Final version in preparation	Spring 2000	EPS 1/RM/34
5.	Statistics for the Determination of Toxicity Test Endpoints	Second draft in preparation	Early 2001	—

[a] Documents available in French and English, copies of published documents can be obtained from EPS Publication Section, ETAD, Environment Canada, Fax: (819)953-7253) Tel: (819)953-5921.

Table D.2 ASTM Standards on Toxicity Testing

Std. No.	
	Aquatic Toxicity Testing — Water
General	
E 1850-97	Guide for Selection of Resident Species as Test Organisms for Aquatic and Sediment Toxicity Tests
E 1203-98	Practice for Using Brine Shrimp Nauplii as Food for Test Animals in Aquatic Toxicity
E 1733-95	Guide for Use of Light in Laboratory Testing
Phytoplankton	
D 3978-80	Practice for Algal Growth Potential Testing with *Selenastrum capricornutum*
E1218-97a	Guide for Conducting Static 96-hour Toxicity Testing with Microalgae
E1913-97	Guide for Conducting Toxicity Tests with Bioluminescent Dinoflagellates
Plant	
E 1841-96	Guide for Conducting Renewal Phytotoxicity Tests with Freshwater Emergent Macrophytes
E 1498-92 (1998)	Guide for Conducting Sexual Reproduction Tests with Seaweeds
E 1415-91 (1998)	Guide for Conducting Static Toxicity Tests with *Lemna gibba* G3
E 1913-97	Guide for Conducting Static, Axenic, 14-Day Phytotoxicity Tests in Test Tubes with the Submerged Aquatic Macrophyte, *Myriophyllum sibiricum* Komarov
Invertebrates	
E 1440-91 (1998)	Guide for Acute Toxicity Test with the Rotifer Brachionus
E 1562-94	Guide for Conducting Acute, Chronic, and Life Cycle Aquatic Toxicity Tests with Polychaetous Annelids
E 724-98	Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs
E 1193-97	Guide for Conducting *Daphnia magna* Life Cycle Toxicity Tests
E 1191-97	Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysids
E 1463-92 (1998)	Guide for Conducting Static and Flow-Through Acute Toxicity Tests with Mysids from the West Coast of the United States
E 1295-89 (1995)	Guide for Conducting Three-Brood, Renewal Toxicity Tests with *Ceriodaphnia dubia*
E 1563-98	Guide for Conducting Static Acute Toxicity Tests with Echinoid Embryos
Vertebrate	
E 729-96	Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians
E 1192-97	Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians
E 1241-98	Guide for Conducting Early Life-Stage Toxicity Tests with Fishes
E 1439-98	Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (Fetax)
General	
E 1022-94	Practice for Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Molluscs
E 1242-97	Practice for Using Octanol-Water Partition Coefficient to Estimate Median Lethal Concentrations for Fish Due to Narcosis
Microcosm	
E 1366-96	Practice for Standardized Aquatic Microcosm; Freshwater
Behavior	
E 1604-94	Guide for Behavioral Testing in Aquatic Toxicology
E 1711-95	Guide for Measurement of Behavior during Fish Toxicity Tests
E 1768-95	Guide for Ventilatory Behavioral Toxicology Testing of Freshwater Fishes
	Aquatic Toxicity Testing — Sediment
General	
E 1391-94	Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing
E 1525-94a	Guide for Designing Biological Tests with Sediments
Marine Sediment Toxicity Tests	
E 1611-94	Guide for Conducting Sediment Toxicity Tests with Marine and Estuarine Polychaetous Annelids
E 1367-99	Guide for Conducting 10-day Static Sediment Toxicity Tests with Marine and Setuarine Amphipods
E 1688-97a	Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminates by Benthic Invertebrates
Freshwater Sediment Toxicity Tests	
E 1706-95b	Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates

Table D.3 Toxicity and Bioaccumulation Testing Categories

Site	Type Assay	Media	Organisms (Examples)
Laboratory	Acute/Screening Toxicity Short-term Chronic	Low Flow High Flow Outfalls	*P. promelas*, *C. dubia*, *Daphnia magna*
	Acute or Chronic	Sediments	*Hyalella azteca*, *Chironomus tentans*, *Chironomus riparius*
	Bioaccumulation	Sediments	*Lumbriculus variegatus*
Field	Acute to Chronic Toxicity	Low Flow High Flow Mixing Zones	*P. promelas*, *C. dubia*, *D. magna*, *H. azteca*, *Gammarus*, *C. tentans*, or *C. riparius*, bivalves
		Sediment	*H. azteca*, *Gammarus*, *C. tentans* or *C. riparius*, *P. promelas*, *D. magna*, Bivalves
	Bioaccumulation	Low Flow High Flow Mixing Zones Sediment	*Lumbriculus variegatus*, bivalves, fish
	Bioaccumulation Surrogate	Low Flow High Flow Mixing Zones Interstitial water?	Semipermeable membrane devices

Acclimation

Test organisms are cultured and tested at the same temperature. Test organisms are cultured in the same water that is used in testing, as recommended by EPA (EPA 1994); therefore, no acclimation will be necessary.

Placing Organisms in Test Chambers

Amphipods are introduced into the overlying water below the air–water interface. Weight is measured on a subset of 20 amphipods used to start the test.

Feeding

For each beaker, 1.0 mL of YCT is added daily from Day 0 to Day 42. The amount of food added to the test chambers is kept to a minimum to avoid microbial growth and water fouling. If excess food collects on the sediment, a fungal or bacterial growth may develop on the sediment surface, in which case feeding is suspended for 1 or more days. A drop in dissolved oxygen below 2.5 mg/L during a test may indicate that the food added is not being consumed. Feeding is suspended for the amount of time necessary to increase the dissolved oxygen concentration. If feeding is suspended in one treatment, it should be suspended in all treatments. Detailed records of feeding rates and the appearance of the sediment surface are made daily.

Monitoring a Test

All chambers are checked daily and observations made to assess test organism behavior such as sediment avoidance. However, monitoring effects on burrowing activity of test organisms may be difficult because the test organisms are often not visible during the exposure. The operation of the exposure system is monitored daily.

Measurement of Overlying Water Quality Characteristics

Conductivity, hardness, alkalinity, and ammonia is measured in all treatments at the beginning and at the end of the sediment exposure portion of the test. Water quality characteristics are also

Table D.4 Recommended Toxicity Test Conditions and Test Acceptability Criteria for *Ceriodaphnia dubia* Screening and Definitive Acute Tests

	Test Conditions	Recommended
1.	Test type:	Static non-renewal, static renewal or flow through
2.	Test duration:	24, 48, or 96 h
3.	Temperature:	20°C ± 1°C; or 25°C ± 1°C
4.	Light quality:	Ambient laboratory illumination
5.	Light intensity:	10–20 μE/m²/s (50–100 ft-c) (ambient laboratory levels)
6.	Photoperiod:	16 h light, 8 h darkness
7.	Test chamber size:	30 mL (minimum)
8.	Test solution volume:	25 mL (minimum) – For whole sediment tests use 5 mL sediment, 20 mL water
9.	Renewal of test solutions:	Minimum, after 48 h
10.	Age of test organisms:	Less than 24 h old
11.	No. organisms per test chamber:	Minimum, 5 for effluent and receiving water tests
12.	No. replicate chambers per concentration:	Minimum, 4 for effluent and receiving water tests
13.	No. organisms per concentration:	Minimum, 20 for effluent and receiving water tests
14.	Feeding regime:	Feed YCT and *Selenastrum* while holding prior to the test; newly-released young should have food available a minimum of 2 h prior to use in a test; add 0.1 mL each of YCT and *Selenastrum* 2 h prior to test solution renewal at 48 h
15.	Test chamber cleaning:	Cleaning not required
16.	Test solution aeration:	None
17.	Dilution water:	Moderately hard synthetic water is prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW, receiving water, groundwater, or synthetic water, modified to reflect receiving water hardness
18.	Test concentrations:	Effluents: minimum of five effluent concentrations and a control Receiving waters: 100% receiving water and a control
19.	Dilution series:	Effluents: ≥ 0.5 dilution series Receiving Waters: None, or ≥ 0.5 dilution series
20.	Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (significant difference from control)
21.	Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period
22.	Sample volume required:	1 L
23.	Test acceptability criterion:	90% or greater survival in controls

measured at the beginning and end of the reproductive phase (Day 29 to Day 42). Conductivity will be measured weekly and DO and pH three times/week

Dissolved oxygen is measured a minimum of three times/week and should be at a minimum of 2.5 mg/L. Aeration is used to maintain dissolved oxygen in the overlying water above 2.5 mg/L.

Temperature is measured at least daily in at least one test chamber from each treatment. The daily mean test temperature must be within 1°C of 23°C. The instantaneous temperature must always be within 3°C of 23°C.

Ending a Test

Endpoints monitored include 28-d survival and growth of amphipods and 35-day and 42-day survival, growth, and reproduction (number of young/female) of amphipods. Growth or reproduction of amphipods may be a more sensitive toxicity endpoint compared to survival.

On Day 28, four of the replicate beakers/sediment are sieved with a #40 mesh sieve (425-μm mesh; U.S. standard size sieve) to remove surviving amphipods for growth determinations. Growth

Table D.5 Recommended Toxicity Test Conditions and Test Acceptability Criteria for *Daphnia pulex* and *D. magna* Screening and Definitive Acute Tests

	Test Conditions	Recommended
1.	Test type:	Static non-renewal, static renewal or flow through
2.	Test duration:	24, 48 or 96 h
3.	Temperature:	20°C ± 1°C; or 25°C ± 1°C
4.	Light quality:	Ambient laboratory illumination
5.	Light intensity:	10–20 μE/m²/s (50–100 ft-c) (ambient laboratory levels)
6.	Photoperiod:	16 h light, 8 h darkness
7.	Test chamber size:	30 mL (minimum)
8.	Test solution volume:	25 mL (minimum) — for whole sediment tests, use 10 mL, sediment, 40 mL water
9.	Renewal of test solutions:	Minimum, after 48 h
10.	Age of test organisms:	Less than 24 h old
11.	No. organisms per test chamber:	Minimum, 5 for effluent and receiving water tests
12.	No. replicate chambers per concentration:	Minimum, 4 for effluent and receiving water tests
13.	No. organisms per concentration:	Minimum, 20 for effluent and receiving water tests
14.	Feeding regime:	Feed YCT and *Selenastrum* while holding prior to the test; newly released young should have food available a minimum of 2 h prior to use in a test; add 0.1 mL each of YCT and *Selenastrum* 2 h prior to test solution renewal at 48 h
15.	Test chamber cleaning:	Cleaning not required
16.	Test solution aeration:	None
17.	Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q or equivalent deionized water and reagent grade chemicals, or 20% DMW, receiving water, groundwater, or synthetic water, modified to reflect receiving water hardness
18.	Test concentrations:	Effluents: minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19.	Dilution series:	Effluents: ≥ 0.5 dilution series Receiving Waters: None, or ≥ 0.5 dilution series
20.	Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (significant difference from control)
21.	Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period
22.	Sample volume required:	1 L
23.	Test acceptability criterion:	90% or greater survival in controls

of amphipods are reported as weight. Dry weight of amphipods in each replicate are determined on Days 28 and 42. Dry weight of amphipods are determined by: (1) transferring rinsed amphipods to a preweighed aluminum pan; (2) drying these samples for 24 hours at 60°C; and (3) weighing the pan and dried amphipods on a balance to the nearest 0.01 mg. Average dry weight of individual amphipods in each replicate is calculated from these data.

On Day 28, the remaining eight beakers/sediment are sieved and the surviving amphipods in each sediment beaker are placed in 300-mL water-only beakers containing 150 to 275 mL of overlying water and a 5 × 5 cm piece of Nitex screen or 3M fiber mat. Each water-only beaker receives 1.0 mL of YCT stock solution and about two volume additions of water daily.

Reproduction of amphipods is measured on Day 35 and Day 42 in the water-only beakers by removing and counting the adults and young in each beaker. On Day 35, the adults are then returned to the same water-only beakers. Adult amphipods surviving on Day 42 are preserved in sugar formalin. The number of adult females is determined by simply counting the adult males (mature male amphipods will have an enlarged second gnathopod) and assuming all other adults are females.

Table D.6 Recommended Toxicity Test Conditions and Test Acceptability Criteria for the Fathead Minnow (*Pimephales promelas*) Screening and Definitive Acute Tests

	Test Conditions	Recommended
1.	Test type:	Static non-renewal, static renewal or flow through
2.	Test duration:	24, 48 or 96 h
3.	Temperature:	20°C ± 1°C or 25°C ± 1°C
4.	Light quality:	Ambient laboratory illumination
5.	Light intensity:	10–20 μE/m²/s (50–100 ft-c) (ambient laboratory levels)
6.	Photoperiod:	16 h light, 8 h darkness
7.	Test chamber size:	250 mL (minimum)
8.	Test solution volume:	200 mL (minimum) — for whole sediment tests, use 150 mL sediment, 600 mL water
9.	Renewal of test solutions:	Minimum, after 48 h
10.	Age of test organisms:	1–14 days: 24 h range in age
11.	No. organisms per test chamber:	Minimum, 10 for effluent test
12.	No. replicate chambers per concentration:	Minimum, 2 for effluent tests Minimum, 4 for receiving water tests
13.	No. organisms per concentration:	Minimum, 20 for effluents tests Minimum, 40 for receiving waters tests
14.	Feeding regime:	*Artemia nauplii* are made available while holding prior to the test; add 0.2 mL *Artemia nauplii* concentrate 2 h prior to test solution renewal at 48 h
15.	Test chamber cleaning:	Cleaning not required
16.	Test solution aeration:	None, unless DO concentration falls below 40% saturation; rate should not exceed 100 bubbles/min
17.	Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI -Q or equivalent deionized water and reagent grade chemicals or 20% DMW, receiving water, groundwater, or synthetic water, modified to reflect receiving water and hardness
18.	Test concentrations:	Effluents: minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19.	Dilution series:	Effluents: ≥ 0.5 dilution series Receiving Waters: None, or ≥ 0.5 dilution series
20.	Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (significant difference from control)
21.	Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period
22.	Sample volume required:	2 L for effluents and receiving waters
23.	Test acceptability criterion:	90% or greater survival in controls

The number of females is used to determine number of young/female/beaker from Day 28 to Day 42. Growth will also be measured for these adult amphipods.

Interpretation of Results

Endpoints measured in the 42-day *H. azteca* test include survival (Days 28, 35, and 42), growth (Days 28 and 42), and reproduction (number of young/female produced from Days 28 to 42). Reproduction is often more variable than growth. Some investigators have shown growth provides unique information that can help discriminate toxic effects of exposure to contaminants in sediment, while others have not seen differences from survival information.

On rare occasions, test organism responses in control sediments may exhibit responses which are less than reference or test sediments. This may be due to the poor nutritional content of the control sediment or other unknown physicochemical factors. Currently, there are no standard control sediments which can be strongly recommended for chronic toxicity testing due to a lack of testing

Table D.7 Recommended Toxicity Test Conditions and Test Acceptability Criteria for the Rainbow Trout (*Oncorhynchus mykiss*) and Brook Trout (*Salvelinus fontinalis*) Screening and Definitive Acute Tests

	Test Conditions	Recommended
1.	Test type:	Static non-renewal, static-renewal or flow-through
2.	Test duration:	24, 48 or 96 h
3.	Temperature:	12 ± 2°C
4.	Light quality:	Ambient laboratory illumination
5.	Light intensity:	10–20 μE/m²/s (50–100 ft-c) (ambient laboratory levels)
6.	Photoperiod:	16 h light, 8 h darkness. Light intensity should be raised gradually over a 15 min period at the beginning of the photoperiod, and lowered gradually at the end of the photoperiod, using a dimmer switch or other suitable control device.
7.	Test chamber size:	5 L (minimum) (test chamber should be covered to prevent fish from jumping out)
8.	Test solution volume:	4 L (minimum) — for whole sediment tests, use 80 mL sediment, and 320 mL water
9.	Renewal of test solutions:	Minimum, after 48 h
10.	Age of test organisms:	Rainbow Trout: 5–30 days, "24 h (after yolk sac absorption to 30 days) Brook Trout: 30–60 days
11.	No. organisms per test chamber:	Minimum, 10 for effluent and receiving water tests
12.	No. replicate chambers per concentration:	Minimum, 2 for effluent tests Minimum, 4 for receiving water tests
13.	No. organisms per concentration:	Minimum, 20 for effluent tests Minimum 40 for receiving water tests
14.	Feeding regime:	Feeding not required
15.	Test chamber cleaning:	Cleaning not required
16.	Test solution aeration:	None, unless DO concentration falls below 6.0 mg/L; rate should not exceed 100 bubbles/min
17.	Dilution water:	Moderately hard synthetic water is prepared using MILLIPORE MILLI-Q or equivalent deionized water and reagent grade chemicals or 20% DMW, receiving water, groundwater, or synthetic water, modified to reflect receiving water hardness
18.	Test concentrations:	Effluents: minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19.	Dilution series:	Effluents: ≥ 0.5 dilution series Receiving Waters: None, or ≥ 0.5 dilution series
20.	Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (significant difference from control)
21.	Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period
22.	Sample volume required:	20 L for effluents 40 L for receiving waters
23.	Test acceptability criterion:	90% or greater survival in controls

and research. Should poor responses be observed in a control sediment, a secondary control or reference sediment may be substituted for comparisons of significance. This will not invalidate the test, but simply adds some degree of uncertainty in the determination of ecological significance.

Recently, the U.S. EPA conducted interlaboratory variance testing with the 42-day *H. azteca* assay. In these tests, the draft standard methods were used. The minimum detectable differences for amphipod survival at 28 and 42 days ranged from 8 to 12% in moderately contaminated sediments. Minimum detectable differences for reproductive endpoints were higher, as expected, ranging from 19 to 25%.

Table D.8 Recommended Toxicity Test Conditions and Test Acceptability Criteria for the *Ceriodaphnia dubia* Survival and Reproduction Test

	Test Conditions	Recommended
1.	Test type:	Static renewal
2.	Test duration:	Until 60% of control females have three broods (maximum test duration 8 days)
3.	Temperature:	25 ± 1°C
4.	Light quality:	Ambient laboratory illumination
5.	Light intensity:	10–20 μE/m²/s (50–100 ft-c) (ambient laboratory levels)
6.	Photoperiod:	16 h light, 8 h darkness.
7.	Test chamber size:	30 mL (minimum)
8.	Test solution volume:	15 mL (minimum) — for whole sediment assays, use 5 mL sediments and 20 mL water
9.	Renewal of test solutions:	Daily
10.	Age of test organisms:	Less than 24 h and all released within an 8 h period
11.	No. neonates per test chamber:	1
12.	No. replicate test chambers per concentration:	10
13.	No. neonates per concentration:	10
14.	Feeding regime:	Feed 0.1 mL each of YCT and 0.1 mL of algal suspension per test chamber daily
15.	Test solution aeration:	None
16.	Dilution water:	Uncontaminated source of receiving water or other natural water, synthetic water prepared using MILLIPORE MILLI-Q or equivalent deionized water and reagent grade chemicals or DMW
17.	Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving water: 100% receiving water or minimum of five concentrations and a control
18.	Dilution factor:	Effluents ≥ 0.5 Receiving waters: None or ≥ 0.5
19.	Endpoints:	Survival and reproduction
20.	Sampling and sample holding requirements:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples collected on days one, three, and five with a maximum holding time of 36 h before first use
21.	Sample volume required:	1 L
22.	Test acceptability criteria:	80% or greater survival and an average of 15 or more young per surviving female in the control solutions; 60% of surviving control organisms must produce three broods

METHODS FOR CONDUCTING LONG-TERM SEDIMENT TOXICITY TESTS WITH *CHIRONOMUS TENTANS*

Conditions for conducting a long-term sediment toxicity test with *C. tentans* are summarized in Table D.17. A general activity schedule is outlined in Table D.18.

The long-term sediment toxicity test with *C. tentans* is conducted at 23°C with a 16L:8D photoperiod at an illuminance of about 500 to 1000 lux. Test chambers, sediment addition, water renewal, and water quality monitoring are as described above for *H. azteca.*

A total of 16 replicates, each containing 12, <24-hour-old larvae are tested for each sample. For the total of 16 replicates, the assignment of beakers is as follows: initially, 12 replicates are set up on Day –1, of which 4 replicates are used for 20-day growth and survival endpoints and 8 replicates for determination of emergence and reproduction. It is typical for males to begin emerging 4 to 7 days before females. Midges in each test chamber are fed 1.5 mL of a 4-g/L Tetrafin™ suspension daily. Endpoints monitored include 20-day survival and ash-free dry weight, emergence; and time to death (adults). Reproduction and egg hatchability are not assessed.

Table D.9 Recommended Toxicity Test Conditions and Test Acceptability Criteria for the Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Test

	Test Conditions	Recommended
1.	Test type:	Static renewal
2.	Test duration:	7 days
3.	Temperature:	25 ± 1°C
4.	Light quality:	Ambient laboratory illumination
5.	Light intensity:	10–20 μE/m²/s (50–100 ft-c) (ambient laboratory levels)
6.	Photoperiod:	16 h light, 8 h darkness.
7.	Test chamber size:	500 mL (minimum)
8.	Test solution volume:	250 mL (minimum) — for whole sediment tests use 50 mL sediment and 200 mL water
9.	Renewal of test solutions:	Daily
10.	Age of test organisms:	Newly hatched larvae less than 24 h old. If shipped, not more than 48 h old, 24 h range in age
11.	No. larvae per test chamber:	15 (minimum of 10)
12.	No. replicate test chambers per concentration:	4 (minimum of 3)
13.	No. larvae per concentration:	60 (minimum of 30)
14.	Source of food:	Newly hatched *Artemia nauplii* (less than 24 h old)
15.	Feeding regime:	Feed 0.1 mL newly hatched (less than 24 h old) brine shrimp nauplii three times daily at 4 h intervals or, as a minimum 0.15 mL twice daily, 6 h between feedings (at the beginning of the work day following renewal). Sufficient larvae are added to provide an excess. Larvae are not fed during the final 12 h of the test
16.	Test chamber cleaning:	Siphon daily, immediately before test solution renewal
17.	Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L. Rate should not exceed 100 bubbles/min.
18.	Dilution water:	Uncontaminated source of receiving water or other natural water, synthetic water prepared using MILLIPORE MILLI-Q or equivalent deionized water and reagent grade chemicals or DMW
19.	Test concentrations:	Effluents: Minimum of five effluent concentration and a control Receiving water: 100% receiving water or minimum of five concentrations and a control
20.	Dilution factor:	Effluents: ≥ 0.5 Receiving waters: none or ≥ 0.5
21.	Endpoints:	Survival and growth (weight)
22.	Sampling and sample handling requirements:	For on-site tests, samples are collected daily, and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples are collected on days one, three, and five with a maximum holding time of 36 h before first use
23.	Sample volume required:	2.5 L/day
24.	Test acceptability criteria:	80% or greater survival in controls: average dry weight per surviving organism in control chambers equals or exceeds 0.25 mg

Collection of Egg Cases

Egg cases are obtained from adult midges held in a sex ratio of 1:3 male:female. Adults are collected 4 days before starting a test. The day after collection of adults, 6 to 8 of the larger "C"-shaped egg cases are transferred to a petri dish with culture water and incubated (at 23°C). Hatching typically begins around 48 hours and larvae typically leave the egg case 24 hours after the first hatch.

Hatching of Eggs

Hatching of eggs should be complete by about 72 hours. Hatched larvae remain with the egg case for about 24 hours and appear to use the gelatinous component of the egg case as an initial

Table D.10 Toxicity Test Conditions and Test Acceptability Criteria for the Fathead Minnow (*Pimephales promelas*) Embryo-Larval Survival and Teratogenicity Test

	Test Conditions	Recommended
1.	Test type:	Static renewal
2.	Test duration:	7 days
3.	Temperature:	25 ± 1°C
4.	Light quality:	Ambient laboratory illumination
5.	Light intensity:	10–20 μE/m²/s (50–100 ft-c) (ambient laboratory levels)
6.	Photoperiod:	16 h light, 8 h darkness.
7.	Test chamber size:	150–500 mL
8.	Test solution volume:	70 mL (minimum) — for whole sediment tests, use 50 mL sediment and 200 mL water
9.	Renewal of test solutions:	Daily
10.	Age of test organisms:	Less than 36 h old embryos (maximum 48 h if shipped)
11.	No. embryos per test chamber:	15 (minimum of 10)
12.	No. replicate test chambers per concentration:	4 (minimum of 3)
13.	No. embryos per concentration:	60 (minimum of 30)
14.	Feeding regime:	Feeding not required
15.	Test solution aeration:	None, unless DO concentration falls below 4 mg/L
16.	Dilution water:	Uncontaminated source of receiving water or other natural water, synthetic water prepared using MILLIPORE MILLI-Q or equivalent deionized water and reagent grade chemicals or DMW
17.	Test concentrations:	Effluents: Minimum of five effluent concentration and a control Receiving water: 100% receiving water or minimum of five concentrations and a control
18.	Dilution factor:	Effluents: ≥ 0.5 Receiving waters: none or ≥ 0.5
19.	Endpoint:	Combined mortality (dead and deformed organisms)
20.	Sampling and sample handling requirements:	For on-site tests, samples are collected daily, and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples are collected on days one, three, and five with a maximum holding time of 36 h before first use
21.	Sample volume required:	1.5 to 2.5 L/day depending on the volume of test solutions used
22.	Test acceptability criteria:	80% or greater survival in controls

source of food. After the first 24-hour period with larvae hatched, egg cases are transferred from the incubation petri dish to another dish with clean test water. The action of transferring the egg case stimulates the remaining larvae to leave the egg case within a few hours. These are the larvae that are used to start the test.

Placing Organisms in Test Chambers

To start the test, larvae are collected with a Pasteur pipette from the bottom of the incubation dish with the aid of a dissecting microscope. Test organisms are pipetted directly into overlying water. Larvae are transferred to exposure chambers within 4 hours of emerging from the egg case.

Feeding

Each beaker received a daily addition of 1.5 mL of Tetrafin (4 mg/mL dry solids). Feeding is curtailed under circumstances described in the amphipod methods.

Dissolved Oxygen

Routine chemistries on Day 0 should be taken before organisms are placed in the test beakers. Excursions of DO as low as 1.5 mg/L did not seem to have an effect on midge survival and

Table D.11 Toxicity Test Conditions and Test Acceptability Criteria for the Algal (*Selenastrum capricornutum*) Growth Test

	Test Conditions	Recommended
1.	Test type:	Static non-renewal
2.	Test duration:	48–96 h
3.	Temperature:	25 ± 1°C
4.	Light quality:	"Cool white" fluorescent lighting
5.	Light intensity:	86 ± 8.6 μE/m²/s (400 ± 40 ft-c or 4306 lux)
6.	Photoperiod:	Continuous illumination
7.	Test chamber size:	125 or 250 mL
8.	Test solution volume:	50 or 100 mL
9.	Renewal of test solutions:	None
10.	Age of test organisms:	4 to 7 days
11.	Initial cell density in test chamber:	10,000 cells/mL
12.	No. replicate chambers per sample:	4 (minimum or 3)
13.	Shaking rate:	100 rmp continuous, or twice daily by hand
14.	Test solution aeration:	None
15.	Dilution water:	Algal stock culture medium, enriched uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q or equivalent deionized water and reagent grade chemicals, or DMW without EDTA or enriched surface water
16.	Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving water: 100% receiving water or minimum of five concentrations and a control
17.	Test dilution factor:	Effluents: ≥ 0.5 Receiving waters: None or ≥ 0.5
18.	Endpoint:	Growth (cell counts, chlorophyll fluorescence, absorbance, biomass)
19.	Sample requirements:	For on-site tests, one sample collected at test initiation, and used within 24 h of the time being removed from the sampling device. For off-site tests, holding time must not exceed 36 h
20.	Sample volume required:	1 or 2 L depending on test volume
21.	Test acceptability criteria:	1×10^6 cells/mL with EDTA or 2×10^5 cells/mL without EDTA in the controls: Variability of controls should not exceed 20%

development (P.K. Sibley, University of Guelph, Guelph, Ontario, personal communication). Based on these findings, periodic depressions of DO below 2.5 mg/L (but not below 1.5 mg/L) are not likely to adversely affect test results, and thus should not be a reason to discard test data. Nonetheless, tests should be managed toward a goal of DO > 2.5 mg/L to ensure satisfactory performance. If the DO level of the water falls below 2.5 mg/L for any one treatment, aeration is conducted in all replicates for the duration of the test.

Monitoring Survival and Growth

At 20 days, four of the initial 12 replicates are selected for use in growth and survival measurements. Using a #40 sieve (425-μm mesh) to remove larvae from sediment, *C. tentans* is collected. Surviving larvae are kept separated by replicate for weight measurements; if pupae are recovered, these organisms are included in survival data but not included in the growth data.

The AFDW of midges is determined for the growth endpoint. All living larvae per replicate are combined and dried to a constant weight (e.g., 60°C for 24 hours). All weigh boats are ashed before use to eliminate weighing errors due to the pan oxidizing during ashing. The sample is brought to room temperature in a desiccator and weighed to the nearest 0.01 mg to obtain mean weights per surviving organism per replicate. The dried larvae in the pan are then ashed at 550°C for 2 hours. The pan with the ashed larvae is then reweighed and the tissue mass of the larvae is determined as the difference between the weight of the dried larvae plus pan and the weight of the ashed larvae plus pan.

Table D.12 Toxicity Test Conditions and Test Acceptability Criteria for the Amphipod (*Hyalella azteca*) Survival Test

	Test Conditions	Recommended
1.	Test type:	Whole sediment toxicity test with renewal of overlying water
2.	Test duration:	10 d
3.	Temperature:	23 ± 1°C
4.	Light quality:	Wide-spectrum fluorescent lights
5.	Illuminance:	About 100 to 1000 lux
6.	Photoperiod:	16 h light, 8 h dark
7.	Test chamber size:	300 mL high-form lipless beaker
8.	Sediment volume:	100 mL
9.	Overlying water volume:	175 mL
10.	Renewal of overlying water:	2 volumes additions/d; continuous or intermittent (e.g., 1 volume addition every 12 h)
11.	Age of test organisms:	7 to 14 d old at the start of the test (1 to 2 d range in age)
12.	No. organisms per test chamber:	10
13.	No. replicate chambers per treatment:	Depends on the objective of the test. Eight replicates are recommended for routine testing
14.	Feeding regime:	YCT food, fed 1.0 mL daily (1800 mg/L stock) to each test chamber
15.	Test solution aeration:	None, unless DO in overlying water falls below 2.5 mg/L
16.	Overlying water:	Culture water, well water, surface water, site water, or reconstituted water
17.	Test chamber cleaning:	If screens become clogged during a test, gently brush the outside of the screen
18.	Overlying water quality:	Hardness, alkalinity, conductivity, pH, and ammonia at the beginning and end of a test; temperature and dissolved oxygen daily
19.	Endpoint:	Survival and growth
20.	Test acceptability criterion:	Minimum mean control survival of 80% and measurable growth of test organisms in the control sediment

Monitoring Emergence

Emergence traps are placed on the reproductive replicates on Day 20 (emergence traps for the auxiliary beakers are added at the corresponding 20-day time interval for those replicates. At 23°C, emergence in control sediments typically begins on or about Day 23 and continues for about 2 weeks. However, in contaminated sediments, the emergence period may be extended by weeks.

Two categories are recorded for emergence: complete emergence and partial emergence. Complete emergence occurs when an organism has shed the pupal exuviae completely and escapes the surface tension of the water. If complete emergence has occurred but the adult has not escaped the surface tension of the water, the adult will die within 24 hours. Therefore, 24 hours will elapse before this death is recorded. Partial emergence occurs when an adult has only partially shed the pupal exuvia. These adults will also die, an event which can be recorded after 24 hours.

Between Day 23 and the end of the test, emergence of males and females, pupal and adult mortality, and time to death for adults is recorded daily for the reproductive replicates.

Ending a Test

The point at which the life cycle test is ended depends upon the sediments being evaluated. In clean sediments, the test typically requires 40 to 50 days from initial setup to completion if all possible measurement endpoints are evaluated. However, test duration will increase in the presence of environmental stressors that act to reduce growth and delay emergence. Where a strong gradient of sediment contamination exists, emergence patterns between treatments will likely become asynchronous, in which case each treatment needs to be ended separately. For this reason, emergence is used as a guide to decide when to end a test. Testing will be terminated with completion of emergence.

Table D.13 Toxicity Test Conditions and Test Acceptability Criteria for the Midge (*Chironomus tentans*) Survival Test

	Test Conditions	Recommended
1.	Test type:	Whole sediment toxicity test with renewal of overlying water
2.	Test duration:	10 d
3.	Temperature:	23 ± 1°C
4.	Light quality:	Wide-spectrum fluorescent lights
5.	Illuminance:	About 100 to 1000 lux
6.	Photoperiod:	16 h light, 8 h dark
7.	Test chamber size:	300 mL high-form lipless beaker
8.	Sediment volume:	100 mL
9.	Overlying water volume:	175 mL
10.	Renewal of overlying water:	2 volumes additions/d; continuous or intermittent (e.g., 1 volume addition every 12 h)
11.	Age of test organisms:	Second to third instar larvae (about 10 d old larvae; all organisms must be third instar or younger with at least 50% of the organisms at third instar)
12.	No. organisms per test chamber:	10
13.	No. replicate chambers per treatment:	Depends on the objective of the test. Eight replicates are recommended for routine testing
14.	Feeding regime:	Tetrafin goldfish food, fed 1.5 ml daily to each test chamber (1.5 mL contains 6.0 mg of dry solids)
15.	Test solution aeration:	None, unless DO in overlying water falls below 2.5 mg/L
16.	Overlying water:	Culture water, well water, surface water, site water, or reconstituted water
17.	Test chamber cleaning:	If screens become clogged during a test, gently brush the outside of the screen
18.	Overlying water quality:	Hardness, alkalinity, conductivity, pH, and ammonia at the beginning and end of a test; temperature and dissolved oxygen daily
19.	Endpoint:	Survival and growth (ash-free dry weight, AFDW)
20.	Test acceptability criterion:	Minimum mean control survival *must* be *70%*, with *minimum* mean *weight/surviving* control organisms of *0.48* mg *AFDW*

For treatments in which emergence has occurred, the treatment (not the entire test) is ended when no further emergence is recorded over a period of 7 days (the 7-day criterion). At this time, all beakers of the treatment are sieved through a #40 mesh screen (425 μm) to recover remaining larvae, pupae, or pupal casts. When no emergence is recorded in a treatment at any time during the test, that treatment can be ended once emergence in the control sediment has ended using the 7-day criterion.

Interpretation of Results

Endpoints measured in the *C. tentans* test include survival, growth, and emergence. On rare occasions, test organisms in control sediments may exhibit responses which are less than reference or test sediments. This may be due to the poor nutritional content of the control sediment or other unknown physicochemical factors. Currently, there are no standard control sediments that can be strongly recommended for chronic toxicity testing due to a lack of testing and research. Should poor responses be observed in a control sediment, a secondary control or reference sediment may be substituted for comparisons of significance. This will not invalidate the test, but simply adds a degree of uncertainty to the determination of ecological significance.

Recently, the U.S. EPA conducted interlaboratory variance testing with the chronic *C. tentans* assay. In these tests, the draft standard methods were used. The minimum detectable differences have not been calculated at this time, but will be available in the near future to provide a point of comparison for the test assays. It is expected that the minimum detectable difference for 28-day survival and emergence endpoints will be in the 15 to 30% range.

Table D.14 Toxicity Test Conditions and Test Acceptability Criteria for the Oligochaete (*Lumbriculus variegatus*) Survival Test

	Test Conditions	Recommended
1.	Test type:	Whole sediment toxicity test with renewal of overlying water
2.	Test duration:	23 d
3.	Temperature:	23 ± 1°C
4.	Light quality:	Wide-spectrum fluorescent lights
5.	Illuminance:	About 100 to 1000 lux
6.	Photoperiod:	16 h light, 8 h dark
7.	Test chamber size:	4 to 6 L aquaria with stainless steel screens or glass stand pipes
8.	Sediment volume:	1 L or more depending on TOC
9.	Overlying water volume:	1 L or more depending on TOC
10.	Renewal of overlying water:	2 volumes additions/d; continuous or intermittent (e.g. 1 volume addition every 12 h)
11.	Age of test organisms:	Adults
12.	No. organisms per test chamber:	Ratio of total organic carbon in sediment to organism dry weight should be no less than 50:1. Minimum of 1 g/ replicate, preferably 5 g/replicate
13.	No. replicate chambers per treatment:	Depends on the objective of the test. Five replicates are recommended for routine testing
14.	Feeding regime:	None
15.	Test solution aeration:	None, unless DO in overlying water falls below 2.5 mg/L
16.	Overlying water:	Culture water, well water, surface water, site water, or reconstituted water
17.	Test chamber cleaning:	If screens become clogged during a test, gently brush the outside of the screen
18.	Overlying water quality:	Hardness, alkalinity, conductivity, pH, and ammonia at the beginning and end of a test; temperature and dissolved oxygen daily
19.	Endpoint:	Bioaccumulation
20.	Test acceptability criterion:	Performance-based criteria specifications

Four test species will be evaluated *in situ* in exposure chambers. The exposure chambers are constructed on plastic core tubes of ~3-in diameter and 4-in length. Two windows are cut on opposite sides of the chamber and covered with nylon mesh. The mesh size varies with the experimental treatment, ranging from 10- to 1000-µm openings. For high flow testing, only water column chambers will be exposed. One duplicate set of chambers will have reduced mesh size openings to allow determinations of flow and suspended solids effects. Chambers are placed in the stream, either in the overlying water or partially buried in the sediment, with exposures varying with the treatment. Organisms are slowly acclimated to site water temperatures and then added to each test chamber (10 organisms/chamber). The age of the organisms, handling, and culturing follow U.S. EPA toxicity test methods for short-term chronic toxicity testing. For bioaccumulation testing, additional organisms are placed to provide enough tissue mass. For the oligochaete assay, 5 g of tissue are used in each chamber. Chambers are placed in the stream in replicates of four and secured with netting and steel stakes. At Days 2 and 10, chambers will be retrieved and organisms enumerated within 2 hours of collection. Test endpoints are shown in Table D.20.

The effects of water quality during high flow events will be measured at all test sites. This will involve exposures using chambers with small and large mesh sizes to vary the organism exposure to suspended solids. Exposures will be for 48 hours and include *D. magna, H. azteca,* and *C. tentans.* Testing will only be conducted when organisms can be exposed to a significant first flush event.

IN SITU TESTING USING CONFINED ORGANISMS

There are many reasons for evaluating toxicity and bioaccumulation *in situ*, such as those shown in Table D.19 and discussed in Section 6. Numerous assessments of stormwater quality have found

Table D.15 Test Conditions for Conducting a 42-day Sediment Toxicity Test with *Hyalella azteca*

	Parameter	Conditions
1.	Test type:	Whole-sediment toxicity test with renewal of overlying water
2.	Temperature:	23 ± 1°C
3.	Light quality:	Wide-spectrum fluorescent lights
4.	Illuminance:	About 500 to 1000 lux
5.	Photoperiod:	16L:8D
6.	Test chamber:	300-mL high-form lipless beaker
7.	Sediment volume:	100 mL
8.	Overlying water volume:	175 mL in the sediment exposure from Day 0 to Day 28 (175 to 275 mL in the water-only exposure from Day 28 to Day 42)
9.	Renewal of overlying water:	2 volume additions/d; continuous or intermittent (e.g., one volume addition every 12 h)
10.	Age of organisms:	7- to 8-d old at the start of the test
11.	Number of organisms/chamber:	10
12.	Number of replicate chambers/treatment:	12 (4 for 28-d survival and growth and 8 for 35- and 42-d survival, growth, and reproduction). Reproduction is more variable than growth or survival; hence, more replicates might be needed to establish statistical differences among treatments
13.	Feeding:	YCT food, fed 1.0 mL (1800 mg/L stock) daily to each test chamber
14.	Aeration:	None, unless dissolved oxygen in overlying water drops below 2.5 mg/L
15.	Overlying water:	Culture water, well water, surface water, or site water. Use of reconstituted water is not recommended
16.	Test chamber cleaning:	If screens become clogged during a test; gently brush the *outside* of the screen
17.	Overlying water quality:	Hardness, alkalinity, conductivity, and ammonia at the beginning and end of a sediment exposure (Day 0 and 28). Temperature daily. Conductivity weekly. Dissolved oxygen (DO) and pH three times/ week. Concentrations of DO should be measured more often if DO drops more than 1 mg/L since the previous measurement.
18.	Test duration:	42 d
19.	Endpoints:	28-d survival and growth; 35- and 42-d survival, growth, reproduction, and number of adult males and females on Day 42
20.	Test acceptability:	Minimum mean control survival of 80% on Day 28

the following study design example useful. The typical assessment will be an upstream–downstream evaluation of an outfall with an additional reference site. The assessment must include both low and high flow periods to separate the role of stormwater and nonpoint source runoff from low flow conditions that may include point sources and groundwater upwelling inputs. For *in situ* toxicity and/or bioaccumulation tasks, a variety of exposure periods can be used, depending on several issues, such as species resilience, meteorological conditions, concern over acute vs. chronic effects, and available resources (longer assessments are more expensive). A great challenge in any stormwater assessment is detecting chronic toxicity effects. The literature has documented (see Chapter 6) that delayed effects may occur days to weeks after pulse exposures to pesticides or metals. This is obviously difficult to determine in routine receiving water assessments. However, given the reality that chronic toxicity may be occurring, it is important to try and assess effects for as long a period as possible. Some test species, such as the cladocerans *C. dubia* and *D. magna* and early life stages of the fathead minnow *P. promelas*, do not survive well within typical *in situ* chambers for more than 4 days. The benthic macroinvertebrates, such as the amphipods *H. azteca* and *Gammarus,* midge *C. tentans*, and bivalves, can be exposed for periods of over a week (Brooker 2000). Fish may also be exposed for longer periods, but often require routine feeding. When determining bioaccumulation potential, the oligochaete worm *L. variegatus* is recommended. It accumulates nonpolar organic chemicals relatively quickly, so exposures as short as 4 days are acceptable.

Table D.16 General Activity Schedule for Conducting a 42-d Sediment Toxicity Test with *Hyalella azteca*

Day	Activity
	Pre-Test
–7	Remove adults and isolate <24-h-old amphipods (if procedures outlined in Section 12.3.4 are followed).
–8	Separate known-age amphipods from the cultures and place in holding chambers. Begin preparing food for the test. The <24-h amphipods are fed 10 mL of YCT (1800 mg/L stock solution) and 10 mL of *Selenastrum capricornutum* (about 3.0 x 107 cells/mL) on the first day of isolation and 5 mL of both YCT and *S. capricornutum* on the 3rd and 5th d after isolation.
–6 to –2	Feed and observe isolated amphipods, monitor water quality (e.g., temperature and dissolved oxygen).
–1	Feed and observe isolated amphipods, monitor water quality. Add sediment into each test chamber, place chambers into exposure system, and start renewing overlying water.
	Sediment Test
0	Measure total water quality (pH, temperature, dissolved oxygen, hardness, alkalinity, conductivity, ammonia). Transfer ten 7- to 8-day-old amphipods into each test chamber. Release organisms under the surface of the water. Add 1.0 mL of YCT (1800 mg/L stock) into each test chamber. Archive 80 amphipods for dry weight determination. Observe behavior of test organisms.
1 to 27	Add 1.0 mL of YCT to each test beaker. Measure temperature daily, conductivity weekly, and dissolved oxygen (DO) and pH three times/week. Observe behavior of test organisms.
28	Measure temperature, dissolved oxygen, pH, hardness, alkalinity, conductivity and ammonia. End the sediment-exposure portion of the test by collecting the amphipods with a #40 mesh sieve (425-µm mesh; U.S. standard size sieve). Use four replicates for growth measurements: count survivors and preserve organisms in sugar formalin for growth measurements. Eight replicates for reproduction measurements: Place survivors in individual replicate water-only beakers and add 1.0 mL of YCT to each test beaker/d and 2 volume additions/d of overlying water.
	Reproduction Phase
29 to 35	Feed daily. Measure temperature daily, conductivity weekly, DO and pH three times a week. Measure hardness and alkalinity weekly. Observe behavior of test organisms.
35	Record the number of surviving adults and remove offspring. Return adults to their original individual beakers and add food.
36 to 41	Feed daily. Measure temperature daily, conductivity weekly, DO and pH three times a week. Measure hardness and alkalinity weekly. Observe behavior of test organisms.
41	Same as Day 1. Measure total water quality (pH, temperature, dissolved oxygen, hardness, alkalinity, conductivity, ammonia).
42	Record the number of surviving adults and offspring. Surviving adult amphipods on Day 42 are preserved in sugar formalin solution. The number of adult males in each beaker is determined from this archived sample. This information is used to calculate the number of young produced per female per replicate from Day 28 to Day 42.

A routine assessment of *in situ* toxicity and bioaccumulation requires that organisms be deployed during low flow conditions; once when the entire exposure period is at baseflow and a second time that captures a high flow event. The organisms at baseflow should be exposed for a period of time greater than or equal to the period of the high flow exposure period (usually 2 to 4 days). Another useful design is to deploy a large number of replicates on Day 0 and then subsample every 2 days for an extended period (such as 14 days). Between one and four species can be evaluated simultaneously, depending on available resources. Often two species are used in each test chamber (as described below). The *in situ* chambers are constructed of clear core sampling tubes (cellulose acetate butyrate) cut to a length of approximately 15 cm. Polyethylene closures cap each end. Two rectangular windows (~85% of the core surface area) are usually covered with 80 µm Nitex® mesh and silicon glued opposite each other. The mesh size varies with the experimental treatment, ranging from 10 to 1000 µm openings. For high flow testing, only water column chambers need be exposed. Duplicate sets of chambers having small vs. large mesh size openings (e.g., 10 vs. 250 µm) allow determinations of flow and suspended solids effects. The source of toxicity/bioaccumulation can also be measured as originating from sediments or overlying water by varying the chamber posi-

Table D.17 Test Conditions for Conducting a Long-Term Sediment Toxicity Test with *Chironomus tentans*

	Parameter	Conditions
1.	Test type:	Whole-sediment toxicity test with renewal of overlying water
2.	Temperature:	23 ± 1°C
3.	Light quality:	Wide-spectrum fluorescent lights
4.	Illuminance:	About 500 to 1000 lux
5.	Photoperiod:	16L:8D
6.	Test chamber:	300-mL high-form lipless beaker
7.	Sediment volume:	100 mL
8.	Overlying water volume:	175 mL
9.	Renewal of overlying water:	2 volume additions/d; continuous or intermittent (e.g., one volume addition every 12 h)
10.	Age of organisms:	<24-hour-old larvae
11.	Number of organisms/chamber:	10
12.	Number of replicate chambers/treatment:	16
13.	Feeding:	Tetrafin goldfish food, fed 1.5 mL daily to each test chamber (1.5 mL contains 6.0 mg of dry solids); starting Day –1
14.	Aeration:	None, unless dissolved oxygen in overlying water drops below 2.5 mg/L
15.	Overlying water:	Culture water, well water, surface water, site water, or reconstituted water
16.	Test chamber cleaning:	If screens become clogged during a test; gently brush the *outside* of the screen
17.	Overlying water quality:	Hardness, alkalinity, conductivity, and ammonia at the beginning and end of a test. Temperature daily (ideally continuously). Dissolved oxygen (DO) and pH three times/week. Concentrations of DO should be measured more often if DO has declined by more than 1 mg/L since previous measurement.
18.	Test duration:	About 40 to 50 d; each treatment is ended separately when no additional emergence has been recorded for seven consecutive days. When no emergence is recorded from a treatment, termination of that treatment should be based on the control sediment using this 7-d criterion.
19.	Endpoints:	20-d survival and AFDW; female and male emergence, adult mortality
20.	Test acceptability:	Minimum average size of *C. tentans* in the control sediment at 20 d must be at least 0.6 mg/surviving organism as dry weights or 0.48 mg/surviving organism as AFDW. Emergence should be ≥ 50%. Time to death after emergence is <6.5 d for males and <5.1 d for females.

tioning and design. Prior to chamber deployment, 10 of each organism (*H. azteca, C. tentans*, and *D. magna*) were gently added to 50-mL test tubes of culture water for ease of transport to field locations (one test tube contained one species only). Transportation of organisms to field sites by this method has proven to minimize handling and travel-related stressors (Chappie and Burton 1997). Upon acclimation, *in situ* chambers capped on one end were immersed into the river, allowing water to fill the chamber by infiltration through the mesh, and test organisms were slowly delivered from the test tubes into the open end and the chambers then capped. Before placement into *in situ* baskets, chambers were held below the water surface and purged of all internal air. Chambers exposed to the sediment interface are secured under wire baskets (see Figure 6.161) and placed with the mesh windows against the sediment. Quadruplicate chambers exposed to overlying waters are secured on top of the wire baskets. The baskets were weighted down with bricks and anchored to the stream bed with rebar. Organisms are acclimated to site water temperatures slowly (1 to 2 degree/hour) and then added to each test chamber (10 organisms/chamber). For example, *C. tentans* and *H. azteca* were placed together in replicate chambers for a total of 20 organisms per chamber. Ground-up laboratory paper toweling is provided as a substrate to reduce stress on these benthic species. Test water for laboratory controls should be the organism culture water. These controls

Table D.18 General Activity Schedule for Conducting a Long-Term Sediment Toxicity Test with *Chironomus tentans*

Day	Activity
–4	Start reproduction flask with cultured adults (1:3 male:female ratio). For example for 15 to 25 egg cases, 10 males and 30 females are typically collected. Egg cases typically range from 600 to 1500 egg/case.
–3	Collect egg cases (a minimum of 6 to 8) and incubate at 23°C.
–2	Check egg cases for viability and development.
–1	1. Check egg cases for hatch and development. 2. Add 100 mL of homogenized test sediment to each replicate beaker and place in corresponding treatment holding tank. After sediment has settled for at least 1 h, add 1.5 mL Tetrafin slurry (4g/L solution) to each beaker. Overlying water renewal begins at this time.
0	1. Transfer all egg cases to a crystallizing dish containing control water. Discard larvae that have already left the egg cases in the incubation dishes. Add 1.5 mL food to each test beaker with sediment before the larvae are added. Add 12 larvae to each replicate beaker (beakers are chosen by random block assignment). Let beakers sit (outside the test system) for 1 h following addition of the larvae. After this period, gently immerse all beakers into their respective treatment holding tanks. 2. Measure temperature, pH, hardness, alkalinity, dissolved oxygen, conductivity and ammonia at start of test.
1–End	On a daily basis, add 1.5 mL food to each beaker. Measure temperature daily. Measure the pH and dissolved oxygen three times a week during the test. If the DO has declined more than 1 mg/L since previous reading, increase frequency of DO measurements and aerate if DO continues to be less than 2.5 mg/L. Measure hardness, alkalinity, conductivity, ammonia weekly.
6	For auxiliary male production, start reproduction flask with culture adults (e.g., 10 males and 30 females; 1:3 male to female ratio).
7–10	Set up schedule for auxiliary male beakers (4 replicates/treatment) same as that described above for Day –3 to Day 0.
19	In preparation for weight determinations, ash weigh-pans at 550°C for 2 h. Note that the weigh boats should be ashed before use to eliminate weighing errors due to the pan oxidizing during ashing of samples.
20	Randomly select four replicates from each treatment and sieve the sediment to recover larvae for growth and survival determinations. Pool all living larvae per replicate and dry the sample to a constant weight (e.g., 60°C for 24 h). Install emergence traps on each reproductive replicate beakers.
21	The sample with dried larvae is brought to room temperature in a desiccator and weighed to the nearest 0.01 mg. The dried larvae in the pan are then ashed at 550°C for 2 h. The pan with the ashed larvae is then reweighed and the tissue mass of the larvae determined as the difference between the weight of the dried larvae plus pan and the weight of the ashed larvae plus pan.
23–End	On a daily basis, record emergence of males and females, pupal, and adult mortality, and time to death for previously collected adults.
33–End	Transfer males emerging from the auxiliary male replicates to individual inverted petri dishes. The auxiliary males are used for mating with females from corresponding treatments from which most of the males had already emerged or in which no males emerged.
40–End	After 7 d of no recorded emergence in a given treatment, end the treatment by sieving the sediment to recover larvae, pupae, or pupal exuviae. When no emergence occurs in a test treatment, that treatment can be ended once emergence in the control sediment has ended using the 7-d criterion.

are typically maintained in a hotel room during field assessments. The age of the organisms, handling, and culturing follow U.S. EPA toxicity test methods for short-term chronic toxicity testing. For bioaccumulation testing, additional organisms are placed to provide enough tissue mass. For the oligochaete assay, 1 to 5 g of tissue (equal to approximately 1:10 animal wet wt:sediment organic carbon) is used in each chamber, depending on analytical requirements. After exposures of 1 to 30 days depending on species and objectives, chambers were gently lifted out of the river and placed into coolers of site water and returned to the field laboratory for enumeration. Upon arrival at the lab, chambers were checked for damage, the outsides rinsed, then individually emptied into crystallizing dishes and the survivors of each species enumerated and logged. Typical measurement endpoints are shown in Table D.20.

Table D.19 *In Situ* Stressor and Sediment Toxicity Tasks and Outcomes

Task	Rationale and Outcome
1. Sediment toxicity: *H. azteca*, *C. tentans*	Laboratory measure of sediment chronic toxicity. Trigger for comprehensive sediment toxicity survey. Determine the potential for adverse effects on benthic organisms.
2. *In situ* toxicity and uptake: *D. magna, H. azteca*, *C. tentans*, *L. variegatus*	Realistic field exposures to water, suspended solids, and sediments. Determine low and high flow responses. Relate to storm flow and food web modeling. Assess the potential for, and source of, adverse effects on the ecosystem.
3. *In situ* partitioning of exposure and *D. magna, H. azteca*	In field exposures, determine and rank primary stressors: flow and stressors: turbidity, photoinduced toxicity, ammonia, metals, nonpolar organics, overlying water, pore water. Relate to transport and food web modeling. Assess the contribution and source of various stressors that produce adverse effects.
4. *In situ* assessment of bioaccumulation and transport potential: SPMDs and peepers.	In field exposures, determine presence and potential for uptake of nonpolar organics through time with SPMDs in surficial waters and pore waters. Assess the presence and transport of contaminants through time with peepers. Target side channel seepage to support transport and food web modeling.

Table D.20 *In situ* Toxicity and Bioaccumulation Measurement Endpoints

Test Organism	Endpoints
Daphnia magna	Survival (2 d)
Hyalella azteca	Survival (2, 7 d) Tissue concentration (7 d)
Chironomus tentans	Survival (2, 7 d), growth (7 d), tissue concentration (7 d)
Lumbriculus variegatus	Tissue concentration (7 d)

The effects of water quality during high flow events should be measured at all test sites. Physicochemical water quality parameters are measured as often as is practical. Preferably, continuous measures of flow and general water quality parameters are made using a data sonde-type instrument. At a minimum, however, measures are made at test initiation, then again at test termination at each field site for each of the following: temperature (°C), dissolved oxygen (mg/L), pH, hardness (mg/L $CaCO_3$), alkalinity (mg/L $CaCO_3$), turbidity (NTU), conductivity (µmhos), and flow. Samples for other potentially useful parameters, such as ammonia, pathogen indicators, BOD, and nutrients, are also collected.

Organisms sampled for tissue analyses are allowed to depurate in culture for several hours. Following that time, organisms are counted, weighed, and frozen. Tissue analyses should be conducted by a laboratory capable of low detection limits with small quantities of tissues.

TOXICITY IDENTIFICATION EVALUATIONS

The toxicity identification evaluation (TIE) is a process by which effluent or pore water samples are fractionated into various classes of contaminants and then tested for toxicity. This allows one to characterize which class of contaminants is primarily responsible for toxicity (EPA 1991a,b). These groups of contaminants include: pH-sensitive and volatile compounds (such as ammonia), metals, oxidant/reductants, and nonpolar organics. Toxicity is determined by exposing *C. dubia* for 24 hours to the various treatment fractionations and then measuring survival. A TIE was conducted following modified draft EPA guidelines for TIEs of sediments (EPA 1991b). Pore water aliquots were used for initial toxicity tests (within 24 hours of sample receipt), baseline ambient pore water, pH adjusted with aeration, pH adjusted with filtration, pH adjusted with C18 filtration, sodium thiosulfate addition, and EDTA addition fractions. If toxicity is removed in any fraction, subsequent chemical analyses will be conducted to confirm the removal of compounds which may be contributing to pore water toxicity. These manipulations and data interpretation can be quite involved and should only be conducted by a laboratory with documented experience.

TOXICITY — MICROTOX SCREENING TEST

Scope and Application

This test measures the reduction of light output at a specific time during the run by bacteria exposed to a water sample. This light output is compared to that of a control sample to calculate relative toxicity. The Microtox Screening Procedure has a range of relative toxicities between 0 and 100% (light output reduction, as compared to the control).

Summary of Method

The Microtox Screening Procedure uses a bioluminescent marine bacteria, *Photobacterium phosphoreum*, to measure the toxicity of a sample relative to a control sample at three times during the 25-min run. At each of the three reading times, the light output of each sample and each control is measured on a chart recorder and recorded as the height of the peak light output on a scale of 0 to 100.

P. phosphoreum emit light as a by-product of respiration. If a sample contains one or more components that interfere with respiration, then the bacteria's light output is reduced proportional to the amount of interference with respiration, or toxicity. The light output reduction is proportional to the toxicity of the sample. The relative toxicity of a sample to the control can then be calculated. These relative toxicities can be compared to toxicity test results using standard reagents specified by this procedure.

For samples that are calculated to be more than 50% toxic, an EC50 concentration is calculated. The EC50 concentration is the fraction of sample, using the Microtox diluent as the dilution solution, that causes a light output from the sample that is 50% of the light output of the control. It is also called the 50% effective concentration.

Sample Handling and Preservation

Glass sample containers must be clean and free of soap residues, and stoppers and lids must not be made of cork. Detergents, cork, and other materials may add chemicals to the sample and may add to its toxicity.

Tap water and distilled water are fatal to the bacteria due to high levels of chlorine. Sample storage containers must be rinsed with deionized or ultra-pure water prior to use, with ultra-pure water being preferable.

Samples should be analyzed soon after arrival at the laboratory. Until they are analyzed, samples should be stored at 4°C. Stored samples may be kept up to 1 week in the refrigerator. Freshwater samples should not be salted until the samples are ready to be analyzed, as salt–metal complexes seem to readily form, reducing the toxicity of the sample. Salted samples can only be stored for approximately 15 to 30 min.

Interferences

Samples having pH values outside the range of 6.3 to 7.8 may be toxic to the bacteria. Normally, the pH of the sample is not adjusted because pH may be the parameter causing toxicity in a natural environment. Color and turbidity will interfere with, and probably reduce, the amount of emitted light leaving the cuvette and reaching the photomultiplier. Organic matter may provide a second food source for the bacteria and may result in a sample whose relative toxicity is calculated to be less than zero.

Apparatus

- Microtox 2055 Analyzer
- 500 µL pipettor (with disposable tips)
- 10 µL pipettor (with disposable tips)
- Glass cuvettes (disposable)

Reagents

- Microtox bacterial reagent
- Microtox reconstitution solution
- Microtox diluent
- Microtox osmotic adjusting solution
- Reagent grade sodium chloride

Procedure

Sampling, Sample Preparation

Note: The older Microtox 2055 instrument has space in its incubator for 15 cuvettes. We label these positions with letters for each of the three rows (A, B, and C) and label the five columns with numbers (1 to 5), giving each position a letter and number, such as A1 for the first position and C5 for the last position. For a normal run, three of the cuvettes (A1, B1, and C1) are reserved for the control solution. One of the remaining 12 cuvettes is reserved for the standard solution whose concentration is approximately the predetermined $ZnSO_4 \cdot 7H_2O$ EC50 concentration. The remaining 11 cuvettes contain the samples to be tested using this screening procedure.

1. Rinse clean 40-mL sample vials, vial caps, and Teflon septa with ultra-pure water.
2. Mix the sample by inverting the container several times.
3. Pour 10 mL of sample into the vial.
4. Add 0.2 g NaCl (reagent grade) to the vial.
5. Mix the sample and salt by inverting the vial until the salt is completely dissolved.

Preparation of Apparatus

1. Discard the cuvettes remaining in the incubator and pre-cool slots from any prior run (used cuvettes are normally left in the incubator to reduce condensation problems).
2. Put new cuvettes into the 15 slots in the incubator and one in the pre-cool slot.
3. Pipette 1.0 mL of diluent into the cuvettes in positions A1, B1, and C1.
4. Pipette 1.0 mL of reconstitution solution into a cuvette in the pre-cool position.
5. Pipette 1.0 mL of each sample (already adjusted for salinity, as specified above) into separate cuvettes in positions A2 through A5, B2 through B5, or C2 through C5.
6. Set the timer for 5 min to allow for temperature stabilization of the reconstitution solution.
7. Get a vial of the Microtox reagent bacteria out of the freezer. (Must be stored in a freezer at no warmer than –20°C.)
8. Tap the reagent vial on the countertop gently several times to break up the contents.
9. After the 5 min temperature stabilization period has expired, open the vial.
10. Quickly, pour the reconstitution solution in the pre-cool slot into the reagent vial.
11. Swirl the contents to mix (all solid reagent should go into solution).
12. Pour the reagent solution back into the pre-cool cuvette.
13. Mix the reagent solution approximately 20 times with a 500 µL pipette.
14. Set the timer for 15 min.

Analysis of Samples

1. Pipette 10 µL of reagent solution into each cuvette in the following order: A1, B1, C1, A2 through A5, B2 through B5, and C2 through C5. Do not immerse the pipette tip in the solutions.
2. Gently mix each cuvette's contents 20 times with a 500 µL pipette. Mix the cuvettes in the same order in which reagent solution was added. Use a single pipette tip for the three controls, but a new tip for each sample and the standard.
3. Push in the "HV" and "HV Check" buttons on the front of the Microtox analyzer. The panel on the front should read between –700 and –800.
4. Push in the "HV Check" button (so it toggles back out) and push in the "Sensitivity X10" and "Run" buttons.
5. Turn on the strip chart recorder.
6. Zero the chart recorder using the knob located on the right side of the machine.
7. Make sure the speed setting is for 1 in/min.
8. Make sure the pen is touching the recorder paper by putting the pen arm down.
9. Place the cuvette in A1 into the turret and close the turret to get a reading on A1.
10. After the reading is obtained, remove the cuvette from the turret.
11. Read the cuvettes in B1 and C1 also to determine which of the three has the largest reading. Place that cuvette back in the turret and close.
12. Adjust the chart reading to between 90 and 100 using the Scan knob on the front of the analyzer. If display reads "1" (not "001"), change the sensitivity setting to "Sensitivity X1."
13. Open the turret and check the zero point again on the chart recorder. Adjust as necessary.
14. Close the turret.
15. Set the timer for 5 min.
16. When the timer rings, read the samples in the following order: A1, B1, C1, A1 through A5, B1 through B5, C1 through C5, A1, B1, and C1.
17. Place the control cuvette (A1, B1, or C1) which has the highest reading in the turret and close.
18. Set the timer for 10 min.
19. When the timer rings, read the samples in the following order: A1, B1, C1, A1 through A5, B1 through B5, C1 through C5, A1, B1 and C1.
20. Place the control cuvette (A1, B1, or C1) which has the highest reading in the turret and close.
21. Set the timer for 10 min.
22. When the timer rings, read the samples in the following order: A1, B1, C1, A1 through A5, B1 through B5, C1 through C5, A1, B1 and C1.
23. Shut off the chart recorder and cap the pen.
24. Return the C1 cuvette to the incubator and close the turret.
25. Push in the "HV" and "Turret" buttons on the front of the analyzer (toggle them off).
26. If, at the end of the test, the light output of any sample is less than half of the light output of the controls, the EC50 concentration of that sample must be found. This is done by rerunning the Microtox test using three to four dilutions of that sample (including one at 100% strength). The previously prepared (salted) sample cannot be used either to create the dilutions or as the 100% strength sample.

Calculations

At each of the three times that a sample is read, each of the three control samples is read three times. The results of these nine analyses are averaged and their standard deviation and coefficient of variation calculated. If the coefficient of variation for the control samples at any time in the run is greater than 0.05 (5%), the run is rejected.

Relative toxicity is calculated as follows:

$$\%\text{Reduction [at time t]} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100\%$$

where Control = average peak height of the control samples at t
Sample = peak height of sample at t

Precision and Accuracy

The Microtox Analyzer is calibrated using solutions of either zinc sulfate or phenol. A standard solution of approximately 10 mg/L zinc sulfate or of approximately 50 mg/L phenol is made. Four dilutions of the standard solution, with three replicates of each dilution, are used in place of the 12 samples in the normal Microtox screening procedure. The four dilutions should bracket the expected EC50 concentration of the standard solution. However, instead of using sodium chloride to adjust the ionic strength of the sample, the Microtox osmotic adjusting solution (MOAS) should be used. The amount of MOAS used should be 10% of the volume of the standard.

During each run, one of the 12 sample positions is occupied by the standard solution at the EC50 concentration. If the relative toxicity of the standard sample is outside the range of 45 to 55%, the run is rejected and repeated with freshly made standard solution. If the EC50 on the repeat again falls outside the range of 45 to 55%, the calibration is repeated. If the calibrated EC50 is significantly higher than the previous calibrations on that box of reagent, then a new box of reagent is opened and the calibration screening procedure is performed on one of the reagents in that box.

Extensive work has been done to establish the precision and accuracy of this procedure. Please refer to A. Ayyoubi, *Physical Treatment of Urban Stormwater Runoff Toxicants*, pp. 11–23.

Health and Safety Information

Refer to the MSDSs for information regarding the use of the reagents in this procedure.

None of the reagents and materials has OSHA PEL(s), AGGIH TLV(s), or other limits. Oral rat LD50 data have not been established for any of the reagents supplied by Microtox.

Sodium chloride, which is one of the reagents and is a component of most of the reagents supplied by Microtox, has an LD50 of 3000 mg/kg. The sodium chloride, either as a reagent or as a component of the other reagents, may cause eye irritation, and ingestion of large quantities may cause vomiting, diarrhea, and dehydration.

No special storage requirements are needed beyond keeping the freeze-dried bacteria culture in a freezer. Reagents are not considered to be a fire or explosion hazard (water may be used to extinguish a fire), and have no hazardous decomposition products. The reagents are stable under ordinary conditions of use and storage. Spilled reagent, whether reacted or not, may be cleaned up by adsorption with paper towels, and excess fluid may be flushed down a regular sewer drain.

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APPENDIX E

Laboratory Safety, Waste Disposal, and Chemical Analyses Methods

CONTENTS

INTRODUCTION

The laboratory safety discussion included in this appendix is summarized from the *Laboratory Safety and Standard Operating Procedures* manual prepared for use in the Water Quality Laboratories of the Department of Civil and Environmental Engineering at the University of Alabama at Birmingham. It was prepared by Shirley Clark and Robert Pitt to ensure safe laboratory practices during our research. The manual and the excerpted information in this appendix include information concerning safe laboratory practices, the use of personal protective equipment, emergency procedures, use and storage of chemicals, and the proper method of waste disposal. This manual also covers hazard communication and incident response. This information is intended to help those in the laboratory to minimize hazards to themselves and their colleagues.

In view of the wide variety of chemical products handled in laboratories, it should not be assumed that the precautions and requirements stated here are all-inclusive. This information should be updated as needed with supplementary information to better protect the health and safety of anyone working in or visiting the laboratories.

Also included in this appendix is a summary of analytical test kits that have been reviewed as to their ability to be used in the field by a variety of users. These kits were reviewed during projects funded by the EPA (Pitt et al. 1993) and by the telecommunications industry (Day 1996; Pitt and Clark 1999). In addition, comments pertaining to needed stormwater extraction methods for organic analyses are also presented, along with information pertaining to the various methods available for analyzing heavy metals. The appendix concludes with a detailed description of calibration and setup procedures for the YSI 6000 water quality sonde that is frequently referenced in the text.

FUNDAMENTALS OF LABORATORY SAFETY

Procurement of Chemicals

Before a chemical is received, information on proper handling, storage, and disposal must be known to those involved. Refer to the appropriate MSDS for further information. No container may be accepted into a laboratory without an adequate identifying label. This label cannot be removed, defaced, or damaged in any way. All substances should be received in a central location. The date of receipt should be noted on all chemicals. Receipt of all chemicals must be noted in the chemical inventory, as well as the laboratory in which the chemical shall be located.

Distribution of Chemicals

When chemicals are hand-carried between laboratories, place the chemical in an outside (secondary) container or bucket. These secondary containers provide protection to the bottle and help keep it from breaking. They also help minimize spillage if the bottle does break. It is recommended that transport of chemicals inside a building be done using a cart where feasible.

Laboratory Chemical Storage

a. Read the label carefully before storing a chemical. All chemicals must be stored according to the Chemical Storage Segregation Scheme. Note that this is a simplified scheme and that in some instances, chemicals in the same category may be incompatible.
b. Store all chemicals by their hazard class. Only within segregation groups can chemicals be stored in alphabetical order. If a chemical exhibits more than one hazard, segregate by using the characteristic that exhibits the *primary hazard*.
c. Do not store chemicals near heat sources such as ovens or steam pipes. Also, do not store chemicals in direct sunlight.
d. Date chemicals when received and first opened. This will ensure that the oldest chemicals are used first, which will decrease the amount of chemicals for disposal. If a particular chemical can become unsafe while in storage, an expiration date should also be included. Keep in mind that expiration dates set by the manufacturer do not necessarily imply that the chemical is safe to use up to that date.
e. Do not use lab benches as permanent storage for chemicals. In these locations, the chemicals can easily be knocked over, incompatible chemicals can be stored alongside one another, and the chemicals are unprotected in the event of a fire. Each chemical must have a proper designated storage location and be returned to it after use.
f. Inspect chemicals and their containers for any signs of deterioration and for the integrity of the label.
g. Do not store any chemicals in glass containers on the floor.
h. Do not use fume hoods as a permanent storage location for chemicals, with the exception of particularly odorous chemicals that may require ventilation. The more containers, boxes, equipment, and other items that are stored in a fume hood, the greater likelihood of having chemical vapors drawn back into the room.
i. Promptly dispose of any old, outdated, or unused chemicals.
j. Chemicals that require refrigeration must be sealed with tight-fitting caps and kept in lab-safe refrigerators. Lab-safe refrigerators/freezers must be used for cold storage of flammables.
k. Do not store chemicals above eye level. If the container breaks, the contents can easily fall on the face and body.
l. Do not store excessive amounts of chemicals in the lab.

Storage Cabinets

Flammable Material Storage Cabinets

Flammables not in active use must be stored in safe containers inside fire-resistant storage cabinets specifically designed to hold them. Flammable material storage cabinets must be specified for all labs that use flammable chemicals. The cabinets must meet NFPA 30 and OSHA 1910.106 standards. Flammable material storage cabinets are designed to protect the contents of the cabinet from the heat and flames of external fire rather than to confine burning liquids within. They can perform their protective function only if used and maintained properly. Cabinets are generally designed with double-walled construction and doors that are 2 in above the base (the cabinet is liquid-proof up to that point).

Acid Storage Cabinets

Acids should be kept in acid storage cabinets specifically designed to hold them. Such cabinets have the same construction features as flammable materials storage cabinets but are coated with epoxy enamel to guard against chemical attack, and use polyethylene trays to collect small spills and provide additional protection from corrosion for the shelves. Periodically check shelves and support for corrosion. Nitric acid should always be stored by itself or in a separate cabinet compartment.

BASIC RULES AND PROCEDURES FOR WORKING WITH CHEMICALS

Laboratory Protocol

Everyone in the lab is responsible for his or her own safety and for the safety of others. Before starting any work in the lab, make it a point to become familiar with the procedures and equipment that are to be used. Work only with chemical products when you know their flammability, reactivity, toxicity, safe handling, storage, and emergency procedures. If you do not understand or are unclear about something, ASK!

Personal Safety Practices

1. Lab coats and safety glasses are required of all persons in laboratories where chemicals are used. This includes visitors, as well as all laboratory personnel. Safety glasses can be found in a case just inside the door to each laboratory. Safety equipment must be donned before a person crosses the tape line separating the entryway to the lab from the working area. Personal protective equipment is only required in the areas designated.
2. Never wear shorts, short skirts, sandals, or open-toed or perforated shoes in the lab.
3. Minimize skin contact. Disposable gloves are available in all labs. Their use is recommended, especially when handling dangerous chemicals or samples whose properties are unknown. This is especially important since we often work with stormwater samples that may be contaminated by raw sewage. Wash exposed skin before leaving the laboratory.
4. Keep the work area clean and uncluttered.
5. Do not smell or taste chemicals.
6. No horseplay in laboratories. Do not engage in behavior that may distract another worker.
7. Always make sure that the exits from the laboratory are free of obstruction.
8. Do not allow children or pets in the lab.
9. Never pipette anything by mouth.
10. Be aware of dangling jewelry, loose clothing, or long hair that might get caught in the equipment.

11. Store food and drinks in refrigerators that are designated for that use only. Food and drinks shall not be carried into the work areas in the lab. Do not consume food or drinks using glassware or utensils that are used for laboratory procedures.
12. Never work alone in the lab if it is avoidable. If you must work alone, make someone aware of your location and have him or her call or check on you periodically. If you must work alone, do not use large containers of any dangerous chemical (such as acids or solvents).
13. Wash your hands frequently throughout the day and before leaving the lab for the day.
14. Do not wear contact lenses in the lab because chemicals or particulates may get caught behind them and cause severe damage to the eye.

Housekeeping

1. Work areas must be kept clean and free of unnecessary chemicals. Clean your work area throughout the day and before you leave at the end of the day.
2. If necessary, clean equipment after use to avoid the possibility of harming the next person who uses it or of contaminating his/her samples.
3. Keep all aisles and walkways in the lab clear to provide a safe walking surface and an unobstructed exit.
4. Do not block access to emergency equipment and utility controls.

Personal Protection — Protective Eyewear

1. Goggles provide the best all-around protection against chemical splashes, vapors, dusts, and mists.
2. Goggles that have indirect vents or are not vented provide the most protection, but an anti-fog agent might be needed.
3. Standard safety glasses provide protection against impact.
4. If using a laser or strong UV light sources (such as photodegradation equipment), wear safety glasses or goggles that provide protection against the specific wavelengths involved.
5. Prescription glasses are generally not appropriate in a laboratory setting. If you wear prescription glasses, either get and wear a pair of prescription safety glasses from your optician or wear the "over-the-glasses" safety glasses when working in the laboratory.
6. Contact lenses should not be worn in a laboratory because they can trap contaminants behind them and reduce or eliminate the effectiveness of flushing with water from an eyewash. Contact lenses may also increase the amount of chemicals trapped on the surface of the eye and decrease removal of the chemical by tearing. If it is necessary to wear contact lenses in a lab, wear protective goggles at all times.

Personal Protection — Protective Gloves

1. Chemicals can permeate any glove. The vapor form of the liquid chemical will break through to the skin side of the glove in most cases within a matter of minutes. The rate at which this occurs depends on the composition of the glove, the chemicals present and their concentration, and the exposure time. While for most chemicals this vapor exposure will not be particularly harmful, for some of the more toxic chemicals, it can be. In addition, once chemicals reach the skin, the glove then acts as a barrier which aids in the penetration of the chemicals through the skin. Effectively, a process called "occlusion" can occur, by which the chemical penetrates the skin more easily when trapped between the glove and the skin than if the skin were exposed without a glove. Consult glove and chemical compatibility charts (such as Table E.1) to ensure that you are using the most appropriate glove. Be sure to check the most up-to-date recommendations from the glove vendors.
2. If direct chemical contact occurs, replace gloves regularly throughout the day. Wash hands regularly and remove gloves before answering the telephone or opening doors. Make sure that hands are clean before using gloves. If chemicals have contaminated the skin prior to the glove being put on, the glove will then speed up the process of skin penetration.
3. Check gloves for cracks, tears, and holes. If the gloves are not in good condition, replace them.

Table E.1 Chemical Resistance of Glove Materials (E = Excellent, G = Good, F = Fair, P = Poor)

Chemical	Natural Rubber	Neoprene	Nitrile	Vinyl
Acetaldehyde	G	G	E	G
Acetic acid	E	E	E	E
Acetone	G	G	G	F
Acrylonitrile	P	G	N/A	F
Ammonium hydroxide	G	E	E	E
Aniline	F	G	E	G
Benzaldehyde	F	F	E	G
Benzene*	P	F	G	F
Benzyl chloride*	F	P	G	P
Bromine	G	G	N/A	G
Butane	P	E	N/A	P
Butyraldehyde	P	G	N/A	G
Calcium hypochlorite	P	G	G	G
Carbon disulfide	P	P	G	F
Carbon tetrachloride*	P	F	G	F
Chlorine	G	G	N/A	G
Chloroacetone	F	E	N/A	P
Chloroform	P	F	G	P
Chromic acid	P	F	F	E
Cyclohexane	F	E	N/A	P
Dibenzyl ether	F	G	N/A	P
Dibutyl phthalate	F	G	N/A	P
Diethanolamine	F	E	N/A	E
Diethyl ether	F	G	E	P
Dimethyl sulfoxide**	N/A	N/A	N/A	N/A
Ethyl acetate	F	G	G	F
Ethylene dichloride*	P	F	G	P
Ethylene glycol	G	G	E	E
Ethylene trichloride*	P	P	N/A	P
Fluorine	G	G	N/A	G
Formaldehyde	G	E	E	E
Formic acid	G	E	E	E
Glycerol	G	G	E	E
Hexane	P	E	N/A	P
Hydrobromic acid (40%)	G	E	N/A	E
Hydrochloric acid	G	G	G	E
Hydrofluoric acid (30%)	G	G	G	E
Hydrogen peroxide	G	G	G	E
Iodine	G	G	N/A	G
Methylamine	G	G	E	E
Methyl cellosolve	E	E	N/A	P
Methyl chloride*	P	E	N/A	P
Methyl ethyl ketone	F	G	G	P
Methylene chloride*	F	F	G	F
Monoethaloamine	F	E	N/A	E
Morpholine	F	E	N/A	E
Naphthalene*	G	G	E	G
Nitric acid	P	P	P	G
Perchloric acid	F	G	F	E
Phosphoric acid	G	E	N/A	E
Potassium hydroxide	G	G	G	E
Propylene dichloride*	P	F	N/A	P
Sodium hydroxide	G	G	G	E
Sodium hypochlorite	G	P	F	G
Sulfuric acid	G	G	F	G
Toluene*	P	F	G	F
Trichloroethylene*	P	F	G	F

Table E.1 Chemical Resistance of Glove Materials (continued) (E=Excellent, G=Good, F=Fair, P=Poor)

Chemical	Natural Rubber	Neoprene	Nitrile	Vinyl
Tricresyl phosphate	P	F	N/A	F
Triethanolamine	F	E	E	E
Trinitrotoluene	P	E	N/A	P

* Aromatic/halogenated hydrocarbons attack all types of glove. Should glove swelling occur, change to fresh gloves.
** No data available regarding resistance to DMSO by natural rubber, neoprene, nitrile, or vinyl; use butyl rubber gloves.

4. Butyl, neoprene, and nitrile gloves are resistant to most chemicals, e.g., alcohols, aldehydes, ketones, most inorganic acids, and most caustics.
5. Disposable latex and vinyl gloves protect against some chemicals, most aqueous solutions, and microorganisms, and reduce the risk of product contamination. DO NOT WEAR LATEX GLOVES IF YOU SHOW SIGNS OF A LATEX ALLERGY.
6. Leather and some knit-gloves will protect against cuts, abrasions, and scratches, but not against chemicals.
7. Temperature-resistant gloves protect against cryogenic liquids, flames, and high temperatures.
8. If the above guidelines are followed and gloves are changed frequently, particularly when liquid comes in contact with the glove, then any of the thin rubber gloves available on the market should serve general laboratory purposes.

Personal Protection — Other Protective Clothing

1. The primary purpose of a lab coat is to protect against splashes and spills. A lab coat should be nonflammable, where necessary, and easily removed.
2. Rubber-coated aprons can be worn to protect against chemical splashes and may be worn over a lab coat for additional protection.
3. Face shields can protect the face, eyes, and throat against impact, dust, particulates, and chemical splashes. However, always wear protective eyewear underneath a face shield. Always wear a face shield when handling large quantities of hazardous chemicals, such as when preparing an acid bath.
4. Shoes that fully cover the feet should always be worn in a lab. If work is going to be performed that includes moving large and heavy objects, steel-toed shoes must be worn.

Avoidance of Routine Exposure

Develop and encourage safe habits. Avoid unnecessary exposure to chemicals by any route. Do not smell or taste chemicals. Vent apparatus that may discharge toxic chemicals (e.g., vacuum pumps, microwaves) into local exhaust devices. Inspect gloves before use. Do not allow release of toxic substances in cold rooms or warm rooms, since these have contained recirculated atmospheres.

Fume Hoods

1. Use the fume hood for all procedures that might result in the release of hazardous chemical vapors or dust. Confirm that the hood is working by holding a Kimwipe® (or other lightweight paper) up to the opening of the hood. The paper should be pulled inward. Leave the hood “on” when it is not in active use if toxic substances are stored inside or if it is uncertain whether adequate general laboratory ventilation will be maintained when it is “off.”
2. Equipment and other materials should be placed at least 6 in behind the sash. This will reduce the exposure of personnel to chemical vapors that may escape into the lab due to air turbulence.
3. When the hood is not in use, pull the sash all the way down.

4. While personnel are working in the hood, pull the sash down as far as is practical. The sash is protection against fires, explosions, chemical splashes, and projectiles. Never put the sash above the line marked as the maximum allowable height for safe use.
5. Do not keep loose papers, paper towels, or tissues in the hood. These material can be drawn into the blower and adversely affect the performance of the hood.
6. Do not use a fume hood as a storage cabinet for chemicals. Excessive storage of chemicals and other items will disrupt the designed airflow in the hood. In particular, do not store chemicals against the baffle at the back of the hood because this will interfere with the laminar air flow.
7. Do not place objects directly in front of a fume hood.
8. Minimize the amount of foot traffic immediately in front of a hood. Walking past hoods causes turbulence that can draw contaminants out of the hood and into the room.

Choice of Chemicals

Use only those chemicals for which the quality of the available ventilation system is appropriate. Do not begin any experiment that requires a fume hood if the hood is not working. If the hood is not working, call Maintenance immediately.

Equipment and Glassware

1. Inspect all glassware before use. Repair or discard any broken, cracked, or chipped glassware.
2. Transport all glass chemical containers in rubber or polyethylene bottle carriers.
3. Inspect laboratory apparatus before use. Use only equipment that is free from cracks, chips, or other defects.
4. If possible, place a pan under a reaction vessel or other container to contain the liquid if the glassware breaks.
5. Do not allow burners or any other ignition source nearby when working with flammable liquids.
6. Properly support and secure laboratory apparatus before use.
7. Either work in the fume hood or ensure that the apparatus is venting to the fume hood if there is a possibility of hazardous vapors being evolved.
8. Always work in a fume hood if there is a possibility of an implosion or explosion.
9. If possible, vent vacuum pump exhaust into a fume hood.
10. When using a vacuum pump, place a trap between the pump and the apparatus.
11. Lubricate pump regularly if possible. Check belt condition and do not operate in a fume hood cabinet that is used for storage of flammables.

Labels and Signs

All hazardous chemicals are required by law to be labeled by the manufacturer. The chemical hygiene officer must ensure that each existing container and any incoming containers are properly labeled. The label must provide the following information:

- The identity of the chemical
- Any warnings
- The manufacturer's name and address

Temporary or transfer containers intended for immediate use by the person who transferred the chemical need not be labeled. However, if the chemical is left unattended (such as premade standards), the container must be labeled. Temporary labels must include:

- The identity of the chemical
- Any warnings
- The target organs affected, if applicable

Signs are intended to warn employees of chemical and physical dangers, such as designated areas where carcinogens or highly toxic chemicals are used or stored. All high hazard areas or hazardous chemical storage should be posted with the proper signs.

Unattended Operations

If an experiment/operation is left unattended, place an appropriate sign on the door and provide for containment of toxic substances in the event of equipment or utility service.

Electrical Safety

1. Examine all electrical cords periodically for signs of wear and damage. If damaged electrical cords are discovered, unplug the equipment and repair (or send the equipment out for repair).
2. Properly ground all electrical equipment.
3. If sparks are noticed while plugging in or unplugging equipment or if the cord feels hot, do not use the equipment until it has been serviced.
4. Do not run electrical cords along the floor where they will be a tripping hazard and subject to wear. If a cord must be run along the floor, protect it with a cord cover.
5. Do not run electrical cords along the floor where liquid spills may be a problem (such as around sinks).
6. Do not run electrical cords above the ceiling if possible. The cord should be visible at all times to ensure that it is in good condition.
7. Do not plug too many items into a single outlet. Multistrip plugs can be used only if they are protected with a circuit breaker and if they are not overused.
8. Do not use extension cords for permanent wiring.

USE AND STORAGE OF CHEMICALS IN THE LABORATORY

Procurement of Chemicals

Material Safety Data Sheets (MSDS) must accompany all initial incoming shipments of all chemicals. MSDSs must be readily available to all personnel in the labs where the chemicals are stored and where they are used. MSDSs shall be kept in three-ring binders near the door so that personnel can familiarize themselves with new chemicals before getting them out and using them.

Before ordering a new chemical, laboratory personnel should obtain information on proper handling, storage, and disposal methods for that chemical.

Consumer products used as they would be at home (such as dishwashing detergent) do not require an MSDS.

Sources of MSDSs include:

- Chemical supplier
- Chemical manufacturer
- Internet resources, such as the UAB Department of Occupational Health and Safety webpage http://www.healthsafe.uab.edu

Working with Allergens

A wide variety of substances can elicit skin and lung hypersensitivity. Examples include common substances such as diazomethane, chromium, nickel, bichromates, formaldehyde, isocyanates, and certain phenols. Because of this variety and the varying responses of individuals, suitable gloves should be used whenever there is a potential for contact with chemicals that may cause skin irritation.

Working with Embryotoxins

Embryotoxins are substances that cause adverse effects on a developing fetus. These effects may include embryolethality, malformations, retarded growth, and postnatal function deficits.

A few substances have been demonstrated to be embryotoxic in humans. These include:

Acrylic acid
Aniline
Benzene
Cadmium
Carbon sulfide
N,N-dimethylacetamide
Dimethylformamide
Dimethyl sulfoxide
Diphenylamine
Estradiol
Formaldehyde
Formamide
Hexachlorobenzene
Iodoacetic acid
Lead compounds
Mercury compounds
Nitrobenzene
Nitrous oxide
Phenol
Thalidomide
Toluene
Vinyl chloride
Xylene
Polychlorinated and polybrominated biphenyls

Embryotoxins requiring special controls should be stored in an adequately ventilated area. The container should be labeled in a clear manner such as the following: EMBRYOTOXIN: READ SPECIFIC PROCEDURES FOR USE. If the storage container is breakable, it should be kept in an impermeable, unbreakable secondary container having sufficient capacity to retain the material, should the primary container fail.

Working with Chemicals of Moderate or High Acute Toxicity or High Chronic Toxicity

Before beginning a laboratory operation, each worker is strongly advised to consult the standard compilations that list toxic properties of known substances and learn what is known about the substance to be used. The precautions and procedures described in this section should be followed if any of the substances to be used in significant quantities is known to be moderately or highly toxic. If any of the substances being used is known to be highly toxic, it is desirable to have two people present in the area at all times.

These procedures should be followed if the toxicological properties of any of the substances being used or prepared are UNKNOWN. If any of the substances to be used or prepared are known to have high chronic toxicity (e.g., compounds of heavy metals and other potent carcinogens), then the precautions and procedures described in this section should be supplemented with additional precautions to aid in containing and ultimately destroying the substances having high chronic toxicity.

If you are considering pregnancy, handle these substances only in a hood with a confirmed satisfactory performance, using appropriate protective apparel to prevent skin contact. If you are pregnant, notify your supervisor and consult your physician before working with these materials.

In addition to the safety protocols discussed earlier, the following three steps must be followed when working with one or more of these substances:

1. Label containers of substances having high chronic toxicity as follows: **WARNING! HIGH ACUTE OR CHRONIC TOXICITY OR CANCER SUSPECT AGENT.**
2. Protect the hands and forearms by wearing either gloves and a laboratory coat or suitable long gloves to avoid contact of the toxic material with the skin.
3. Procedures involving volatile toxic substances and those involving solid or liquid toxic substances that may result in the generation of aerosols should be conducted in a fume hood or other suitable containment device.
4. After working with toxic materials, wash the hands and arms immediately. Never eat, drink, chew gum, apply cosmetics, take medicine, or store foods in areas where toxic substances are being used.

These standard precautions will provide laboratory workers with good protection from most toxic substances. In addition, records that include amounts of material used and names of workers involved should be kept as part of the laboratory notebook record of the experiment. For strong carcinogens, an accurate record of such substances being stored and the amounts used, dates of use, and names of users must be maintained.

To minimize hazards from accidental breakage of apparatus or spills of toxic substances in the hood, containers of such substances should be stored in pans or trays made of polyethylene or other chemical-resistant material, and the apparatus should be mounted above trays of the same material. Alternatively, the working surface of the hood can be fitted with a removable liner of adsorbent, plastic-backed paper. Such procedures will make clean up of accidental spills easier. Areas where toxic substances are being used and stored must have restricted access, and warning signs should be posted if a special toxicity hazard exists. If the substance is suspected of having a high chronic toxicity, the storage area must be maintained under negative pressure with respect to its surroundings.

In general, the waste materials and solvents containing toxic substances should be stored in closed, impervious containers so that personnel handling the containers will not be exposed to their contents.

The laboratory worker must be prepared for potential accidents or spills involving toxic substances. If a toxic substance contacts the skin, the area should be washed with water. If there is a major spill outside the hood, the room or appropriate area should be evacuated and necessary measures should be taken to prevent exposures to other workers. Spills must be cleaned by personnel wearing suitable personal protective equipment.

Some examples of potent carcinogens (substances known to have high chronic toxicity), along with their corresponding chemical class, are:

Alkylating Agents:
- α-Halo ethers
  - Bis(chloromethyl)ether and chloromethyl ether
  - Methyl chloromethyl ether
- Aziridines
  - Ethylene imine
  - 2-Methylaziridine
- Diazo, azo, and azoxy compounds
  - 4-Dimethylaminobenzene
- Electrophilic alkenes and alkynes
  - Acrylonitrile
  - Acrolein
  - Ethyl acrylate
- Epoxides
  - Ethylene oxide
  - Diepoxybutane
  - Epichlorohydrin

- Propylene oxide
- Styrene oxide

Acylating Agents:

- β-Propiolactone
- Dimethylcarbamoyl chloride
- β-Butyrolactone

Organohalogen compounds:

- 1,2-Dibromo-3-chloropropane
- Vinyl chloride
- Chloroform
- Methyl iodide
- 2,4,6-Trichlorophenol
- Bis(2-chloroethyl)sulfide
- Carbon tetrachloride
- Hexachlorobenzene
- 1,4-Dichlorobenzene

Natural products:

- Adriamycin
- Bleomycin
- Progesterone
- Aflatoxins
- Reserpine
- Safrole

Inorganic compounds:

- Cisplatin

Aromatic amines:

- 4-Aminobiphenyl
- Aniline
- *o*-Anisidine
- Benzidine and derivatives
- 1,1-Bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT)
- *o*-Toluidine

Other Extremely Hazardous Chemicals:

- Arsenic, organic arsenic, and derivatives
- Arsine and gaseous derivatives
- Asbestos
- Azathioprine
- Bromodeoxyuridine
- 1,4-Butanediol dimethylsulfonate (Myleran)
- *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN)
- Chlorambucil
- Chloropicrin in gas mixtures
- Cyanogen
- Cyanogen chloride
- Cyclophosphamide
- Diborane
- Diisopropylfluorophosphate
- 9,10-Dimethyl-1,2-benzanthracene (DMBA)
- Erionite
- Germane
- Hexaethyltetraphosphate
- Hydrogen cyanide
- Hydrogen selenide
- Melphalan
- *N*-Methyl-*N*-benzylnitrosamine
- *N*-Methyl-*N*-nitrosourea

Mustard gas
2-Naphthylamine
Nitric oxide
Nitrogen dioxide
Nitrogen tetroxide
Parathion
Phosgene
Phosphine
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
Thorium dioxide

Some examples of compounds normally classified as strong carcinogens include the following:

2-Acetylaminofluorene
Benzo[*a*]pyrene
7,12-Dimethylbenz[*a*]anthracene
Dimethylcarbamoyl chloride
Hexamethylphosphoramide
3-Methylcholanthrene
2-Nitronaphthalene
Propane sultone
Various *N*-nitrosamides

The above substances (in both lists) must be used and stored in areas with restricted access. Special warning signs must be posted in these areas. Containers should be stored in chemical-resistant trays, and work must be performed within or above these trays. Cover surfaces where these substances are used with absorbent, plastic-backed paper. Performance-certified hood or other containment devices must be used when generation of toxic vapor, gases, dusts, or aerosols might occur.

Chemical Storage

The chemical storage area should be posted with an appropriate sign. Chemicals must be stored in appropriate containers and correctly labeled. Chemical compatibility must be determined to reduce the likelihood of hazardous reactions. The following steps should be followed when assessing chemical compatibility:

1. Identify the chemical
2. Determine the hazard class of the chemical: toxic, flammable, reactive, corrosive, oxidizer, low hazard.
3. Segregate the chemicals according to the above classifications. If there is a potential for hazardous interactions within a specific class, further separation is warranted. Label the area for each class of chemical.
4. General rules for compatibility:
   a. Highly toxic or carcinogenic chemicals should be ordered and stored in the smallest practical amount.
   b. Flammable or combustible liquids must be stored in approved containers, flammable material storage cabinets, or in properly designed under-hood storage areas. No more than 10 gallons of flammable liquids may be stored outside an approved flammable material storage cabinet. No more than 60 gallons of flammable liquids may be stored in a laboratory.
   c. Water-reactive chemicals should be located in a cool, dry area away from potential sources of water.
   d. Corrosives should be separated into acid and base subclasses. Large containers of corrosives should be stored on the lowest shelf or in special cabinets. Acids and bases should be separated from active metals and substances that can generate toxic gases upon contact. **NITRIC ACID MUST BE STORED SEPARATELY.**
   e. Oxidizers must be separated from combustible and flammable chemicals as well as reducing agents.

Compressed gas cylinders must be stored in well-ventilated areas where the temperature does not exceed 125°F. Cylinders must be stored in an upright position. Cylinders not in use should have the valve protection caps in place. Cylinders must be chained down to a fixed structure using the appropriate brackets and chains.

Never mix chemicals unless such mixing is part of a documented and approved procedure.

Transportation

1. All chemicals should be labeled before being transported.
2. When chemicals are hand-carried, they should be placed in an outside container or acid-carrying bucket to protect against breakage and spillage.
3. When chemicals are transported by wheeled cart, the cart should be stable under the load and have wheels large enough to negotiate uneven surfaces (such as expansion joints and floor drain depressions) without tipping or stopping suddenly. Incompatible chemicals should never be transported on the same cart.
4. Laboratory moves and transfers of large amounts of chemicals should be coordinated through the Hazardous Materials Facility.
5. Secondary containment should always be used to contain substances if there is a break in the primary container.

The following are conditions for chemical transport in elevators:

Chemicals should be labeled and carried in secure, break-resistant containers with tight-fitting caps. The packing systems supplied by manufacturers are excellent at preventing breakage during transport and may be reused for this purpose. The individual transporting the hazardous chemicals should operate the elevator alone, whenever possible.

The safe transport of small quantities of flammable liquids should include provisions that include the use of rugged, pressure-resistant, nonventing containers, storage during transport in a well-ventilated vehicle, and elimination of potential ignition sources.

If there is a spill or accident, contact the University Chemical Safety Director and state your name, telephone number, location of incident, name and quantity of material involved, and the extent of injuries, if any. Take all necessary emergency measures, such as removing contaminated clothing, washing any chemicals from the skin with soap and water, and seeking prompt medical attention. If it is necessary for the individual transporting the chemicals to leave the scene of an accident or spill, he/she should delegate someone to remain at the scene until emergency personnel arrive. The responsible party should return as soon as possible.

Cylinders that contain compressed gases are primarily shipping containers and should not be subjected to rough handling or abuse. Such misuse can seriously weaken the cylinder and render it unfit for further use or transform it into a missile with sufficient energy to propel it through masonry walls. To protect the valve during transport, the cover cap should be left screwed on hand-tight until the cylinder is in place and ready for actual use. The preferred transport method, even for short distances, is by suitable hand truck with the cylinder strapped into place. Only one cylinder should be handled at a time. After a cylinder has been relocated, straps, chains, or a suitable stand to keep it from falling must restrain it.

PROCEDURES FOR SPECIFIC CLASSES OF HAZARDOUS MATERIALS

This section will address the rules and procedures for handling chemicals that fall into one or more of five fundamental classes of laboratory chemicals: flammables, corrosives, oxidizers, reactives, and compressed gases.

Flammable Solvents

Flammable liquids are the most common chemicals found in a laboratory. The primary hazard associated with flammable liquids is their ability to readily ignite and burn. One should note that it is the vapor of a flammable liquid, not the liquid itself, which ignites and causes a fire.

The rate at which a liquid vaporizes is a function of its *vapor pressure*. In general, liquids with a high vapor pressure evaporate at a higher rate compared to liquids of lower vapor pressure. It should be noted that vapor pressure increases rapidly as the temperature rises, as does the evaporation rate. A reduced-pressure environment also accelerates the rate of evaporation.

The *flash point* of a liquid is the lowest temperature at which a liquid gives off a vapor at a rate sufficient to form an air–vapor mixture that will ignite, but will not sustain ignition. Many common flammable solvents have flash points significantly lower than room temperature.

The limits of *flammability* or *explosivity* define the range of fuel–air mixtures that will sustain combustion. The lower limit of this range is called the *lower explosive limit* (*LEL*), and the higher limit of this range is called the *upper explosive limit* (*UEL*). Materials with very broad flammability ranges are particularly treacherous due to the fact that virtually any fuel–air combination may form an explosive atmosphere.

The *vapor density* of a flammable material is the density of the corresponding vapor relative to air under specific temperature and pressure conditions. Flammable vapors with densities greater than one (and thus "heavier" than air) are potentially lethal because they will accumulate at floor level and flow with remarkable ease, in much the same manner that a liquid would. The obvious threat is that these mobile vapors may eventually reach an ignition source, such as an electrical outlet or a lit Bunsen burner.

Examples of Flammable Liquids

Acetone
Ethyl ether
Toluene
Methyl formate

Use and Storage of Flammables

1. Flammable liquids that are not in active use must be stored in safe containers inside fire-resistant storage cabinets designed for flammables, or inside storage rooms.
2. Minimize the amount of flammable liquids stored in the lab.
3. Use flammables only in areas free of ignition sources.
4. Never heat flammables with an open flame. Instead, use steam baths, water baths, oil baths, hot air baths, sand baths, or heating mantles.
5. Never store flammable chemicals in a standard household refrigerator. There are several ignition sources located inside a standard refrigerator that can set off a fire or violent explosion. Flammables can only be stored cold in a lab safe or explosion-proof refrigerator. Another alternative is to use an ice bath to chill the chemicals. Remember, there is no safety benefit in storing a flammable chemical in a refrigerator if the flash point of that chemical is below the temperature of that refrigerator.
6. The transfer of material to or from a metal container is generally accompanied by an accumulation of static charge on the container. This fact must be kept in mind when transferring flammable liquids, since the discharge of this static charge could generate a spark, thereby igniting the liquid. To make these transfers safer, flammable liquid dispensing and receiving containers must be bonded together before pouring. Large containers such as drums must also be grounded when used as dispensing or receiving vessels. All grounding and bonding connections must be metal to metal.

Health Effects Associated with Flammables

In general, the vapors of many flammables are irritating to mucous membranes of the respiratory system and eyes, and in high concentrations are narcotic. The following symptoms are typical for the respective routes of entry:

Acute Health Effects:
- Inhalation — headache, fatigue, dizziness, drowsiness, narcosis (stupor and unresponsiveness)
- Ingestion — slight gastrointestinal irritation, dizziness, fatigue
- Skin Contact — dry, cracked, and chapped skin
- Eye Contact — stinging, watery eyes, inflammation of the eyelids

Chronic Health Effects:
- The chronic health effects will vary depending on the specific chemical, the duration of the exposure, and the extent of the exposure. However, damage to the lungs, liver, kidneys, heart, and/or central nervous system may occur. Cancer and reproductive effects are also possible.

Flammable Groups Exhibiting These Health Effects:
- Hydrocarbons — aliphatic hydrocarbons are narcotic but their systemic toxicity is relatively low. Aromatic hydrocarbons are all potential narcotic agents, and overexposure to the vapors can lead to loss of muscular coordination, collapse, and unconsciousness. Benzene is toxic to bone marrow and can cause leukemia.
- Alcohols — vapors are only moderately narcotic.
- Ethers — exhibit strong narcotic properties but for the most part are only moderately toxic.
- Esters — vapors may result in irritation to the eyes, nose, and upper respiratory tract.
- Ketones — systemic toxicity is generally not high.

First-Aid Procedures for Exposures to Flammable Materials

Inhalation Exposure — remove person from contaminated area if it is safe to do so. Get medical attention and do not leave person unattended.

Ingestion Exposures — remove the person, if possible, from source of contamination. Get medical attention.

Dermal Exposures — remove person from source of contamination. Remove clothing, jewelry, and shoes from the affected areas. Flush the affected areas with water for at least 15 min and obtain medical attention.

Eye Contact — remove person from source of contamination. Flush the eyes with water for at least 15 min. Obtain medical attention.

Personal Protective Equipment

Always use a fume hood while working with flammable liquids. Nitrile and neoprene gloves are effective against most flammables. Wear a nonflammable lab coat to provide a barrier to your skin, and goggles if splashing is likely to occur.

Oxidizers

Oxidizers or oxidizing agents present fire and explosion hazards on contact with combustible materials. Depending on the class, an oxidizing material may increase the burning rate of combustibles with which it comes in contact; cause the spontaneous ignition of combustibles with which it comes in contact; or undergo an explosive reaction when exposed to heat, shock, or friction. Oxidizers are generally corrosive.

Examples of Common Oxidizers

Peroxides
Nitrites
Nitrates
Chlorates
Perchlorates
Chlorites
Hypochlorites
Dichromates

Use and Storage of Oxidizers

1. In general, store oxidizers away from flammables, organic compounds, and combustible materials.
2. Strong oxidizing agents like chromic acid should be stored in glass or some other inert container, preferably unbreakable. Corks and rubber stoppers should not be used.
3. Reaction vessels containing appreciable amounts of oxidizing materials should never be heated in oil baths, but rather on a heating mantle or sand bath.

Use and Storage of Perchloric Acid

1. Perchloric acid is an oxidizing agent of particular concern. The oxidizing power of perchloric acid increases as concentration and temperature increase. Cold, 70% perchloric acid is a strong, nonoxidizing corrosive. A 72% perchloric acid solution at elevated temperatures is a strong oxidizing agent. An 85% perchloric acid solution is a strong oxidizer at room temperature.
2. Do not attempt to heat perchloric acid if you do not have access to a properly functioning perchloric acid fume hood. Perchloric acid can only be heated in a hood specially equipped with a wash down system to remove any perchloric acid residue. The hood should be washed down after each use and it is preferred to dedicate the hood to perchloric acid use only.
3. Whenever possible, substitute a less hazardous chemical for perchloric acid.
4. Perchloric acid can be stored in a perchloric acid fume hood. Keep only the minimum amount necessary for your work. Another acceptable storage site for perchloric acid is on a metal shelf or in a metal cabinet away from organic or flammable materials. A bottle of perchloric acid should also be stored in a glass secondary container to contain leakage.
5. Do not allow perchloric acid to come in contact with any strong dehydrating agents such as sulfuric acid. The dehydration of perchloric acid is a severe fire and explosion hazard.
6. Do not order or use anhydrous perchloric acid. It is unstable at room temperature and can decompose spontaneously with a severe explosion. Anhydrous perchloric acid will explode upon contact with wood.

Health Effects Associated with Oxidizers

Oxidizers are covered here primarily due to their potential to add to the severity of a fire or to initiate a fire. But there are some generalizations that can be made regarding the health hazards of an oxidizing material. In general, oxidizers are corrosive and many are highly toxic.

Acute Health Effects

Some oxidizers, such as nitric and sulfuric acid vapors, chlorine, and hydrogen peroxide, act as irritant gases. All irritant gases can cause inflammation in the surface layer of tissues when in direct contact. They can also cause irritation of the upper airways, conjunctiva, and throat.

Some oxidizers, such as fluorine, can cause severe burns of the skin and mucous membranes. Chlorine trifluoride is extremely toxic and can cause severe burns to tissue.

Nitrogen trioxide is very damaging to tissue, especially the respiratory tract. The symptoms from an exposure to nitrogen trioxide may be delayed for hours, but fatal pulmonary edema may result.

Osmium tetroxide, another oxidant commonly employed in the laboratory, is also dangerous due to its high degree of acute toxicity. It is a severe irritant of both the eyes and the respiratory tract. Inhalation can cause headache, coughing, dizziness, lung damage, difficulty breathing, and may be fatal.

Chronic Health Effects

Nitrobenzene and chromium compounds can cause hematological and neurological changes. Compounds of chromium and manganese can cause liver and kidney disease. Chromium (VI) compounds have been associated with lung cancer.

First Aid for Oxidizers

In general, if a person has inhaled, ingested, or come into direct contact with these materials, the person must be removed from the source of contamination as quickly as possible when it is safe to do so. Medical help must be summoned. In the case of an exposure directly to the skin or eyes, it is imperative that the exposed person be taken to an emergency shower or eyewash immediately. Flush the affected areas for a minimum of 15 minutes and then get medical attention.

Personal Protective Equipment

1. In many cases, the glove of choice will be neoprene, polyvinyl chloride (PVC), or nitrile. Be sure to consult a glove compatibility chart to ensure that the glove material is appropriate for the particular chemical you are working with.
2. Goggles must be worn if the potential for splashing exists or if exposure to vapor or gas is likely.
3. Always use these materials in a chemical fume hood as most pose a hazard via inhalation.

Corrosives

General Characteristics

1. Corrosives are most commonly acids or alkalis, but many other materials can be severely damaging to living tissue.
2. Corrosives can cause visible destruction or irreversible alterations at the site of contact. Inhalation of the vapor or mist can cause severe bronchial irritation. Corrosives are particularly damaging to the skin and eyes.
3. Certain substances considered noncorrosive in their natural dry state are corrosive when wet, such as when in contact with moist skin or mucous membranes. Examples of these materials are lithium chloride, halogen fluorides, and allyl iodide.
4. Sulfuric acid is a very strong dehydrating agent and nitric acid is a strong oxidizing agent. Dehydrating agents can cause severe burns to the eyes due to their affinity for water.

Examples of Corrosives

Sulfuric acid
Chromic acid
Stannic chloride
Ammonium bifluoride
Bromine
Ammonium hydroxide

Use and Storage of Corrosives

1. Always store acids separately from bases. Also, store acids in acid storage cabinets away from flammables since many acids are also strong oxidizers.
2. Do not work with corrosives unless an emergency shower and continuous flow eyewash are available.
3. Add acid to water, but never water to acid. This is to prevent splashing from the acid due to the generation of excessive heat as the two substances mix.
4. Never store corrosives above eye level. Store on a low shelf or cabinet.
5. It is a good practice to store corrosives in a tray or bucket to contain any leakage.
6. When possible, purchase corrosives in containers that are coated with a protective plastic film that will minimize the danger to personnel if the container is dropped.
7. Store corrosives in a wood cabinet or one that has a corrosion-resistant lining. Corrosives stored in an ordinary metal cabinet will quickly damage it. If the supports that hold up the shelves become corroded, the result could be serious. Acids should be stored in acid storage cabinets specially designed to hold them, and nitric acid should be stored in a separate cabinet or compartment.

Use and Storage of Hydrofluoric Acid

1. Hydrofluoric acid is extremely hazardous. Hydrofluoric acid can cause severe burns, and inhalation of anhydrous hydrogen fluoride can be fatal.
2. Initial skin contact with hydrofluoric acid may not produce any symptoms.
3. Only persons fully trained in the hazards of hydrofluoric acid should use it.
4. Always use hydrofluoric acid in a properly functioning fume hood. Be sure to wear personal protective clothing.
5. If you suspect that you have come in direct contact with hydrofluoric acid: wash the area with water for at least 15 minutes, remove clothing, and then promptly seek medical attention. If hydrogen fluoride vapors are inhaled, move the person immediately to an uncontaminated atmosphere (if safe to do so), keep the person warm, and seek prompt medical attention.
6. NEVER STORE HYDROFLUORIC ACID IN A GLASS CONTAINER BECAUSE IT IS INCOMPATIBLE WITH GLASS.
7. Store hydrofluoric acid separately in an acid storage cabinet and keep only the amount necessary in the lab.
8. Creams for treatment of hydrofluoric acid exposure are commercially available and should be kept on site.

Health Effects Associated with Corrosives

All corrosives are severely damaging to living tissues and also attack other materials, such as metal.

Skin contact with alkali metal hydroxides, e.g., sodium hydroxide and potassium hydroxide, is more dangerous than with strong acids. Contact with alkali metal hydroxides normally causes deeper tissue damage because there is less pain than with an acid exposure. The exposed person may not wash it off thoroughly enough or seek prompt medical attention.

All hydrogen halides are acids that are serious respiratory irritants and also cause severe burns. Hydrofluoric acid is particularly dangerous. At low concentrations, hydrofluoric acids do not immediately show any signs or symptoms upon contact with skin. It may take several hours for the hydrofluoric acid to penetrate the skin before you would notice a burning sensation. However, by this time permanent damage, such as second and third degree burns with scarring, can result.

Acute Health Effects

Inhalation — irritation of mucous membranes, difficulty in breathing, fits of coughing, pulmonary edema
Ingestion — irritation and burning sensation of lips, mouth, and throat; pain in swallowing; swelling of the throat; painful abdominal cramps; vomiting; shock; risk of perforation of stomach
Skin Contact — burning, redness and swelling, painful blisters, profound damage to tissues; and with alkalis, a slippery, soapy feeling
Eye Contact — stinging, watery eyes, swelling of eyelids, intense pain, ulceration of eyes, loss of eyes or eyesight

Chronic Health Effects

Symptoms associated with a chronic exposure vary greatly depending on the chemical. The chronic effect of hydrochloric acid is damage to the teeth; the chronic effects of hydrofluoric acid are decreased bone density, fluorosis, and anemia; the chronic effects of sodium hydroxide are unknown.

First Aid for Corrosives

Inhalation — remove person from source of contamination if safe to do so. Get medical attention. Keep person warm and quiet and do not leave unattended.
Ingestion — remove person from source of contamination if safe to do so. Get medical attention and inform emergency responders of the name of the chemical swallowed.
Skin Contact — remove person from source of contamination if safe to do so and take immediately to an emergency shower or source of water. Remove clothing, shoes, socks, and jewelry from affected areas as quickly as possible, cutting them off if necessary. Be careful to not get any chemical on your skin or to inhale the vapors. Flush the affected area with water for a minimum of 15 minutes. Get medical attention.
Eye Contact — remove person from source of contamination if safe to do so and take immediately to an eyewash or source of water. Rinse the eyes for a minimum of 15 minutes. Have the person look up and down and from side to side. Get medical attention. Do not let the person rub the eyes or keep them tightly shut.

Personal Protective Equipment

Always wear proper gloves when working with acids. Neoprene and nitrile gloves are effective against most acids and bases. Polyvinyl chloride (PVC) is also effective for most acids. A rubber-coated apron and goggles should also be worn. If splashing is likely to occur, wear a face shield over the gloves. Always use corrosives in a chemical fume hood.

Reactives

General Characteristics

Polymerization Reactions

Polymerization is a chemical reaction in which two or more molecules of a substance combine to form repeating structural units of the original molecule. This can result in an extremely high or uncontrolled release of heat. An example of a chemical that can undergo a polymerization reaction is styrene.

Water-Reactive Molecules

When water-reactive materials come in contact with water, one or more of the following can occur:

- Liberation of heat, which may cause ignition of the chemical itself if it is flammable, or ignition of flammables that are stored nearby
- Release of a flammable, toxic, or strong oxidizing gas; release of metal oxide fumes
- Formation of corrosive acids

Water-reactive chemicals can be particularly hazardous to firefighting personnel responding to a fire in a lab, because water is the most commonly used fire-extinguishing medium. Examples of water-reactive materials:

Alkali metals: lithium, sodium, potassium
Magnesium
Silanes
Alkylaluminums
Zinc
Aluminum

Pyrophoric material can ignite spontaneously in the presence of air. Examples of pyrophoric materials:

Diethylzinc
Triethylaluminum
Many organometallic compounds

Peroxide-Forming Materials

Peroxides are very unstable and some chemicals that can form them are commonly used in laboratories. This makes peroxide-forming materials some of the most hazardous substances found in a lab. Peroxide-forming materials are chemicals that react with air, moisture, or impurities to form peroxides. The tendency to form peroxides by most of these materials is greatly increased by evaporation or distillation. Organic peroxides are extremely sensitive to shock, sparks, heat, friction, impact, and light. Many peroxides formed from materials used in laboratories are more shock sensitive than TNT. Just the friction from unscrewing the cap of a container of ether that has peroxides in it can provide enough energy to cause a severe explosion.

Examples of peroxide-forming materials:

Diisopropyl ether
Sodium amide
Dioxane
Tetrahydrofuran
Butadiene
Acrylonitrile
Divinylacetylene
Potassium amide
Diethyl ether
Vinyl ethers
Vinylpyridine
Styrene

Other Shock-Sensitive Materials

These materials are explosive and sensitive to heat and shock. Examples of shock-sensitive materials:

Chemicals containing nitro groups
Fulminates
Hydrogen peroxide (30+%)
Ammonium perchlorate
Benzoyl peroxide (when dry)
Compounds containing the functional groups: acetylide, azide, diazo, halamine, nitroso, and ozonide

Use and Storage of Reactives

1. A good way to reduce the potential risks is to minimize the amount of material used in the experiment. Use only the amount of material necessary to achieve the desired results.
2. Always substitute a less hazardous chemical for a highly reactive chemical whenever possible. If it is necessary to use a highly reactive chemical, order only the amount that is necessary for the work.
3. Store water-reactive materials in an isolated part of the lab. A cabinet far removed from any water sources, such as sinks, emergency showers, and chillers, is an appropriate location. Clearly label the cabinet "**Water-Reactive Chemicals — No Water**."
4. Store pyrophorics in an isolated part of the lab and in clearly marked cabinets. Be sure to routinely check the integrity of the container and dispose of materials in corroded or damaged containers.
5. Do not open the chemical container if peroxide formation is suspected. The act of opening the container could be sufficient to cause a severe explosion. Visually inspect liquid peroxide-forming materials for crystals or unusual viscosity before opening. Pay special attention to the area around the cap. Peroxides usually form upon evaporation, so they will most likely be formed on the threads under the cap.
6. Date all peroxide-forming materials with the date received and the expected shelf life. Chemicals such as diisopropyl ether, divinyl acetylene, sodium amide, and vinylidene chloride should be discarded after 3 months. Chemicals such as dioxane, diethyl ether, and tetrahydrofuran should be discarded after 1 year.
7. Store all peroxide-forming chemicals away from heat, sunlight, and sources of ignition. Sunlight accelerates the formation of peroxides.
8. Secure the lids and caps on these containers to discourage the evaporation and concentration of these chemicals.
9. Never store peroxide-forming chemicals in glass containers with screw cap lids or glass stoppers. Friction and grinding must be avoided. Also, never store these chemicals in a clear glass bottle where they would be exposed to light.
10. Contamination of an ether by peroxides or hydroperoxides can be detected simply by mixing the ether with 10% (w/w) aqueous potassium iodide solution — a yellow color change due to oxidation of iodide to iodine confirms the presence of peroxides. Small amounts of peroxides can be removed from contaminated ethers via distillation from lithium aluminum hydride ($LiAlH_4$), which both reduces the peroxide and removes contaminating water and alcohols. However, if you suspect that peroxides may be present, it is wise to dispose of the material. If you notice crystal formation in the container or around the cap, do not attempt to open or move the container.
11. Never distill an ether unless it is known to be free of peroxides.
12. Store shock-sensitive materials separately from other chemicals and in a clearly labeled cabinet.
13. Never allow picric acid to dry out, as it is extremely explosive. Always store picric acid in a wetted state.

Health Hazards Associated with Reactives

Reactive chemicals are grouped as a category primarily because of the safety hazards associated with their use and storage and not because of similar acute or chronic health effects. For health hazard information on specific reactive materials, consult the MSDS or the manufacturer. However,

there are some hazards common to the use of reactive materials. Injuries can occur due to heat or flames, inhalation of fumes, vapors and reaction products, and flying debris.

First Aid for Reactives

If someone is seriously injured, the most important step is to contact emergency responders as quickly as possible. Explain the situation and describe the location clearly and accurately.

If someone is bleeding severely, apply a sterile dressing, clean cloth, or handkerchief to the wound. Then put protective gloves on and place the palm of your hand directly over the wound and apply pressure and keep the person calm. Continue to apply pressure until help arrives.

If a person's clothes are on fire, he or she should drop immediately to the floor and roll. If a fire blanket is available, put it over the individual. An emergency shower, if one is immediately available, can also be used to douse the flames.

If a person goes into shock, have the individual lie down on his/her back, if safe to do so, and raise the feet about 1 ft above the floor.

Personal Protective Equipment

Wear appropriate personal protective clothing while working with highly reactive materials. This might include impact-resistant safety glasses or goggles, a face shield, gloves, a lab coat (to minimize injuries from flying glass or an explosive flash), and a shield. Conduct work within a chemical fume hood as much as possible and pull down the sash as far as is practical. When the experiment does not require you to reach into the fume hood, keep the sash closed.

Barriers can offer protection of personnel against explosion and should be used. Many safety catalogs offer commercial shields that are commonly polycarbonate and are weighted at the bottom for stability. It may be necessary to secure the shields firmly to the work surface.

Compressed Gas Cylinders

Cylinders of compressed gas can pose a chemical as well as a physical hazard. If the valve were to break off a cylinder, the amount of force present could propel the cylinder through a block wall. For example, a small cylinder of compressed breathing air used by SCUBA divers has the explosive force of 1.5 lb of TNT.

Use and Storage of Compressed Gas Cylinders

1. Whenever possible, use flammable and reactive gases in a fume hood or other well-ventilated enclosure. Certain categories of toxic gases must always be stored and used in well-ventilated enclosures.
2. Always use the appropriate regulator on a cylinder. If a regulator will not fit a cylinder's valve, do not attempt to adapt or modify it to fit a cylinder it was not designed for. Regulators are designed to fit only specific cylinders to avoid improper use.
3. Inspect regulators, pressure-relief valves, cylinder connections, and hose lines frequently for damage.
4. Never use a cylinder that cannot be positively identified. Color-coding is not a reliable way to identify cylinders since the color can vary from supplier to supplier.
5. Do not use oil or grease on any cylinder component of an oxidizing gas because a fire or explosion can result.
6. Never transfer gases from one cylinder to another. The gas may be incompatible with the residual gas remaining in the cylinder or may be incompatible with the cylinder material.
7. Never completely empty cylinders during lab operations; rather, leave approximately 25 PSI of pressure. This will prevent any residual gas in the cylinder from becoming contaminated.

8. Place all cylinders so the main valve is accessible.
9. Close the main cylinder valve whenever the cylinder is not in use.
10. Remove regulators from unused cylinder and always put the safety cap in place to protect the valve.
11. Always secure cylinder, whether empty or full, to prevent it from falling over and damaging the valve (or falling on your foot). Secure cylinders by chaining or strapping them to a wall, lab bench, or other fixed support.
12. Oxygen should be stored in an area that is at least 20 feet away from any flammable or combustible materials or separated from them by a noncombustible barrier at least 5 ft high and having a fire-resistant rating of at least $^1/_2$ hour.
13. To transport a cylinder, put on the safety cap and strap the cylinder to a hand truck in an upright position. Never roll a cylinder.
14. Always clearly mark empty cylinders and store them separately (using chalk to write "MT" on a cylinder in big letters is satisfactory for noting an empty cylinder).
15. Open cylinder valves slowly.
16. Only compatible gases should be stored together in a gas cylinder cabinet.
17. Flammable gases must be stored in properly labeled, secured areas away from possible ignition sources and kept separate from oxidizing gases.
18. Do not store compressed gas cylinders in areas where the temperature can exceed 125°F.

EMERGENCY PROCEDURES

All accidents, hazardous materials spills, or other dangerous incidents should be reported. A list of telephone numbers must be posted on the door to each laboratory (and must be kept up to date). Telephone numbers shall also be posted beside every telephone in the laboratories. The list of telephone numbers must include 24-hour numbers for the following personnel:

Laboratory Supervisor
Principal Investigator(s)
Emergency Medical Services
Police Department
Maintenance
Chemical Response Unit

Callers should explain any emergency situation clearly, calmly, and in detail.

Primary Emergency Procedures for Fires, Spills, and Accidents

1. In the event of a fire, pull the nearest fire alarm. If you are in the laboratory and a fire alarm sounds, quickly secure your work (cap bottles, etc.) so that it is not dangerous to a passer-by, lock the laboratory, and evacuate the building per the fire evacuation instructions. If the emergency is not in the laboratory where you are located, the last person to leave should turn off the lights.
2. If you are unable to control or extinguish a fire, follow the building evacuation procedure.
3. Attend to any person who may have been contaminated and/or injured if it is safe to reach them.
4. Use safety showers and eye washes as appropriate. In the case of eye contact, promptly flush eyes with water for a minimum of 15 minutes and seek immediate medical attention. For ingestion cases, contact the Poison Control Center at 1-800-POISON1. In the case of skin contact, promptly flush the affected area with water and remove any contaminated clothing or jewelry. If symptoms persist after washing, seek medical attention.
5. Notify persons in the immediate area about the spill, evacuating all nonessential personnel from the spill area and adjoining areas that may be impacted by vapors or a potential fire.
6. If the spilled material is flammable, turn off all potential ignition sources. Avoid breathing vapors of the spilled materials. Be aware that some materials either have no odor or create olfactory fatigue, so that you stop smelling the odor very quickly.

7. Leave on or establish exhaust ventilation if it is safe to do so. Close doors to slow the spread of odors.
8. Notify the appropriate authorities (Laboratory Supervisor, Principal Investigator, Chemical Health and Safety) about the spill and the required documentation.
9. IF THERE IS AN IMMEDIATE THREAT TO LIFE OR HEALTH, call Emergency Services at 911.

Building Evacuation Procedures

1. Building evacuation may be necessary if there is a chemical release, fire, explosion, natural disaster, or medical emergency.
2. Be aware of the marked exits from your area and building.
3. To activate the building alarm system, pull the handle on one of the red boxes located in the hallway.
4. Call the appropriate authorities.
5. Walk quickly to the nearest marked exit and ask others to do the same.
6. Outside, proceed to a clear reassembly area that is at least 150 ft from the affected building and that does not interfere with the work of emergency personnel.
7. DO NOT RETURN TO THE BUILDING UNTIL YOU ARE TOLD THAT IT IS SAFE TO DO SO.

Minor Spills

1. Trained personnel should use the spill control kit appropriate to the material spilled to clean up the spill.
2. If the spill is minor and of known limited danger, clean it up immediately. Determine the appropriate cleaning method by referring to the material's MSDS. During cleanup, wear the appropriate protective gear.
3. Cover liquid spills with compatible adsorbent material such as spill pillows or a kitty litter/vermiculite mix, if it is compatible. If appropriate materials are available, corrosives should be neutralized prior to adsorption. Clean spills from the outer area first, cleaning toward the center.
4. Place the spilled material into an appropriate impervious container and seal. Schedule its disposal.
5. If appropriate, wash the affected surface with soap and water. Mop up the residues and place them in an appropriate container for disposal.
6. If the spilled material is not water soluble, a solvent such as xylene may be necessary to clean the surface(s). Check the solubility of the spilled material in various solvents and use the least toxic effective solvent available. Wear appropriate personal protective equipment.
7. Notify the Laboratory Supervisor about the need to replace the used items from the spill control kit.

Mercury Spills

Mercury is commonly used in many technical procedures. When contained properly, it is of little threat to our health. Immediate attention to mercury spills is important because spilled mercury can accumulate over time, resulting in exposure to mercury vapor.

When a spill occurs, use the following procedure:

1. Restrict the area. Allow no one to enter the room except for trained personnel to help with containment of the spill.
2. Contact the Chemical Safety Director.
3. Broken thermometers that contain small amounts of mercury may be safely collected by trained laboratory personnel in a container that can be sealed. Always wear disposable gloves when cleaning up mercury and dispose of all mercury and mercury contaminated waste through the chemical waste program. Anyone handling mercury or cleaning up mercury spills should wash hands thoroughly using soap and water when finished. Report all mercury spills to the Chemical Safety Director.

CHEMICAL WASTE DISPOSAL PROGRAM

Chemical Waste Containers

Containers used for the accumulation of hazardous waste must be in good condition, free of leaks and compatible with the waste being stored in them. A waste accumulation container should be opened only when it is necessary to add waste, and should otherwise be capped. Hazardous waste must not be placed in unwashed containers that previously held incompatible materials.

If a hazardous waste container is not in good condition (i.e., it leaks), either transfer the waste from the bad container into a good container, pack the container in a larger and nonleaking container, or manage the waste in some other way that prevents the potential for a release of contamination.

A storage container holding a hazardous waste that is incompatible with any waste or other materials stored nearby in other containers must be separated from the other materials or protected from them by means of a wall, partition, or other secondary containment device.

Guidelines for Waste Containers

- Must be marked with the words "waste" or "spent" and its contents indicated. NO container should be marked with the words "hazardous" or "nonhazardous." Paint over or remove old labels from waste containers.
- Must be kept at or near (immediate vicinity) the site of generation and under control of the generator.
- Must be compatible with the contents (i.e., acid should not be stored in metal cans).
- Must be closed at all times except when actively receiving waste.
- Must be properly identified before disposal.
- Must be safe to transport with nonleaking screw-on caps.
- Must be filled to a safe level (not beyond the bottom of the neck of the container or a 2-in headspace for a 55-gallon drum).

NOTE: Do not use RED BAGS or SHARPS CONTAINERS (Biohazard) for hazardous waste collection.

Labeling Containers

Before chemicals can be disposed of, a waste tag is required. It should be filled out by the waste generator and attached to each container. The information on the tag is used to categorize and treat the waste. A manifest is also required. Fill out all paperwork legibly, accurately, and completely.

Waste Minimization

Avoid purchasing and using large quantities when it is not necessary. Implement microscale techniques whenever possible.

Flammable Organic Solvents

Collection for Reuse

Many flammable organics can be reused for fuel unless they are extremely toxic or give off toxic products of combustion. *Do not* combine any other chemicals with the flammable organic solvents listed below. *Halogenated solvents* (solvents containing chlorine, fluorine, or bromine), acutely toxic flammables, acids, bases, heavy metals, oxidizers, and pesticides should be collected

in separate containers. The following is a list of the most frequently encountered compounds that are suitable for heat recovery:

Acetone	Methyl alcohol
2-Butanol	Methyl cellosolve
Butyl alcohol	Pentane
Cyclohexane	Petroleum ether
Diethyl ether	2-Propanol
Ethyl acetate	Sec-butyl alcohol
Ethyl alcohol	Tert-butyl alcohol
Heptane	Tetrahydrofuran
Hexane	Xylene

Disposal of Chemicals down the Sink or Sanitary Sewer System

Very few chemical wastes produced in laboratories are acceptable for disposal down the sink or sanitary sewer system. The local Sewer Use/Pretreatment Ordinance establishes uniform requirements for all users of the wastewater treatment system. Many chemicals can interfere with the proper function of the treatment facility and can render them unable to comply with state and federal regulations under the Clean Water Act of 1977.

Generators of laboratory waste are advised to exercise caution with respect to sink disposal of chemical wastes. In general, small-scale research activities (100 mL or less) of certain types of water-soluble, nontoxic, and nonflammable chemicals may be poured if they have been approved by the Chemical Safety Director. It is recommended that such materials be disposed of through the Department of Occupational Health and Safety, even in small quantities.

Chemical Substitution

Whenever possible, it is desirable to substitute nonhazardous, biodegradable chemicals for hazardous chemicals. Use of these chemicals will reduce the volume of hazardous waste generated. Examples of acceptable substitutes include:

1. Citric acid-based cleaning solutions for xylene-, benzene-, and toluene-containing cleaning solutions.
2. Nonhalogenated solvents in parts washers or other solvent processes.
3. Detergent and enzymatic cleaners can be substituted for sulfuric acid/potassium dichromate (chromerge) cleaning solutions and ethanol/potassium hydroxide cleaning solutions.

Neutralization and Deactivation

Certain hazardous chemical wastes can be rendered nonhazardous by specific neutralization or deactivation laboratory procedures. Contact the Chemical Safety Officer to see if the waste you generate is suitable for neutralization.

Elimination of Nonhazardous Waste from Hazardous Waste

The following items *are not* considered to be hazardous. They should be collected in disposable containers or plastic bags, clearly labeled as nonhazardous waste, and put in the wastebasket. All compounds identified by the two letter code "NH" are nonhazardous and should not be disposed of via the chemical waste program unless they are components of a mixture with hazardous materials or are suitable for chemical recycling.

Nonhazardous Waste

Organic Chemicals

Acetates: calcium (Ca), sodium (Na), ammonium (NH_4), and potassium (K)
Amino acids and their salts
Citric acid and salts of sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), and ammonium (NH_4)
Lactic acid and salts of sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), and ammonium (NH_4)
Sugars: glucose, lactose, fructose, sucrose, maltose

Inorganic Chemicals

Bicarbonates: sodium (Na), potassium (K)
Borates: sodium (Na), potassium (K), magnesium (Mg), calcium (Ca)
Bromides: sodium (Na), potassium (K)
Carbonates: sodium (Na), potassium (K), magnesium (Mg), calcium (Ca)
Chlorides: sodium (Na), potassium (K), magnesium (Mg), calcium (Ca)
Fluorides: calcium (Ca)
Iodides: sodium (Na), potassium (K)
Oxides: boron (B), magnesium (Mg), calcium (Ca), aluminum (Al), silicon (Si), iron (Fe)
Phosphates: sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), ammonium (NH_4)
Silicates: sodium (Na), potassium (K), magnesium (Mg), and calcium (Ca)
Sulfates: sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), ammonium (NH_4)

Laboratory Materials

Chromatographic adsorbents
Filter paper without hazardous chemical residue
Non-contaminated glassware
Rubber gloves

Waste Disposal

All laboratories are required to comply with federal and state regulations regarding the packing, labeling, and transport of hazardous materials. Before contacting the Hazardous Materials Facility for waste removal, the following procedures must be completed. *Improperly packed or labeled waste cannot be removed.*

Step One: Packing the Waste

Containers

Collect each chemical waste in a separate screw-top container. *Do not mix* wastes. Use the smallest container size to match the amount of chemical waste generated. The container the chemical was originally shipped in is an ideal waste collection container, if it is an appropriate size. All waste containers must be tightly capped. *Each container must be labeled as to chemical content*. For mixtures, give approximate percentages of each chemical compound. *Milk jugs are not acceptable for chemical storage*. If using a container that originally contained another chemical, completely remove the original label prior to relabeling. Completely fill chemical waste collection containers.

Shock-Sensitive and Water-Reactive Compounds and Lecture Bottles

Shock-sensitive and water-reactive compounds and lecture bottles require special handling. These materials should always be packed separately from other chemicals.

Packing Filled Containers in Boxes

Chemicals that have the potential to react with each other should *not* be packed in the same box.

Determine the packing hazard class for each chemical waste. When determining the class for a mixture of chemicals, *reactivity* has priority over *toxicity.* If you have difficulty determining the packing class of a mixture, call the Hazardous Materials Manager.

Segregate the wastes according to the hazard class and pack them into cardboard boxes. *Do not pack different classes in the same box*. Place dividers and shock absorbing materials (newspapers, vermiculite) between the containers.

Step Two: Completing the Manifest

The label for the chemical waste is called a packing *manifest*. A manifest must be completed and attached to *each* box. Laboratory personnel should complete the manifest following the directions below:

1. Laboratory Information: Fill in the generator's name (i.e., principal investigator, lab director), telephone number, department, building, room number, and the date.
2. Waste Information: The contents of each container must be identified on the manifest. *Nonspecified chemical waste* items are extremely difficult for hazardous materials personnel to handle. Good laboratory record keeping and labeling of all chemicals and chemical wastes prevents unknown waste items. Any chemical material that is potentially recyclable should *not* be contaminated with other chemicals for disposal. Where appropriate, note on the manifest if material is unopened.
3. The generator should check the information on the manifest, sign his or her name, and attach it to the corresponding box.

Step Three: Chemical Waste Removal

Attach one copy to the box and retain a copy for laboratory records. Specify where the waste is to be picked up. If your waste is not picked up in a reasonable period of time, call to inquire. Any incomplete or improperly completed manifest will be returned to the generator with an explanation for its return.

MATERIAL SAFETY DATA SHEETS (MSDS)

Since Material Safety Data Sheets (MSDS) are centrally related to the safe handling of hazardous substances, it is imperative that laboratory workers have easy access to them. There are three basic means of obtaining an MSDS:

Chemical manufacturer
Chemical supplier
Internet, such as through the UAB Department of Occupational Health and Safety webpage at: http://www.healthsafe.uab.edu

In general, the preferred source for the MSDS is the chemical manufacturer, primarily because these files are actively updated to accurately reflect all that is known about the hazardous material in question.

MSDSs are the cornerstone of chemical hazard communication. They provide most of the information you should know to work with chemicals safely. The following sections describe the information normally contained in an MSDS:

Product Name and Identification

Name of the chemical as it appears on the label
Manufacturer's name and address
Emergency telephone numbers for obtaining further information about a chemical in the event of an emergency
Chemical name or synonym
C.A.S. # — the Chemical Abstract Service Registry number, which identifies the chemical
Date of preparation of the MSDS

Hazardous Ingredients/Identity Information

Hazardous Ingredients

Substances which, in sufficient concentration, can produce physical or acute or chronic health hazards to persons exposed to the product. Physical hazards include fire, explosion, corrosion, and projectiles. Health hazards include any health effect, even irritation or development of allergies.

Threshold Limit Value (TLV)

A TLV is the highest airborne concentration of a substance to which nearly all adults can be repeatedly exposed, day after day, without experiencing adverse effects. These are usually based on an 8-hour time-weighted average.

Permissible Exposure Limit (PEL)

The PEL is an exposure limit established by OSHA.

Short-Term Exposure Limit (STEL)

The STEL is a 15-min time-weighted average exposure which should not be exceeded at any time during a workday. A STEL exposure should not occur more than four times per day, and there should be at least 60 min between exposures.

Lethal Dose 50 (LD50)

Lethal single dose (usually oral) in mg/kg (milligrams of chemical per kilogram of animal body weight) of a chemical that results in the death of 50% of a test animal population.

Lethal Concentration 50 (LC50)

Concentration dose expressed in ppm for gases or micrograms per liter of air for dusts or mists that results in the death of 50% of a test animal population administered in one exposure.

Physical/Chemical Characteristics

Boiling point, vapor pressure, vapor density, specific gravity, melting point, appearance, and odor are given in this section and all provide useful information about the chemical. Boiling point and vapor pressure provide a good indication of the volatility of the material. Vapor density indicates whether vapors will sink, rise, or disperse throughout the area. The farther the values are from 1 (the value assigned to atmospheric air), the faster the vapors will sink or rise.

Fire and Explosion Hazard Data

Flashpoint — refers to the lowest temperature at which a liquid gives off enough vapor to form an ignitable mixture with air.

Flammable or Explosive Limits — the range of concentrations over which a flammable vapor mixed with air will flash or explode if an ignition source is present.

Extinguishing Media — the fire-fighting substance that is suitable for use on the substance which is burning.

Unusual Fire and Explosive Hazards — hazards that might occur as the result of overheating or burning of the specific material.

Reactivity Data

Stability — indicates whether the material is stable or unstable under normal conditions of storage, handling, and use.

Incompatibility — lists any materials that would, upon contact with the chemical, cause the release of large amounts of energy, flammable gas or vapor, or toxic vapor or gas.

Hazardous Decomposition Products — any materials that may be produced in dangerous amounts if the specific material is exposed to burning, oxidation, heating, or allowed to react with other chemicals.

Hazardous Polymerization — a reaction with an extremely high or uncontrolled release of energy, caused by the material reacting with itself.

Health Hazard Data

Routes of Entry

Inhalation — breathing in of a gas, vapor, fume, mist, or dust.

Skin Absorption — a possible significant contribution to overall chemical exposure by way of absorption through the skin, mucous membranes, and eyes by direct or airborne contact.

Ingestion — the taking up of the substance through the mouth.

Injection — having the material penetrate the skin through a cut or by mechanical means.

Health Hazards (Acute and Chronic)

Acute — an adverse effect with symptoms developing rapidly

Chronic — an adverse effect that can be the same as an acute effect, except that the symptoms develop slowly over a long period of time or with recurrent exposures.

Carcinogen

A substance that is determined to be cancer producing or potentially cancer producing.

Signs and Symptoms of Overexposure

The most common symptoms or sensations a person could expect to experience from overexposure to a specific material. It is important to remember that only some symptoms will occur with exposures in most people.

Emergency and First-Aid Procedures

Instructions for treatment of a victim of acute inhalation, ingestion, and skin or eye contact with a specific hazardous substance. The victim should be examined by a physician as soon as possible.

Specific HACH MSDS Information

This information is presented here because of the large number of specialized HACH Co. reagents and procedures used in environmental laboratories. HACH MSDSs describe the hazards of their chemical products. Each of their MSDSs has 10 sections.

Header Information

Typically provides the vendor name, company address and telephone number, emergency telephone numbers, vendor's catalog number, date of the MSDS, and version of the MSDS.

Product Information

Product name
Chemical Abstract Services (CAS) number
Chemical name
Chemical formula, where appropriate
Chemical family to which the material belongs

Ingredients (lists all components)

PCT:	Percent by weight of each component in product (unless trade secret)
CAS NO:	Chemical Abstract Services (CAS) registry number for component
SARA:	If component is listed in SARA 313 and more is used than amount listed, must notify EPA.
TLV:	Threshold Limit Value. Maximum airborne concentration for 8-hour exposure that is recommended by the American Conference for Governmental Industrial Hygienists (ACGIH).
PEL:	Permissible Exposure Limit. Maximum airborne concentration for 8-hour exposure that is regulated by the Occupational Health and Safety Administration (OSHA).
HAZARD:	Physical and health hazards of component explained.

Physical Data

Physical state, color, odor, solubility, boiling point, melting point, specific gravity, pH, vapor density, evaporation rate, corrosivity, stability, and storage precautions.

Fire, Explosion Hazard, and Reactivity Data

Flashpoint: Temperature at which liquid will give off enough vapor to ignite. Used to define flammability and ignitability

Lower Flammable Limit (LFL or LEL): Lowest concentration that will produce flash or fire when ignition source is present

Upper Flammable Limit (UFL or UEL): Vapor concentration in air above which the vapor concentration is too great to burn

NFPA Codes: The National Fire Protection Association (NFPA) has a system to rate the degree of hazard presented by a chemical. Codes usually found in colored diamond and range from 0 (minimal hazard) to 4 (extreme hazard). They are grouped into the following hazards: health (blue), flammability (red), reactivity (yellow), and special hazards (white).

Health Hazard Data

Describes how a chemical can enter body (ingestion, inhalation, skin contact), its acute and chronic effects, and lists if a component is a carcinogen, mutagen, or teratogen.

Precautionary Measures

Special storage instructions
Handling instructions
Conditions to avoid
Protective equipment needed

First Aid

Spill and disposal procedures.

Transportation Data

Shipping name, hazard class, and ID number of the product.

References

Supporting references are also included in the HACH MSDS sheets.

SUMMARY OF FIELD TEST KITS

Field test kits can be important analytical tools during receiving water investigations. Chapter 6, among others, described how they can be used to obtain rapid and cost-effective data. However, the careful selection of the test kits to be used is critical. It is important to consider several factors, specifically the sensitivity of the procedure, safety hazards associated with the method, the cost (both capital and expendables) to conduct the analyses, and the time and expertise needed to conduct the test. Table E.2 summarizes these attributes, including results of conducting sensitivity tests using ultra-clean water and stormwater (Pitt and Clark 1999). The useful range is the minimum detection limit found during our tests to the upper limit that does not require dilution. The precision is the coefficient of variation based on replicate analyses, and the recovery is the slope of the regression line comparing analyses of spiked samples using these procedures and standard methods. The recovery tests were conducted using both ultra-clean water prepared using reverse osmosis (RO) and stormwater to identify any matrix interference problems. Any problems noted during the tests are also indicated, especially safety concerns, unusual amounts of expertise needed, and storage requirements.

These tests represent several classes of analytical procedures. The following sets of photos illustrate some of the simpler test kit methods. Figure E.1 illustrates the basic colorimetric procedure with a color wheel to analyze basic water color using a HACH test kit, while Figures E.2 and E.3 show simple color indicator paper strips for alkalinity. Vacuum vials are also used in several test

Table E.2 Summary of All Field Test Kits Evaluated

Method	Manufacturer and Kit Name	Capital Cost	Expendable Cost (per sample)	Time Reqd. (min)	Useful Range	Precision (COV)	Recovery (RO/runoff)	Problems with Test (safety hazards, expertise required, etc.)
				Ammonia				
Colorimetric determination of ammonia using Nessler's reaction	CHEMetrics *Ammonia 1 DCR Photometer*	$435 for kit	$0.63	5	0.03–2.5 mg/L	0.15	0.85/1.27	6-month shelf life, with refrigeration; sharps and mercury in waste
Colorimetric determination of ammonia using salicylate	HACH *Nitrogen, Ammonia: Salicylate Method without Distillation*	$1495 for DR/2000	$2.88	20	0.10–0.7	0.17	1.15/1.10	
Colorimetric determination of ammonia using Nessler's reaction	La Motte *Ammonia Nitrogen, High Range*	$895 for Smart Color.	$0.33	10	0.38–3	na	1.22/1.21	Waste contains a mercury compound; high detection limit (0.4 mg/L)
Colorimetric determination of ammonia using salicylate	La Motte *Ammonia Nitrogen, Low Range*	$895 for Smart Color.	$0.76	20	0.17–1.5	na	1.04/0.96	
				Bacteria				
Colorimetric	IDEXX *Colilert*			24 hr	na	na	na	24-hour test period required
Colorimetric	Industrial Municipal Equipment, Inc. *IME Test KoolKount Assayer*	$0.00	$4.00	30 min to 13 hr	na	na	na	Not a selective test, but sensitive to a mixed microbial population
				BTEX				
Immunoassay	Dtech (EM Science) *BTEX Test Kit*	$500	$25	30–60	na	na	na	Reagents expire in 1 to 2 months and require refrigeration; requires 30–60 min to conduct test; requires extensive expertise; $25 per test
	PetroSense	$6900		5	na	na	na	Expensive instrument ($6900)

Chlorides								
Silver nitrate titration	HACH *silver nitrate titration*	$94 for digital titrator	$0.66	not evaluated	na	na	na	Unclear titration endpoint, no useful data obtainable; recommended that conductivity analyses be used as a better indicator of chlorides in a sample
Conductivity								
Electronic probe	YSI *Model 33 SCT*	$600 for kit	$0.00	1	98–? µS/cm	na	0.90/0.93	
Electronic probe	Horiba *Twin*	$250 for kit	$0.00	1	75–50,000 µS/cm	0.04	1.08/1.02	Replace sensor every 6 months for $60
Electronic probe	Horiba *U-10 (Cond., temp., DO, turb., pH)*	$2800 for kit	$0.00	1	87–? µS/cm	na	0.95/0.96	Expensive instrument, but multiparameter
Copper								
Colorimeter	CHEMetrics *Copper 1 DCR Photometer Kit*	$435 for kit	$0.63	15	0.3–3.5 mg/L	na	0.64/0.52	Sharps and poor recovery; not very repeatable
Colorimeter	La Motte *Copper (Diethyldithio-carbamate)*	$895 for Smart Color.	$0.41	10	0.1–3.5	na	1.11/0.93	
Colorimeter	La Motte *Copper (Bicinchoninic Acid)*	$895 for Smart Color.	$0.23	20	0.6–3.5	na	0.94/0.93	Extra time required to dissolve reagent; not very repeatable
Colorimeter	HACH *Bicinchonate Copper Method Using AccuVac Ampoules*	$1495 for DR/2000	$0.28	20	0.5–5.0	na	0.97/0.96	Sharps
Detergents								
Colorimetric	CHEMetrics *Detergents (Anionic Surfactants)*	$60 for 1st 30 tests and standards	$2.38	10	0.15–3 mg/L	na	1.66/1.82	Sharps; chloroform extraction (very small volume and well contained)
Colorimetric	HACH *Surfactants, Anionic, Crystal Violet Method*	$1495 for DR/2000	$1.10	30	na	na	na	Large amounts of benzene required; require laboratory hood; waste disposal problem

Table E.2 Summary of All Field Test Kits Evaluated (continued)

Method	Manufacturer and Kit Name	Capital Cost	Expendable Cost (per sample)	Time Reqd. (min)	Useful Range	Precision (COV)	Recovery (RO/runoff)	Problems with Test (safety hazards, expertise required, etc.)
				Fluoride				
Ion selective electrode	Cole-Parmer *Fluoride Tester*	$600 for electrode, meter and calib. kit	$0.25	5–10	0.1–20 mg/L	0.22	0.97/0.96	Requires frequent and time consuming calibration; too fragile for field use
Spectrophotometric determination of bleaching by fluoride	HACH *Fluoride SPADNS Reagent*	$1495 for DR/2000	$0.37	10	0.3–2	na	1.10/1.07	Should use automatic pipettes, hard to use in field; SPADNS Reagent is hazardous
Spectrophotometric determination of bleaching by fluoride	HACH *Fluoride SPADNS Reagent Using AccuVac Ampoules*	$1495 for DR/2000	$1.17	5	0.1–2	0.05	0.97/0.94	Sharps; SPADNS Reagent is hazardous
				Hardness				
EDTA titration	CHEMetrics *Hardness, Total 20–200 ppm*	$0.00	$2.25	5–10	na	0.01	na	Sharps
EDTA titration	HACH *Total Hardness Using Digital Titrator*	$94 for digital titrator	Varies with sample strength	Varies with sample strength	na	na	na	
				Lead				
Solid phase extraction, colorimeter	HACH *LeadTrak System*	$395 for DR/100 kit or $1495 for DR/2000	$4.61	45	0.005–0.15	na	0.84/0.87	Requires extensive expertise; complex kit; time-consuming (45 min), but only kit with useful sensitivity
Sulfide staining	Innovative Synthesis Corporation *The Lead Detective*	$3.00		5	na	na	na	Poor sensitivity
	HybriVet Systems *Lead Check Swabs*	$3.00		5	na	na	na	Poor sensitivity
	Carolina Environment Company *KnowLead*	$3.00		5	na	na	na	Poor sensitivity

Test strips	EM Science *Lead*	$500 for Reflecto-Quant Meter-	$1.11 -	10	na	na	na	Not sensitive enough
				Nitrate*				
Colorimeter	La Motte *Nitrate*	$895 for Smart Color.	$1.22	20	0.8–3 mg/L	na	0.81/1.06	
ISE	Horiba *CARDY*	$235 for kit	$60/ sensor (per 6 months)	na	4.9–?	0.97	0.90/0.70	Designed for high concentrations; poor recoveries and precision at lower concentrations
Test strips	EM Science *Nitrate Quant Test Strips*	$500 for Reflecto Quant Meter	$0.49	2	1.7–500	na	1.00/1.61	Reagents must be refrigerated; more scatter than most other tests
Spectrophotometric	HACH *Nitrate, LR*	$1495 for DR/2000			na	na	na	Sharps; too sensitive of a test
Spectrophotometric	HACH *Nitrate, MR*	$1495 for DR/2000	$0.56	7	2.8–16	na	0.93/1.06	Sharps
Colorimeter	CHEMetrics *Nitrate (Nitrogen)*	$48 for 1st 30 tests and standards	$0.73	30	0.5–22	na	1.06/1.02	Sharps

* Nitrite and nitrate tests have a Cd-based reagent that is hazardous.

				PAH				
Immunoassay	EM Science *Dtech PAH Test Kit*	$500	$25	30–60	na	na	na	Reagents expire in 1 to 2 months and require refrigeration; requires 30–60 min to conduct test; requires extensive expertise; $25 per test
				pH				
Electrode	Cole-Parmer *pH Wand*	$155 for kit	$92/ electro.	5	0–14	0.01	na	Daily calibration; fragile meter
Electrode	Horiba *Twin pH*	$235 for kit	$70 for sensor. $25 for stand.	1	0–12	<0.01	na	Daily calibration
Electrode	Sentron *pH Probe*	$595 for meter and electrode-	None-	1	0–14	<0.01	na	Expensive, but rugged instrument ($595)

Table E.2 Summary of All Field Test Kits Evaluated (continued)

Method	Manufacturer and Kit Name	Capital Cost	Expendable Cost (per sample)	Time Reqd. (min)	Useful Range	Precision (COV)	Recovery (RO/runoff)	Problems with Test (safety hazards, expertise required, etc.)
Test paper	EM Science *ReflectoQuant pH*	$500 for Reflecto-Quant Meter	$0.89	2	4–9	0.08	na	Optics of expensive instrument ($500) are difficult to keep clean
Spectrophotometric	La Motte *pH*	$895 for Smart Color.	$0.22	5	5–9.5	na	na	
Test paper	Fisher Scientific *Alkacid Test Strips*	$0.00		1	0–12	0.07	na	Only readable to within ±1 pH unit, poor comparison to pH meters for actual samples
				Potassium				
Spectrophotometric	HACH *Potassium Tetraphenylborate*	$1495 for DR/2000	$3	30	0.5–7 mg/L	na	0.81/0.90	
ISE	Horiba *CARDY*	$235 for kit	$60/ sensor (per 6 months)	5	2.0–?	0.04	0.53/0.46	Method designed for much higher concentrations; more scatter than other tests
Colorimeter	La Motte *Potassium*	$895 for Smart Color.	$0.29	15	3.3–10	na	1.35/1.05	
Spectrophotometric	*La Motte Potassium Reagent Set*	$895 for Smart Color.	$0.29	15	1.3–7	0.06	?/0.90	
				Zinc				
Spectrophotometric	La Motte *Zinc*	$895 for Smart Color.	$0.59	5	0.14–3 mg/L	na	0.88/0.85	Dilute indicator expires in a month; uses dilute cyanide
Spectrophotometric	HACH *Zinc, Zincon Method*	$1495 for DR/2000	$0.37	10	na	na	na	Uses granular cyanide and is unacceptable for field use
Test strips	EM Science *ReflectoQuant Zinc*	$500 for Reflecto-Quant Meter	$0.56	5	na	na	na	Reflectoquant requires frequent cleaning and test has high detection limit

From Day, J. *Selection of Appropriate Analytical Procedures for Volunteer Field Monitoring of Water Quality.* MSCE thesis, Department of Civil and Environmental Engineering, University of Alabama at Birmingham. 1996. With permission.

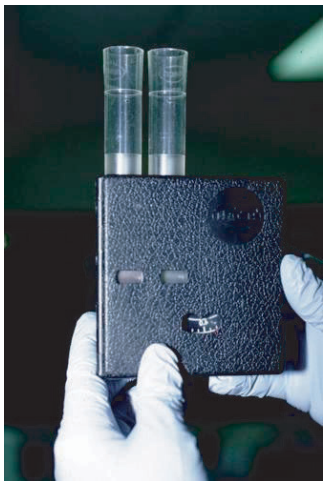

Figure E.1 HACH color test kit.

Figure E.2 Quantistrip method for alkalinity.

Figure E.3 Comparing Quantistrip against color standards.

Figure E.4 CHEMetrics copper test kit.

Figure E.5 CHEMetrics color reader.

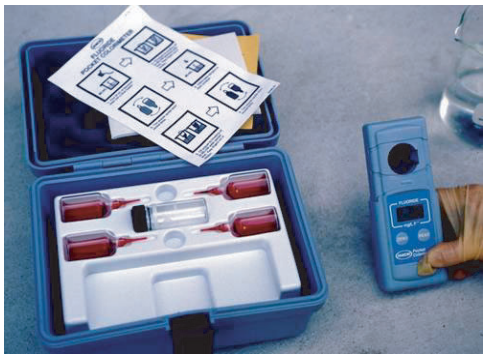

Figure E.6 HACH AccuVac kit for fluoride.

Figure E.7 Reading AccuVac absorbance.

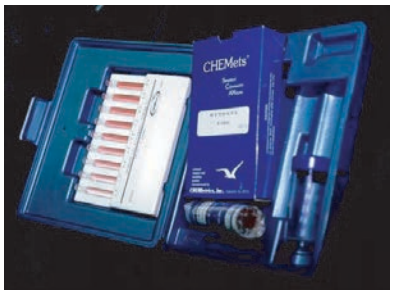

Figure E.8 CHEMetrics nitrate test kit.

Figure E.9 Cole Parmer ORP probe.

kits to automatically draw a sample into an evacuated ampoule that contains a specific amount of reagent. Figures E.4 through E.8 are different examples of these types of kits. Figure E.9 is an example of a simple probe used to directly measure ORP of a water sample (a necessary field analysis because of changes occurring after sample collection and transport to the laboratory). Many of other types of test kits are more complex and require several steps for the analyses. Some of the most complex procedures may require as many as 10 steps and more than 30 min for analyses.

While many of the simple methods are quite useful for field monitoring, the more complex (and expensive) procedures must be more carefully weighed against traditional (and more accurate) laboratory methods. In general, we found that the field test kits were more accurate than we had originally expected. However, the sensitivities of many of the field test kits were much poorer than expected, making them much less useful. In addition, numerous safety hazards can exist with these kits, sharps and hazardous reagents and wastes being the most serious.

SPECIAL COMMENTS PERTAINING TO HEAVY METAL ANALYSES

The above discussion on field test kits points out the obvious shortcomings of trying to obtain meaningful heavy metal data using simple procedures. There are a number of methods available for heavy metals, with the traditional methods restricted to the laboratory. The following discussion summarizes these available methods, especially their sensitivities.

Table E.3 lists the metals and associated methods included in the 1995 version of *Standard Methods for the Examination of Water and Wastewater*. Other listings of environmental analytical methods are published by ASTM (American Society of Testing Materials) and by the U.S. Environmental Protection Agency (in the *Code of Federal Regulations*, especially 40 CFR, 136 "Guidelines Establishing Test Procedures for the Analysis of Pollutants"). Methods listed in these references are generally taken as approved for many purposes. Table E.3 lists about 40 different metals and 12 different basic analytical methods. Most all of the metals can be analyzed using atomic absorption spectrometry (AAS) and inductively coupled plasma emission spectrometry (ICP). In addition, many of the metals have specific chemical tests that use spectrophotometric or titration methods. For most stormwater investigations, only a relatively few of these metals are routinely evaluated, including arsenic, cadmium, chromium, copper, lead, mercury, nickel, selenium, and zinc.

Table E.3 Metal Methods Included in the 1995, 19th Edition of *Standard Methods for the Examination of Water and Wastewater*

	Color	AAS	Flame	C-V AAS	ET AAS	Hydride	ICP	ASV	Other
Aluminum	×	×					×		
Antimony		×					×		
Arsenic	×					×	×		
Barium		×					×		
Beryllium	×	×					×		
Bismuth		×							
Cadmium	×	×					×	×	
Calcium	×	×					×		
Cesium		×							
Chromium	×	×					×		IC
Cobalt		×					×		
Copper	×	×					×		
Gold		×							
Iridium		×							
Iron	×	×					×		
Lead	×	×					×	×	
Lithium		×	×				×		
Magnesium		×					×		grav
Manganese	×	×					×		
Mercury	×			×					
Molybdenum		×					×		
Nickel		×					×		
Osmium		×							
Palladium		×							
Platinum		×							
Potassium		×	×				×		ISE
Rhenium		×							
Rhodium		×							
Ruthenium		×							
Selenium	×				×	×	×		fluro
Silver	×	×					×		
Sodium		×	×				×		
Strontium		×	×				×		
Thallium		×					×		
Thorium		×							
Tin		×							
Titanium		×							
Vanadium	×	×					×		
Zinc	×	×					×		

Note:° Color: Specific chemical colorimetric methods; AAS: Atomic absorption spectrometry; Flame: Flame emission photometry; ASV: Anodic stripping voltammetry; C-V AAS: Cold-vapor AAS; ET AAS: Electrothermal AAS; ICP: Inductively coupled plasma emission spectrometry; Hydride: Hydride generation AAS; Other: IC (ion chromatography), grav (gravimetric), ISE (ion selective electrode), and fluro (fluorometric)

Table E.4 compares the optimal metal concentration ranges for AAS and ICP, the most commonly used instrumentation (*Standard Methods* 1995). Instrument detection limits are about 15 times less than the lower values shown on this table, which represent the lower limits of quantification. The lower limits of the flame AAS optimal concentration ranges are generally about the same as for the plasma AES, while the electrothermal AAS lower limits are 10 to 1000 times lower. However, the plasma AES instrument has a much greater dynamic range than either AAS instrument. The plasma AES also has fewer interferences and can analyze many elements simultaneously. Because of these differences, many laboratories use a plasma AES for general

Table E.4 Optimal Concentration Ranges of Metals in Samples

	Flame AAS (mg/L)	Electrothermal AAS (mg/L)	Inductively Coupled Plasma AES (mg/L)
Aluminum	5–100	0.02–0.2	0.6–100
Antimony	1–40	0.02–0.3	0.45–100
Arsenic		0.005–0.1	0.75–100
Barium	1–20	0.01–0.2	0.030–50
Beryllium	0.05–2	0.001–0.03	0.005–10
Bismuth	1–5		
Cadmium	0.05–2	0.0005–0.01	0.06–50
Calcium	0.2–20		0.15–100
Cesium	0.5–15		
Chromium	0.2–10	0.005–0.1	0.1–50
Cobalt	0.5–10	0.005–0.1	0.1–50
Copper	0.2–10	0.005–0.1	0.1–50
Gold	0.5–20		
Iron	0.3–10	0.005–0.1	0.1–100
Lead	1–20	0.005–0.1	0.6–100
Lithium	0.1–2		0.06–100
Magnesium	0.02–2		0.45–100
Manganese	0.1–10	0.001–0.03	0.06–50
Molybdenum	1–20	0.003–0.06	0.12–100
Nickel	0.3–10	0.005–0.1	0.2–50
Platinum	5–75		
Potassium	0.1–2		1.5–100
Selenium		0.005–0.1	1.0–100
Silver	0.1–4	0.001–0.025	0.1–50
Sodium	0.03–1		
Strontium	0.3–5		0.03–50
Thallium			0.6–100
Tin	10–200	0.02–0.3	
Titanium	5–100		
Vanadium	2–100		0.1–50
Zinc	0.05–2		0.03–100

Data from *Standard Methods for the Examination of Water and Wastewater. 19th edition.* Water Environment Federation. Washington, D.C. 1995.

analytical work and an electrothermal AAS for individual samples for single elements at very low concentrations.

Table E.5 lists various operational and cost attributes of these metal analysis methods (Pitt et al. 1997). The trade-offs between the various types of equipment are obvious. The instruments with greater sensitivity cost more. Only an electrothermal AAS instrument can analyze many samples quickly (with an autosampler) with good sensitivity, but with only a few metals being analyzed at a time, at the most. The instruments that can analyze many metals at a time include the ICP units. However, only the ICP/MS units are capable of similar low sensitivities as the electrothermal AAS units. These units are mostly still being used in research environments and are not typically used in production laboratories, as they require well-trained specialized operators and are the most costly alternative shown.

In flame AAS, a sample is aspirated directly into a flame (typically air–acetylene) and is atomized. A light beam (from a hollow cathode lamp designed for a specific wave length) is directed through the flame and into a monochromator, and finally into a detector. The detector measures the amount of light absorbed by the atomized element. The lamp operating at the specific wavelength of the metal makes the method relatively free from spectral and radiation interferences. However, different schemes (continuum-source, Zeeman, or Smith-Hieftje) to correct for molecular absorption and light scattering interferences are typically used.

Table E.5 Attributes of Metal Analysis Methods

	Flame AAS	Electrothermal AAS	Plasma ICP	Plasma ICP/MS	Anodic Stripping Voltammetry	X-Ray Fluorescence
Capital cost ($US)	10,000–30,000	25,000–80,000	40,000–80,000	150,000–250,000	8000–25,000	25,000–60,000
Operational cost[a]	Low	Moderate	Moderate–high	High	Low to moderate	Low
Sensitivity	Good	Very good	Poor–good	Very good	Excellent	Poor (solid matrices only)
Operation (number of metals at a time)	Single	Single–few	Many	Many	Few	Few
Sample throughput	High	High	High	Low	Moderate	Moderate
Ease of use	Good	Moderate	Good–moderate	Poor	Moderate–poor	Moderate
External sample preparation	Acid digestion	Acid digestion	Acid digestion	Acid digestion	Filtration	Possibly grind and sieve to obtain uniform particles

[a]° Approximate operational costs, including expendable supplies (gases, acids, filters, graphite tubes, etc.), but not labor ($/sample): low: 3–10; moderate: 10–25; high: >25.

From Pitt, R., S. Mirov, K. Parmer, and A. Dergachev. Laser applications for water quality analyses, in *ALT'96 International Symposium on Laser Methods for Biomedical Applications.* Edited by V. Pustovoy. SPIE — The International Society for Optical Engineering. Volume 2965, pp. 70–82. 1997.

Cold-vapor AAS is used for very sensitive determinations of mercury. In this scheme, the sample (modified with H_2SO_4, HNO_3, $KMnO_4$, and $SnCl_2$ to volatilize the mercury) is purged with air, which is then directed into an absorption cell placed in the light pathway where the flame unit is normally located.

Electrothermal (graphite furnace) AAS is much more sensitive than flame AAS because it can place a much greater density of atoms in the light pathway. Contamination is therefore much more critical than with flame units. Electrothermal AAS is subject to more interferences than flame AAS and is only recommended for very low concentrations of metals. However, because of the relatively low concentrations of many heavy metals found in stormwater, especially the dissolved fraction, graphite furnace AAS (Figure E.10) is the preferred method in this area of research (using a suitable background corrector to minimize most interferences).

Inductively coupled plasma atomic emission spectroscopy uses a controlled plasma from argon gas ionized by an applied radio frequency. A sample aerosol is directed into the plasma, which is at an extremely high temperature (6000 to 8000 K). This results in almost complete dissociation of the metal molecules and significantly reduced chemical interferences compared to most other metal analyses techniques. Another important advantage of the ICP is the extremely wide dynamic range of the instrument, as shown in Table E.4. An emission light emitted from the sample and plasma combination is focused in a monochromator and is detected using a series of photomultipliers set at specific wavelengths for the elements of interest.

The ICP/MS uses a mass spectrophotometer to separate the analyte ions emitted by the plasma and sample mixture according to their mass-to-charge ratios. This results in a much more sensitive unit (comparable to the electrothermal AAS), and it can detect multiple elements simultaneously.

Anodic stripping voltammetry is rarely used in a production laboratory, but it is a relatively common research instrument (Figure E.11). ASV is one of the most sensitive metal analysis techniques, even more sensitive than electrothermal AAS. Cyclic ASV is also capable of identifying

Figure E.10 Graphite furnace AAS used for stormwater analyses at the University of Alabama at Birmingham.

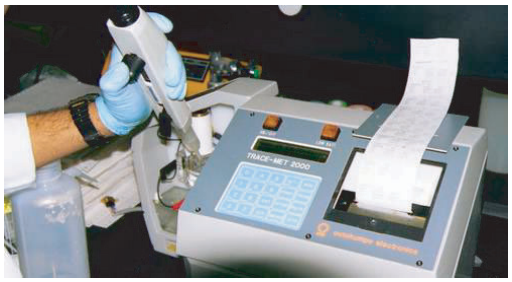

Figure E.11 Anodic stripping voltammeter (Outo kompku) for heavy metal analyses.

different characteristics of the metals in the sample. The analyzer uses a three-step process. The first step typically plates a mercury film on a glossy carbon electrode. The second step plates the metals on the mercury film, and the third step strips the metals from the film as a function of increasing oxidizing potential. This last step allows the individual metals to be identified and quantified. Only metals that form an amalgam can be determined (such as cadmium, copper, lead, and zinc, metals of great interest in most environmental investigations). Because the instrument is so sensitive, great care must be taken to avoid contamination. Interferences may be caused by complexes that form between metals in the sample (such as between high concentrations of copper and zinc). ASV is especially well suited for analyzing heavy metals in saline waters (such as snowmelt) where graphite furnace procedures are subject to many interferences from the high salt concentrations.

X-ray fluorescence (Figure E.12) can also be used to detect heavy metals in solid samples, such as sediments and soils, including particulates trapped on filters (from water or air samples). The sample is irradiated with low-intensity X rays causing the elements in the sample to fluoresce. The emitted X rays from the irradiated sample are sorted by their energy level and are used to identify and quantify the metals of interest. Relatively little sample preparation is needed, especially for homogeneous samples. The technique is commonly used as a screening tool in the field to guide sampling for more accurate and sensitive laboratory analyses. Its relatively poor sensitivity limits its use for most environmental investigations, except for evaluating heavily contaminated sites.

Sample preparation is very critical for all of these metal analysis procedures. Typical sample preparation requires acid digestion using a combination of acids to reduce interferences by organic matter and to convert the metal associated with particulates (and colloids) to the free metal forms that can be detected. Nitric acid digestion with heat is adequate for most samples. However, hydrofluoric acid is also needed if the digestion is to completely release metals that may be tied up in a silica matrix. Unfortunately, hydrofluoric acid forms volatile compounds with some metals, resulting in their partial loss upon storage if not analyzed immediately. Almost all of the stormwater heavy metals can be released from the particulates using just nitric acid, especially considering metal losses from using a hydrofluoric acid digestion. A nitric acid and perchloric acid mixture may be needed to digest organic material in the samples. Microwave-assisted digestion (Figure E.13) has become more common recently because of improved metal recovery, much faster digestion, and better repeatability.

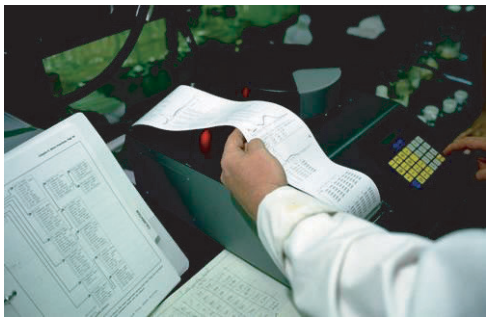

Figure E.12 X-ray fluorescence unit for analyses of heavy metals in solids.

Figure E.13 Microwave digestion of stormwater samples for heavy metal analyses.

STORMWATER SAMPLE EXTRACTIONS FOR EPA METHODS 608 AND 625

The following paragraphs outline the modified organic extraction methods that have been used by UAB for the analysis of wet-weather flows (Pitt and Clark 1999). These modifications are necessary because of the large amount of particulates in the samples and the large particulate fraction of the organics of greatest interest. These particulates interfere with solid-phase extraction procedures, for example, resulting in very little recovery of organic toxicants using that method.

1. Samples are extracted using a liquid–liquid separatory funnel technique. This has been found to give the most reliable results, especially compared to solid phase extraction or critical fluid extraction methods, for stormwater samples (and most surface water samples). The problem with stormwater organics is that a substantial fraction of many of the organic compounds of interest are associated with particulates. This particulate fraction needs to be quantified, as stormwater has been shown to have significant effects on receiving water sediments. If emulsions prevent achieving acceptable solvent recovery with separatory funnel extraction, continuous extraction is used. The separatory funnel extraction scheme described below assumes a sample volume of 250 mL. Serial extraction of the base/neutrals uses 10 mL additions of methylene chloride, as does the serial extraction of the acids. Prior to the extraction, all glassware is oven baked at 300°C for 24 hours.
2. A sample volume of 250 mL is collected in a 400-mL beaker and poured into a 500-mL glass separation funnel. For every 12 samples extracted, an additional four samples are extracted for quality control and quality assurance. These include three 250-mL composite samples made of equal amounts of the 12 samples, and one 250-mL sample of reverse osmosis water. Standard solution additions consisting of 25 µL of 1000 µg/mL base/neutral spiking solution, 25 µL of 1000 µg/mL base/neutral surrogates, 12.5 µL of 2000 µg/mL acid spiking solution, and 12.5 µL of 2000 µg/mL acid surrogates are made to the separation funnels of two of the three composite samples and mixed well. Sample pH is measured with wide-range pH paper and adjusted to pH > 11 with sodium hydroxide solution.
3. A 10-mL volume of methylene chloride is added to the separatory funnel and sealed by capping. The separatory funnel is gently shaken by hand for 15 s and vented to release pressure (Figure E.14). The cap is removed from the separatory funnel and replaced with a vented snorkel stopper. The separatory funnel is then placed on a mechanical shaker and shaken for 2 min. After returning the separatory funnel to its stand and replacing the snorkel stopper with the cap, the organic layer is allowed to separate from the water phase for a minimum of 10 min, longer if an emulsion develops

(Figure E.15). The extract and any emulsion present is then collected into a 125-mL Erlenmeyer flask (Figure E.16).

4. A second 10-mL volume of methylene chloride is added to the separatory funnel, and the extraction method is repeated, combining the extract with the previously collected extract in the Erlenmeyer flask. For persistent emulsions, those with emulsion interface between layers more than one third the volume of the solvent layer, the extract including the emulsion is poured into a 50-mL centrifuge vial, capped, and centrifuged at 2000 rpm for 2 min to break the emulsion (Figures E.17 and E.18). Water phase separated by the centrifuge is collected from the vial and returned to the separatory funnel using a disposable pipette. The centrifuge vial with the extract is recapped before performing the extraction of the acid portion.
5. The pH of the remaining sample in the separatory funnel is adjusted to pH < 2 using sulfuric acid. The acidified aqueous phase is serially extracted twice with 10-mL aliquots of methylene chloride, as in the previous base/neutral extraction procedure. Extract and any emulsions are again collected in the 125-mL Erlenmeyer flask.
6. The base/neutral extract is poured from the centrifuge vial though a drying column of at least 10 cm of anhydrous sodium sulfate and is collected in a 50-mL beaker (Figure E.19). The Erlenmeyer flask is rinsed with 5 mL of methylene chloride, which is then used to rinse the centrifuge vial and then to rinse the drying column and complete the quantitative transfer.

Figure E.14 Initial hand shaking the separatory funnel and venting gas.

Figure E.15 Separation of organic solvent extract from water sample.

Figure E.16 Collecting solvent extract and emulsion after separation.

Figure E.17 Extract in centrifuge vial.

7. The base/neutral extract is transferred into a 50-mL concentration vial and is placed in an automatic vacuum/centrifuge concentrator from Savant (Figure E.20). (Vacuum concentration is used in place of the Kuderna–Danish method; Figure E.21.) Extract is concentrated to approximately 0.5 mL.
8. The acid extract collected in the 125-mL Erlenmeyer flask is placed in the 50-mL centrifuge vial. Again, if emulsions persist, the extract is centrifuged at 2000 rpm for 2 min. Water is drawn from the extract and discarded. Extract is poured through the 10 cm anhydrous sodium sulfate drying column and collected in the 50-mL beaker as before. The Erlenmeyer flask is then rinsed with 5 mL of methylene chloride, which is then poured into the centrifuge vial and finally through the drying column.
9. The acid extract is then poured into the 50-mL concentration vial combining it with the evaporated base/neutral extract. The combined extract is then concentrated to approximately 0.5 mL in the automatic vacuum/centrifuge concentrator.
10. Using a disposable pipette, extract is transferred to a graduated Kuderna–Danish concentrator. Approximately 1.5 mL of methylene chloride is placed in the concentration vial for rinsing. This rinse solvent is then used to adjust the volume of extract to 2.0 mL. Extract is then poured into a labeled Teflon-sealed screw-cap vial and freezer stored until analysis (Figure E.22).

Notes for method 608: under the alkaline conditions of the extraction step, α-BHC, γ-BHC, endosulfan I and II, and endrin are subject to partial decomposition. Florisil cleanup is not utilized unless the sample matrix creates excessive background interference.

When sediments are being analyzed for organic compounds, we use a semiautomated method in place of the traditional Soxlet extraction method. A Dionex ASE (accelerated solvent extractor) (Figure E.23) is used to extract organic compounds from the sediment, while an OI gel permeation chromatograph (Figure E.24) is used to clean up the extracts.

Figure E.18 Extract placed in centrifuge.

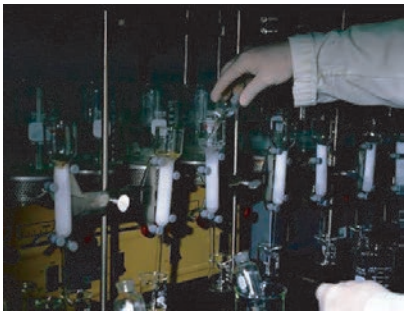

Figure E.19 Drying columns containing anhydrous sodium sulfate.

Figure E.20 Automatic vacuum/centrifuge concentrator (Savant AS 160).

Figure E.21 Alternative micro Kuderna–Danish concentration method.

Figure E.22 GC/MSD used for organic analyses.

Figure E.23 Dionex ASE for automatic extractions of organics from sediment samples.

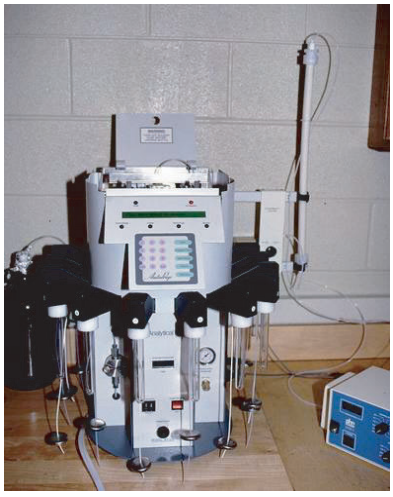

Figure E.24 OI GPC used to clean sed iment extracts.

CALIBRATION AND DEPLOYMENT SETUP PROCEDURE FOR YSI 6000UPG WATER QUALITY MONITORING SONDE

This discussion on calibration and deployment setup procedures for the YSI 6000 is presented here due to the reliance on this water quality monitoring sonde for many different applications presented in this book. This discussion was prepared by John Easton, Ph.D. candidate, University of Alabama at Birmingham, who has used this equipment extensively during his research. These procedures are therefore a compilation of the instructions given by YSI, in addition to his field and lab experience with this equipment.

The YSI 6000upg Environmental Monitoring System is a multiparameter, water quality measurement and data logging system. It is intended for use in research, assessment, and regulatory compliance applications. This instrument, or sonde, is ideal for profiling and monitoring water conditions in lakes, rivers, wetlands, estuaries, coastal waters, and monitoring wells. It can be left unattended for weeks at a time with measurement parameters sampled at a user-defined setup interval and data saved securely in the unit's internal memory. The Model 6000upg is designed to house four field-replaceable probes (six sensors) and a depth sensor module in the sonde body. The 6000upg communicates with a computer with a terminal emulation program, or via the Ecowatch for Windows software. The data is easily exported to any spreadsheet program for sophisticated data analysis. The unit operates on eight C-size alkaline batteries. Depending upon the activated sensor configuration and frequency of data collection, the unit can provide up to 90 days of battery life.

The Environmental Research Area at UAB has four 6000upgs configured to collect the following measurement parameters: dissolved oxygen, conductivity, specific conductance, salinity, total dissolved solids, resistivity, temperature, pH, ORP, depth, level, and turbidity. Table E.6 gives the reported performance specifications for each sensor.

This method details how to calibrate the sonde for the following measurement parameters: specific conductivity, dissolved oxygen, depth, pH, and turbidity for freshwater monitoring, plus routine maintenance of the DO and conductivity probes. The temperature and ORP probes require no calibration, but should be checked against known standards. This method also describes how to configure the sonde for unattended deployment or sampling.

All calibration standards should be prepared fresh, and this procedure should be done at approximately 25°C. The following lists the materials and supplies needed for calibrations:

Materials

- One or more containers to hold calibration standards. YSI calibration cup or 800-mL beaker
- Large (5-gallon) bucket filled with tap water for rinsing the sonde between calibration solutions

Table E.6 Performance Specifications and Sensor Types in the YSI 6000 Sonde

Parameter	Sensor Type	Range	Accuracy	Resolution
Dissolved oxygen % saturation	Rapid Pulse – Clark-type, polarographic	0–200% air saturation	±2% air saturation	0.1% air saturation
Conductivity[a]	4 electrode cell with autoranging	0–100 mS/cm	±0.5% of reading + 0.001 mS/cm	0.01 mS/cm
Temperature	Thermistor	–5–45°C	±0.15°C	0.01°C
pH	Glass combination electrode	2–14 units	±0.2 units	0.01 units
ORP	Platinum ring	–999–999 mV	±20 mV	0.1 mV
Turbidity	Optical, 90° scatter, mechanical cleaning	0–1000 NTU	±5%	0.1 NTU
Depth — Medium	Stainless steel strain gauge	0–61 m	±0.12 m	0.001 m
Depth — Shallow	Stainless steel strain gauge	0–9.1 m	±0.06 m	0.001 m

[a] Report outputs of specific conductance (conductivity corrected to 25°C)

- Volumetric flasks, graduated cylinders, pipette, and pipette tips for preparation of calibration solutions
- Barometer. NOTE: *Remember that barometer readings which appear in meteorological reports are generally corrected to sea level and are not useful for your calibration procedure unless they are uncorrected and at the elevation and location of the sonde*.
- Dissolved oxygen probe maintenance kit, contains: O-rings, DO membranes, pencil eraser (or very fine sandpaper), electrode filling solution
- Several clean, absorbent paper towels or cotton cloths for drying the sonde between rinses and calibration solutions
- Computer (with Ecowatch software), connection cable for interfacing computer with sonde, AC power supply, and eight C-size alkaline batteries
- Allen wrench for removing sonde guard and battery compartment cover

Reagents

- Deionized water (diH_2O)
- pH buffers: 7.00, 4.01, and/or 10.01 (either 4.01 or 10.01, in addition to the 7.00 solution is suitable for two-point calibration)
- Conductivity standard, e.g., NaCl solution at 16,640 µS/cm @ 25°C
- Turbidity standard, e.g., Formazin solution at 4000 NTU

Initial Calibration Procedure

- Remove sonde guard
- Check to see if DO electrode is bright silver; if not, clean by gently rubbing with the pencil eraser. Clean eraser particles off probe completely. Fill probe well with filling solution and replace membrane. Put probe guard back onto sonde.
- Connect computer to sonde and connect sonde to external AC power supply

Conductivity Probe Calibration

- Prepare conductivity standard. Use a 1 mS/cm (1000 µS/cm) standard if the sonde is to be deployed in fresh water. For example, dilute typically available 16.640 mS/cm standard solution 1:16.64 with diH_2O (to prepare 500 mL, add 30 mL of 16.640 mS/cm standard and QS to 500 mL with diH_2O).
- Decant 1 mS/cm solution into calibration cup and immerse sonde into cup.
- Launch Ecowatch software. Open communications with sonde, and type "menu." From the sonde main menu select **2. Calibrate**. From the calibrate menu, select **1. Conductivity** to access the conductivity calibration procedure and then **1. SpCond** to access the specific conductance calibration procedure. Enter the calibration value of the standard you are using (1.000 mS/cm at 25°C) and press ENTER.
- The current values of all enabled sensors will appear on the screen and will change with time as they stabilize. Observe the readings under SpCond and when they show no significant change for approximately 30 s, press ENTER.
- The screen will indicate that the calibration has been accepted and prompt you to HIT ANY KEY to return to the **Calibrate** menu.

- If you receive an error message indicating that the calibration is out of range, assure yourself that the calibration solution was prepared correctly. If it was, remove sonde guard, and using small brush (located in pocket in user's manual) clean out the channel on the conductivity probe. BE GENTLE. Replace sonde guard and repeat calibration steps.
- Rinse the sonde in tap or purified water and dry.

DO Probe (and depth) Calibration

- Place approximately 1/8-in (3 mm) of water or a saturated sponge in the bottom of the calibration cup. Make sure the DO and temperature probes are *not* immersed in the water. Wait approximately 10 minutes for the air in the cup to become water saturated. NOTE: *if the transport cup is used, make certain that the cup is vented to the atmosphere by loosening the vent screw.*
- From the **Calibrate** menu, select **2. DO%** to access the DO% calibration procedure.
- Enter the current barometric pressure in mm Hg. (inches of Hg × 25.4 = mm Hg).
- Press ENTER and the computer will indicate that the calibration procedure is in progress.
- After approximately 1 min, the calibration will be complete. Press any key as instructed, and the screen will display the percent saturation value which corresponds to your local barometric pressure input. For example, if your local barometer reads 742 mm Hg, the screen will display 97.6% (742/760) at this point. If an error message is received, proceed to the diagnostics step; otherwise, press any key to return to the **Calibrate** menu (and skip the following diagnostic step).
- If an error message was received, conduct a diagnostics test. From the **Main** menu, chose **8. Diagnostics**. Check the DO charge. This value should read between 25 and 75 during calibration. If out of this range, then the probe needs to be cleaned (pencil eraser) or replaced. After cleaning, repeat the above DO calibration procedure.
- Following the DO calibration, leave the sonde in water-saturated air. From the **Calibrate** menu, select **3. Depth** to access the depth calibration procedure.
- Input 0.00 or some known sensor offset in feet. (The depth sensor is about 0.46 ft above the bottom of the probe compartment, and this offset value could be used if installing the unit vertically and depth in relation to the sonde bottom was desired.) Press ENTER and monitor the stabilization of the depth readings with time.
- When no significant change occurs for approximately 30 s, press ENTER to confirm the calibration and zero the sensor with regard to the current barometric pressure.
- Press any key to return to the **Calibrate** menu.

pH Probe Calibration

- Place approximately 400 mL of pH 7 buffer in a clean calibration cup. Allow at least 1 min for temperature equilibrium before proceeding.
- Immerse probe into solution. From the **Calibrate** menu, select **6. pH** to access the pH calibration choices and then **2. 2-Point**.
- Press ENTER and input the value of the buffer (7.00) at the prompt. Press ENTER, and observe the values under pH until the readings are stable for 30 s.
- Press ENTER. The display will indicate that the calibration is accepted. (If an error message is received, repeat with fresh buffer.)
- Press any key to continue.
- Rinse the sonde in water and dry before proceeding.
- Place approximately 400 mL of a second pH buffer solution in a clean calibration cup. The second buffer might be pH 4.01 if the monitored water is expected to be acidic, or pH 10.01 if the monitored water is expected to be basic. Allow at least 1 min for temperature equilibrium before proceeding.
- Press ENTER and input the value of the second buffer (4.01 or 10.01) at the prompt. Press ENTER, and observe the values under pH until the readings are stable for 30 s.
- Press ENTER. After the second value calibration is complete, press any key to return to the **Calibrate** menu.
- Rinse the sonde in water and dry before proceeding.

Turbidity Probe Calibration

- Prepare 100 NTU solution. Dilute 4000 NTU formazin solution 1:40 with diH_2O (pipette 25 mL of 4000 NTU formazin solution into 1-L volumetric flask and qs to 1 L). **Formazin is a hazardous material, and special care needs to be taken. Read and follow all precautions**.

- Select **9. Turbidity** from the **Calibrate** menu, and then **2. 2-Point**.
- To begin the calibration, immerse the sonde in approximately 300 mL of 0 NTU standard (clear, deionized water), and press ENTER.
- Input the value 0.00 NTU at the prompt, and press ENTER.
- After calibration of the mechanical wiper speed, the screen will display real-time readings, which will allow you to determine when turbidity values have stabilized. If the readings appear unusually high or low or are unstable, there are probably bubbles on the optical surface. Activate the mechanical wiper by pressing the "3" key to remove the bubbles.
- After stable readings are observed for approximately 40 s, press ENTER to confirm the first calibration. Press any key to continue.
- Dry the sonde and probes carefully and then place the sonde in approximately 300 mL of the second turbidity standard (100 NTU). Input the value 100.0 NTU, press ENTER, and view the stabilization of the values on the screen.
- As described previously, if the readings appear unusually high or low or are unstable, activate the wiper to remove bubbles and be sure to wait 40 s before confirming the calibration.
- After the readings have stabilized, press ENTER to confirm the calibration. Press any key to return to the **Calibrate** menu. Input "0" to return to the **Main** menu.
- Proceed to the deployment setup procedure.

Deployment Setup Procedure (for unattended monitoring)

- Unplug the AC power source, and continue this procedure using the sonde's internal (battery) power.
- Select **1. Run** from the sonde **Main** menu. The **Run** menu will be displayed.
- Select **3. Unattended** sample from the **Run** menu.
- The current time and date, all active sensors, battery voltage, and free flash disk space will be displayed.
- Note: if the current time and date are not correct, your unattended sampling study will not begin or end when you desire. To correct the time and date, see Section 2.5 in the instruction manual.
- You will be asked to enter the starting date. Use the following format: XX/XX/XX. For example to start on 1 January, 1999, enter "01/01/99."
- Enter the starting time. Use the following format: XX/XX/XX. You must include not only hours and minutes, but seconds. For example, if you want to start a study at 8 AM, you must enter "08:00:00."
- Enter the study duration in days. For example, for a 2-week study, enter "14."
- Enter interval in minutes. For example, to collect data every 15 minutes, enter "15."
- Enter the site description.
- You will be asked if all start-up information is correct. Check the information carefully (especially the estimated battery life) and, if you want to change something, press "N." If all information is correct, press "Y." The following message will be displayed briefly: *INSTRUMENT IS IN UNATTENDED MODE*.
- Continue to press "zero" until the Ecowatch software breaks communication with the sonde (after exit from the **Main** menu).
- Remove the communication cable from the sonde and screw on the waterproof connector cap. The sonde is now ready for deployment.

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Pitt, R.E., R.I. Field, M.M. Lalor, D.D. Adrian, and D. Barbe. *Investigation of Inappropriate Pollutant Entries into Storm Drainage Systems: A User's Guide.* Rep. No. EPA/600/R-92/238, NITS Rep. No. PB93-131472/AS, U.S. EPA, Storm and Combined Sewer Pollution Control Program (Edison, NJ), Risk Reduction Engineering Lab., Cincinnati, OH. 1993

Pitt, R., S. Mirov, K. Parmer, and A. Dergachev. Laser applications for water quality analyses, in *ALT'96 International Symposium on Laser Methods for Biomedical Applications.* Edited by V. Pustovoy. SPIE — The International Society for Optical Engineering. Vol. 2965, pp. 70–82. 1997.

Pitt, R. and S. Clark. *Communication Manhole Water Study: Characteristics of Water Found in Communications Manholes*. Final Draft. Office of Water, U.S. Environmental Protection Agency. Washington, D.C. July 1999.

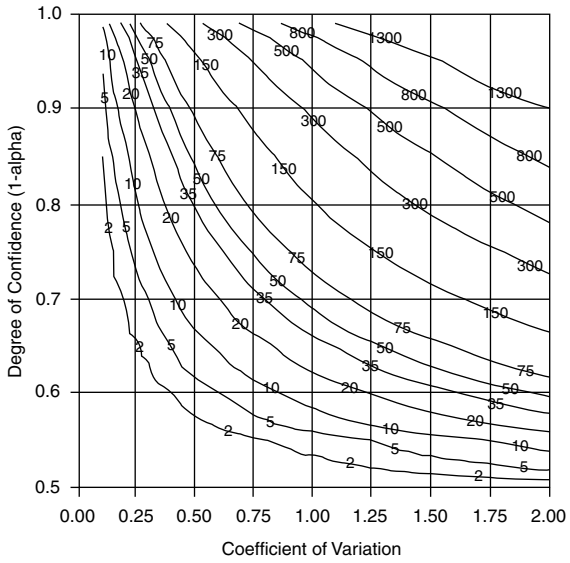
Standard Methods for the Examination of Water and Wastewater. 19th edition. Water Environment Federation. Washington, D.C. 1995.

APPENDIX F

Sampling Requirements for Paired Tests*

* From R. Pitt and K. Parmer. *Quality Assurance Project Plan (QAPP) for EPA Sponsored Study on Control of Stormwater Toxicants*. Department of Civil and Environmental Engineering, University of Alabama at Birmingham. 1995.

Number of Sample Pairs Needed
(Power = 0.5 Difference = 10%)



Number of Sample Pairs Needed
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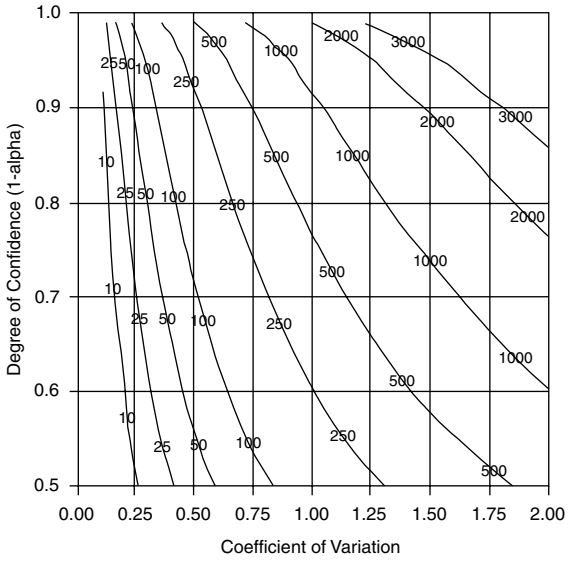
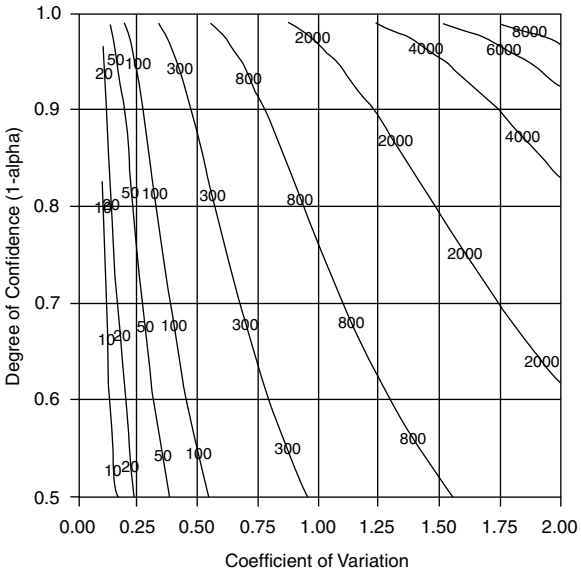


Figure F.1

Number of Sample Pairs Needed
(Power = 0.9 Difference = 10%)



Number of Sample Pairs Needed
(Power = 0.5 Difference = 25%)

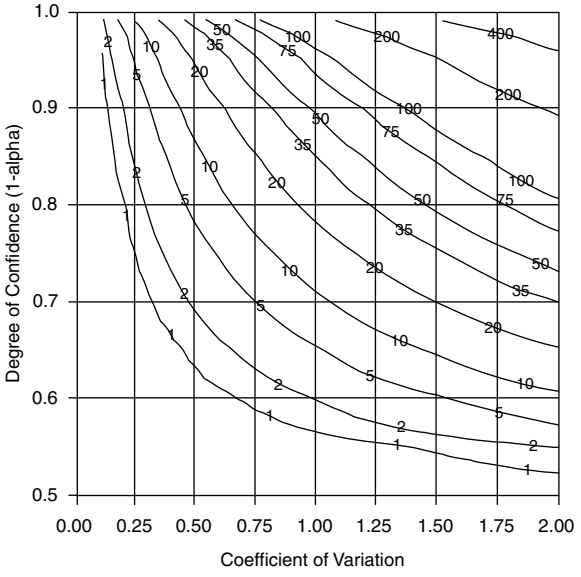


Figure F.2

Number of Sample Pairs Needed
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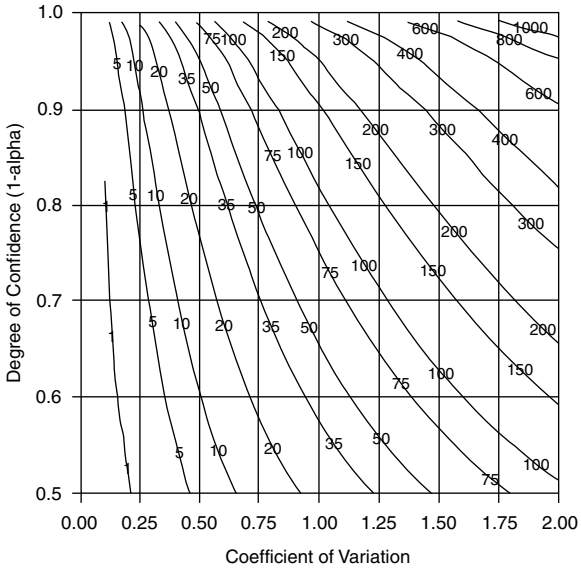

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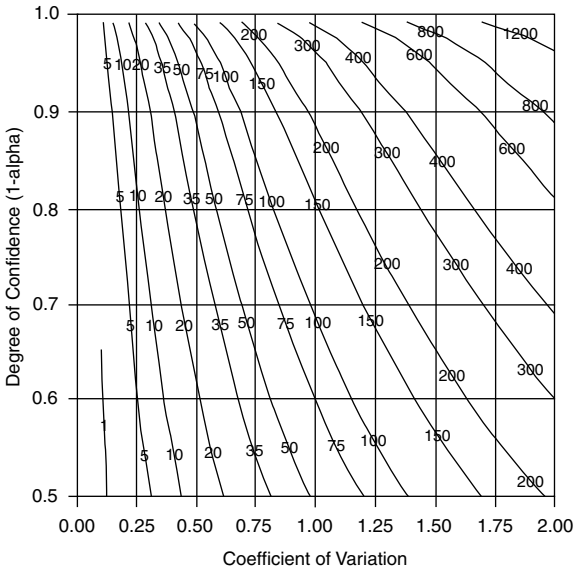
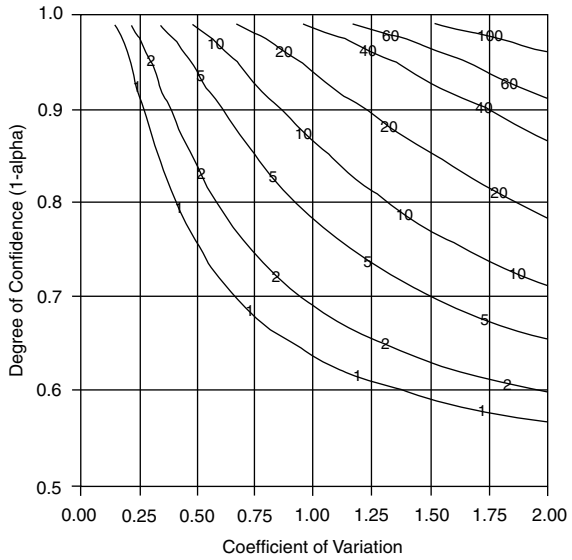


Figure F.3

Number of Sample Pairs Needed
(Power = 0.5 Difference = 50%)



Number of Sample Pairs Needed
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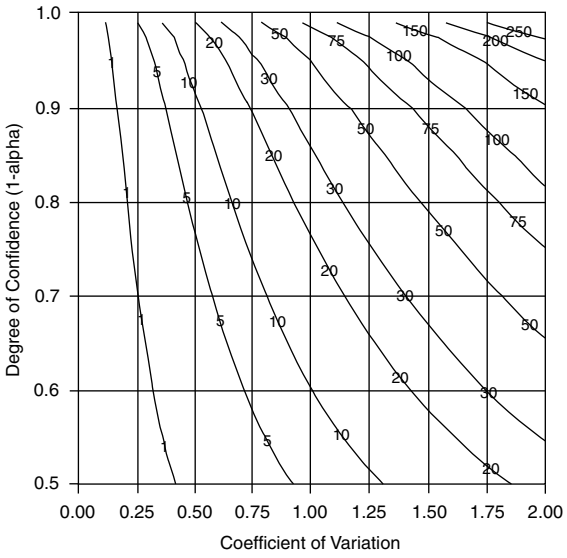
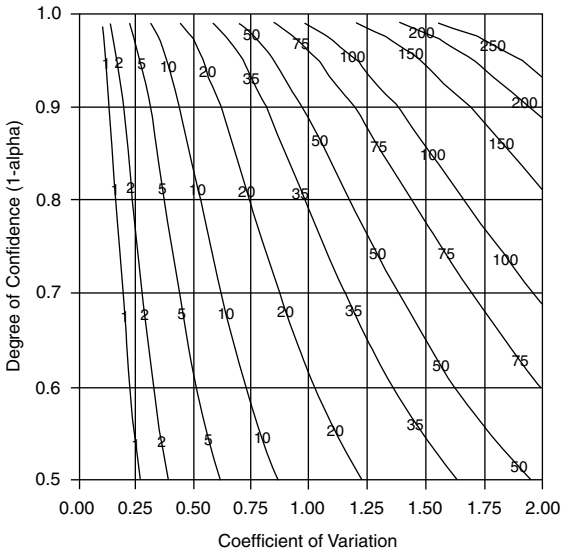


Figure F.4

Number of Sample Pairs Needed
(Power = 0.9 Difference = 50%)



Number of Sample Pairs Needed
(Power = 0.5 Difference = 75%)

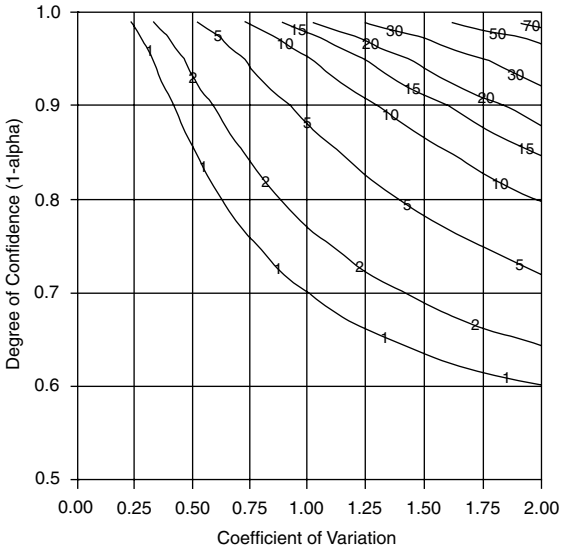


Figure F.5

Number of Sample Pairs Needed
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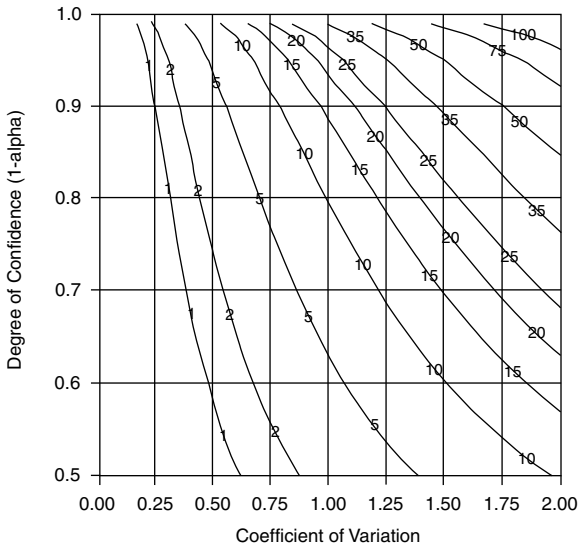

Number of Sample Pairs Needed
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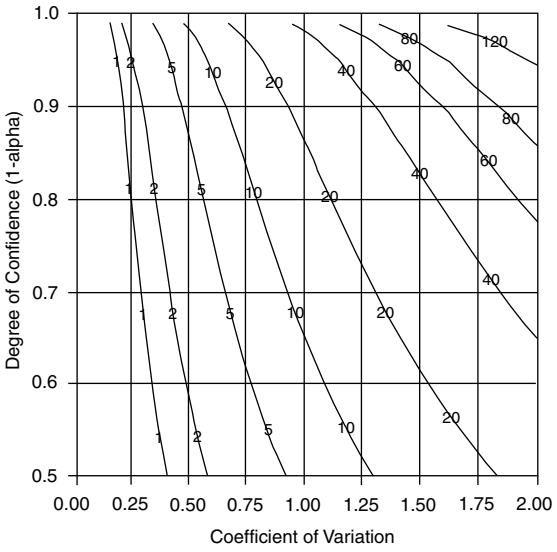
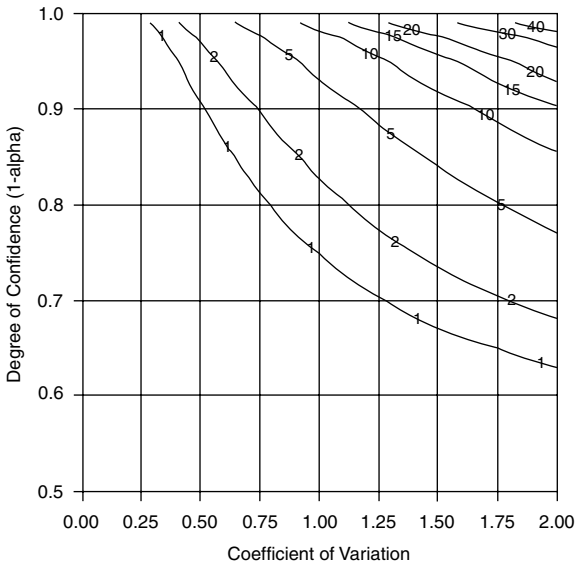


Figure F.6

Number of Sample Pairs Needed
(Power = 0.5 Difference = 95%)



Number of Sample Pairs Needed
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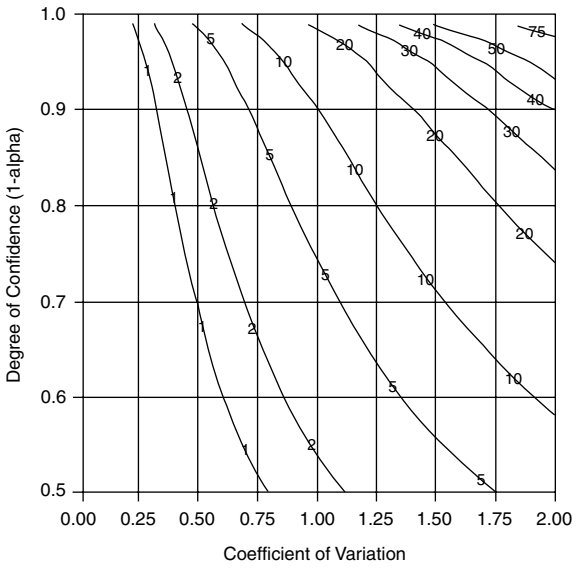

Figure F.7

Number of Sample Pairs Needed
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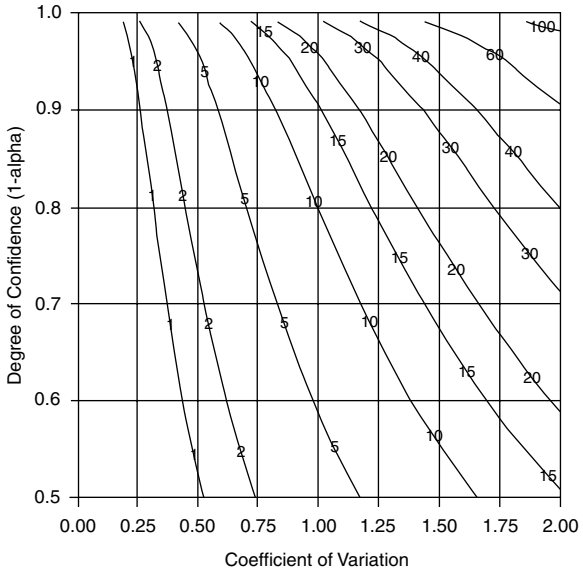

Number of Sample Pairs Needed
(Power = 90% Confidence = 95%)

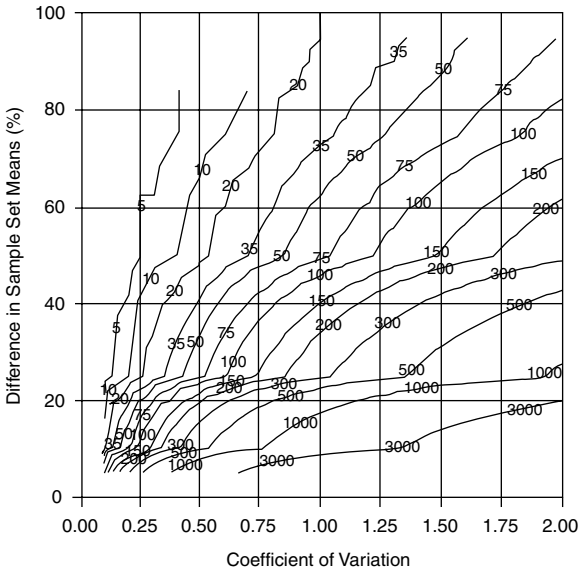

Figure F.8

Number of Sample Pairs Needed
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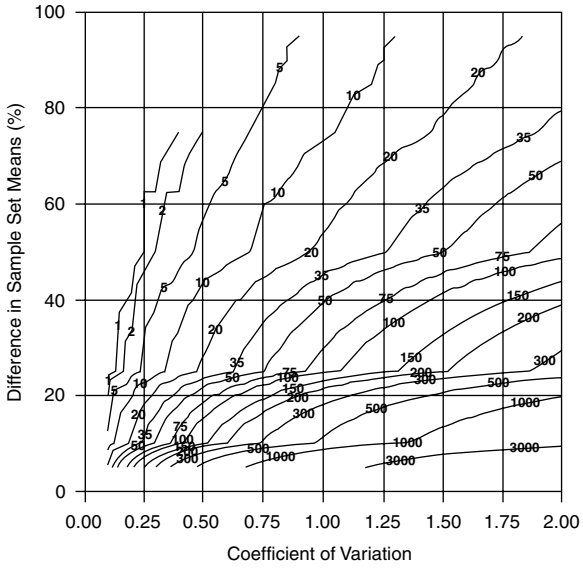

Figure F.9

APPENDIX G

Water Quality Criteria

CONTENTS

INTRODUCTION

One of the most confusing aspects of conducting a receiving water study is attempting to compare acquired water quality data to appropriate standards and criteria. In many cases, available data have been obtained haphazardly without specific project objectives in mind. Inappropriate constituents also may have been measured, based more on convenience (and expense) than usefulness. The user is then left with trying to understand if a problem exists and determining the extent of the problem. This book has emphasized the need for careful experimental design (with clear objectives) and the need for a multidisciplinary approach in receiving water studies.

In all cases, the user will still need to compare acquired data with some type of objective. As stated in Chapter 8, however, care must be taken when comparing measured values with available criteria. In addition, many of the most commonly measured constituents (such as turbidity, Secchi disk transparency, and specific conductivity) are not directly comparable to water quality criteria, and are best evaluated through long experience at a monitoring location and through comparisons with observations obtained at reference sites. Finally, Chapter 8 (and elsewhere) lists reasons why water quality criteria are not directly applicable to stormwater-related conditions. Nevertheless, water quality criteria are important tools that cannot be overlooked. If measured conditions exceed established criteria, then problems may occur, requiring that the conditions be investigated further. However, the most serious problem associated with water quality criteria applied to stormwater is the likelihood of false negative conclusions, based on the observation of no, or few, exceedances. As noted elsewhere, problems caused by stormwater in receiving waters may more likely be associated with habitat disturbances and contaminated sediment than by elevated water quality concentrations. In addition, few receiving water studies include broad representations of toxicants and conventional pollutants, especially in sufficient numbers and sampling frequencies, to make statistically valid comparisons with the criteria.

The following sections of this appendix summarize U.S. Environmental Protection Agency water quality standards and criteria for selected constituents of concern when conducting a receiving water investigation. These criteria and standards are subject to periodic change, and it is important to review the most current listing from the EPA at: http://www.epa.gov/OST/standards. Much of the background discussion in this Appendix is summarized directly from EPA (1986b).

EPA'S WATER QUALITY CRITERIA AND STANDARDS PLAN — PRIORITIES FOR THE FUTURE

In September 1998, the EPA announced a plan (URL: http://www.epa.gov/OST/standards/planfs.html) for working together with the states and tribes to enhance and improve the water quality criteria and standards program across the country. This plan describes new criteria and standards program initiatives that EPA and the states and tribes will take over the next decade. The development and implementation of criteria and standards will provide a basis for enhancements to the total maximum daily load (TMDL) program, National Pollutant Discharge Elimination System (NPDES) permitting, nonpoint source control, wetlands protection, and other water resources management efforts.

The EPA's Office of Water will emphasize and focus on the following priority areas for the Criteria and Standards Program over the next decade:

- Developing nutrient criteria and assessment methods to better protect aquatic life and human health
- Developing criteria for microbial pathogens to better protect human health during water recreation
- Completing the development of biocriteria as an improved basis for aquatic life protection
- Maintaining and strengthening the existing ambient water quality criteria for water and sediments
- Evaluating possible criteria initiatives for excessive sedimentation, flow alterations, and wildlife
- Developing improved water quality modeling tools to better translate water quality standards into implementable control strategies
- Ensuring implementation of these new initiatives and improvements by the states and tribes in partnership with EPA

Over the past two decades, state and tribal water quality standards and water quality-based management approaches have relied upon aquatic life use designations and protective criteria based primarily upon narrative, chemical-specific, and whole-effluent toxicity methodologies. Using these approaches, much progress has been made. However, not all of the nation's waters have achieved the Clean Water Act goal of "fishable and swimmable," and significant water pollution problems still exist. The EPA concludes that there is an essential need for improved water quality standards. Adding nutrient criteria and biological criteria to the water quality criteria and standards program ensures further improvements in maintaining and restoring aquatic life. Improved human health criteria will better protect against bioaccumulative pollutants, and new microbial pathogen controls will better protect human health (especially that of children) during water-related recreation. Better tools are also needed for controlling excessive sedimentation, flow alterations, and for protecting wildlife.

COMPILATION OF RECOMMENDED WATER QUALITY CRITERIA AND EPA'S PROCESS FOR DERIVING NEW AND REVISED CRITERIA

Section 304(a) of the Clean Water Act, 33 U.S.C. 1314(a)(1), requires the EPA to publish and periodically update ambient water quality criteria. These criteria are to "… accurately reflect the latest scientific knowledge … on the kind and extent of all identifiable effects on health and welfare including, but not limited to, plankton, fish, shellfish, wildlife, plant life … which may be expected from the presence of pollutants in any body of water. …" Water quality criteria developed under section 304(a) are based solely on data and scientific judgments on the relationship between pollutant concentrations and environmental and human health effects. These recommended criteria provide guidance for states and tribes in adopting water quality standards under section 303(c) of the CWA. The compilation was published in the *Federal Register* and can be accessed on the Office of Science and Technologies Home-page: http://www.epa.gov/OST/

The following tables are from the April 1999 compilation report (EPA 822-Z-99-001). In these tables, CMC refers to the "criterion maximum concentration" with an exposure period of 1 hour (generally corresponding to the earlier "acute" criterion), and CCC refers to the "criterion continuous concentration" with an averaging period of 4 days (generally corresponding to the earlier "chronic" criterion). "Freshwater" and "saltwater" refer to aquatic life uses in these waters.

Following these tables are discussions for many constituents of concern when conducting a receiving water investigation. These discussions, which briefly outline specific problems associated with different concentrations of the pollutants, are mostly from the 1986 EPA *Water Quality Criteria* report. Some of the criteria have been modified since that time, specifically for ammonia and bacteria, and those discussions have been modified to reflect these newer guidelines.

U.S. Recommended Water Quality Criteria for Priority Toxic Pollutants

	Priority Pollutant	CAS Number	Freshwater CMC (µg/L)	Freshwater CCC (µg/L)	Saltwater CMC (µg/L)	Saltwater CCC (µg/L)	Human Health For Consumption of: Water + Organism (µg/L)	Human Health For Consumption of: Organism Only (µg/L)	*Federal Register* Cite/Source
1	Antimony	7440360					14[B,Z]	4300[B]	57FR60848
2	Arsenic	7440382	340[A,D,K]	150[A,D,K]	69[A,D,bb]	36[A,D,bb]	0.018[C,M,S]	0.14[C,M,S]	62FR42160 57FR60848
3	Beryllium	7440417					J,Z	J	62FR42160
4	Cadmium	7440439	4.3[D,E,K]	2.2[D,E,K]	42[D,bb]	9.3[D,bb]	J,Z	J	62FR42160
5a	Chromium III	16065831	570[D,E,K]	74[D,E,K]			J,Z Total	J	EPA820/B-96-001 62FR42160
5b	Chromium VI	18540299	16[D,K]	11[D,K]	1,100[D,bb]	50[D,bb]	J,Z Total	J	62FR42160
6	Copper	7440508	13[D,E,K,cc]	9.0[D,E,K,cc]	4.8[D,cc,ff]	3.1[D,cc,ff]	1300[U]		62FR42160
7	Lead	7439921	65[D,E,bb,gg]	2.5[D,E,bb,gg]	210[D,bb]	8.1[D,bb]	J	J	62FR42160
8	Mercury	7439976	1.4[D,K,hh]	0.77[D,K,hh]	1.8[D,ee,hh]	0.94[D,ee,hh]	0.050[B]	0.051[B]	62FR42160
9	Nickel	7440020	470[D,E,K]	52[D,E,K]	74[D,bb]	8.2[D,bb]	610[B]	4,600[B]	62FR42160
10	Selenium	7782492	[L,R,T]	5.0[T]	290[D,bb,dd]	71[D,bb,dd]	170[Z]	11,000	62FR42160 IRIS 09/01/91
11	Silver	7440224	3.4[D,E,G]		1.9[D,G]				62FR42160
12	Thallium	7440280					1.7[B]	6.3[B]	57FR60848
13	Zinc	7440666	120[D,E,K]	120[D,E,K]	90[D,bb]	81[D,bb]	9100[U]	69,000[U]	62FR42160 IRIS 10/01/92
14	Cyanide	57125	22[K,Q]	5.2[K,Q]	1[Q,bb]	1[Q,bb]	700[B,Z]	220,000[B,H]	EPA820/B-96-001 57FR60848
15	Asbestos	1332214					7 million fibers/L[I]		57FR60848
16	2,3,7,8-TCDD dioxin	1746016					1.3E-8[C]	1.4E-8[C]	62FR42160
17	Acrolein	107028					320	780	57FR60848
18	Acrylonitrile	107131					0.059[B,C]	0.66[B,C]	57FR60848
19	Benzene	71432					1.2[B,C]	71[B,C]	62FR42160
20	Bromoform	75252					4.3[B,C]	360[B,C]	62FR42160
21	Carbon tetrachloride	56235					0.25[B,C]	4.4[B,C]	57FR60848
22	Chlorobenzene	108907					680[B,Z]	21,000[B,H]	57FR60848
23	Chlorodibromomethane	124481					0.41[B,C]	34[B,C]	62FR42160
24	Chloroethane	75003							
25	2-Chloroethylvinyl ether	110758							

26	Chloroform	67663					5.7[B,C]	470[B,C]	62FR42160
27	Dichlorobromomethane	75274					0.56[B,C]	46[B,C]	62FR42160
28	1,1-Dichloroethane	75343							
29	1,2-Dichloroethane	107062					0.38[B,C]	99[B,C]	57FR60848
30	1,1-Dichloroethylene	75354					0.057[B,C]	3.2[B,C]	57FR60848
31	1,2-Dichloropropane	78875					0.52[B,C]	39[B,C]	62FR42160
32	1,3-Dichloropropene	542756					10[B]	1700[B]	57FR60848
33	Ethylbenzene	100414					3100[B,Z]	29,000[B]	62FR42160
34	Methyl bromide	74839					48[B]	4000[B]	62FR42160
35	Methyl chloride	74873					J	J	62FR42160
36	Methylene chloride	75092					4.7[B,C]	1600[B,C]	62FR42160
37	1,1,2,2-Tetrachloroethane	79345					0.17[B,C]	11[B,C]	57FR60848
38	Tetrachloroethylene	127184					0.8[C]	8.85[C]	57FR60848
39	Toluene	108883					6800[B,Z]	200,000[B]	62FR42160
40	1,2-*trans*-Dichloroethylene	156605					700[B,Z]	140,000[B]	62FR42160
41	1,1,1-Trichloroethane	71556					J,Z	J	62FR42160
42	1,1,2-Trichloroethane	79005					0.60[B,C]	42[B,C]	57FR60848
43	Trichloroethane	79016					2.7[C]	81[C]	57FR60848
44	Vinyl chloride	75014					2.0[C]	525[C]	57FR60848
45	2-Chlorophenol	95578					120[B,U]	400[B,U]	62FR42160
46	2,4-Dichlorophenol	120832					93[B,U]	790[B,U]	57FR60848
47	2,4-Dimethylphenol	105679					540[B,U]	2300[B,U]	62FR42160
48	2-Methyl-4,6-dinitrophenol	534521					13.4	765	57FR60848
49	2,4-Dinitrophenol	51285					70[B]	14,000[B]	57FR60848
50	2-Nitrophenol	88755							
51	4-Nitrophenol	100027							
52	3-Methyl-4-Chlorophenol	59507					U	U	
53	Pentachlorophenol	87865	19[F,K]	15[F,K]	13[bb]	7.9[bb]	0.28[B,C]	8.2[B,C,H]	62FR42160
54	Phenol	108952					21,000[B,U]		62FR42160
								4,600,000[B,H,U]	57FR60848
55	2,4,6-Trichlorophenol	88062					2.1[B,C,U]	6.5[B,C]	62FR42160
56	Acenaphthene	83329					1200[B,U]	2700[B,U]	62FR42160
57	Acenaphthylene	208968							
58	Anthracene	120127					9600[B]	110,000[B]	62FR42160
59	Benzidine	92875					0.00012[B,C]	0.00054[B,C]	57FR60848
60	Benzoaanthracene	56553					0.0044[B,C]	0.049[B,C]	62FR42160
61	Benzoapyrene	50328					0.0044[B,C]	0.049[B,C]	62FR42160
62	Benzobfluoranthene	205992					0.0044[B,C]	0.049[B,C]	62FR42160
63	Benzoghiperylene	191242							

U.S. Recommended Water Quality Criteria for Priority Toxic Pollutants (continued)

	Priority Pollutant	CAS Number	Freshwater CMC (µg/L)	Freshwater CCC (µg/L)	Saltwater CMC (µg/L)	Saltwater CCC (µg/L)	Human Health For Consumption of: Water + Organism (µg/L)	Human Health For Consumption of: Organism Only (µg/L)	*Federal Register* Cite/Source
64	Benzokfluoranthene	207089					0.0044[B,C]	0.049[B,C]	62FR42160
65	Bis-2-chloroethoxymethane	111911							
66	Bis-2-chloroethylether	111444					0.031[B,C]	1.4[B,C]	57FR60848
67	Bis-2-chloroisopropylether	39638329					1400[B]		62FR42160
								170,000[B]	57FR60848
68	Bis-2-ethylhexylphthalate[X]	117817					1.8[B,C]	5.9[B,C]	57FR60848
69	4-Bromophenyl phenyl ether	101553							
70	Butylbenzyl phthalate[W]	85687					3000[B]	5200[B]	62FR42160
71	2-Chloronaphthalene	91587					1700[B]	4300[B]	62FR42160
72	4-Chlorophenyl phenyl ether	7005723							
73	Chrysene	218019					0.0044[B,C]	0.049[B,C]	62FR42160
74	Dibenzoa,hanthracene	53703					0.0044[B,C]	0.049[B,C]	62FR42160
75	1,2-Dichlorobenzene	95501					2700[B,Z]	17,000[B]	62FR42160
76	1,3-Dichlorobenzene	541731					400	2600	62FR42160
77	1,4-Dichlorobenzene	106467					400[Z]	2600	62FR42160
78	3,3′-Dichlorobenzidine	91941					0.04[B,C]	0.077[B,C]	57FR60848
79	Diethyl phthalate[W]	84662					23,000[B,C]	120,000[B]	57FR60848
80	Dimethyl phthalate[W]	131113					313,000	2,900,000	57FR60848
81	Di-*n*-Butyl phthalate[W]	84742					2700[B]	12,000[B]	57FR60848
82	2,4-Dinitrotoluene	121142					0.11[C]	9.1[C]	57FR60848
83	2,6-Dinitrotoluene	606202							
84	Di-*n*-octyl phthalate	117840							
85	1,2-Diphenylhydrazine	122667					0.040[B,C]	0.54[B,C]	57FR60848
86	Fluoranthene	206440					300[B]	370[B]	62FR42160
87	Fluorene	86737					1300[B]	14,000[B]	62FR42160
88	Hexachlorobenzene	118741					0.00075[B,C]	0.00077[B,C]	62FR42160
89	Hexachlorobutadiene	87683					0.44[B,C]	50[B,C]	57FR60848
90	Hexachlorocyclopentadiene	77474					240[B,U,Z]	17,000[B,H,U]	57FR60848
91	Hexachloroethane	67721					1.9[B,C]	8.9[B,C]	57FR60848
92	Idenol1,2,3-cdpyrene	193395					0.0044[B,C]	0.049[B,C]	62FR42160
93	Isophorone	78591					36[B,C]	2600[B,C]	IRIS 11/01/97
94	Naphthalene	91203							

95	Nitrobenzene	98953					17[B]	1900[B,H,U]	57FR60848
96	*N*-Nitrosodimethylamine	62759					0.00069[B,C]	8.1[B,C]	57FR60848
97	*N*-Nitrosodi-*n*-propylamine	621647					0.005[B,C]	1.4[B,C]	62FR42160
98	*N*-Nitrosodiphenylamine	86306					5.0[B,C]	16[B,C]	57FR60848
99	Phenanthrene	85018							
100	Pyrene	129000					960[B]	11,000[B]	62FR42160
101	1,2,4-Trichlorobenzene	120821					260[Z]	940	IRIS 11/01/96
102	Aldrin	309002	3.0[G]		1.3[G]		0.00013[B,C]	0.00014[B,C]	62FR42160
103	α-BHC	319846					0.0039[B,C]	0.013[B,C]	62FR42160
104	β-BHC	319857					0.014[B,C]	0.046[B,C]	62FR42160
105	γ-BHC (Lindane)	58899	0.95[K]		0.16[G]		0.019[C]	0.063[C]	62FR42160
106	δ-BHC	319868							
107	Chlordane	57749	2.4[G]	0.0043[G,aa]	0.09[G]	0.004[G,aa]	0.0021[B,C]	0.0022[B,C]	62FR42160 IRIS 02/07/98
108	4,4′-DDT	50293	1.1[G]	0.001[G,aa]	0.13[G]	0.001[G,aa]	0.00059[B,C]	0.00059[B,C]	62FR42160
109	4,4′-DDE	72559					0.00059[B,C]	0.00059[B,C]	62FR42160
110	4,4′-DDD	72548					0.00083[B,C]	0.00084[B,C]	62FR42160
111	Dieldrin	60571	0.24[K]	0.056[K,O]	0.71[G]	0.0019[G,aa]	0.00014[B,C]	0.00014[B,C]	62FR42160
112	α-Endosulfan	959988	0.22[G,Y]	0.056[K,O]	0.034[G,Y]	0.0087[G,Y]	110[B]	240[B]	62FR42160
113	β-Endosulfan	33213659	0.22[G,Y]	0.056[G,Y]	0.034[G,Y]	0.0087[G,Y]	110[B]	240[B]	62FR42160
114	Endosulfan sulfate	1031078					110[B]	240[B]	62FR42160
115	Endrin	72208	0.086[K]	0.036[K,O]	0.037[G]	0.0023[G,aa]	0.76[B]	0.81[B,H]	62FR42160
116	Endrin aldehyde	7421934					0.76[B]	0.81[B,H]	62FR42160
117	Heptachlor	76448	0.52[G]	0.0038[G,aa]	0.053[G]	0.0036[G,aa]	0.00021[B,C]	0.00021[B,C]	62FR42160
118	Heptachlor epoxide	1024573	0.52[G,V]	0.0038[G,V,aa]	0.053[G,V]	0.0036[G,V,aa]	0.00010[B,C]	0.00011[B,C]	62FR42160
119	Polychlorinated biphenyls			0.014[N,aa]		0.03[N,aa]			62FR42160
							0.00017[B,C,P]	0.00017[B,C,P]	63FR16182
120	Toxaphene	8001352	0.73	0.0002[aa]	0.21	0.0002[aa]	0.00073[B,C]	0.00075[B,C]	62FR42160

A This recommended water quality criterion was derived from data for arsenic (III), but is applied here to total arsenic, which might imply that arsenic (III) and arsenic (V) are equally toxic to aquatic life and that their toxicities are additive. In the arsenic criteria document (EPA 440/5-84-033, January 1985), Species Mean Acute Values are given for both arsenic (III) and arsenic (V) for five species and the ratios of the SMAVs for each species range from 0.6 to 1.7. Chronic values are available for both arsenic (III) and arsenic (V) for one species; for the fathead minnow, the chronic value for arsenic (V) is 0.29 times the chronic value for arsenic (III). No data are known to be available concerning whether the toxicities of the forms of arsenic to aquatic organisms are additive.

B This criterion has been revised to reflect the Environmental Protection Agency's q1* or RfD, as contained in the Integrated Risk Information System (IRIS) as of April 8, 1998. The fish tissue bioconcentration factor (BCF) from the 1980 Ambient Water Quality Criteria document was retained in each case.

C This criterion is based on carcinogenicity of 10^{-6} risk. Alternate risk levels may be obtained by moving the decimal point (e.g., for a risk level of 10^{-5}, move the decimal point in the recommended criterion one place to the right).

U.S. Recommended Water Quality Criteria for Priority Toxic Pollutants (continued)

D Freshwater and saltwater criteria for metals are expressed in terms of the dissolved metals in the water column. The recommended water quality criteria value was calculated by using the previous 304(a) aquatic life criteria expressed in terms of total recoverable metal, and multiplying it by a conversion factor (CF). The term "Conversion Factor" (CF) represents the recommended conversion factor for converting a metal criterion expressed as the total recoverable fraction in the water column to a criterion expressed as the dissolved fraction in the water column. (Conversion Factors for saltwater CCCs are not currently available. Conversion factors derived for saltwater CMCs have been used for both saltwater CMCs and CCCs). See "Office of Water Policy and Technical Guidance on Interpretation and Implementation of Aquatic Life Metals Criteria," October 1, 1993, by Martha G. Prothro, Acting Assistant Administrator for Water, available from the Water Resource center, USEPA, 401 M St., SW, mail code RC4100, Washington, DC 20460; and 40CFR§131.36(b)(1). Conversion Factors applied in the table can be found in Appendix A to the Preamble- Conversion Factors for Dissolved Metals.

E The freshwater criterion for this metal is expressed as a function of hardness (mg/L) in the water column. The value given here corresponds to a hardness of 100 mg/L. Criteria values for other hardness may be calculated from the following: CMC (dissolved) = $\exp\{m_A[\ln(\text{hardness})] + b_A\}$ (CF), or CCC (dissolved) = $\exp\{m_C[\ln(\text{hardness})] + b_C\}$ (CF) and the parameters specified in Appendix B to the Preamble- *Parameters for Calculating Freshwater Dissolved Metals Criteria That Are Hardness-Dependent*.

F Freshwater aquatic life values for pentachlorophenol are expressed as a function of pH, and are calculated as follows: CMC = exp(1.005(pH) – 4.869); CCC = exp(1.005(pH) – 5.134). Values displayed in table correspond to a pH of 7.8.

G This Criterion is based on 304(a) aquatic life criterion issued in 1980, and was issued in one of the following documents: Aldrin/Dieldrin (EPA 440/5-80-019), Chlordane (EPA 440/5-80-027), DDT (EPA 440/5-80-038), Endosulfan (EPA 440/5-80-046), Endrin (EPA 440/5-80-047), Heptachlor (EPA 440/5-80-019), Hexachlorocyclohexane (EPA 440/5-80-054), Silver (EPA 440/5-80-071). The Minimum Data Requirements and derivation procedures were different in the 1980 Guidelines than in the 1985 Guidelines. For example, a "CMC" derived using 1980 Guidelines was derived to be used as an instantaneous maximum. If assessment is to be done using an average period, the values given should be divided by 2 to obtain a value that is more comparable to a CMC derived using the 1985 Guidelines.

H No criterion for protection of human health from consumption of aquatic organisms excluding water was presented in the 1980 criteria document or in the *1986 Quality Criteria for Water*. Nevertheless, sufficient information was presented in the 1980 document to allow the calculation of a criterion, even though the results of such a calculation were not shown in the document.

I This criterion for asbestos is the Maximum Contaminant Level (MCL) developed under the Safe Drinking Water Act (SDWA).

J EPA has not calculated human health criterion for this contaminant. However, permit authorities should address this contaminant in NPDES permit actions using the State's existing narrative criteria for toxics.

K This recommended criterion is based on a 304(a) aquatic life criterion that was issued in the *1995 Updates: Water Quality Criteria Documents for the Protection of Aquatic Life in Ambient Water*, (EPA-820-B-96-001, September 1996). This value was derived using the GLI Guidelines (60FR15393-15399, March 23, 1995; 40CFR132 Appendix A); the difference between the 1985 Guidelines and the GLI Guidelines are explained on page iv of the 1995 Updates. None of the decisions concerning the derivation of this criterion were affected by any considerations that are specific to the Great Lakes.

L The CMC = 1/[(f1/CMC1) + (f2/CMC2)] where f1 and f2 are the fractions of total selenium that are treated as selenite and selenate, respectively, and CMC1 and CMC2 are 185.9 µg/l and 12.83 µg/l, respectively.

M EPA is currently reassessing the criteria for arsenic. Upon completion of the reassessment the Agency will publish revised criteria as appropriate.

N PCBs are a class of chemicals which include Aroclors, 1242, 1254, 1221, 1232, 1248, 1260, and 1016, CAS numbers 53469219, 11097691, 11104282, 11141165, 12672296, 11096825 and 12674112 respectively. The aquatic life criteria apply to this set of PCBs.

O The derivation of the CCC for this pollutant did not consider exposure through the diet, which is probably important for aquatic life occupying upper trophic levels.

P This criterion applies to total pcbs, i.e., the sum of all congener or all isomer analyses.

Q This recommended water criterion is expressed as µg free cyanide (as CN)/L.

R This value was announced (61FR58444-58449, November 14, 1996) as a proposed GLI 303(c) aquatic life criterion. EPA is currently working on this criterion and so this value might change substantially in the near future.

S This recommended water quality criterion refers to the inorganic form only.

T This recommended water quality criterion is expressed in terms of total recoverable metal in the water column. It is scientifically acceptable to use the conversion factor of 0.922 that was used in the GLI to convert this to a value that is expressed in terms of dissolved metal.

U The organoleptic effect criterion is more stringent than the value for priority toxic pollutants.

V This value was derived from data for heptachlor and the criteria document provides insufficient data to estimate the relative toxicities of heptachlor and heptachlor epoxide.

W Although EPA has not published a final criteria document for this compound it is EPA's understanding that sufficient data exist to allow calculation of aquatic criteria. It is anticipated that industry intends to publish in the peer reviewed literature draft aquatic life criteria generated in accordance with EPA Guidelines. EPA will review such criteria for possible issuance as national WQC.

X There is a full set of aquatic life toxicity data that show that DEHP is not toxic to aquatic organisms at or below its solubility limit.

Y This value was derived from data for endosulfan and is most appropriately applied to the sum of alpha-endosulfan and beta-endosulfan.

Z A more stringent MCL has been issued by the EPA. Refer to drinking water regulations (40 CFR 141) or Safe Drinking Water Hotline (1-800-426-4791) for values.

aa This CCC is based on the Final Residue Value procedure in the 1985 Guidelines. Since the publication of the Great Lakes Aquatic Life Criteria Guidelines in 1995 (60FR15393-15399, March 23, 1995), the Agency no longer uses the Final Residue Value procedure for deriving CCCs for new or revised 304(a) aquatic life criteria.

bb This water quality criterion is based on a 304(a) aquatic life criterion that was derived using the 1985 Guidelines (*Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses,* PB85-227049, January 1985) and was issued in one of the following criteria documents: Arsenic (EPA 440/5-84-033), Cadmium (EPA 440/5-84-032), Chromium (EPA 440/5-84-029), Copper (EPA 440/5-84-031), Cyanide (EPA 440/5-84-028), Lead (EPA 440/5-84-027), Nickel (EPA 440/5-86-004), Pentachlorophenol (EPA 440/5-86-009), Toxaphene (EPA 440/5-86-006), Zinc (EPA 440/5-87-003).

cc When the concentration of dissolved organic carbon is elevated, copper is substantially less toxic and use of Water-Effect Ratios might be appropriate.

dd The selenium criteria document (EPA 440/5-87-006, September 1987) provides that if selenium is as toxic to saltwater fishes in the field as it is to freshwater fishes in the field, the status of the fish community should be monitored whenever the concentration of selenium exceeds 5.0 µg/L in salt water because the saltwater CCC does not take into account uptake via the food chain.

ee This recommended water quality criterion was derived on page 43 of the mercury criteria document (EPA 440/5-84-026, January 1985). The saltwater CCC of 0.025 µg/L given on page 23 of the criteria document is based on Final Residue Value procedure in the 1985 Guidelines. Since the publication of the Great Lakes Aquatic Life Criteria Guidelines in 1995 (60FR15393-15399, March 23, 1995), the Agency no longer uses the Final Residue Value procedure for deriving CCCs for new or revised 304(a) aquatic life criteria.

ff This recommended water quality criterion was derived in *Ambient Water Quality Criteria Saltwater Copper Addendum* (Draft, April 14, 1995) and was promulgated in the Interim final National Toxics Rule (60FR22228-222237, May 4, 1995).

gg EPA is actively working on this criterion and so this recommended water quality criterion may change substantially in the near future.

hh This recommended water quality criterion was derived from data for inorganic mercury (II), but is applied here to total mercury. If a substantial portion of the mercury in the water column is methylmercury, this criterion will probably be under protective. In addition, even though inorganic mercury is converted to methylmercury and methylmercury bioaccumulates to a great extent, this criterion does not account for uptake via the food chain because sufficient data were not available when the criterion was derived.

U.S. Recommended Water Quality Criteria for Nonpriority Pollutants

	Nonpriority Pollutant	CAS Number	Freshwater CMC (µg/L)	Freshwater CCC (µg/L)	Saltwater CMC (µg/L)	Saltwater CCC (µg/L)	Human Health For Consumption of: Water + Organism (µg/L)	Human Health For Consumption of: Organism Only (µg/L)	*Federal Register* Cite/Source
1	Alkalinity	—		20000[F]					Gold Book
2	Aluminum pH 6.5–9.0	7429905	750[G,I]	87[G,I,L]					53FR33178
3	Ammonia	7664417	FRESHWATER CRITERIA ARE pH DEPENDENT — SEE DOCUMENT[D] SALTWATER CRITERIA ARE pH AND TEMPERATURE DEPENDENT						EPA822-R-98-008 EPA440/5-88-004
4	Aesthetic qualities	—	NARRATIVE STATEMENT — SEE DOCUMENT						Gold Book
5	Bacteria	—	FOR PRIMARY RECREATION AND SHELLFISH USES — SEE DOCUMENT						Gold Book
6	Barium	7440393					1000[A]		Gold Book
7	Boron	—	NARRATIVE STATEMENT — SEE DOCUMENT						Gold Book
8	Chloride	16887006	860000[G]	230000[G]					53FR19028
9	Chlorine	7782505	19	11	13	7.5	C		Gold Book
10	Chlorophenoxy herbicide 2,4,5,-TP	93721					10[A]		Gold Book
11	Chlorophenoxy herbicide 2,4-D	94757					100[A,C]		Gold Book
12	Chloropyrifos	2921882	0.083[G]	0.041[G]	0.011[G]	0.0056[G]			Gold Book
13	Color	—	NARRATIVE STATEMENT — SEE DOCUMENT[F]						Gold Book
14	Demeton	8065483		0.1[F]		0.1[F]			Gold Book
15	Ether, Bis Chloromethyl	542881					0.00013[E]	0.00078[E]	IRIS 01/01/91
16	Gases, Total Dissolved	—	NARRATIVE STATEMENT — SEE DOCUMENT[F]						Gold Book
17	Guthion	86500		0.01[F]		0.01[F]			Gold Book
18	Hardness	—	NARRATIVE STATEMENT — SEE DOCUMENT						Gold Book
19	Hexachlorocyclo-hexane-Technical	319868					0.0123	0.0414	Gold Book
20	Iron	7439896		1000[F]			300[A]		Gold Book
21	Malathion	121755		0.1[F]		0.1[F]			Gold Book
22	Manganese	7439965					50[A]	100[A]	Gold Book
23	Methoxychlor	72435		0.03[F]		0.03[F]	100[A,C]		Gold Book
24	Mirex	2385855		0.001[F]		0.001[F]			Gold Book
25	Nitrates	14797558					10,000[A]		Gold Book
26	Nitrosamines	—					0.0008	1.24	Gold Book
27	Dinitrophenols	25550587					70	14,000	Gold Book
28	Nitrosodibutylamine,N	924163					0.0064[A]	0.587[A]	Gold Book
29	Nitrosodiethylamine,N	55185					0.0008[A]	1.24[A]	Gold Book
30	Nitrosopyrrolidine,N	930552							Gold Book
31	Oil and grease	—	NARRATIVE STATEMENT — SEE DOCUMENT[F]						Gold Book
32	Oxygen, dissolved	7782447	NARRATIVE STATEMENT — SEE DOCUMENT[O]						Gold Book

33	Parathion	56382	0.065[J]	0.013[J]					Gold Book
34	Pentachlorobenzene	608935					3.5[E]	4.1[E]	IRIS 03/01/88
35	pH	—		6.5–9[F]		6.5–8.5[F,K]	5–9		Gold Book
36	Phosphorus elemental	7723140				0.1[F,K]			Gold Book
37	Phosphate phosphorus	—		NARRATIVE STATEMENT — SEE DOCUMENT					Gold Book
38	Solids dissolved and salinity	—					250,000[A]		Gold Book
39	Solids suspended and turbidity	—		NARRATIVE STATEMENT — SEE DOCUMENT[F]					Gold Book
40	Sulfide-hydrogen sulfide	7783064		2.0[F]		2.0[F]			Gold Book
41	Tainting substances	—		NARRATIVE STATEMENT — SEE DOCUMENT					Gold Book
42	Temperature	—		NARRATIVE STATEMENT — SEE DOCUMENT[M]					Gold Book
43	Tetrachlorobenzene,1,2,4,5-	95943					2.3[E]	2.9[E]	IRIS 03/01/91
44	Tributyltin TBT	—	0.46[N]	0.063[N]	0.37[N]	0.010[N]			62FR42554
45	Trichlorophenol,2,4,5-	95954					2600[B,E]	9800[B,E]	IRIS 03/01/88

A This human health criterion is the same as originally published in the Red Book which predates the 1980 methodology and did not utilize the fish ingestion BCF approach. This same criterion value is now published in the Gold Book.

B The organoleptic effect criterion value is more stringent than the value presented in the non priority pollutants table.

C A more stringent Maximum Contaminant Level (MCL) has been issued by EPA under the Safe Drinking Water Act. Refer to drinking water regulations 40CFR141 or Safe Drinking Water Hotline (1-800-426-4791) for values.

D According to the procedures described in the *Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses*, except possibly where a very sensitive species is important at a site, freshwater aquatic life should be protected if both conditions specified in Appendix C to the Preamble- Calculation of Freshwater Ammonia Criterion are satisfied.

E This criterion has been revised to reflect the Environmental Protection Agency's q1* or RfD, as contained in the Integrated Risk Information System (IRIS) as of April 8, 1998. The fish tissue bioconcentration factor (BCF) used to derive the original criterion was retained in each case.

F The derivation of this value is presented in the Red Book (EPA 440/9-76-023, July, 1976).

G This value is based on a 304(a) aquatic life criterion that was derived using the 1985 Guidelines (*Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses*, PB85-227049, January 1985) and was issued in one of the following criteria documents: Aluminum (EPA 440/5-86-008); Chloride (EPA 440/5-88-001), Chloropyrifos (EPA 440/5-86-005).

I This value is expressed in terms of total recoverable metal in the water column.

J This value is based on a 304(a) aquatic life criterion that was issued in the *1995 Updates: Water Quality Criteria Documents for the Protection of Aquatic Life in Ambient Water* (EPA-820-B-96-001). This value was derived using the GLI Guidelines (60FR15393-15399, March 23, 1995; 40CFR132 Appendix A); the differences between the 1985 Guidelines and the GLI Guidelines are explained on page iv of the 1995 Updates. No decision concerning this criterion was affected by any considerations that are specific to the Great Lakes.

K According to page 181 of the Red Book:

> For open ocean waters where the depth is substantially greater than the euphotic zone, the pH should not be changed more than 0.2 units from the naturally occurring variation of any caes outside the range of 6.5 to 8.5. For shallow, highly productive coastal and estuarine areas where naturally occurring pH variations approach the lethal limits of some species, changes in pH should be avoided but in any case should not exceed the limits established for fresh water, i.e., 6.5–9.0.

U.S. Recommended Water Quality Criteria for Nonpriority Pollutants (continued)

L There are three major reasons why the use of Water-Effect Ratios might be appropriate. (1) The value of 87 µg/l is based on a toxicity test with the striped bass in water with pH = 6.5–6.6 and hardness <10 mg/L. Data in "Aluminum Water-Effect Ratio for the 3M Plant Effluent Discharge, Middleway, West Virginia" (May 1994) indicate that aluminum is substantially less toxic at higher pH and hardness, but the effects of pH and hardness are not well quantified at this time. (2) In tests with the brook trout at low pH and hardness, effects increased with increasing concentrations of total aluminum even though the concentration of dissolved aluminum hydroxide particles. In surface waters, however, the total recoverable procedure might measure aluminum associated with clay particles, which might be less toxic than aluminum associated with aluminum hydroxide. (3) EPA is aware of field data indicating that many high quality waters in the U.S. contain more than 87 µg aluminum/L, when either total recoverable or dissolved is measured.

M U.S. EPA. 1973. Water Quality Criteria 1972. EPA-R3-73-033. National Technical Information Service, Springfield, VA.; U.S. EPA. 1977. Temperature Criteria for Freshwater Fish: Protocol and Procedures. EPA-600/3-77-061. National Technical Information Service, Springfield, VA.

N This value was announced (62FR42554, August 7, 1997) as a proposed 304(a) aquatic life criterion. Although EPA has not responded to public comment, EPA is publishing this as a 304(a) criterion in today's notice as guidance for States and Tribes to consider when adopting water quality criteria.

O U.S. EPA. 1986. Ambient Water Quality Criteria for Dissolved Oxygen. EPA 440/5-86-003. National Technical Information Service, Springfield, VA.

U.S. Recommended Water Quality Criteria for Organoleptic Effects

	Pollutant	CAS Number	Organoleptic Effect Criteria (μg/L)	*Federal Register* Cite/Source
1	Acenaphthene	83329	20	Gold Book
2	Monochlorobenzene	108907	20	Gold Book
3	3-Chlorophenol	—	0.1	Gold Book
4	4-Chlorophenol	106489	0.1	Gold Book
5	2,3-Dichlorophenol	—	0.04	Gold Book
6	2,5-Dichlorophenol	—	0.5	Gold Book
7	2,6-Dichlorophenol	—	0.2	Gold Book
8	3,4-Dichlorophenol	—	0.3	Gold Book
9	2,4,5-Trichlorophenol	95954	1	Gold Book
10	2,4,6-Trichlorophenol	88062	2	Gold Book
11	2,3,4,6-Tetrachlorophenol	—	1	Gold Book
12	2-Methyl-4-Chlorophenol	—	1800	Gold Book
13	3-Methyl-4-Chlorophenol	59507	3000	Gold Book
14	3-Methyl-6-Chlorophenol	—	20	Gold Book
15	2-Chlorophenol	95578	0.1	Gold Book
16	Copper	7440508	1000	Gold Book
17	2,4-Dichlorophenol	120832	0.3	Gold Book
18	2,4-Dimethylphenol	105679	400	Gold Book
19	Hexachlorocyclopentadiene	77474	1	Gold Book
20	Nitrobenzene	98953	30	Gold Book
21	Pentachlorophenol	87865	30	Gold Book
22	Phenol	108952	300	Gold Book
23	Zinc	7440666	5000	45FR79341

1. These criteria are based on organoleptic (taste and odor) effects. Because of variations in chemical nomenclature systems, this listing of pollutants does not duplicate the listing in Appendix A of 40 CFR Part 423. Also listed are the Chemical Abstracts Service (CAS) registry numbers, which provide a unique identification for each chemical.

Additional Notes:

1. Criteria Maximum Concentration and Criterion Continuous Concentration

The Criteria Maximum Concentration (CMC) is an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed briefly without resulting in an unacceptable effect. The Criterion Continuous Concentration (CCC) is an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect. The CMC and CCC are just two of the six parts of aquatic life criterion; the other four parts are the acute averaging period, chronic averaging period, acute frequency of allowed exceedance, and chronic frequency of allowed exceedance. Because 304(a) aquatic life criteria are national guidance, they are intended to be protective of the vast majority of the aquatic communities in the United States.

2. Criteria Recommendations for Priority Pollutants, Nonpriority Pollutants, and Organoleptic Effects

This compilation lists all priority toxic pollutants and some non priority toxic pollutants, and both human health effect and organoleptic effect criteria issued pursuant to CWA §304(a). Blank spaces indicate that EPA has no CWA §304(a) criteria recommendations. For a number of nonpriority toxic pollutants not listed, CWA §304(a) "water + organism" human health criteria are not available, but, EPA has published MCLs under the SDWA that may be used in establishing water quality standards to protect water supply designated uses. Because of variations in chemical nomenclature systems, this listing of toxic pollutants does not duplicate the listing in Appendix A of 40 CFR Part 423. Also listed are the Chemical Abstracts Service CAS registry numbers, which provide a unique identification for each chemical.

3. Human Health Risk

The human health criteria for the priority and nonpriority pollutants are based on carcinogenicity of 10^{-6} risk. Alternate risk levels may be obtained by moving the decimal point (e.g., for a risk level of 10^{-5}, move the decimal point in the recommended criterion one place to the right).

4. Water Quality Criteria published pursuant to Section 304(a) or Section 303(c) of the CWA

Many of the values in the compilation were published in the proposed California Toxics Rule (CTR, 62FR42160). Although such values were published pursuant to Section 303(c) of the CWA, they represent the Agency's most recent calculation of water quality criteria and thus are published today as the Agency's 304(a) criteria. Water quality criteria published in the proposed CTR may be revised when EPA takes final action on the CTR.

5. Calculation of Dissolved Metals Criteria

The 304(a) criteria for metals, shown as dissolved metals, are calculated in one of two ways. For freshwater metals criteria that are hardness-dependent, the dissolved metal criteria were calculated using a hardness of 100 mg/L as $CaCO_3$ for illustrative purposes only. Saltwater and freshwater metals' criteria that are not hardness-dependent are calculated by multiplying the total recoverable criteria before rounding by the appropriate conversion factors. The final dissolved metals' criteria in the table are rounded to two significant figures. Information regarding the calculation of hardness dependent conversion factors are included in the footnotes.

6. Correction of Chemical Abstract Services Number

The Chemical Abstract Services number (CAS) for Bis(2-Chloroisoprpyl) Ether, has been corrected in the table. The correct CAS number for this chemical is 39638-32-9. Previous publications listed 108-60-1 as the CAS number for this chemical.

7. Maximum Contaminant Levels

The compilation includes footnotes for pollutants with Maximum Contaminant Levels (MCLs) more stringent than the recommended water quality criteria in the compilation. MCLs for these pollutants are not included in the compilation, but can be found in the appropriate drinking water regulations (40 CFR 141.11-16 and 141.60-63), or can be accessed through the Safe Drinking Water Hotline (800-426-4791) or the Internet (http://www.epa.gov/ost/tools/dwstds-s.html).

8. Organoleptic Effects

The compilation contains 304(a) criteria for pollutants with toxicity-based criteria as well as non-toxicity based criteria. The basis for the non-toxicity based criteria are organoleptic effects for 23 pollutants. Pollutants with organoleptic effect criteria more stringent than the criteria based on toxicity (e.g., included in both the priority and non-priority pollutant tables) are footnoted as such.

9. Category Criteria

In the 1980 criteria documents, certain recommended water quality criteria were published for categories of pollutants rather than for individual pollutants within that category. Subsequently, in a series of separate actions, the Agency derived criteria for specific pollutants within a category. Therefore, in this compilation EPA is replacing criteria representing categories with individual pollutant criteria (e.g., 1,3-dichlorobenzene, 1,4-dichlorobenzene and 1,2-dichlorobenzene).

10. Specific Chemical Calculation

A. Selenium

(1) Human Health

In the 1980 Selenium document, a criterion for the protection of human health from consumption of water and organisms was calculated based on a BCF of 6.0 L/kg and a maximum water-related contribution of 35 μg Se/day. Subsequently, the EPA Office of Health and Environmental Assessment issued an errata notice (February 23, 1982), revising the BCF for selenium to 4.8 L/kg. In 1988, EPA issued an addendum (ECAO-CIN-668) revising the human health criteria for selenium. Later in the final National Toxic Rule (NTR, 57 FR 60848), EPA withdrew previously published selenium human health criteria, pending Agency review of new epidemiological data.

This compilation includes human health criteria for selenium, calculated using a BCF of 4.8 L/kg along with the current IRIS RfD of 0.005 mg/kg/day. EPA included these recommended water quality criteria in the compilation because the data necessary for calculating a criteria in accordance with EPA's 1980 human health methodology are available.

(2) Aquatic Life

This compilation contains aquatic life for selenium that are the same as those published in the proposed CTR. In the CTR, EPA proposed an acute criterion for selenium based on the criterion proposed for selenium in the Water Quality Guidance for the Great Lakes System (61 FR 58444). The GLI and CTR proposals take into account data showing that selenium's two most prevalent oxidation states, selenite and selenate, present differing potentials for aquatic toxicity, as well as new data indicating that various forms of selenium are additive. The new approach produces a different selenium acute criterion concentration, or CMC, depending upon the relative proportions of selenite, selenate, and other forms of selenium that are present.

EPA notes its currently undertaking a reassessment of selenium, and expects the 304(a) criteria for selenium will be revised based on the final reassessment (63FR26186). However, until such time as revised water quality criteria for selenium are published by the Agency, the recommended water quality criteria in this compilation are EPA's current 304(a) criteria.

U.S. Recommended Water Quality Criteria for Organoleptic Effects (continued)

B. 1,2,4-Trichlorobenzene and Zinc

Human health criteria for 1,2,4-trichlorobenzene and zinc have not been previously published. Sufficient information is now available for calculating water quality criteria for the protection of human health from the consumption of aquatic organisms and the consumption of aquatic organisms and water for both these compounds. Therefore, EPA is publishing criteria for these pollutants in this compilation.

C. Chromium (III)

The recommended aquatic life water quality criteria for chromium (III) included in the compilation are based on the values presented in the document titled: *1995 Updates: Water Quality Criteria Documents for the Protection of Aquatic Life in Ambient Water*; however, this document contains criteria based on the total recoverable fraction. The chromium (III) criteria in this compilation were calculated by applying the conversion factors used in the Final Water Quality Guidance for the Great Lakes System (60 FR 15366) to the 1995 Update document values.

D. Ether, Bis (Chloromethyl), Pentachlorobenzene, Tetrachlorobenzene 1,2,4,5-, Trichlorophenol

Human health criteria for these pollutants were last published in EPA's *Quality Criteria for Water 1986* or "*Gold Book*." Some of these criteria were calculated using Acceptable Daily Intake (ADIs) rather than RfDs. Updated q1*s and RfDs are now available in IRIS for ether, bis (chloromethyl), pentachlorobenzene, tetrachlorobenzene 1,2,4,5-, and trichlorophenol, and were used to revise the water quality criteria for these compounds. The recommended water quality criteria for ether, bis (chloromethyl) were revised using an updated q1*, while criteria for pentachlorobenzene, and tetrachlorobenzene 1,2,4,5-, and trichlorophenol were derived using an updated RfD value.

E. PCBs

In this compilation EPA is publishing aquatic life and human health criteria based on total PCBs rather than individual arochlors. These criteria replace the previous criteria for the seven individual arochlors. Thus, there are criteria for a total of 102 of the 126 priority pollutants.

Appendix A — Conversion Factors for Dissolved Metals

Metal	Conversion Factor (freshwater CMC)	Conversion Factor (freshwater CCC)	Conversion Factor (saltwater CMC)	Conversion Factor (saltwater CCC)[1]
Arsenic	1.000	1.000	1.000	1.000
Cadmium	1.136672–[(ln hardness) (0.041838)]	1.101672–[(ln hardness) (0.041838)]	0.994	0.994
Chromium III	0.316	0.860	—	—
Chromium VI	0.982	0.962	0.993	0.993
Copper	0.960	0.960	0.83	0.83
Lead	1.46203–[(ln hardness) (0.145712)]	1.46203–[(ln hardness) (0.145712)]	0.951	0.951
Mercury	0.85	0.85	0.85	0.85
Nickel	0.998	0.997	0.990	0.990
Selenium	—	—	0.998	0.998
Silver	0.85	—	0.85	—
Zinc	0.978	0.986	0.946	0.946

Appendix B — Parameters for Calculating Freshwater Dissolved Metals Criteria That Are Hardness Dependent

Chemical	m_A	b_A	m_C	b_C	Freshwater Conversion Factor (CF) Acute	Chronic
Cadmium	1.128	–3.6867	0.7852	–2.715	1.136672–[(ln hardness) (0.041838)]	1.101672–[(ln hardness) (0.041838)]
Chromium II	0.8190	3.7256	0.8190	0.6848	0.860	0.860
Copper	0.9422	–1.700	0.8545	–1.702	0.960	0.960
Lead	1.273	–1.460	1.273	–4.705	1.46203–[(ln hardness) (0.145712)]	1.46203–[(ln hardness) (0.145712)]
Nickel	0.8460	2.255	0.8460	0.0584	0.998	0.997
Silver	1.72	–6.52	—	—	0.85	—
Zinc	0.8473	0.884	0.8473	0.884	0.978	0.986

Appendix C — Calculation of Freshwater Ammonia Criterion

1. The one-hour average concentration of total ammonia nitrogen (in mg N/L) does not exceed, more than once every three years on the average, the CMC calculated using the following equation:

$$CMC = \frac{0.275}{1 + 10^{7.204 - pH}} + \frac{39.0}{1 + 10^{pH - 7.204}}$$

In situations where salmonids do not occur, the CMC may be calculated using the following equation:

$$CMC = \frac{0.411}{1 + 10^{7.204 - pH}} + \frac{58.4}{1 + 10^{pH - 7.204}}$$

2. The 30-day average concentration of total ammonia nitrogen (in mg N/L) does not exceed, more than once every 3 years on the average, the CCC calculated using the following equation:

$$CCC = \frac{0.0858}{1 + 10^{7.688 - pH}} + \frac{3.70}{1 + 10^{pH - 7.688}}$$

and the highest 4-day average within the 30-day period does not exceed twice the CCC.

AMMONIA

The ammonia criteria are only for the protection of aquatic life, as no criteria have been developed for the protection of human health (consumption of contaminated fish or drinking water). The water quality criteria are for general guidance only and do not constitute formal water quality

standards. However, the criteria reflect the scientific knowledge concerning the effects of the pollutants and are recommended EPA acceptable limits for aquatic life.

The data used in deriving the EPA criteria are predominantly from flow-through tests in which ammonia concentrations were measured. Ammonia was reported to be acutely toxic to freshwater organisms at concentrations (uncorrected for pH) ranging from 0.53 to 22.8 mg/L NH_3 for 19 invertebrate species representing 14 families and 16 genera and from 0.083 to 4.60 mg/L NH_3 for 29 fish species from 9 families and 18 genera. Among fish species, reported 96-hour LC50 values ranged from 0.083 to 1.09 mg/L for salmonids and from 0.14 to 4.60 mg/L NH_3 for other fish. Reported data from chronic tests on ammonia with two freshwater invertebrate species, both daphnids, showed effects at concentrations (uncorrected for pH) ranging from 0.304 to 1.2 mg/L NH_3, and with nine freshwater fish species, from five families and seven genera, ranging from 0.0017 to 0.612 mg/L NH_3.

Concentrations of ammonia acutely toxic to fishes may cause loss of equilibrium, hyperexcitability, increased breathing, cardiac output and oxygen uptake, and, in extreme cases, convulsions, coma, and death. At lower concentrations, ammonia has many effects on fishes, including a reduction in hatching success, reduction in growth rate and morphological development, and pathologic changes in tissues of gills, livers, and kidneys.

Several factors have been shown to modify acute NH_3 toxicity in fresh water. Some factors alter the concentration of un-ionized ammonia in the water by affecting the aqueous ammonia equilibrium, and some factors affect the toxicity of un-ionized ammonia itself, either ameliorating or exacerbating the effects of ammonia. Factors that have been shown to affect ammonia toxicity include dissolved oxygen concentration, temperature, pH, previous acclimation to ammonia, fluctuating or intermittent exposures, carbon dioxide concentration, salinity, and the presence of other toxicants.

The most well studied of these is pH; the acute toxicity of NH_3 has been shown to increase as pH decreases. However, the percentage of the total ammonia that is un-ionized decreases with decreasing pH. Sufficient data exist from toxicity tests conducted at different pH values to formulate a relationship to describe the pH-dependent acute NH_3 toxicity. The very limited amount of data regarding effects of pH on chronic NH_3 toxicity also indicate increasing NH_3 toxicity with decreasing pH, but the data are insufficient to derive a broadly applicable toxicity/pH relationship. Data on temperature effects on acute NH_3 toxicity were limited and somewhat variable, but indications are that NH_3 toxicity to fish is greater as temperature decreases. There was no information available regarding temperature effects on chronic NH_3 toxicity. Examination of pH and temperature-corrected acute NH_3 toxicity values among species and genera of freshwater organisms showed that invertebrates are generally more tolerant than fishes, a notable exception being the fingernail clam. There is no clear trend among groups of fish; the several most sensitive tested species and genera include representatives from diverse families (Salmonidae, Cyprinidae, Percidae, and Centrarchidae). Available chronic toxicity data for freshwater organisms also indicate invertebrates (cladocerans, an insect species) to be more tolerant than fishes, again with the exception of the fingernail clam. When corrected for the presumed effects of temperature and pH, there was no clear trend among groups of fish for chronic toxicity values. The most sensitive species, including representatives from five families (Salmonidae, Cyprinidae, Ictaluridae, Centrarchidae, and Catostomidae), have chronic values ranging by not much more than a factor or two. Available data indicate that differences in sensitivities between warm- and cold-water families of aquatic organisms are inadequate to warrant discrimination in the national ammonia criterion between bodies of water with “warm-” and “cold-water” fishes; rather, effects of organism sensitivities on the criterion are most appropriately handled by site-specific criteria derivation procedures.

Data for concentrations of NH_3 toxic to freshwater phytoplankton and vascular plants, although limited, indicate that freshwater plant species are appreciably more tolerant to NH_3 than are invertebrates or fishes. The ammonia criterion appropriate for the protection of aquatic animals will therefore in all likelihood be sufficiently protective of plant life.

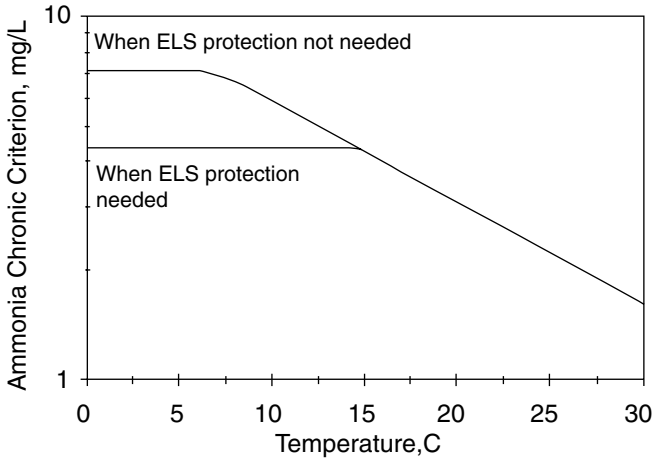

Figure G.1 Chronic criterion values for early life stages (ELS) of fish in the 1999 update; pH = 7.5.

National Ammonia Water Quality Criteria

The U.S. EPA has published a 1999 Update of Ambient Water Quality Criteria for Ammonia (1999 Ammonia Update). The 1999 Ammonia Update contains EPA's most recent freshwater aquatic life criteria for ammonia, superseding all previous EPA-recommended freshwater criteria for ammonia. The 1999 Ammonia Update pertains only to fresh waters and does not change or supersede the aquatic life criterion for ammonia in salt water, published in Ambient Water Quality Criteria for Ammonia (Saltwater) in 1989. The new criteria reflect recent research and data since 1984, and are a revision of several elements in the 1984 criteria, including the pH and temperature relationship of the acute and chronic criteria and the averaging period of the chronic criterion. As a result of these revisions, the acute criterion for ammonia is now dependent on pH and fish species, and the chronic criterion is dependent on pH and temperature. At lower temperatures, the dependency of chronic criterion is also dependent on the presence or absence of early life stages of fish (ELS). The effect of temperature and expected presence of early life stages of fish on the chronic criterion in the 1999 Update is shown in Figure G.1. The temperature dependency in the 1999 Update results in a gradual increase in the criterion as temperature decreases, and a criterion that is more stringent, at temperatures below 15°C, when early life stages of fish (ELS) are expected to be present.

EPA's recommendations in the 1999 Update represent a change from both the 1984 chronic criterion, which was dependent mainly on pH, and from the 1998 Update, in which the chronic criterion was dependent on pH and the presence of early life stages of fish. The temperature dependency of ammonia toxicity at temperatures below 20°C is incorporated directly into the criterion of the 1999 Update. The other significant revision in the 1999 Update is EPA's recommendation of 30 days as the averaging period for the ammonia chronic criterion. EPA recommends the 30B3 (the lowest 30-day average flow based on a 30-year return interval when flow records are analyzed using EPA's 1986 DFLOW procedure), the 30Q10 (the lowest 30-day average flow based on a 10-year return interval when flow records are analyzed using extreme-value statistics), or the 30Q5 as the appropriate design flows associated with the 30-day averaging period of the ammonia chronic criterion. In addition, EPA recommends that within the 30-day averaging period, no 4-day average concentration should exceed 2.5 times the chronic criterion, or criterion continuous concentration (CCC). Consequently, the design flow should also be protective of any 4-day average at 2.5 times the CCC. EPA believes that in the vast majority of cases, the 30Q10 is protective of both the CCC and any 4-day average at 2.5 times the CCC. However, if a state or tribe specifies the use of the 30Q5, then the state or tribe should demonstrate that a 7Q10 (the lowest average 7-day once-in-10-year flow using extreme-

value statistics) is protective of 2.5 times the CCC, to ensure that any short-term (4-day) flow variability within the 30-day averaging period does not lead to shorter-term chronic toxicity.

BACTERIA

Development of Bathing Beach Bacteriological Criteria

Dufour (1984) presents an excellent overview of the history of bacterial standards and water contact recreation, summarized here. Total coliforms were initially used as indicators for monitoring outdoor bathing waters, based on a classification scheme presented by W.J. Scott in 1934. Scott had proposed four classes of water, with total coliform upper limits of 50, 500, 1000, and >1000 MPN/100 mL for each class. He had developed this classification based on an extensive survey of the Connecticut shoreline where he found that about 93% of the samples contained less than 1000 total coliforms per 100 mL. A sanitary survey classification also showed that only about 7% of the shoreline was designated as poor. He therefore concluded that total coliform counts of <1000 MPN/100 mL probably indicated acceptable waters for swimming. This standard was based on the principle of attainment, where very little control or intervention would be required to meet this standard. In 1943, the State of California independently adopted an arbitrary total coliform standard of 1000 MPN/100 mL for swimming areas. This California standard was not based on any evidence, but it was assumed to relate well with the drinking water standard at the time.

Dufour points out that a third method used to develop a standard for bathing water quality used an analytical approach adopted by H.W. Streeter in 1951. He used a ratio between *Salmonella* and total coliforms, the number of bathers exposed, the approximate volume of water ingested by bathers daily, and the average total coliform density. Streeter concluded that water containing <1000 MPN total coliforms/100 mL would pose no great *S. typhosa* health hazard. Dufour points out that it is interesting that all three approaches in developing a swimming water criterion resulted in the same numeric limit.

One of the earliest bathing beach studies to measure actual human health risks associated with swimming in contaminated water was directed by Stevenson (1953), of the U.S. Public Health Service's Environmental Health Center, in Cincinnati, OH, and was conducted in the late 1940s. They studied swimming at Lake Michigan at Chicago (91 and 190 MPN/100 mL median total coliform densities), the Ohio River at Dayton, KY (2700 MPN/100 mL), at Long Island Sound at New Rochelle and at Mamaroneck, NY (610 and 253 MPN/100 mL). They also studied a swimming pool in Dayton, KY. Two bathing areas were studied in each area, one with historically poorer water quality than the other. Individual home visits were made to participating families in each area to explain the research program and to review the calendar record form. Follow-up visits were made to each participating household to ensure completion of the forms. Total coliform densities were monitored at each bathing area during the study. More than 20,000 persons participated in the study in the three areas. Almost a million person-days of usable records were obtained. The percentage of the total person-days when swimming occurred ranged from about 5 to 10%. The number of illnesses of all types recorded per 1000 person-days varied from 5.3 to 8.8. They found an appreciably higher illness incidence rate for the swimming group, compared to the nonswimming group, regardless of the bathing water quality (based on total coliform densities). A significant increase in gastrointestinal illness was observed among the swimmers who used one of the Chicago beaches on 3 days when the average coliform count was 2300 MPN/100 mL. The second instance of positive correlation was observed in the Ohio River study where swimmers exposed to the median total coliform density of 2700 MPN/100 mL had a significant increase in gastrointestinal illness, although the illness rate was relatively low. They suggested that the strictest bacterial quality requirements that existed then (as indicated above, based on Scott's 1934 work) might be relaxed without significant detrimental effect on the health of bathers.

It is interesting to note that in 1959, the Committee on Bathing Beach Contamination of the Public Health Laboratory Service of the U.K. concluded that "bathing in sewage-polluted seawater carries only a negligible risk to health, even on beaches that are aesthetically very unsatisfactory" (Alexander et al. 1992).

Dufour (1984) pointed out that total coliforms were an integral element in establishing fecal coliform limits as an indicator for protecting swimming uses. As a result of the Stevenson (1953) study, reported above, a geometric mean fecal coliform level of 200 MPN per 100 mL was recommended by the National Technical Advisory Committee (NTAC) of the Federal Water Pollution Control Administration in 1968 and was adopted by the U.S. Environmental Protection Agency in 1976 as a criterion for direct water contact recreation (Cabelli et al. 1979). This criterion was adopted by almost all states by 1984. It was felt that fecal coliform levels were more specific to sewage contamination and had less seasonal variation than total coliform levels. Since fecal coliform exposures at swimming beaches had never been linked to disease, the NTAC reviewed the USPHS studies, as published by Stevenson (1953). The 2300 MPN/100 mL total coliform count association with gastrointestinal disease was used in conjunction with a measured ratio of fecal coliform to total coliform counts (18%) obtained at the Ohio River site studied earlier. It was therefore assumed that a health effect could be detected when the fecal coliform count was 400 MPN/100 mL (18% of 2300 = 414). Dufour (1984) notes that a detectable health effect was undesirable and that the NTAC therefore recommended a limit of 200 MPN/100 mL for fecal coliforms. Dufour (1984) mentions that, although likely coincidental, the 1968 proposed limit for fecal coliforms (200 MPN/100 mL) was very close to being theoretically equivalent to the total coliform limit of 1000 MPN/100 mL that was being replaced (200/0.18 = 1100).

The Cabelli et al. (1979) study was undertaken to address many remaining questions pertaining to bathing in contaminated waters. Their study examined conditions in New York (at a Coney Island beach, designated as barely acceptable, and at a Rockaway beach, designated as relatively unpolluted). About 8000 people participated in the study, approximately evenly divided between swimmers and nonswimmers at the two beaches. Total and fecal coliforms, *Escherichia*, *Klebsiella, Citrobacter–Enterobacter*, enterococci, *Pseudomonas aeruginosa*, and *Clostridium perfringens* were evaluated in water samples obtained from the beaches during the epidemiological study. The most striking findings were the increases in the rates of vomiting, diarrhea, and stomach ache among swimmers relative to nonswimmers at the barely acceptable beach, but not at the relatively unpolluted beach. Ear, eye, nose, and skin symptoms, as well as fever, were higher among swimmers compared to nonswimmers at both beaches. They concluded that measurable health effects do occur at swimming beaches that meet the existing health standards. Children, Hispanic Americans, and low-middle socioeconomic groups were identified as the most susceptible portions of the population.

Cabelli et al. (1982) presented data from the complete EPA-sponsored swimming beach study, conducted in New York, New Orleans, and Boston. The study was conducted to address issues from prior studies conducted in the 1950s (including Stevenson's 1953 study noted above) that were apparently contradictory. They observed a direct, linear relationship between highly credible gastrointestinal illness and enterococci. The frequency of gastrointestinal symptoms also had a high degree of association with distance from known sources of municipal wastewater. Table G.1 shows correlation coefficients for total gastrointestinal (GI) and highly credible gastrointestinal (HCGI) symptoms and mean indicator densities found at the New York beaches from 1970 to 1976. The best correlation coefficients were found for enterococci. In contrast, the correlation coefficients for fecal coliforms (the basis for most federal and state guidelines) were poor. Very low levels of enterococcus and *Escherichia coli* in the water (about 10 MPN/100 mL) were associated with appreciable attack rates (about 10/10,000 persons).

They concluded that swimming in even marginally polluted marine bathing water is a significant route of transmission for observed gastrointestinal illness. They felt that the gastrointestinal illness was likely associated with the Norwalk-like virus that had been confirmed in 2000 cases in a shellfish-associated outbreak in Australia and in several outbreaks associated with contaminated drinking water.

Table G.1 Correlation Coefficients between Gastrointestinal Symptoms and Bacterial Densities at New York City Beaches

Indicator	HCGI Correlation Coefficient	GI Correlation Coefficient	Number of Observations
Enterococci	0.96	0.81	9
Escherichia coli	0.58	0.51	9
Klebsiella	0.61	0.47	11
Enterobacter-Citrobacter	0.64	0.54	13
Total coliforms	0.65	0.46	11
Clostridium perfringens	0.01	–0.36	8
Pseudomonas aeruginosa	0.59	0.35	11
Fecal coliforms	0.51	0.36	12
Aeromonas hydriphila	0.60	0.27	11
Vibrio parahemolyticus	0.42	0.05	7

From Cabelli et al. 1982.

Table G.2 Correlation Coefficients for Bacterial Parameters and Gastrointestinal Disease (Freshwater Swimming Beaches)

	Highly Credible Gastrointestinal Illness	Total Gastrointestinal Illness	Number of Study Units
Enterococci	0.774	0.673	9
E. coli	0.804	0.528	9
Fecal coliforms	–0.081	0.249	7

From Dufour 1984.

Dufour (1984) also reviewed a series of studies conducted at freshwater swimming beaches from 1979 to 1982, at Tulsa, OK, and at Erie, PA. Only enterococci, *E. coli,* and fecal coliforms were monitored, based on the results of the earlier studies. Table G.2 shows the correlation coefficients for these three bacterial parameters and gastrointestinal disease.

These results are quite different from the results of the marine studies in that both enterococci and *E. coli* had high correlation coefficients between the bacterial levels and the incidence of gastrointestinal illness. However, the result was the same for fecal coliforms, in that there was no association between fecal coliform levels and gastrointestinal illness. Dufour (1984) concluded that enterococci would be the indicator of choice for gastrointestinal illness, based on scientific dependability. *E. coli* could also be used, if only fresh waters were being evaluated. Fecal coliforms would be a poor choice for monitoring the safety of bathing waters. However, he concluded that numeric standards should be different for fresh and saline waters because of different die-off rates for the bacteria and viruses for differing salinity conditions.

Other studies examined additional illness symptoms associated with swimming in contaminated water, besides gastrointestinal illness, and identified other potentially useful bacterial indicators. Seyfried et al. (1985), for example, examined users of swimming beaches in Toronto for respiratory illness, skin rashes, plus eye and ear problems, in addition to gastrointestinal illness. They found that total staphylococci correlated best with swimming-associated total illness, plus ear, eye, and skin illness. However, fecal streptococci and fecal coliforms also correlated (but not as well) with swimming-associated total illness. Ferley et al. (1989) examined illnesses among swimmers during the summer of 1986 in the French Ardèche river basin, during a time when untreated domestic sewage was entering the river. They examined total coliforms, fecal coliforms, fecal streptococci, *Pseudomonas aeruginosa,* and *Aeromonas* spp., but only two samples per week were available for each swimming area. The total morbidity ratio for swimmers compared to nonswimmers was 2.1 (with a 95% confidence interval of 1.8 to 2.4), with gastrointestinal illness the major illness observed. They found that fecal streptococci (FS) was the best indicator of gastrointestinal illness. A critical FS value of 20 MPN/100 mL indicated significant differences between the swimmers and nonswimmers. Skin

ailments were also more common for swimmers than for nonswimmers and were well correlated with the concentrations of fecal coliforms, *Aeromonas* spp., and *P. aeruginosa*. They noted that a large fraction (about 60%) of the fecal coliforms corresponded to *E. coli,* and that their definition of fecal streptococci essentially was what North American researchers termed enterococci.

Many of the available epidemiological studies have been confined to healthy adult swimmers, in relatively uncontaminated waters. However, it is assumed that those most at risk would be children, the elderly, and those chronically ill, especially in waters known to be degraded. Obviously, children are the most likely of this most-at-risk group to play in, or by, water. Alexander et al. (1992) therefore specifically examined the risk of illness associated with swimming in contaminated sea water for children, aged 6 to 11 years old. This study was based on parental interviews for 703 child participants during the summer of 1990 at Blackpool beach, U.K. Overall, 80% of the samples at the Blackpool Tower site and 93% of the samples at the South Pier site failed to meet the European Community standards for recreational waters. All of the 11 designated beaches in Lancashire (including Blackpool beach), in the northwest region of England, continually failed the European directive imperative standards for recreational waters. During this study, statistically significant increases in disease were found in children who had water contact compared to those who did not. Diarrhea and loss of appetite had strong associations with the water contact group, while vomiting and itchy skin had moderate associations. No other variables examined (household income, sex of the child, sex of the respondent, general health, chronic or recurring illness in the child, age of the child, foods eaten, including ice cream, other dairy products, chicken, hamburgers, shellfish, or ice cubes, acute symptoms in other household members, presence of children under 5 in the household, and other swimming activities) could account for the significant increases in the reported symptoms for the children who had water contact.

Santa Monica Bay Project

This study was the first large-scale epidemiological study in the U.S. to investigate possible adverse health effects associated with swimming in ocean waters affected by discharges from separate storm drains (SMBRP 1996). This was a follow-up study after previous investigations found that human fecal waste was present in the stormwater collection systems (*Water Environment & Technology* 1996b; *Environmental Science & Technology* 1996b; Haile et al. 1996). This subsection was previously considered in Chapter 4 of this book, but is repeated here for comparison with the other discussions on the development of the standards for bacteria exposure from stormwater.

During a 4-month period in the summer of 1995, about 15,000 ocean swimmers were interviewed on the beach and during telephone interviews 1 to 2 weeks later. They were queried concerning illnesses since their beach outing. The incidence of illness (such as fever, chills, ear discharge, vomiting, coughing with phlegm, and credible gastrointestinal illness) was significantly greater (from 44 to 127% increased incidence) for oceangoers who swam directly off the outfalls, compared to those who swam 400 yards away, as shown on Table G.3. As an example, the rate ratio (RR) for fever was 1.6, while it was 2.3 for ear discharges, and 2.2 for highly credible gastrointestinal illness (HCGI) comprised of vomiting and fever. The approximated associations were weak for any of the symptoms, and moderate for the others listed. Disease incidence dropped significantly with distance from the storm drain. At 400 yards, and beyond, upcoast or downcoast, elevated disease risks were not found. The results did not change when adjusted for age, beach, gender, race, socioeconomic status, or worry about health risks associated with swimming at the beach.

These interviews were supplemented with indicator and pathogen bacteria and virus analyses in the waters. The greatest health problems were associated with times of highest concentrations (*E. coli* > 320 cfu/100 mL, enterococcus > 10^6 cfu/100 mL, total coliforms > 10,000 cfu/100 mL, and fecal coliforms > 400 cfu/100 mL). Bacteria populations greater than these are common in urban runoff and in urban receiving waters. Symptoms were found to be associated with swimming in areas where bacterial indicator levels were greater than these critical counts. Table G.4 shows the health outcomes associated with swimming in areas having bacterial counts greater than these

Table G.3 Comparative Health Outcomes for Swimming in Front of Storm Drain Outfalls, Compared to Swimming at Least 400 Yards Away

Health Outcome	Relative Risk	Rate Ratio	Estimated Association	Estimated No. of Excess Cases per 10,000 Swimmers (rate difference)
Fever	57%	1.57	Moderate	259
Chills	58%	1.58	Moderate	138
Ear discharge	127%	2.27	Moderate	88
Vomiting	61%	1.61	Moderate	115
Coughing with phlegm	59%	1.59	Moderate	175
Any of the above symptoms	44%	1.44	Weak	373
HCGI-2	111%	2.11	Moderate	95
SRD (significant respiratory disease)	66%	1.66	Moderate	303
HCGI-2 or SRD	53%	1.53	Moderate	314

From SMBRP 1996.

Table G.4 Heath Outcomes Associated with Swimming in Areas Having High Bacterial Counts

Indicator (and critical cutoff count)	Health Outcome	Increased Risk	Risk Ratio	Estimated Association	Excess Cases per 10,000 Swimmers
E. coli	Earache and	46%	1.46	Weak	149
(>320 cfu/100 mL)	nasal congestion	24%	1.24	Weak	211
Enterococci	Diarrhea w/blood	323%	4.23	Strong	27
(>106 cfu/100 mL)	and HCGI-1	44%	1.44	Weak	130
Total coliform bacteria (>10,000 cfu/100 mL)	Skin rash	200%	3.00	Moderate	165
Fecal coliform bacteria (>400 cfu/100 mL)	Skin rash	88%	1.88	Moderate	74

From SMBRP 1996.

critical values. The association for enterococci with bloody diarrhea was strong, and the association of total coliforms with skin rash was moderate, but nearly strong.

The ratio of total coliform to fecal coliform was found to be one of the better indicators for predicting health risks when swimming close to the storm drain. When the total coliforms were greater than 1000 cfu/100 mL, the strongest effects were generally observed when the total to fecal coliform ratio was 2. The risks decreased as the ratio increased. In addition, illnesses were more common on days when enteric viruses were found in the water.

The SMBRP (1996) concluded that less than 2 miles of Santa Monica Bay's 50-mile coastline had problematic health concerns due to the storm drains flowing into the bay. They also concluded that the bacterial indicators currently being monitored do help predict risk. In addition, the total to fecal coliform ratio was found to be a useful additional indicator of illness. As an outcome of this study, the Los Angeles County Department of Health Services will post new warning signs advising against swimming near the outfalls ("Warning! Storm drain water may cause illness. No swimming"). These signs will be posted on both sides of all flowing storm drains in Los Angeles County. In addition, county lifeguards will attempt to warn and advise swimmers to stay away from areas directly in front of storm drain outlets, especially in ponded areas. The county is also accelerating its studies on sources of pathogens in stormwater.

Bacteria Criteria for Water-Contact Recreation

A recreational water quality criterion can be defined as a "quantifiable relationship between the density of an indicator in the water and the potential human health risks involved in the water's recreational use." From such a definition, a criterion can be adopted which establishes upper limits for densities of indicator bacteria in waters that are associated with acceptable health risks for swimmers.

Table G.5 National Bacteria Criteria (Single Sample Maximum Allowable Density, counts per 100 mL)

		Designated Beach[a]	Moderate Full Body Contact Recreation[a]	Lightly Used Full Body Contact[a]	Infrequently Used Full Body Contact[a]	Drinking Water[b]
Freshwater	Enterococci	61	89	108	151	1
	E. coli	235	298	406	576	1
Marine water	Enterococci	104	124	276	500	1

[a] EPA 1986

The Environmental Protection Agency, in 1972, initiated a series of studies at marine and freshwater bathing beaches which were designed to determine if swimming in sewage-contaminated marine and fresh water carries a health risk for bathers, and, if so, to what type of illness. Additionally, the EPA wanted to determine which bacterial indicator is best correlated to swimming-associated health effects and if the relationship is strong enough to provide a criterion (EPA 1986a).

The quantitative relationships between the rates of swimming-associated health effects and bacterial indicator densities were determined using standard statistical procedures. The data for each summer season were analyzed by comparing the bacteria indicator density for a summer bathing season at each beach with the corresponding swimming-associated gastrointestinal illness rate for the same summer. The swimming-associated illness rate was determined by subtracting the gastrointestinal illness rate in nonswimmers from that for swimmers.

The EPA's evaluation of the bacteriological data indicated that using the fecal coliform indicator group at the maximum geometric mean of 200 organisms per 100 mL, as recommended in *Quality Criteria for Water,* would cause an estimated 8 illness per 1000 swimmers at freshwater beaches.

Additional criteria, using *E. coli* and enterococci bacteria analyses, were developed using these currently accepted illness rates. These bacteria are assumed to be more specifically related to poorly treated human sewage than the fecal coliform bacteria indicator. The equations developed by Dufour (1983) were used to calculate new indicator densities corresponding to the accepted gastrointestinal illness rates.

It should be noted that these indicators only relate to gastrointestinal illness, and not other problems associated with waters contaminated with other bacterial or viral pathogens. Common swimming beach problems associated with contamination by stormwater include skin and ear infections caused by *Pseudomonas aeruginosa* and *Shigella*.

National bacteria criteria have been established for contact with bacteria and are shown in Table G.5. State standards usually also exist for fecal coliform bacteria. Typical public water supply standards (Alabama's are shown) are as follows:

1. Bacteria of the fecal coliform group shall not exceed a geometric mean of 2000/100 mL; nor exceed a maximum of 4000/100 mL in any sample. The geometric mean shall be calculated from no less than five samples collected at a given station over a 30-day period at intervals not less than 24 hours. The membrane filter counting procedure will be preferred, but the multiple tube technique (five-tube) is acceptable.
2. For incidental water contact and recreation during June through September, the bacterial quality of water is acceptable when a sanitary survey by the controlling health authorities reveals no source of dangerous pollution and when the geometric mean fecal coliform organism density does not exceed 100/100 mL in coastal waters and 200/100 mL in other waters. When the geometric mean fecal coliform organism density exceeds these levels, the bacterial water quality shall be considered acceptable only if a second detailed sanitary survey and evaluation discloses no significant public health risk in the use of such waters. Waters in the immediate vicinity of discharges of sewage or other wastes likely to contain bacteria harmful to humans, regardless of the degree of treatment afforded these wastes, are not acceptable for swimming or other whole-body water-contact sports.

Standards for fish and wildlife waters are similar to the above standard for a public water supply, except Part 1 has different limits: "Bacteria of the fecal coliform group shall not exceed a geometric mean of 1000/100 mL on a monthly average value; nor exceed a maximum of 2000/100 mL in any sample." Part 2 is the same for both water beneficial uses.

CHLORIDE, CONDUCTIVITY, AND TOTAL DISSOLVED SOLIDS

Total dissolved solids, chlorides, and conductivity observations are typically used to indicate the magnitude of dissolved minerals in the water. The term *total dissolved solids* (or *dissolved solids*) is generally associated with fresh water and refers to the inorganic salts, small amounts of organic matter, and dissolved materials in the water. Salinity is an oceanographic term, and although not precisely equivalent to the total dissolved salt content, it is related (Capurro 1970). Chlorides (not chlorine) are directly related to salinity because of the constant relationship between the major salts in seawater. Conductivity is a measure of the electrical conductivity of water and is also generally related to total dissolved solids, chlorides, or salinity. The principal inorganic anions (negatively charged ions) dissolved in fresh water include the carbonates, chlorides, sulfates, and nitrates (principally in groundwaters); the principal cations (positively charged ions) are sodium, potassium, calcium, and magnesium.

Human Health Criteria for Dissolved Solids

Excess dissolved solids are objectionable in drinking water because of possible physiological effects, unpalatable mineral tastes, and higher costs because of corrosion or the necessity for additional treatment. The physiological effects directly related to dissolved solids include laxative effects principally from sodium sulfate and magnesium sulfate and the adverse effect of sodium on certain patients afflicted with cardiac disease and women with toxemia associated with pregnancy. One study was made using data collected from wells in North Dakota. Results from a questionnaire showed that with wells in which sulfates ranged from 1000 to 1500 mg/L, 62% of the respondents indicated laxative effects associated with consumption of the water. However, nearly one quarter of the respondents to the questionnaire reported difficulties when concentrations ranged from 200 to 500 mg/L (Moore 1952). To protect transients to an area, a sulfate level of 250 mg/L should afford reasonable protection from laxative effects.

As indicated, sodium frequently is the principal component of dissolved solids. Persons on restricted sodium diets may have an intake restricted from 500 to 1000 mg/day (National Research Council 1954). The portion ingested in water must be compensated by reduced levels in food ingested so that the total does not exceed the allowable intake. Using certain assumptions of water intake (e.g., 2 L of water consumed per day) and the sodium content of food, it has been calculated that for very restricted sodium diets, 20 mg/L sodium in water would be the maximum, while for moderately restricted diets, 270 mg/L sodium would be the maximum. Specific sodium levels for entire water supplies have not been recommended by the EPA, but various restricted sodium intakes are recommended because: (1) the general population is not adversely affected by sodium, but various restricted sodium intakes are recommended by physicians for a significant portion of the population, and (2) 270 mg/L of sodium is representative of mineralized waters that may be aesthetically unacceptable, but many domestic water supplies exceed this level. Treatment for removal of sodium in water supplies is also costly (NAS 1974).

A study based on consumer surveys in 29 California water systems was made to measure the taste threshold of dissolved salts in water (Bruvold et al. 1969). Systems were selected to eliminate possible interferences from other taste-causing substances besides dissolved salts. The study revealed that consumers rated waters with 320 to 400 mg/L dissolved solids as "excellent," while those with 1300 mg/L dissolved solids were "unacceptable." A "good" rating was registered for dissolved solids less than 650 to 750 mg/L. The 1962 U.S. Public Health Service Drinking Water

Standards recommended a maximum dissolved solids concentration of 500 mg/L, unless more suitable supplies were unavailable.

Specific constituents included in the dissolved solids in water may cause mineral tastes at lower concentrations than other constituents. Chloride ions have frequently been cited as having a low taste threshold in water. Data from Ricter and MacLean (1939) on a taste panel of 53 adults indicated that 61 mg/L NaCl was the median level for detecting a difference from distilled water. At a median concentration of 395 mg/L chloride, a salty taste was identified. Lockhart et al. (1955) when evaluating the effect of chlorides on water used for brewing coffee, found threshold taste concentrations for chloride ranging from 210 to 310 mg/L, depending on the associated cation. These data indicate that a level of 250 mg/L chlorides is a reasonable maximum level needed to protect consumers.

The causation of corrosion and encrustation of metallic surfaces by water containing dissolved solids is well known. By using water with 1750 mg/L dissolved solids as compared with 250 mg/L, service life was reduced from 70% for toilet flushing mechanisms to 30% for washing equipment. Such increased corrosion was calculated in 1968 to cost the consumer an additional $0.50 per 1000 gallons used.

The U.S. EPA has adopted secondary drinking water standards (40 CFR D143.3) and ambient water quality criteria. The National Secondary Drinking Water Maximum Contaminant Level (MCL) for chloride is 250 mg/L (40 CFR D 143.3). This corresponds roughly to a conductivity measurement of about 1200 $\mu S/cm^2$, but this is never exactly the case. However, the relationship between conductivity and chloride can be established on a site-specific basis. Chloride toxicity is increased when the counter ion of the chloride salt is not sodium.

Aquatic Life Criteria for Dissolved Solids

All species of fish and other aquatic life must tolerate a range of dissolved solids concentrations in order to survive under natural conditions. Studies in Saskatchewan found that several common freshwater species survived 10,000 mg/L dissolved solids, that whitefish and pikeperch survived 15,000 mg/L, but only the stickleback survived 20,000 mg/L dissolved solids. It was concluded that lakes with dissolved solids in excess of 15,000 mg/L were unsuitable for most freshwater fishes (Rawson and Moore 1944). The 1968 NTAC Report also recommended maintaining an osmotic pressure level of less than that caused by a 15,000 mg/L solution of sodium chloride.

Indirect effects of excess dissolved solids are primarily the elimination of desirable food plants and other habitat-forming plants. Rapid salinity changes cause plasmolysis of tender leaves and stems because of changes in osmotic pressure. The 1968 NTAC Report recommended the following limits in salinity variation from natural to protect wildlife habitats:

Natural Salinity (parts per thousand)	Variation Permitted (parts per thousand)
0 to 3.5 (fresh water)	1
3.5 to 13.5 (brackish water)	2
13.5 to 35 (seawater)	4

Alabama is an example of a state that has established a standard for chloride to protect aquatic life. A chloride criterion of 230 mg/L is used to protect aquatic life in the Cahaba River.

CHROMIUM

Aquatic Life Effects of Cr^{3+}

Acute values for Cr^{3+} are available for 20 freshwater animal species in 18 genera ranging from 2.2 mg/L for a mayfly to 71 mg/L for caddisfly. Hardness has a significant influence on toxicity, with Cr^{3+} being more toxic in soft water.

A life-cycle test with *Daphnia magna* in soft water gave a chronic value of 66 µg/L. In a comparable test in hard water, the lowest test concentration of 44 µg/L inhibited reproduction of *D. magna*, but this effect may have resulted from ingested precipitated chromium. In a life-cycle test with the fathead minnow in hard water, the chronic value was 1.0 mg/L. Toxicity data were available for only two freshwater plant species. A concentration of 9.9 mg/L inhibited growth of roots of Eurasian watermilfoil. A freshwater green alga was affected by a concentration of 397 µg/L in soft water. No bioconcentration factor was measured for Cr^{3+} with freshwater organisms.

National Freshwater Aquatic Life Criteria for Cr^{3+}

The procedures described in the guidelines indicate that, except possibly where a locally important species is very sensitive, freshwater aquatic organisms and their uses should not be affected unacceptably if the 4-day average (chronic) concentration (in µg/L) of Cr^{3+} does not exceed the numerical value given by:

$$e^{(0.8190(\ln(\text{hardness}))+1.561)}$$

more than once every 3 years on the average, and if the 1-hour average (acute) concentration (in µg/L) does not exceed the numerical value given by:

$$e^{(0.8190(\ln(\text{hardness}))+3.688)}$$

more than once every 3 years on the average. For example, at hardnesses of 50, 100, and 200 mg/L as $CaCO_3$ the 4-day average concentrations of Cr^{3+} are 120, 210, and 370 µg/L, respectively, and the 1-hour average concentrations are 980, 1700, and 3100 µg/L. Many states have adopted these equations to define the Cr^{3+} standards for freshwater aquatic life uses.

Human Health Criteria for Chromium

For the protection of human health from the toxic properties of Cr^{3+} ingested through water and contaminated aquatic organisms, the ambient water criterion is determined to be 170 mg/L. For the protection of human health from the toxic properties of Cr^{3+} ingested through contaminated aquatic organisms alone, the ambient water criterion is determined to be 3433 mg/L. In contrast, the ambient water quality criterion for total Cr^{6+} is recommended to be identical to the existing drinking water standard, which is 50 µg/L.

COPPER

Effects of Copper on Aquatic Life

Acute toxicity data are available for species in 41 genera of freshwater animals. At a hardness of 50 mg/L, the genera range in sensitivity from 17 µg/L for *Ptychocheilus* to 10 mg/L for *Acroneuria*. Data for eight species indicate that acute toxicity decreases as hardness increases. Additional data for several species indicate that toxicity also decreases with increased alkalinity and total organic carbon.

Chronic values are available for 15 freshwater species and range from 3.9 µg/L for brook trout to 60 µg/L for northern pike. Fish and invertebrate species seem to be about equally sensitive to the chronic toxicity of copper.

Toxicity tests have been conducted on copper with a wide range of freshwater plants and the sensitivities are similar to those of animals. Complexing effects of the test media and a lack of

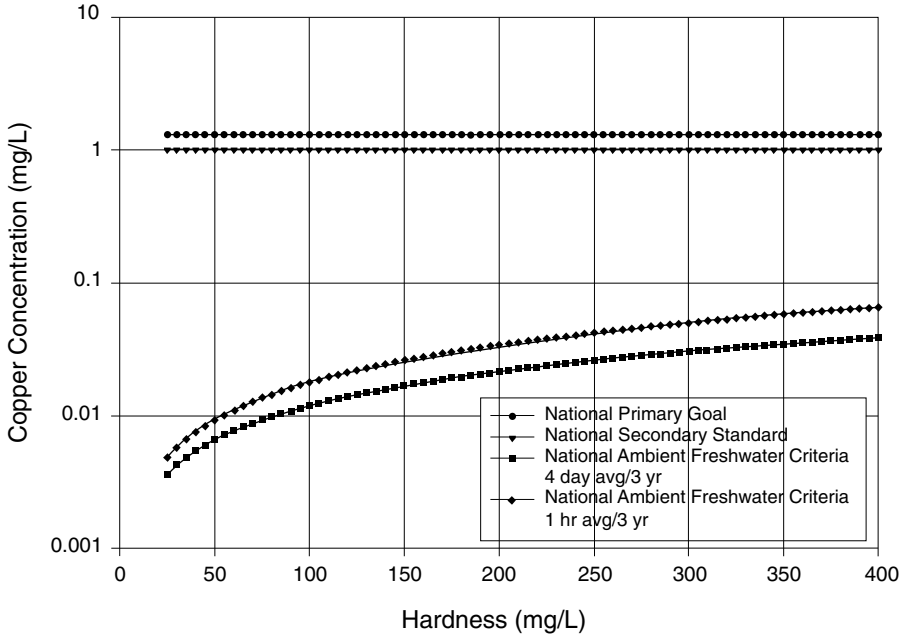

Figure G.2 National copper criteria.

good analytical data make interpretation and application of these results difficult. Protection of animal species, however, appears to offer adequate protection of plants. Copper does not appear to bioconcentrate very much in the edible portion of freshwater aquatic species.

National Aquatic Life Criteria for Copper

The U.S. EPA has established a national ambient water quality criteria for the protection of wildlife (EPA 1986b). The wildlife protection criteria are a function of hardness and are shown in Figure G.2.

Human Health Criteria for Copper

The U.S. EPA has established a primary drinking water goal (40 CFR D Subpart F 141.51) of 1.3 mg/L, a secondary drinking water quality MCL of 1.0 mg/L (40 CFR 143.3).

HARDNESS

Water hardness is caused by the divalent metallic ions (having charges of +2) dissolved in water. In fresh water, these are primarily calcium and magnesium, although other metals such as iron, strontium, and manganese also contribute to the hardness content, but usually to a much lesser degree. Hardness commonly is reported as an equivalent concentration of calcium carbonate ($CaCO_3$).

Concerns about water hardness originated because hard water requires more soap to form a lather and because hard water causes scale in hot water systems. Modern use of synthetic detergents has eliminated the concern of hard water in laundries, but it is still of primary concern for many industrial water users. Many households use water softeners to reduce scale formation in hot water systems and for water taste reasons.

There are no national standards for hardness, but water hardness has a dramatic effect on criteria for a number of heavy metals. “The affects of hardness on freshwater fish and other aquatic life appear

Table G.6 USGS Hardness Scale

Hardness (mg/L as $CaCO_3$)	Classification
<60	Soft
61–120	Moderately hard
121–180	Hard
>180	Very hard

From Leeden et al. 1990.

to be related to the ions causing hardness rather than hardness (EPA 1986b)." The USGS classifies the hardness of waters using the scale in Table G.6.

Natural sources of hardness principally are limestones which are dissolved by percolating rainwater. Groundwaters are therefore generally harder than surface waters. Industrial sources include the inorganic chemical industry and discharges from operating and abandoned mines.

Hardness in fresh water is frequently distinguished in carbonate and noncarbonate fractions. The carbonate fraction is chemically equivalent to the bicarbonates present in water. Since bicarbonates are generally measured as alkalinity, the carbonate hardness is equal to the alkalinity.

The determination of hardness in raw waters subsequently treated and used for domestic water supplies is useful as a parameter to characterize the total dissolved solids present and for calculating chemical dosages for water softening. Because hardness concentrations in water have not been proven to be health related, the final level of hardness to be achieved by water treatment principally is a function of economics. Since water hardness can be removed with treatment by such processes as lime-soda softening and ion exchange systems, a water quality criterion for raw waters used as a public water supply is not given by the EPA.

The effects of hardness on freshwater fish and other aquatic life appear to be related to the ions causing the hardness rather than by hardness as a general indicator. Both the NTAC (1968) and NAS (1974) panels have recommended against the use of the term *hardness* and suggested the use of the concentrations of the specific ions instead. For most existing data, it is difficult to determine whether toxicity of various metal ions is reduced because of the formation of metallic hydroxides and carbonates caused by the associated increases in alkalinity, or because of an antagonistic effect of one of the principal cations contributing to hardness, e.g., calcium, or a combination of both effects. Stiff (1971) presented an example showing that if cupric ions were the toxic form of copper, whereas copper carbonate complexes were relatively nontoxic, then the observed difference in toxicity of copper between hard and soft waters can be explained by the difference in alkalinity rather than hardness. Recent laboratory work has also shown that alkalinity may be more related to heavy metal toxicity than water hardness. As noted previously, however, carbonate hardness and alkalinity are the same.

Doudoroff and Katz (1953), in their review of the literature on toxicity, presented data showing that increasing calcium in particular reduced the toxicity of other heavy metals. Under usual conditions in fresh water and assuming that other bivalent metals behave like copper, it is reasonable to assume that both effects occur simultaneously and explain the observed reduction of toxicity of metals in waters containing carbonate hardness. The amount of reduced toxicity related to hardness, as measured by a 40-hour LC50 for rainbow trout, has been estimated to be about four times for copper and zinc when the hardness was increased from 10 to 100 mg/L as $CaCO_3$ (NAS 1974). As shown in other discussions for specific heavy metals, many of the heavy metal criteria depend on water hardness. The allowable concentrations of cadmium, chromium, lead, and zinc to protect fish and other aquatic life, are much less in soft waters than in hard waters, for example.

HYDROCARBONS

The U.S. EPA has promulgated criteria for several of the organic toxicants that can be found in stormwater or in urban receiving waters. In addition, the EPA has specific criteria for the detection of individual organic molecules. The MCLs (maximum concentration limits) for the individual chemicals are mostly all well below 0.1 mg/L (40 CFR D Subpart F 141.50 and Subpart G 141.61). The following table summarizes several of the criteria for toxic organics:

aldrin+dieldrin	0.002 μg/L (acute freshwater aquatic life) 0.007 ng/L (human health)
chlorodane	2.4 μg/L (maximum conc. for acute freshwater aquatic life) 0.046–4.6 μg/L (human health)
DDT and metabolites	1.1 μg/L (maximum concentration for acute freshwater aquatic life)
DDE	1.05 mg/L (acute freshwater aquatic life)
2,4-dichlorophenol	2.02 mg/L (acute freshwater aquatic life)
2,4-dimethylphenol	2.1 mg/L (acute freshwater aquatic life)
endosulfan	0.05 μg/L (acute freshwater aquatic life)
endrin	0.0023 μg/L (acute freshwater aquatic life)
pentachlorophenol	55 μg/L (acute freshwater aquatic life)
phthalate esters	940 μg/L (acute freshwater aquatic life)
polycyclic aromatic hydrocarbons	0.28–28 ng/L (human health)

Several of the compounds periodically found in urban runoff also have state and/or national standards for the protection of human health, including some that are recognized carcinogens. The following table lists typical limits (for Alabama, at 10^{-5} risk level):

	Water and Fish Consumption	Fish Consumption Only
Noncarcinogens		
2-Chlorophenol	0.12 mg/L	0.40 mg/L
Diethyl phthalate	23	118
Dimethyl phthalate	313	2900
Di-*n*-butyl phthalate	3	12
Isophorone	7	490
Carcinogens		
Benzo(ghi)perylene	0.03 μg/L	0.31 μg/L
Benzo(k)fluoranthene	0.03	0.31
3,3-Dichloro-benzidine	0.39	0.77
Hexachlorobutadiene	4.5	500
N-Nitrosodiphenylamine	50	160

Florida water quality criteria for organic toxicants include the following pesticide limits:

2,4-D	0.1 μg/L(potable water supply)
andrin+dieldrin	0.003 μg/L (potable water supply, recreation, fish and wildlife)
chlordane	0.01 μg/L (potable water supply) 0.01 μg/L (recreation, fish and wildlife)
endosulfan	0.003 μg/L (potable water supply, recreation, fish and wildlife)
endrin	0.004 μg/L (potable water supply, recreation, fish and wildlife)
heptachlor	0.001 μg/L (potable water supply, recreation, fish and wildlife)
lindane	0.01 μg/L (potable water supply, recreation, fish and wildlife)
malathion	0.1 μg/L (potable water supply, recreation, fish and wildlife)
methoxychlor	0.03 μg/L (potable water supply, recreation, fish and wildlife)
mirex	0.001 μg/L (potable water supply, recreation, fish and wildlife)
parathion	0.04 μg/L (potable water supply, recreation, fish and wildlife)
toxaphene	0.005 μg/L (potable water supply, recreation, fish and wildlife)

LEAD

Aquatic Life Summary for Lead

The acute toxicity of lead to several species of freshwater animals has been shown to decrease as the hardness of water increases. At a hardness of 50 mg/L, the acute sensitivities of 10 species

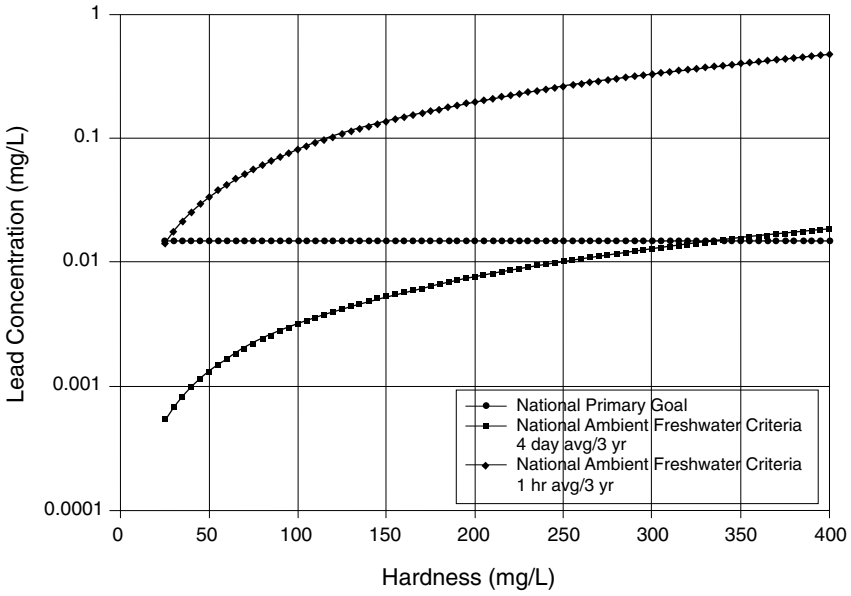

Figure G.3 National lead criteria.

range from 142 µg/L for an amphipod to 236 mg/L for a midge. Data on the chronic effects of lead on freshwater animals are available for two fish and two invertebrate species. The chronic toxicity of lead also decreases as hardness increases and the lowest and highest available chronic values (12.3 and 128 µg/L) are both for a cladoceran, but in soft and hard water, respectively. Freshwater algae are affected by concentrations of lead above 500 µg/L, based on data for four species. Bioconcentration factors are available for four invertebrate and two fish species and range from 42 to 1700.

National Aquatic Life Criteria for Lead

For the protection of wildlife, U.S. EPA has set a national freshwater criteria for lead that is a function of hardness. Figure G.3 shows these standards.

Human Health Criteria for Lead

The U.S. EPA has set the lead National Drinking Water MCL goal at 0 mg/L (40 CFR D Subpart F 141.51) and the National Drinking Action Level at 0.015 mg/L (40 CFR D Subpart I 141.80 (2) (c)).

NITRATE AND NITRITE

Two gases (molecular nitrogen and nitrous oxide) and five forms of nongaseous, combined nitrogen (amino and amide groups, ammonium, nitrite, and nitrate) are important in the nitrogen cycle. The amino and amide groups are found in soil organic matter and as constituents of plant and animal protein. The ammonium ion either is released from proteinaceous organic matter and urea or is synthesized in industrial processes involving atmospheric nitrogen fixation. The nitrite ion is formed from the nitrate or the ammonium ions by certain microorganisms found in soil, water, sewage, and the digestive tract. The nitrate ion is formed by the complete oxidation of ammonium ions by soil or water microorganisms; nitrite is an intermediate product of this nitrification process. In oxygenated natural water systems, nitrite is rapidly oxidized to nitrate. Growing plants assimilate nitrate or ammonium ions and convert them to protein. A process known as denitrification takes

place when nitrate containing soils become anaerobic and the conversion to nitrite, molecular nitrogen, or nitrous oxide occurs. Ammonium ions may also be produced in some circumstances.

Among the major point sources of nitrogen entering water bodies are municipal and industrial wastewaters, septic tanks, and feed lot discharges. Nonpoint sources of nitrogen include farm-site fertilizer and animal wastes, lawn fertilizer, sanitary landfill leachate, atmospheric fallout, nitric oxide and nitrite discharges from automobile exhausts and other combustion processes, and losses from natural sources such as mineralization of soil organic matter. Water reuse systems in some fish hatcheries employ a nitrification process for ammonia reduction; this may result in exposure of the hatchery fish to elevated levels of nitrite (Russo et al. 1974).

Human Health Nitrate and Nitrite Criteria

In quantities normally found in food or feed, nitrates become toxic only under conditions in which they are, or may be, reduced to nitrites. Otherwise, at "reasonable" concentrations, nitrates are rapidly excreted in the urine. High intake of nitrates constitutes a hazard primarily to warm-blooded animals under conditions that are favorable to reduction to nitrite. Under certain circumstances, nitrate can be reduced to nitrite in the gastrointestinal tract. It then reaches the bloodstream and reacts directly with hemoglobin to produce methemoglobin, consequently impairing oxygen transport.

The reaction of nitrite with hemoglobin can be hazardous in infants under 3 months of age. Serious and occasionally fatal poisonings in infants have occurred following ingestion of untreated well waters shown to contain nitrate at concentrations greater than 10 mg/L nitrate nitrogen (as N) (NAS 1974). High nitrate concentrations are frequently found in shallow farm and rural community wells, often as the result of inadequate protection from barnyard drainage or from septic tanks (USPHS 1961; Stewart et al. 1967). Increased concentrations of nitrates also have been found in streams from farm tile drainage in areas of intense fertilization and farm crop production (Harmeson et al. 1971). Approximately 2000 cases of infant methemoglobinemia have been reported in Europe and North America between 1945 and 1950; 7 to 8% of the affected infants died (Walton 1951). Many infants have drunk water in which the nitrate nitrogen content was greater than 10 mg/L without developing methemoglobinemia. The differences in susceptibility to methemoglobinemia are not yet understood, but appear to be related to a combination of factors including nitrate concentration, enteric bacteria, and the lower acidity characteristic of the digestive systems of baby mammals. Methemoglobinemia systems and other toxic effects were observed when high nitrate well waters containing pathogenic bacteria were fed to laboratory mammals (Wolff et al. 1972). Conventional water treatment has no significant effect on nitrate removal from water (NAS 1974).

Because of the potential risk of methemoglobinemia to bottle-fed infants, and in view of the absence of substantiated physiological effects at nitrate concentrations below 10 mg/L nitrate nitrogen, this level is the criterion for domestic water supplies. Waters with nitrite nitrogen concentrations over 1 mg/L should not be used for infant feeding. Waters with a significant nitrite concentration usually would be heavily polluted and probably bacteriologically unacceptable.

The only national criterion for nitrate is 10 mg/L as N (40 CFR D Subpart F 141.51). The criterion applies to domestic water supplies. As noted above, the real danger from nitrate occurs when nitrate occurs in a reducing environment and converts to nitrite. The U.S. EPA set a National Primary Drinking Water MCL for nitrite at 1 mg/L as N (40 CFR D Subpart F 141.51).

Nitrate and Nitrite Aquatic Life Criteria

For fingerling rainbow trout, *Salmo gairdneri*, the respective 96-hour and 7-day LC50 toxicity values were 1360 and 1060 mg/L nitrate nitrogen in fresh water (Westin 1974). Knepp and Arkin (1973) observed that largemouth bass, *Micropterus salmoides*, and channel catfish, *Ictalurus punc-*

tatus, could be maintained at concentrations up to 400 mg/L nitrate without significant effect on their growth and feeding activities.

Nitrite forms of nitrogen were found to be much more toxic than nitrate forms. As an example, the 96-hour and 7-day LC50 values for chinook salmon were found to be 0.9 and 0.7 mg/L nitrite nitrogen in fresh water (Westin 1974). The effects of nitrite nitrogen on yearling rainbow trout, *Oncorhynchus mykiss*, showed that they suffered a 55% mortality after 24 hours at 0.55 mg/L; fingerling rainbow trout suffered a 50% mortality after 24 hours of exposure at 1.6 mg/L; and chinook salmon, *Oncorhynchus tshawytscha*, suffered a 40% mortality within 24 hours at 0.5 mg/L. There were no mortalities among rainbow trout exposed to 0.15 mg/L nitrite nitrogen for 48 hours. These data indicate that salmonids are more sensitive to nitrite toxicity than are other fish species, e.g., minnows, *Phoxinus laevis*, which suffered a 50% mortality within 1.5 hours of exposure to 2030 mg/L nitrite nitrogen, but required 14 days of exposure for mortality to occur at 10 mg/L (Klinger 1957), and carp, *Cyprinus carpio*, when raised in a water reuse system, tolerated up to 1.8 mg/L nitrite nitrogen (Saeki 1965).

The EPA concluded that (1) levels of nitrate nitrogen at or below 90 mg/L would have no adverse effects on warm-water fish (Knepp and Arkin 1973); (2) nitrite nitrogen at or below 5 mg/L should be protective of most warm-water fish (McCoy, 1972); and (3) nitrite nitrogen at or below 0.06 mg/L should be protective of salmonid fishes (Russo et al. 1974; Russo and Thurston 1975). These levels either are not known to occur or would be unlikely to occur in natural surface waters.

Recognizing that concentrations of nitrate or nitrite that would exhibit toxic effects on warm- or cold-water fish could rarely occur in nature, restrictive criteria were not recommended by the EPA.

PHOSPHATE

Phosphorus in the elemental form is very toxic (having an EPA marine life criteria of 0.10 µg/L) and is subject to bioaccumulation in much the same way as mercury. Phosphate forms of phosphorus are a major nutrient required for plant nutrition. In excessive concentrations, phosphates can stimulate plant growth. Excessive growths of aquatic plants (eutrophication) often interfere with water uses and are nuisances.

Generally, phosphates are not the only cause of eutrophication, but frequently it is the key of all the elements required by freshwater plants (generally, it is present in the least amount relative to need). Therefore, an increase in phosphorus allows use of other already present nutrients for plant growth. In addition, of all the elements required for plant growth in the water environment, phosphorus is the most easily controlled by man. In some aquatic systems, however, nitrogen compounds may be the most critical nutrients because of relatively large amounts of treated sewage (which is especially high in phosphates) in relation to other pollution sources, such as agricultural and urban runoff (which are high in nitrogen).

Phosphates enter waterways from several different sources. The human body excretes about one pound per year of phosphorus compounds. The use of phosphate detergents increases the per capita contribution to about 3.5 lb per year of phosphorus compounds. Some industries, such as potato processing, have wastewaters high in phosphates. Many nonpoint sources (crop, forest, and urban lands) contribute varying amounts of phosphorus compounds to watercourses. This drainage may be surface runoff of rainfall, effluent from agricultural tile lines, or return flow from irrigation. Cattle feedlots, birds, tree leaves, and fallout from the atmosphere all are contributing sources.

Evidence indicates that (1) high phosphorus compound concentrations are associated with accelerated eutrophication of waters, when other growth-promoting factors are present; (2) aquatic plant problems develop in reservoirs and other standing waters at phosphorus values lower than those critical in flowing streams; (3) reservoirs and lakes collect phosphates from influent streams and store a portion of them within consolidated sediments, thus serving as a phosphate sink; and (4) phosphorus concentrations critical to noxious plant growth vary and nuisance growths may

result from a particular concentration of phosphate in one geographical area but not in another. The amount or percentage of inflowing nutrients that may be retained by a lake or reservoir is variable and will depend upon: (1) the nutrient loading to the lake or reservoir; (2) the volume of the euphotic zone; (3) the extent of biological activities; (4) the detention time within a lake basin or the time available for biological activities; and (5) the discharge from the lake.

Once nutrients are discharged into an aquatic ecosystem, their removal is tedious and expensive. Phosphates are used by algae and higher aquatic plants and may be stored in excess of use within the plant cells. With decomposition of the plant cell, some phosphorus may be released immediately through bacterial action for recycling within the biotic community, while the remainder may be deposited with sediments. Much of the material that combines with the consolidated sediments within the lake bottom is bound permanently and will not be recycled into the system, but some can be released in harmful quantities.

Aquatic Life Summary for Phosphate

Total phosphate concentrations in excess of 100 µg/L (expressed as total phosphorus) may interfere with coagulation in water treatment plants. When such concentrations exceed 25 µg/L at the time of the spring turnover on a volume-weighted basis in lakes or reservoirs, they may occasionally stimulate excessive or nuisance growths of algae and other aquatic plants. Algal growths cause undesirable tastes and odors in water, interfere with water treatment, become aesthetically unpleasant, and alter the chemistry of the water supply. They contribute to eutrophication.

To prevent the development of biological nuisances and to control accelerated or cultural eutrophication, total phosphates as phosphorus (P) should not exceed 50 µg/L in any stream at the point where it enters any lake or reservoir, nor 25 µg/L within the lake or reservoir. A desired goal for the prevention of plant nuisances in streams or other flowing waters not discharging directly to lakes or impoundments is 100 µg/L total P (Mackenthun 1973). Most relatively uncontaminated lake districts are known to have surface waters that contain from 10 to 30 µg/L total phosphorus as P (Hutchinson 1957).

The majority of the nation's eutrophication problems are associated with lakes or reservoirs, and currently there are more data to support the establishment of a limiting phosphorus level in those waters than in streams or rivers that do not directly impact such water. There are natural conditions, also, that would dictate the consideration of either a more or less stringent phosphorus level. Eutrophication problems may occur in waters where the phosphorus concentration is less than that indicated above and, obviously, such waters would need more stringent nutrient limits. Likewise, there are those waters within the United States where phosphorus is not now a limiting nutrient and where the need for phosphorus limits is substantially diminished.

There are two basic needs in establishing a phosphorus criterion for flowing waters: one is to control the development of plant nuisances within the flowing water and, in turn, to control and prevent animal pests that may become associated with such plants. The other is to protect the downstream receiving waterway, regardless of its proximity in linear distance. It is evident that a portion of that phosphorus that enters a stream or other flowing waterway eventually will reach a receiving lake or estuary either as a component of the fluid mass, as bedload sediments that are carried downstream, or as floating organic materials that may drift just above the stream's bed or float on its water's surface. Superimposed on the loading from the inflowing waterway, a lake or estuary may receive additional phosphorus as fallout from the atmosphere or as a direct introduction from shoreline areas.

Another method to control the inflow of nutrients, particularly phosphates, into a lake is that of prescribing an annual loading to the receiving water. Vollenweider (1973) suggests total phosphorus (P) loadings, in grams per square meter of surface area per year, that will be a critical level for eutrophic conditions within the receiving waterway for a particular water volume. The mean

depth of the lake in meters is divided by the hydraulic detention time in years. Vollenweider's data suggest a range of loading values that should result in oligotrophic lake water quality:

Mean Depth/Hydraulic Detention Time (m/y)	Oligotrophic or Permissible Loading (g/m/yr)	Eutrophic or Critical Loading (g/m/yr)
0.5	0.07	0.14
1.0	0.10	0.20
2.5	0.16	0.32
5.0	0.22	0.45
7.5	0.27	0.55
10.0	0.32	0.63
25.0	0.50	1.00
50.0	0.71	1.41
75.0	0.87	1.73
100.0	1.00	2.00

There may be waterways where higher concentrations, or loadings, of total phosphorus do not produce eutrophication, as well as those waterways where lower concentrations or loadings of total phosphorus may be associated with populations of nuisance organisms. Waters now containing less than the specified amounts of phosphorus should not be degraded by the introduction of additional phosphates.

pH

pH is a measure of the hydrogen ion activity in a water sample. It is mathematically related to hydrogen ion activity according to the expression: $pH = -\log_{10} H^+$, where H^+ the hydrogen ion activity, expressed in moles/L. The pH of natural waters is a measure of the acid–base equilibrium achieved by the various dissolved compounds, salts, and gases. The principal chemical system controlling pH in natural waters is the carbonate system, which is composed of atmospheric carbon dioxide (CO_2) and resulting carbonic acid (H_2CO_3), bicarbonate ions (HCO_3^-) and carbonate ions (CO_3^{2-}) The interactions and kinetics of this system have been described by Stumm and Morgan (1970).

pH is an important factor in the chemical and biological reactions in natural waters. The degree of dissociation of weak acids or bases is affected by changes in pH. This effect is important because the toxicity of many compounds is affected by the degree of dissociation. One such example is for hydrogen cyanide. Cyanide toxicity to fish increases as the pH is lowered because the chemical equilibrium is shifted toward an increased concentration of a more toxic form of cyanide. Similar results have also been shown for hydrogen sulfide (H_2S) (Jones 1964). Conversely, rapid increases in pH can cause increased NH_3 concentrations that are also toxic. Ammonia has been shown to be 10 times as toxic at pH 8.0 as at pH 7.0 (EIFAC 1969).

The solubility of metal compounds contained in bottom sediments, or as suspended material, is also affected by pH. For example, laboratory equilibrium studies under anaerobic conditions indicated that pH was an important parameter involved in releasing manganese from bottom sediments (Delfino and Lee 1971).

Coagulation, used for removal of colloidal color and turbidity through the use of aluminum or iron salts, generally has an optimum pH range of 5.0 to 6.5. The effect of pH on chlorine in water principally concerns the equilibrium between hypochlorous acid (HOCl) and the hypochlorite ion (OCl^-) according to the reaction:

$$HOCl = H^+ + OCl^-$$

High hydrogen ion concentrations (low pH) would therefore cause much more HOCl to be present, than at high pH values. Chlorine disinfection is more effective at values less than pH 7 (favoring HOCl, the more effective disinfectant). Water is therefore adjusted to a pH of between 6.5 and 7 before most water treatment processes. Corrosion of plant equipment and piping in the distribution system can lead to expensive replacement as well as the introduction of metal ions such as copper, lead, zinc, and cadmium. Langelier (1936) developed a method to calculate and control water corrosive activity that employs calcium carbonate saturation theory and predicts whether the water would tend to dissolve metal piping, or deposit a protective layer of calcium carbonate on the metal. Generally, this level is above pH 7 and frequently approaches pH 8.3, the point of maximum bicarbonate/carbonate buffering.

Since pH is relatively easily adjusted before, and during, water treatment, a rather wide range is acceptable for water serving as a source of public water supply. A range of pH from 5.0 to 9.0 would provide a water treatable by typical (coagulation, sedimentation, filtration, and chlorination) treatment plant processes. As the range is extended, the cost of pH-adjusting chemicals increases.

pH Aquatic Life Effects and Criteria

A review of the effects of pH on freshwater fish has been published by the European Inland Fisheries Advisory Commission (1969). The commission concluded:

> There is no definite pH range within which a fishery is unharmed and outside which it is damaged, but rather, there is a gradual deterioration as the pH values are further removed from the normal range. The pH range which is not directly lethal to fish is 5 to 9; however, the toxicity of several common pollutants is markedly affected by pH changes within this range, and increasing acidity or alkalinity may make these poisons more toxic. Also, an acid discharge may liberate sufficient CO_2 from bicarbonate in the water either to be directly toxic, or to cause the pH range of 5 to 6 to become lethal.

Mount (1973) performed bioassays on the fathead minnow, *Pimephales promelas*, for a 13-month, one-generation time period to determine chronic pH effects. Tests were run at pH levels of 4.5, 5.2, 5.9, 6.6, and a control of 7.5. At the two lowest pH values (4.5 and 5.2), behavior was abnormal and the fish were deformed. At pH values less than 6.6, egg production and hatchability were reduced when compared with the control. It was concluded that a pH of 6.6 was marginal for vital life functions.

Bell (1971) performed bioassays with nymphs of caddisflies (two species), stoneflies (four species), dragonflies (two species), and mayflies (one species). All are important fish food organisms. The 30-day TL50 pH values ranged from 2.5 to 5.4, with the caddisflies being the most tolerant and the mayflies being the least tolerant. The pH values at which 50% of the organisms emerged ranged from 4.0 to 6.6 with increasing percentage emergence occurring with the increasing pH values.

Based on present evidence, a pH range of 6.5 to 9.0 appears to provide adequate protection for the life of freshwater fish and bottom-dwelling invertebrates. Outside of this range, fish suffer adverse physiological effects, increasing in severity as the degree of deviation increases until lethal levels are reached:

pH Range	Effect on Fish
5.0–6.0	Unlikely to be harmful to any species unless either the concentration of free CO_2 is greater than 20 ppm, or the water contains iron salts which are precipitated as ferric hydroxide, the toxicity of which is not known
6.0–6.5	Unlikely to be harmful to fish unless free CO_2 is present in excess of 100 ppm
6.5–9.0	Harmless to fish, although the toxicity of other poisons may be affected by changes within this range

From EIFAC 1969

The U.S. EPA set a national drinking water secondary standard limiting pH ranges of domestic water supplies to 6.5 to 8.5 (40 CFR D 143.3). For the protection of fish and bottom-dwelling invertebrates, the U.S. EPA recommends that pH values should be less than 9 and greater than 6.5 (EPA 1986b).

SUSPENDED SOLIDS AND TURBIDITY

Suspended solids (sometimes referred to as nonfilterable residue) and turbidity are related to the solids content that is not dissolved. Turbidity refers to the blockage of light penetration and is measured by examining the backscatter from an intense light beam, while suspended solids are measured by weighing the amount of dried sediment that is trapped on a 0.45-µm filter, after filtering a known sample volume. The suspended solids test therefore measures a broad variety of solids that are contained in the water, including floatable material and settleable matter, in addition to the suspended solids. An Imhoff cone can be used to qualitatively estimate the settleable solids content of water. Subjecting the filter to a high temperature will burn off the more combustible solids. The remaining solids are usually referred to as the nonvolatile solids. The amount burned is assumed to be related to the organic fraction of the wastewater.

Turbidity (and color) can be caused mostly by very small particles (less than 1 µm), while the suspended solids content is usually associated with more moderate-sized particles (10 to 100 µm). Suspended solids can cause water quality problems directly, as discussed in the following paragraphs from *Water Quality Criteria* (EPA 1986b). They may also have other pollutants (such as organics and toxicants) associated with them that would cause additional problems. The control of suspended solids is required in most discharge permits because of potential sedimentation problems downstream of the discharge and the desire to control associated other pollutants.

Turbid water interferes with recreational use and aesthetic enjoyment of water. Turbid waters can be dangerous for swimming, especially if diving facilities are provided, because of the possibility of unseen submerged hazards and the difficulty in locating swimmers in danger of drowning (NAS 1974). The less turbid the water, the more desirable it becomes for swimming and other water contact sports. Other recreational pursuits, such as boating and fishing, will be adequately protected by suspended solids criteria developed for protection of fish and other aquatic life.

Fish and other aquatic life requirements concerning suspended solids can be divided into those whose effect occurs in the water column and those whose effect occurs following sedimentation to the bottom of the water body. Noted effects are similar for both fresh and marine waters. The effects of suspended solids on fish have been reviewed by the European Inland Fisheries Advisory Commission (EIFAC 1969). This review in 1965 identified four effects on the fish and fish food populations.

1. By acting directly on the fish swimming in water in which solids are suspended, and either killing them or reducing their growth rate, resistance to disease, etc.
2. By preventing the successful development of fish eggs and larvae
3. By modifying natural movements and migrations of fish
4. By reducing the abundance of food available to the fish

Settleable materials which blanket the bottom of water bodies damage the invertebrate populations, block gravel spawning beds, and if organic, remove dissolved oxygen from overlying waters (EIFAC 1969; Edberg and Hofstan 1973). In a study downstream from the discharge of a rock quarry where inert suspended solids were increased to 80 mg/L, the density of macroinvertebrates decreased by 60%, while in areas of sediment accumulation, benthic invertebrate populations also decreased by 60% regardless of the suspended solid concentrations (Gammon 1970). Similar effects have been reported downstream from an area which was intensively logged. Major increases in

stream suspended solids (25 mg/L upstream vs. 390 mg/L downstream) caused smothering of bottom invertebrates, reducing organism density to only 7.3 vs. 25.5/ft^2 upstream (Tebo 1955).

When settleable solids block gravel spawning beds which contain eggs, high mortalities result. There is also evidence that some species of salmonids will not spawn in such areas (EIFAC 1969). It has been postulated that silt attached to the eggs prevents sufficient exchange of oxygen and carbon dioxide between the egg and the overlying water. The important variables are particle size, stream velocity, and degree of turbulence (EIFAC 1969). Deposition of organic materials to the bottom sediments can cause imbalances in stream biota by increasing bottom animal density (principally worms), and diversity is reduced as pollution-sensitive forms disappear (Mackenthun 1973). Algae, likewise, flourish in such nutrient-rich areas, although forms may become less desirable (Tarzwell and Gaufin 1953).

Plankton and inorganic suspended materials reduce light penetration into the water body, reducing the depth of the photic zone. This reduces primary production and decreases fish food. The NAS committee in 1974 recommended that the depth of light penetration not be reduced by more than 10% (NAS 1974). Additionally, the near-surface waters are heated because of the greater heat absorbency of the particulate material which tends to stabilize the water column and prevents vertical mixing (NAS 1974). Such mixing reductions decrease the dispersion of dissolved oxygen and nutrients to lower portions of the water body. Increased temperatures also reduce the capacity of the stream to contain dissolved oxygen.

Suspended inorganic material in water also sorbs organic materials, such as pesticides. Following this sorption process, subsequent sedimentation may remove these materials from the water column into the sediments (NAS 1974). However, the sedimentation of these polluted sediments can cause dramatic changes in the benthic microorganism populations, which in turn affect other aquatic life forms. Recent research associated with the effects of polluted sediments in urban streams is summarized in earlier chapters of this book.

Water Quality Criteria for Suspended Solids and Turbidity

The EPA water quality criterion for freshwater fish and other aquatic life is essentially that proposed by the National Academy of Sciences and the Great Lakes Water Quality Board: "Settleable and suspended solids should not reduce the depth of the compensation point for photosynthetic activity by more than 10 percent from the seasonally established norm for aquatic life."

States have selected numeric values for turbidity. Alabama, for example, uses the same standard for all designated uses: "There shall be no turbidity of other than natural origin that will cause substantial visible contrast with the natural appearance of waters or interfere with any beneficial uses which they serve. Furthermore, in no case shall turbidity exceed 50 Nephelometric units (NTU) above background. Background will be interpreted as the natural condition of the receiving waters, without the influence of man-made or man-induced causes. Turbidity levels caused by natural runoff will be included in establishing background levels." In addition, the state of Alabama has minimum conditions applicable to all state waters that includes: "State waters shall be free from substances attributable to sewage, industrial wastes, or other wastes that will settle to form bottom deposits which are unsightly, putrescent, or interfere directly or indirectly with any classified water use."

ZINC

Aquatic Life Criteria for Zinc

The U.S. EPA has set a national ambient water quality for the protection of wildlife as a function of hardness (EPA 1986b), and ambient water quality for the Great Lakes as a function of hardness (40 CFR 132.3 (b)). Figure G.4 shows these criteria.

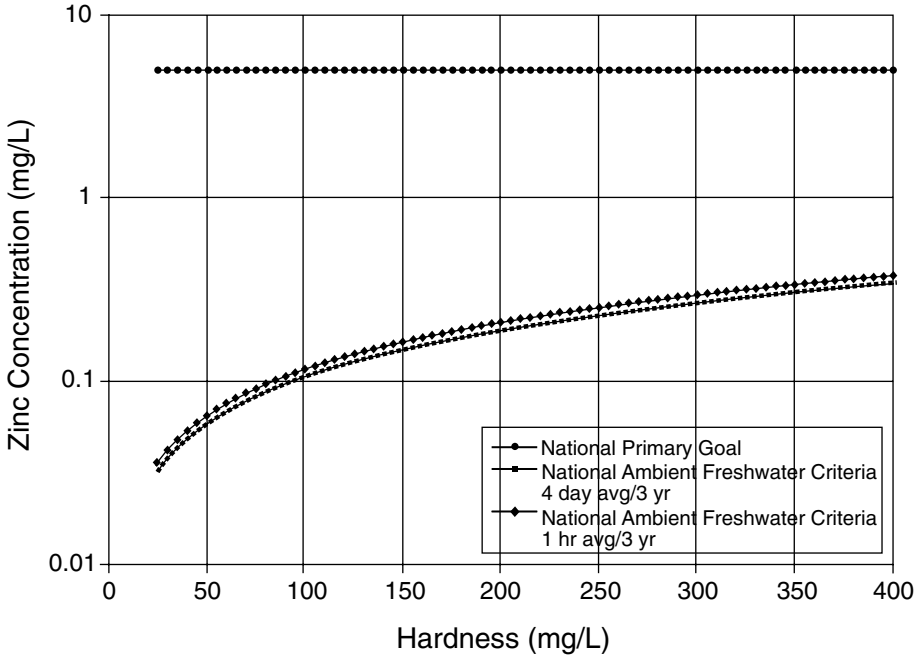

Figure G.4 Zinc criteria.

Human Health Criteria for Zinc

The U.S. EPA has set a national secondary MCL for zinc at 5 mg/L (40 CFR D 143.3), based on available organoleptic data, and to control undesirable taste and odor quality of ambient water. It should be recognized that organoleptic data have limitations as a basis for establishing water quality criteria, and have no demonstrated relationship to potential adverse human health effects.

SEDIMENT GUIDELINES

Water quality criteria and standards are proven to be useful tools for helping to assess receiving water quality and beneficial use attainment. For these reasons, it is logical that sediment quality criteria would also be a useful tool. However, the complexity of sediments has impeded establishing guidelines because of the lack of clear relationships between sediment characteristics and the bioavailability of associated contaminants. Nonetheless, several useful approaches have been proposed for establishing sediment guidelines (also called criteria, standards, guidelines, objectives, or assessment values). In recent years, there has been a tremendous increase in sediment contaminant research and monitoring, which has resulted in improved sediment quality guidelines. The U.S. EPA has proposed guidelines using a theoretical approach known as *equilibrium partitioning guidelines*. Concentrations of contaminants are predicted in interstitial water and compared to the chronic water quality criteria to establish whether the sediments are toxic. Currently there are only criteria for acenaphthene, phenanthrene, fluoranthene, dieldrin, and endrin. This approach normalizes nonpolar organic compounds to the sediment total organic carbon content and metals to the acid volatile sulfide content. Both these sediment parameters have been shown to strongly control bioavailability

Table G.7 Sediment Quality Guidelines for Freshwater Ecosystems

Substance	TEL	PEL	LEL	SEL	MET	TET	ERL	ERM	SQAL
Metals (in mg/kg DW)									
Arsenic	5.9	17	6	33	7	17	33	85	NG
Cadmium	0.596	3.53	0.6	10	0.9	3	5	9	NG
Chromium	37.3	90	26	110	55	100	80	145	NG
Copper	35.7	197	16	110	28	86	70	390	NG
Lead	35	91.3	31	250	42	170	35	110	NG
Mercury	0.174	0.486	0.2	2	0.2	1	0.15	1.3	NG
Nickel	18	36	16	75	35	61	30	50	NG
Zinc	123	315	120	820	150	540	120	270	NG
Polycyclic Aromatic Hydrocarbons (in µg/kg DW)									
Anthracene	NG	NG	220	3700	NG	NG	85	960	NG
Fluorene	NG	NG	190	1600	NG	NG	35	640	540
Naphthalene	NG	NG	NG	NG	400	600	340	2100	470
Phenanthrene	41.9	515	560	9500	400	800	225	1380	1800
Benz[a]anthracene	31.7	385	320	14800	400	500	230	1600	NG
Benzo(a)pyrene	31.9	782	370	14400	500	700	400	2500	NG
Chrysene	57.1	862	340	4600	600	800	400	2800	NG
Dibenz[a,h]anthracene	NG		60		NG		60		NG
Fluoranthene	111	2355	750	10200	600	2000	600	3600	6200
Pyrene	53	875	490	8500	700	1000	350	2200	NG
Total PAHs	NG	NG	4000	100000	NG	NG	4000	35000	NG
Polychlorinated Biphenyls (in µg/kg DW)									
Total PCBs	34.1	277	70	5300	200	1000	50	400	NG
Organochlorine Pesticides (in µg/kg DW)									
Chlordane	4.5	8.9	7	60	7	30	0.5	6	NG
Dieldrin	2.85	6.67	2	910	2	300	0.02	8	110
Sum DDD	3.54	8.51	8	60	10	60	2	20	NG
Sum DDE	1.42	6.75	5	190	7	50	2	15	NG
Sum DDT	NG	NG	8	710	9	50	1	7	NG
Total DDTs	7	4450	7	120	NG	NG	3	350	NG
Endrin	2.67	62.4	3	1300	8	500	0.02	45	42
Heptachlor epoxide	0.6	2.74	5	50	5	30	NG	NG	NG
Lindane (gamma-BHC)	0.94	1.38	3	10	3	9	NG	NG	3.7

PEL = Probable effect level; dry weight (Smith et al. 1996).

SEL = Severe effect level, dry weight (Persaud et al. 1993).

TET = Toxic effect threshold; dry weight (EC and MENVIQ 1992).

ERM = Effects range median; dry weight (Long and Morgan 1991).

NG = No guideline.

(e.g., Ingersoll et al. 1997). It does not appear that the U.S. EPA approach will result in additional guidelines in the near future. There have been several empirical approaches that are based on co-occurrence of adverse biological effects observed in the field or laboratory related to sediment contaminant concentrations. Tables G.7 and G.8 list some of the most reliable sediment quality guidelines available. Included in these are some "consensus" approaches that may be a first priority if one chooses to use a sediment guideline in their assessment. It is interesting to note that the majority of the approaches produce guidelines that are relatively similar; therefore, the consensus approach has added credibility.

Table G.8 Sediment Quality Guidelines for Polycyclic Aromatic Hydrocarbons (μg/g organic carbon)[a]

PAH	ERL[b]	ERM[b]	TEL[b]	PEL[b]	SLC[b]	LAET[b]	HAET[b]	EqP	TEC Mean	MEC Mean	Consensus EEC
Naphthalene	16	210	3	39	41	210	270				
Acenaphthylene	4	64	1	13	5	>56	130				
Acenaphthene	2	50	1	9	6[c]	50	200	230			
Fluorene	2	54	2	14	10	54	360				
Phenanthrene	24	150	9	54	37	150	690	240			
Anthracene	9	110	5	24	16	96	1300				
Low-molecular-weight PAH	57	638	21	153	115	616	2950				
Fluoranthene	60	510	11	149	64	170	3000	300			
Pyrene	66	260	15	140	66	260	1,600				
Benz[a]anthracene	26	160	7	69	26	130	510				
Chrysene	38	280	11	85	38	140	920				
Benzo[b]fluoranthene	32[c]	188[c]	7[c]	71[c]	32[c]	160	445				
Benzo[k]fluoranthene	28[c]	162[c]	6[c]	61[c]	28[c]	160	445				
Benzo[a]pyrene	43	160	9	76	40	160	360				
High-molecular-weight PAH	293	1720	66	651	294	1180	7280				
Total PAH	350	2358	87	804	409	1796	10,230	211	290 (119–461)	1800 (682–2,854)	10,000

[a] ERL = effects range-low;
ERM = effects range-median;
TEL = threshold effects level;
PEL = probable effects level;
SLC = screening level concentration;
LAET = low apparent effects threshold;
HAET = high apparent effects threshold;
EqP = U.S. Environmental Protection Agency criteria derived from equilibrium partitioning theory;
TEC = Threshold effect concentration;
MEC = Median effects concentration;
EEC = Extreme effects concentration.
[b] SQG at 1% OC.
[c] No SQG. Estimate assuming mean ratio to PAH mixture LC50 for other high-molecular-weight PAHs.

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APPENDIX H

Watershed and Receiving Water Modeling

CONTENTS

INTRODUCTION

Models are important tools for watershed and receiving water analyses because they enable comprehensive evaluations of large systems and can predict future conditions. Models always have errors, but these can be reduced through good calibration and verification using locally obtained data, as described in this book.

For stormwater issues, most models can be separated into watershed models and receiving water models. Both are briefly addressed in this appendix. Many (and constantly increasing in numbers) public domain water quality models are available. Periodically, these are available on a CD-ROM from the EPA (*Exposure Models Library and Integrated Model Evaluation System,* EPA Office of Research and Development CD-ROM. EPA-600-C-92-002. Revised March 1996). Numerous specialized Internet sites also have download sites or links to the EPA download sites for acquiring these models and documentation. The main EPA source is through the EPA's Athens, GA, Center for Exposure Assessment Modeling (CEAM), where much of the EPA's water quality modeling support is available (downloads, short courses, etc.). One especially interesting reference available from Athens is *Rates, Constants, and Kinetics Formulations in Surface Water Quality Modeling* (second edition), EPA/600/3-85/040, prepared by Tetra Tech in 1985, but still highly useful. This report is available in PDF format from: http://www.epa.gov/ORD/WebPubs/surfaceH2O/surface.html. Not only does this report contain summaries of the model processes and lab and field data for the different fate processes, it also summarizes many field techniques that can be used to collect the needed local data.

The CEAM Internet site is at: http://www.epa.gov/CEAM/. The major models available at this web site are shown in Table H.1 (as of February 2000). These are all DOS-based, Fortran-coded programs. Very few Windows or Macintosh programs are available, but they will operate in the

Table H.1 DOS Programs Available to Download from the EPA's Center for Exposure Assessment Modeling (CEAMS) Group

File Name/Size (MB)	Description/Abstract/Release Notes	Version Number	Release Date
INSTALAN.EXE / 1.28	ANNIE-IDE tool kit	1.14	Sep 91
INSTALCI.EXE / .5	CEAM information system	3.21	May 95
INSTALCM.EXE / 1.63	CORMIX model / documentation	3.20	Dec 96
INSTALEX.EXE / 1.00	EXAMS model / documentation	2.97.5	Jun 97
INSTALFG.EXE / 1.07	FGETS model system	3.0.18	Sep 94
INSTALFW.EXE / 1.05	FEMWATER model / documentation	1.00	Jul 93
INSTALGC.EXE / 1.16	GCSOLAR model / documentation	1.20	Jul 99
INSTALHC.EXE / 8.44	HSCTM2D model / documentation	1.01	Nov 98
INSTALHS.EXE / 8.66	HSPF model / documentation	11.00	Apr 97
HSP11Y2K.EXE / .84	HSPF model / documentation / Year 2000 (Y2K) Patch	11.00	Dec 99
INSTALLC.EXE / .71	LC50 model / documentation	1.00	Jan 99
INSTALMT.EXE / 2.49	MINTEQ model / documentation	4.01	Dec 99
INSTALMS.EXE / 6.52	MMSOILS model / documentation	4.00	Jun 97
INSTALMM.EXE / 3.49	MULTIMED model / documentation	1.01	Dec 92
INSTALM2.EXE / 4.79	MULTIMED model / documentation	2.00 Beta	Oct 96
INSTALDP.EXE / 3.34	MULTIMDP model / documentation	1.00	Oct 96
INSTALOF.EXE / .34	Sample ANNIE-IDE application	1.61	Sep 91
INSTALOX.EXE / .40	OXYREF data base / documentation	1.00	Dec 98
INSTALP2.EXE / 2.76	PRZM2 model / documentation	2.00	Oct 94
INSTALP3.EXE / 5.15	PRZM3 model / documentation	3.12 Beta	Mar 98
INSTALPL.EXE / 1.44	PLUMES model / documentation	3.00	Dec 94
INSTALPT.EXE / 5.43	PATRIOT model / documentation	1.20	Nov 94
INSTALQ2.EXE / 2.21	QUAL2EU model system / Documentation	3.22	May 96
INSTALSW.EXE / 1.6	SWMM model system	4.30	May 94
INSTALSX.EXE / .39	SMPTOX3 model / documentation	2.01	Feb 93
INSTALWP.EXE / 3.14	WASP model / documentation	5.10	Oct 93

DOS shell of the Windows operating systems. Most of these programs were originally developed many years ago (with the processes reasonably well described in the Tetra Tech "rate" report of 1985, noted above).

There are numerous proprietary Windows "front-ends" for selected programs, along with proprietary versions that have substantial changes in the code. In addition, many private Internet sites also provide downloadable public domain water quality models, or "test" versions of commercial programs. Obviously, it is impossible to develop a complete list of available water quality models, and it is difficult for the user to select which model may be most appropriate for his or her specific use. Excellent model reviews are periodically prepared, such as *Compendium of Tools for Watershed Assessment and TMDL Development,* EPA-841-B-97-006, 1997. In addition, numerous listservers are available to provide excellent user support for specific models. A representative listing of list servers and other water quality modeling support is provided by Dr. Bill James at the University of Guelph at http://www.eos.uoguelph.ca/webfiles/james/homepage/Research/ListServers.html.

A major surface water quality modeling effort at EPA is directed toward supporting the Total Maximum Daily Load (TMDL) program. As part of this support, the BASINS model (*Better Assessment Science Integrating Point and Nonpoint Sources*), a Windows-based structure of several interconnected programs and a geographical information system (GIS), described later, has been developed. The main report is available as EPA-823, R-96001, May, 1996. Extensive Internet support, including downloads of the main program, and regional data, is available at http://www.epa.gov/OST/BASINS/. The structure of BASINS will allow additional models to be added to this framework. The extremely powerful aspect of BASINS is the GIS capabilities where local data can be easily integrated for model use. Individual CD-ROMS are available for each of the 10 EPA regions containing much local data. BASINS has six main components: nationally derived databases with Data Extraction Tools; assessment tools; utilities to facilitate organizing and evaluating the data, including land use

data; Watershed Characterization Reports; water quality models; and the Nonpoint Source Model. It currently uses portions of HSPF for the land-based modeling component (NPSM, the Nonpoint Source Model), and QUAL2E and TOXIROUTE for the stream water quality models. Even though many of model components are older Fortran-coded modules, the Windows and GIS interfaces makes the model relatively easy to use.

BASINS is a large-scale model and may be too complex for focusing on specific smaller areas, or when detailed evaluations are needed. Figure H.1 is an overview of the individual environmental models commonly used (and evaluated in *Compendium of Tools for Watershed Assessment and TMDL Development*). Obviously, BASINS, although extremely powerful and needed for some applications, currently does not offer the flexibility that the wide range of individual models can.

MODELING STORMWATER EFFECTS AND THE NEED FOR LOCAL DATA FOR CALIBRATION AND VERIFICATION

A typical use of stormwater monitoring data is to calibrate and verify a model that will be used to examine many questions. Common uses of models are to determine the major sources of pollutants and to design control programs to effectively reduce the problem discharges. There are three general classes of stormwater models:

- Unit area loadings
- Simple models
- Complex models

Unit Area Loadings

Table 2.5 included unit area loading estimates for stormwater, based on numerous observations from throughout North America (mostly from the EPA's NURP projects, EPA 1983, and from other selected North American studies). Most of the available NURP data are from monitoring medium-density residential areas, but data from Wisconsin and Toronto included data from various land uses. These estimates are most useful when making preliminary assessments on a large scale, especially in preparing an experimental design for site-specific monitoring. As an example, these values can be used to identify the most significant land uses in a watershed and help direct the monitoring effort, as shown in Table 5.4 (repeated below as Table H.2) and Figure 5.7, a marginal benefit analysis. Obviously, the variations of unit area loadings can be very large, depending on specific conditions, but the basic rankings of land use related discharges are still useful for preliminary evaluations.

For most constituents, manufacturing industrial and commercial areas have the largest unit area loadings, while parks and low-density residential areas have the smallest unit area loadings. The importance of the areas in a watershed is obviously dependent on the size of the area. Medium-density residential areas comprise the majority of the land area for most cities, and therefore also for most large urban watersheds. These large areas increase the significance of this land use. However, relatively small amounts of industrial or commercial activity can overwhelm the residential contributions in small and moderate-sized drainages. Chapter 2 presented information showing the relative importance of industrial and residential areas in Toronto (Pitt and McLean 1986), based on a comprehensive monitoring program and measured unit area loadings for the major land uses.

The earlier Toronto discussion in Chapter 2 also showed how dry-weather flows and snowmelt contributions can be very important. That example stresses the need to consider all phases of flows that may be discharged from separate storm drainage systems. Few published unit area stormwater loading values include these other contributions that can have major effects on receiving water conditions.

Unit area loadings for a local area can be determined based on local monitoring data using one of the other modeling methods described below. Unit area loadings are a convenient method to summarize extensive monitoring data and to highlight potential problem areas, especially if integrated

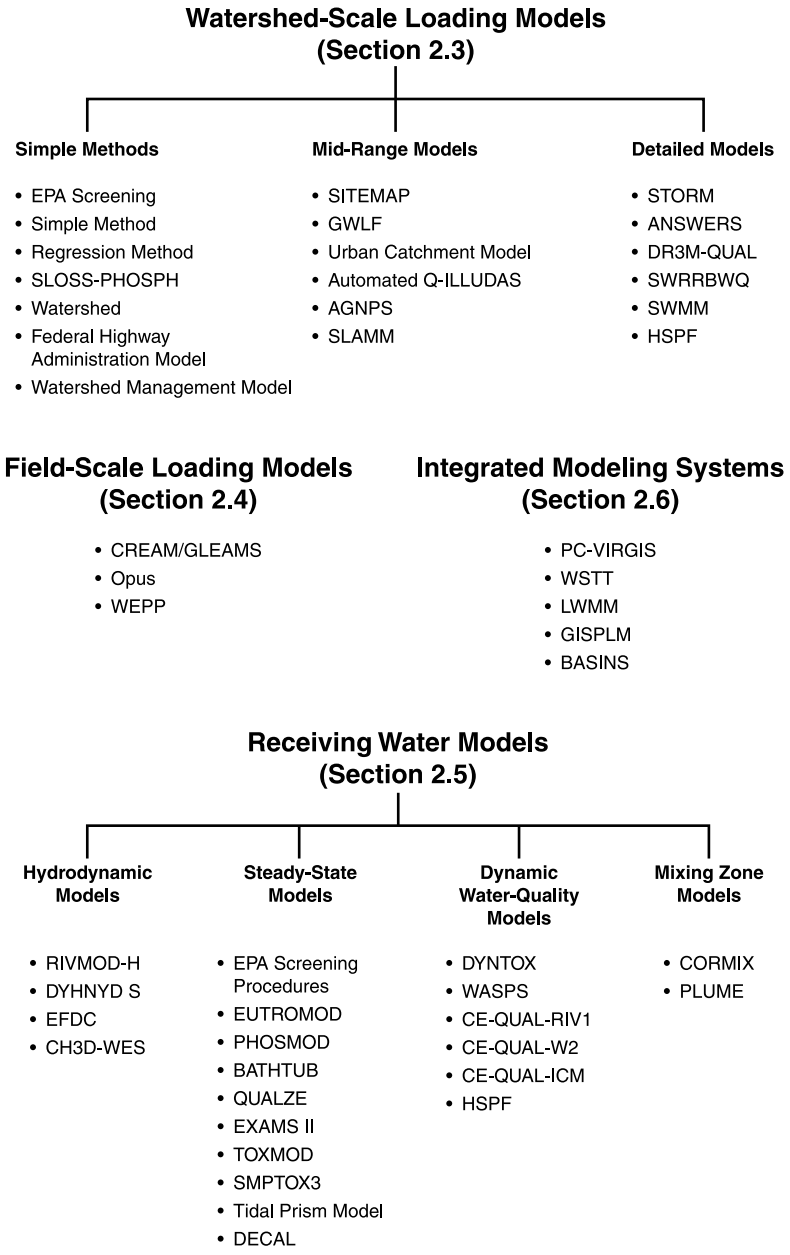

Figure H.1 Environmental models commonly in use. (From EPA. *Compendium of Tools for Watershed Assessment and TMDL Development*. EPA-841-B-97-006. U.S. Environmental Protection Agency. 1997.)

with a GIS. GIS has been successfully used with nonpoint source modeling activities to display the unit area loadings predicted from monitoring and modeling programs for many alternatives. Otherwise, the massive amounts of data generated is difficult to summarize in an easily presentable manner.

Simple Models

Simplified stormwater models usually take the general form:

$$\text{Unit Area Loading} = (\text{EMC}) \times (\text{Rv}) \times (\text{Rain})$$

Table H.2 Example Marginal Benefit Analysis

	Land Use (ranked by % mass per category)	% of Area	Critical Unit Area Loading	Relative Mass	% Mass per Category	Accum. (% mass)	Straight-line Model	Marginal Benefit
1	Older medium-density resid.	24	200	4800	22.8	22.8	6.25	16.5
2	High-density resid.	7	300	2100	10.0	32.7	12.5	20.2
3	Office	7	300	2100	10.0	42.7	18.8	24.0
4	Strip commercial	8	250	2000	9.5	52.2	25.0	27.2
5	Multiple family	8	200	1600	7.6	59.8	31.3	28.5
6	Manufacturing industrial	3	500	1500	7.1	66.9	37.5	29.4
7	Warehousing	5	300	1500	7.1	74.0	43.8	30.3
8	New medium-density resid.	5	250	1250	5.9	80.0	50.0	30.0
9	Light industrial	5	200	1000	4.7	84.7	56.3	28.4
10	Major roadways	5	200	1000	4.7	89.4	62.5	26.9
11	Civic/educational	10	100	1000	4.7	94.2	68.8	25.4
12	Shopping malls	3	250	750	3.6	97.7	75.0	22.7
13	Utilities	1	150	150	0.7	98.5	81.3	17.2
14	Low-density resid. with swales	5	25	125	0.6	99.1	87.5	11.6
15	Vacant	2	50	100	0.5	99.5	93.8	5.8
16	Park	2	50	100	0.5	100.0	100.0	0.0
	Total	100		21075	100			

Table H.3 Median EMCs and COVs for All Sites Monitored during NURP

Pollutant		Residential		Mixed		Commercial		Open/Nonurban	
		Median	COV	Median	COV	Median	COV	Median	COV
BOD_5	mg/L	10.0	0.41	7.8	0.52	9.3	0.31	—	—
COD	mg/L	73	0.55	65	0.58	57	0.39	40	0.78
TSS	mg/L	101	0.96	67	1.14	69	0.85	70	2.92
Total lead	µg/L	144	0.75	114	1.35	104	0.68	30	1.52
Total copper	µg/L	33	0.99	27	1.32	29	0.81	—	—
Total zinc	µg/L	135	0.84	154	0.78	226	1.07	195	0.66
Total Kjeldahl nitrogen	µg/L	1900	0.73	1288	0.50	1179	0.43	965	1.00
NO_2-N + NO_3-N	µg/L	736	0.83	558	0.67	572	0.48	543	0.91
Total P	µg/L	383	0.69	263	0.75	201	0.67	121	1.66
Soluble P	µg/L	143	0.46	56	0.75	80	0.71	26	2.1

From EPA. *Results of the Nationwide Urban Runoff Program*. Water Planning Division, PB 84-185552, Washington, D.C. December 1983.

where EMC is the event mean concentration, Rv is the volumetric runoff coefficient (or the effective impervious area, EIA), and Rain is the total rain depth for the period of concern (usually a year). With the appropriate conversions, this simple equation predicts the unit area loadings for the monitored area. This is the method used in the stormwater permit applications for the EPA's NPDES (Nationwide Pollutant Discharge Elimination System) permit program.

The problems with this simplified model include: typically poor estimates of EMC, the Rv value varies for different rain depths, and the procedure cannot easily distinguish seasonal effects (unless EMC values are available for each season), and it cannot be used to evaluate the effectiveness of stormwater control practices.

The main problem with using this simplified model is obtaining an adequate estimate for the EMC. Table H.3 contains the basic concentration information from the EPA's NURP studies (EPA 1983) that are generally used for these analyses. The coefficient of variation (COV) values for these median values are seen to vary from 0.5 to more than 1.0. Figure H.2, also from the EPA's NURP studies (EPA 1983), illustrates the wide variations in observed concentrations for the common stormwater constituents. Wide concentration variations make it more difficult to distinguish between

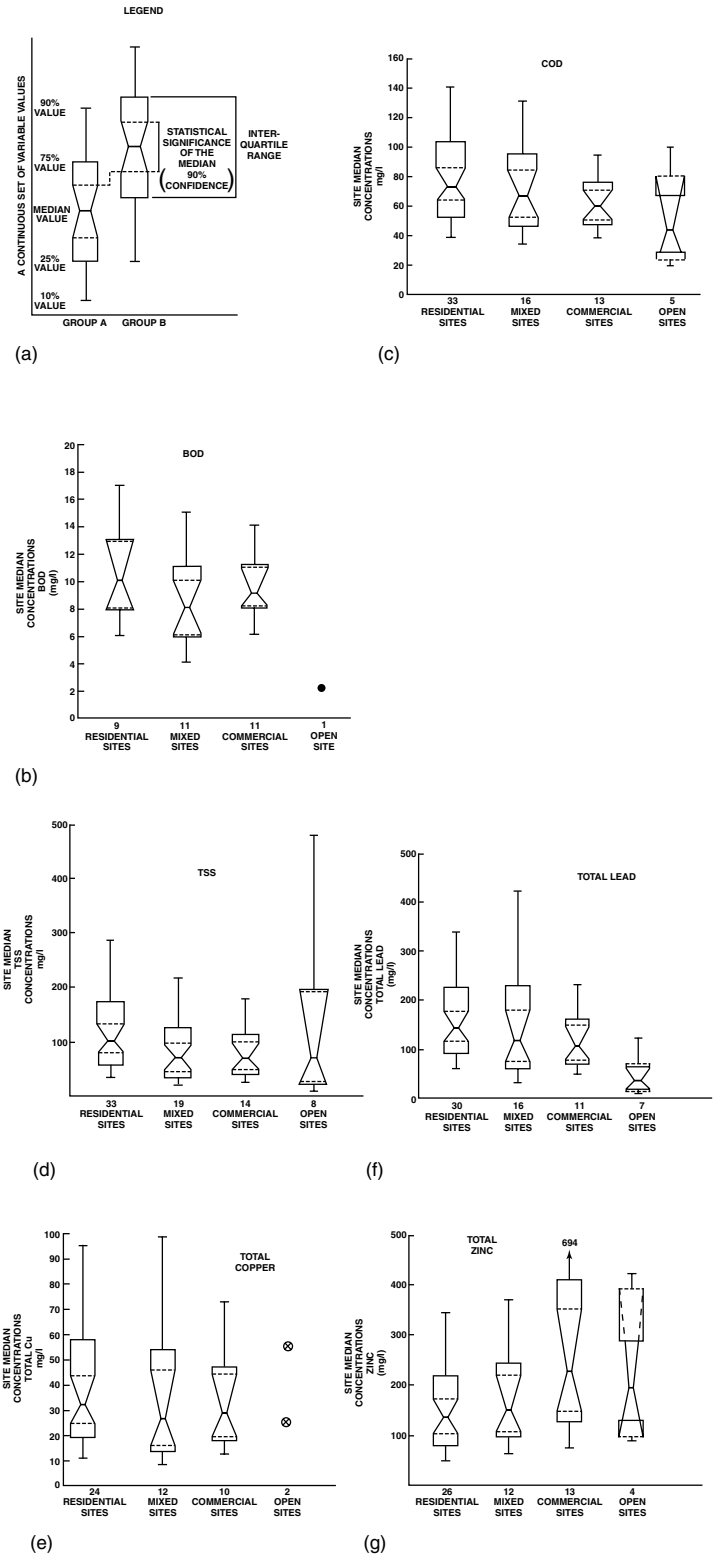

Figure H.2 Box plots of pollutant EMCs for different land uses. (From EPA. *Results of the Nationwide Urban Runoff Program*. Water Planning Division, PB 84-185552, Washington, D.C. December 1983.)

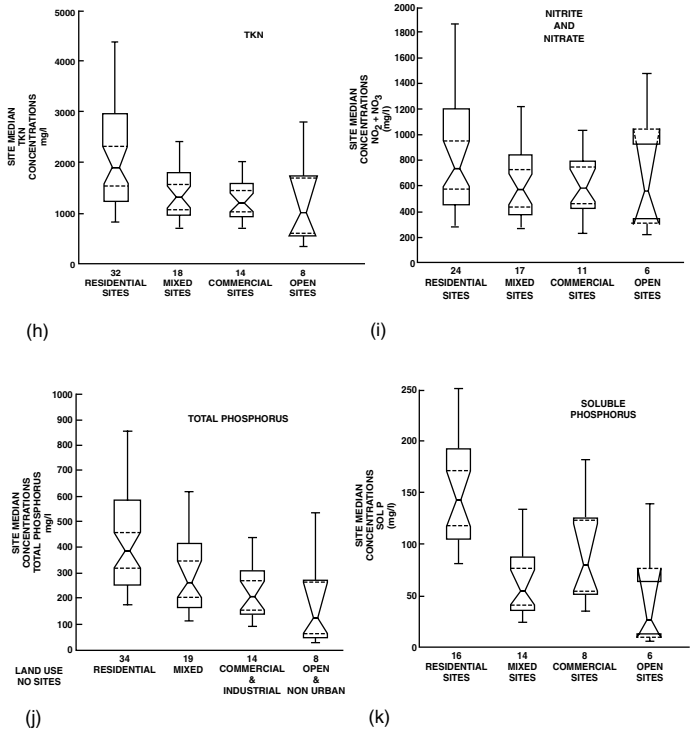

Figure H.2 (continued)

different land uses. As an example, Figure H.2 indicates that suspended solids, BOD, copper, zinc, and nitrite plus nitrate median values are not likely significantly different for any of the four land use categories shown. However, open site COD, phosphorus, and lead median concentrations are likely significantly less than for the other three land uses.

The stormwater permit program typically requires three events to be sampled to determine the EMC value. This small sampling effort likely results in inaccurate EMC estimates because of the relatively large variation in stormwater quality from the same sampling location. As seen in Figure H.3 (a duplicate of Figure 5.3), about 25 samples are required to estimate the EMC with an estimated error of 25% or less, if the COV is 0.5. Most of the time, the COV is even larger, requiring even more samples. The use of only three samples to determine the EMC value would likely result in errors of several hundred percent (using typical levels for confidence of 95% and power of 80%). Such large EMC errors would be reflected in similar errors in the calculated unit area loading values. This could result in incorrect conclusions concerning the relative pollutant sources and inappropriate expenditures of resources for stormwater control.

Errors also occur when selecting the volumetric runoff coefficient (Rv) value. For drainage design, the Rv value is assumed to be equal to the amount of directly connected impervious area. This is sometimes modified to be equal to the "effective" impervious area, as it is obvious that paved areas (and roofs) that drain to pervious areas contribute some runoff, but less than if the paved areas were directly connected to the drainage system. In addition, the Rv is different for different rain depths at the same area. Small rain depths are associated with relatively small Rv values, while larger rains produce larger Rv values, as shown in Figure H.4 (Pitt 1987). Table H.4 (Pitt 1987) illustrates how different urban surfaces contribute increasing fractions of rainfall as runoff. Therefore, if constant Rv values are used for all rains, large errors may occur for individual rains (overpredict for small rains and underpredict for large rains), although the annual average, or annual total, may be acceptable, assuming the monitored rains represent the complete set of annual

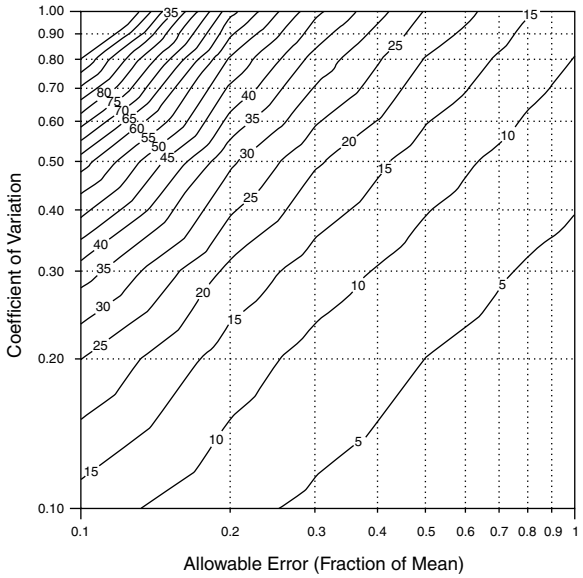

Figure H.3 Sampling requirements for different error goals, alpha of 0.05 and beta of 0.20 (duplicate of Figure 5.3).

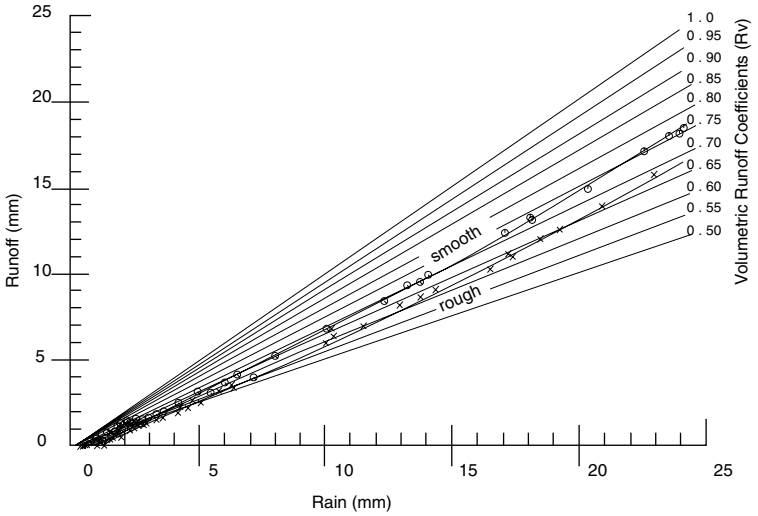

Figure H.4 Rainfall-runoff responses for pavement tests. (From Pitt, R. *Small Storm Urban Flow and Particulate Washoff Contributions to Outfall Discharges*. Ph.D. dissertation submitted to the Department of Civil and Environmental Engineering, University of Wisconsin, Madison. 1987. With permission.)

rains. If only moderate to large rains are monitored (a typical goal), then the averaged Rv for the monitored rains would be larger than the true annual averaged Rv.

Typical estimation methods used for runoff volume were developed for large drainage design storms (several inches in depth) and are not appropriate for the smaller events that are most significant in water quality studies. Table H.4 (Pitt 1987) shows how these runoff coefficients (the fraction of rain that occurs as direct runoff) for impervious areas vary greatly for different rain depths. After several inches of rain (in the range for drainage design studies), the Rv values for all paved and roof areas are between 0.9 and 0.99, resulting in little error if a constant 0.9 value is used. However, at 0.1 to 0.4 in of rain (the rain range where the water pollutants are becoming important), the Rv values for the different paved and roof areas vary greatly (from 0.25 to 0.95).

Table H.4 Observed Directly Connected Runoff Coefficients for Impervious Areas

	0.1 in	0.4 in	1.7 in	Depth When Coefficient Is about 0.9, in
Roads and other small impervious areas	0.4	0.6	0.8	3
Pitched roofs	0.7	0.9	0.98	0.25
Flat roofs	0.25	0.7	0.85	2
Large paved areas and freeways	0.95	0.97	0.99	0.05

From Pitt, R. *Small Storm Urban Flow and Particulate Washoff Contributions to Outfall Discharges*. Ph.D. dissertation submitted to the Department of Civil and Environmental Engineering, University of Wisconsin, Madison. 1987. With permission.

This would result in very large runoff prediction errors if a constant Rv value was assumed for all areas, especially when trying to predict where the runoff water originated.

Most of the annual rainfall is associated with many small individual events and not with the few rarer large rains. Figure H.5a shows measured rain and runoff distributions for Milwaukee during the 1983 NURP monitored rain year. Rains between 0.05 and 5 in were monitored during this period. Two large events (greater than 3 in) occurred during this monitoring period, which greatly bias these curves, compared to typical rain years. During this period:

- The median rain depth was about 0.3 in.
- 66% of all Milwaukee rains were less than 0.5 in in depth.
- For medium-density residential areas, 50% of runoff was associated with rains less than 0.75 in for Milwaukee.
- A 100-year, 24-hour rain of 5.6 in for Milwaukee could produce about 15% of the typical annual runoff volume, but only contributes about 0.15% of the average annual runoff volume when amortized over 100 years.
- Typical 25-year drainage design storms (4.4 in in Milwaukee) produce about 12.5% of the typical annual runoff volume but only about 0.5% of the average runoff volume.

Figure H.5b shows measured Milwaukee pollutant discharges associated with different rain depths for a medium-density residential area. Suspended solids, COD, lead, and phosphates discharges are seen to closely follow the general shape of the runoff distribution shown in Figure H.5a.

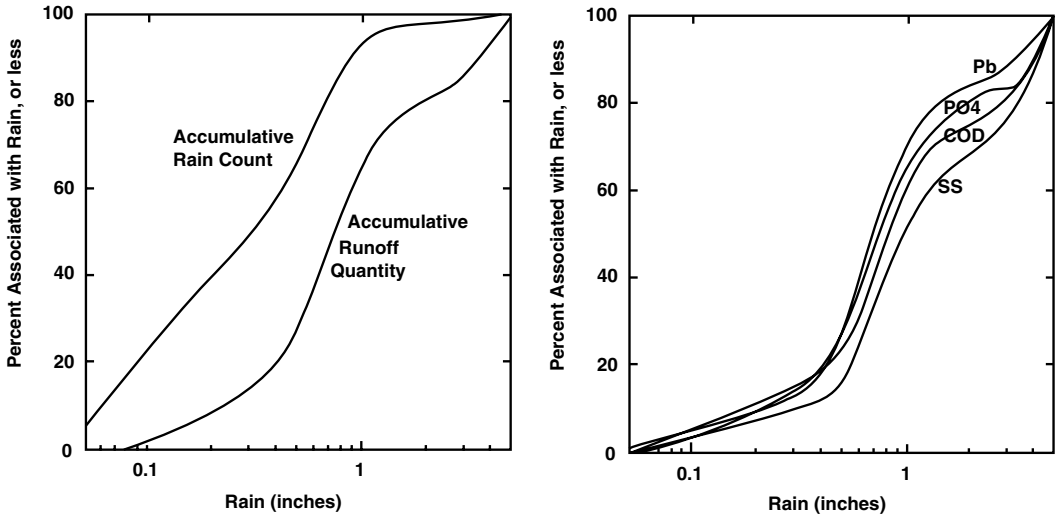

Figure H.5 Accumulative distributions of Milwaukee rain, runoff, and pollutant loadings for medium-density residential areas monitored during 1981 to 1983 (duplicate of Figures 6.1 and 6.2).

Table H.5 Observed Disturbed Urban Soil Volumetric Runoff Coefficients (RV) for Different Rain Depths

	0.1 in	0.4 in	1.7 in	Depth When RV Coefficient Is about 0.1
Clayey soils	0	0.15	0.25	0.2
Sandy soils	0	0	0.05	2.5

From Pitt, R. *Small Storm Urban Flow and Particulate Washoff Contributions to Outfall Discharges.* Ph.D. dissertation submitted to the Department of Civil and Environmental Engineering, University of Wisconsin, Madison. 1987. With permission.

Being able to accurately predict runoff volume is very important in order to reasonable predict runoff pollutant discharges. The shape of the runoff and pollutant runoff curves in Figure H.5 show three distinct regions (values given for Milwaukee):

- Common rains having relatively low pollutant discharges are associated with rains less than about 0.5 in in depth. These are key rains when runoff associated water quality violations, especially for bacteria, are of most concern.
- Rains between 0.5 and 1.5 in are responsible for about 75% of the runoff pollutant discharges and are key rains when addressing mass discharges.
- Rains greater than 1.5 in are associated with drainage design and are only responsible for relatively small portions of the annual pollutant discharges, even with the two unusually large rains that are included in these observations.

Similar relationships are observed for other regions in the country, but the specific rain depths associated with the three specific regions vary. In the southeast, the rain depths separating these three regions are about twice as large as observed for Milwaukee, for example.

Of course, the coefficients shown in Table H.4 can decrease substantially if the paved areas are not directly connected to the drainage system (especially important for roofs and parking areas), or if roadside grass swales are used. It should also be noted that disturbed urban soils contribute much more runoff for moderate rains than the typically expected values (Table H.5).

Complex Models

There are numerous models that fall in the mid-range and detailed model categories that are considered complex. These models all require the use of computers and varying amounts of input data, and they all require calibration and verification for local conditions. These models are constantly changing and new models are continually being developed. The selection of the most appropriate model for a specific situation is therefore important. A good source for model reviews that is periodically updated is the EPA's *Compendium of Watershed-Scale Models for TMDL Development* (EPA 1997). This document was developed for watershed planners and regulators who are responsible for preparing "Total Maximum Daily Load" (TMDL) discharge limitations for receiving waters that are affected by many pollutant sources, including stormwater.

Tables H.6 and H.7 are model summaries from the TMDL report (EPA 1997), while Tables H.8 and H.9 list some of the attributes of many models (including data requirements and overall model complexity). The main distinctions between the mid-range models and the detailed models are that most of the mid-range models are considered "planning" models (for evaluations), while the detailed models are more oriented toward specific design (including greater time-resolution in predicted flows and concentrations). As an example, the mid-range models typically do not require nearly as many details pertaining to specific drainage system layouts as do the detailed models: the mid-range models can operate with more lumped parameters (larger-scale average conditions), while many of the detailed models require detailed drainage system layout information. More of the detailed models can also

Table H.6 Evaluation of Model Capabilities — Mid-Range Models

	Criteria	NPSMAP	GWLF	P8-UCM	SIMPTM	Auto-QI	AGNPS	SLAMM
Land use	Urban	H	H	H	H	H	—	H
	Rural	H	H	—	—	—	H	—
	Point sources	M	M	H	—	—	H	H
Time scale	Annual	—	—	—	—	—	—	—
	Single event	L	—	H	—	—	H	—
	Continuous	H	N	—	—	H	—	H
Hydrology	Runoff	H	H	H	H	H	H	H
	Baseflow	L	H	—	L	L	—	L
Pollutant loading	Sediment	—	H	—	H	H	H	H
	Nutrients	H	H	H	H	H	H	H
	Others	—	—	H	H	H	H	H
Pollutant routing	Transport	L	L	L	L	M	—	M
	Transformation	—	—	—	—	—	H	—
Model output	Statistics	M	L	—	L	—	—	L
	Graphics	M	M	H	—	—	H	L
	Format options	H	H	H	H	L	H	H
Input data	Requirements	M	M	M	M	M	M	M
	Calibration	L	L	L	L	M	L	M
	Default data	H	H	M	M	L	M	M
	User interface	H	H	H	M	M	M	H
BMPS	Evaluation	L	L	H	M	M	M	M
	Design criteria	—	—	H	L	M	M	L
Documentation		H	H	H	L	M	H	M

From EPA. *Compendium of Tools for Watershed Assessment and TMDL Development*. EPA-841-B-97-006. U.S. Environmental Protection Agency. 1997.

include pollutant transformations and nonconservative pollutant behavior, especially for receiving water effects, than the mid-range models. Obviously, there are places where models of each type are needed. In some cases, it is useful to use a mid-range model to predict drainage area runoff conditions and a detailed model to evaluate specific issues pertaining to the drainage system and receiving waters. As an example, the Toronto Area Watershed Management Strategy program (TAWMS) used SLAMM to predict drainage area pollutant and flow discharges, SWMM to predict CSO discharges from the older sections of the city, and HSPF to evaluate receiving water conditions resulting from these discharges (OME 1986). In another example of multiple model use, engineers used SIMPTM in conjunction with SWMM to better predict Portland CSO overflow conditions (Roger Sutherland, personal communication, *Columbia Slough Management Plan*, prepared for the City of Portland's Bureau of Environmental Services). In another example of multiple model use, several cities in Wisconsin have used SLAMM in conjunction with geographical information systems to better prepare the input files required by the program and to display the model results (Thum et al. 1990; Ventura and Kim 1993). The use of a GIS is an especially powerful tool to summarize massive amounts of information, especially when making presentations to the community and to politicians.

Most of the mid-range models were originally developed on personal computers and some have relatively easy-to-use interfaces. The use of "default" values is also common for these models, sometimes restricting the use of locally obtained calibration data. The mid-range models included on Table H.6 are:

- NPSMAP, the Nonpoint Source Model for Analysis and Planning model is a spreadsheet template developed by Omicron Assoc. that predicts nutrient loadings for urban and agricultural areas.
- GWLF, the Generalized Loading Functions model was developed at Cornell University to assess point and nonpoint loadings of nitrogen and phosphorus from relatively large agricultural and urban watersheds. It includes rainfall/runoff processes and erosion predictions. Most of the processes are controlled by default values.

- P8-UCM, the Urban Catchment Model was developed by John Walker for the Narragansett Bay Project to simulate stormwater pollutants in small urban catchments. Evaluations of various management practices are possible with P8, including help in their sizing for specific control objectives. It incorporates many default values from the EPA's Nationwide Urban Runoff Program (EPA 1983).
- SIMPTM, the Simplified Particle Transport Model was developed by Roger Sutherland, of Pacific Water Resources, to simulate runoff, sediment, and yield of other pollutants from urban watersheds, including the evaluation of some control practices. Detailed particulate buildup and washoff processes are included, based on northwest regional data.
- Auto-QI, the Automated Qual-Illudas model was developed by Mike Trestriep at the Illinois State Water Survey to perform continuous simulations of runoff from impervious and pervious urban areas and to evaluate the effectiveness of selected control practices. It also includes components to examine receiving water impacts. A version of the model is linked to the ARC/INFO GIS program.
- AGNPS, the Agricultural Nonpoint Source pollution model was developed by the USDA Agricultural Research Service. It addresses potential impacts from point and nonpoint source pollution on surface and groundwater in agricultural watersheds. Alternative management programs are also evaluated. The spatial (grid) design of the model allows it to be interfaced to GIS and digital terrain models to simplify inputting the model parameters.
- SLAMM the Source Loading and Management Model was developed by Robert Pitt of the University of Alabama at Birmingham to evaluate the effects of urban development characteristics and source and outfall controls on pollutant discharges. It examines runoff from separate drainage areas that may include a wide variety of land uses and control practices. The outfall discharge estimates can then be evaluated in a separate model to evaluate receiving water impacts. Unique small storm hydrology and particulate washoff procedures, based on extensive field measurements, are incorporated in the model to more accurately predict the role of different source areas in generating stormwater pollutant discharges.

Detailed models were all originally developed on mainframe computers, but most have been ported to personal computers over the past several years. Most still have awkward user interfaces and require a group of skilled users to take advantage of most of their comprehensive capabilities, although proprietary Windows-based user interfaces and proprietary modifications of some of the more popular models (especially SWMM) are becoming common. The detailed models shown on Table H.7 include:

- STORM, the Storage, Treatment, Overflow Runoff Model was developed by the U.S. Army Corps of Engineers to continuously simulate urban runoff quantity, sediment, and several conservative pollutants. It has most commonly been used to evaluate the trade-offs between treatment and storage options for the control of CSOs.
- ANSWERS, the Areal Nonpoint Source Watershed Environment Response Simulation Model was developed by the University of Georgia to evaluate the effects of land use, management schemes, and conservation practices on the quantity and quality of watershed runoff. It stresses erosion and sediment transport processes.
- DR3M, the Distributed Routing Rainfall Runoff Model is supported by the U.S. Geological Survey and was developed to study conventional pollutants in predominantly urban areas. It produces detailed hydrographs and pollutant transport plots.
- SWRRBQ, the Simulation for Water Resources in Rural Basins model was developed by the USDA to simulate hydrologic, sedimentation, nutrient, and pesticide movement in large, complex, rural watersheds.
- SWMM the Storm Water Management Model was developed by the EPA to derive design criteria for structural stormwater controls. SWMM is likely the most commonly used detailed stormwater model, especially when evaluating sewerage issues and combined sewer overflows.
- HSPF, the Hydrological Simulation Program–FORTRAN was developed by the U.S. EPA to simulate water quantity and quality for a wide range of organic and inorganic pollutants from agricultural and urban watersheds. It is probably the most comprehensive model available, especially considering receiving water impacts. Chemical, biological, and physical processes are included to account for pollutant transport and transformations. However, much calibration information is required to effectively use all of HSPF's capabilities.

Table H.7 Evaluation of Model Capabilities — Detailed Models

	Criteria	STORM	ANSWERS	DR3M	SWRRBWQ	SWMM	HSPF
Land use	Urban	H	—	H	L	H	H
	Rural	—	H	—	H	L	M
	Point sources	H	—	H	H	H	H
Time scale	Annual	—	—	—	—	—	—
	Single event	L	H	L	L	H	H
	Continuous	H	—	H	H	H	H
Hydrology	Runoff	H	H	H	H	H	H
	Baseflow	L	—	L	H	H	H
Pollutant loading	Sediment	H	H	H	H	H	H
	Nutrients	H	H	H	H	H	H
	Others	H	—	—	H	H	H
Pollutant routing	Transport	—	M	H	H	L	H
	Transformation	—	—	—	—	L	H
Model output	Statistics	L	—	H	H	H	H
	Graphics	—	H	M	M	L	L
	Formal options	H	H	H	H	H	H
Input data	Requirements	M	H	H	M	H	H
	Calibration	L	L	M	M	H	H
	Default data	M	L	H	H	M	M
	User interface	—	—	M	M	—	—
BMPs	Evaluation	M	M	H	M	H	H
	Design criteria	M	M	M	—	H	H
Documentation		H	M	M	H	H	H

From EPA. *Compendium of Tools for Watershed Assessment and TMDL Development*. EPA-841-B-97-006. U.S. Environmental Protection Agency. 1997.

The mid-range models are probably the most commonly used because they are perceived as being easier to use and require less input information. That was certainly true when most of these detailed models were developed, but some of them, most notably SWMM, have a growing industry supporting their use, including the availability of much improved user interfaces. However, the cost of obtaining and using (entering and verifying) detailed information that may be required may not be justified by the intended use of the data generated by the model. The application of detailed models is more cost-effective when applied to address complex situations or objectives.

Dr. Bill James of the University of Guelph has long been an advocate of long-term continuous simulations. The cost of the required computer time has also decreased to the point where the use of long-term continuous simulations is no longer prohibitive, and is strongly recommended in order to obtain a much better understanding of watershed responses under a wide variety of conditions. The modeling of a few "design" storms may be satisfactory for simple drainage design considerations, where the only parameter of interest is peak flow rate. However, limiting simulations to only a few storms falls far short when a wide variety of water quality questions are important. The behavior of different stormwater quality control practices is also dependent on many different hydraulic parameters, not just peak flow rate. The use of several years of rainfall data in continuous simulations should therefore be considered the norm. If a model is not capable of continuous simulations, its usefulness is probably severely restricted to only the most rudimentary preliminary evaluations.

Receiving Water Models

Some of the models listed above include receiving water components (such as HSPF), but there are many additional models available that are specific for receiving waters and are in the public

Table H.8 Listing of Attributes of Commonly Used Urban Models

Attribute	DR3M-QUAL	HSFP	Statistical[a]	STORM	SWMM
Sponsoring agency	USGS	EPA	EPA	HEC	EPA
Simulation type[b]	C,SE	C,SE	N/A	C	C,SE
No pollutants	4	10	Any	6	10
Rainfall/runoff analysis	Y	Y	N[c]	Y	Y
Sewer system flow routing	Y	Y	N/A	N	Y
Full, dynamic flow routing equations	N	N	N/A	N	Y[d]
Surcharge	Y[e]	N	N/A	N	Y[d]
Regulators, overflow structures, e.g., weirs, orifices, etc.	N	N	N/A	Y	Y
Special solids routine	Y	Y	N	N	Y
Storage analysis	Y	Y	Y[f]	Y	Y
Treatment analysis	Y	Y	Y[f]	Y	Y
Suitable for screening (S), design (D)	S,D	S,D	S	S	S,D
Available on microcomputer	N	Y	Y[g]	N	Y
Data and personnel requirements[h]	Medium	High	Medium	Low	High
Overall model complexity[i]	Medium	High	Medium	Medium	High

[a] EPA procedure.
[b] C = continuous simulation, SE = single event simulation.
[c] Runoff coefficient used to obtain runoff volumes.
[d] Full dynamic equations and surcharge calculations only in Extran Block of SWMM.
[e] Surcharge simulated by storing excess inflow at upstream end of pipe. Pressure flow not simulated.
[f] Storage and treatment analyzed analytically.
[g] FHWA study, Driscoll et al. (1989).
[h] General requirements for model installation, familiarization, data requirement, etc. To be interpreted only very generally.
[i] Reflection of general size and overall model capabilities. Note that complex models may still be used to simulate very simple systems with attendant minimal data requirements.

From EPA. *Modeling of Nonpoint Source Water Quality in Urban and Non-urban Areas*. Office of Research and Development. U.S. Environmental Protection Agency. Washington, D.C. EPA 600/3-91/039. 1991.

Table H.9 Listing of Commonly used Non-Urban Runoff Models

Attribute	AGNPS	ANSWERS	CREAMS	HSPF	PRZM	SWRRB	UTM-TOX
Sponsoring agency	USDA	Purdue	USDA	EPA	EPA	USDA	ORNL & EPA
Simulation type	C,SE	SE	C,SE	C,SE	C	C	C, SE
Rainfall/runoff analysis	Y	Y	Y	Y	Y	Y	Y
Erosion modeling	Y	Y	Y	Y	Y	Y	Y
Pesticides	Y	N	Y	Y	Y	Y	N
Nutrients	Y	Y	Y	Y	N	Y	N
User-defined constituents	N	N	N	Y	N	N	Y
Soil processes							
Pesticides	N	N	Y	Y	Y	Y	N
Nutrients	N	N	Y	Y	N	Y	N
Multiple land type capability	Y	Y	N	Y	N	Y	Y
In-stream water quality simulation	N	N	N	Y	N	N	Y
Available on microcomputer	Y	Y	Y	Y	Y	Y	N
Data and personnel requirements	M	M/H	H	H	M	M	H
Overall model complexity	M	M	H	H	M	M/H	H

Y = yes, N = no, M = Moderate, H = High, C = Continuous, SE = Storm Event.

From EPA. *Modeling of Nonpoint Source Water Quality in Urban and Non-urban Areas*. Office of Research and Development. U.S. Environmental Protection Agency. Washington, D.C. EPA 600/3-91/039. 1991.

domain and available through the EPA's CEAM. Some included on the 1996 version of the *Exposure Models Library and Integrated Model Evaluation System* are listed below and in Figure H.6:

Surface Water Models	
Selected for 1st and 2nd Level Reviews:	**Selected for 1st Level Review Only:**
CEQUALRIV1	EXAMS:
CEQUALW2	FATE: fate of organics
CTAP: chemical transport & analysis program	GCSOLAR
DYNTOX: dynamic toxicity model	HEC-5Q & 6
EUTRO4	MICHRIV: transport in water & sediments
GEMS-EXAMS: geographical exposure modeling systems - EXAMs	PCPROUTE-PC: pollutant routing model
HSPF: hydrologic simulation program - fortran	PLUMES:
QUAL2E: enhanced stream water quality model	RESTMP: water temperature model
REACHSCAN	RIVMOD: sediment transport
SERATRA: in-stream sediment-contaminant transport	SEDDEP: settling of wastewater particulates
TOX 14	SMPTOX: stream toxic model
WQRRS: water quality (ecological cycling) in rivers and reservoirs	TERMS: thermal simulation of lakes
WASP5: water quality assessment program	TWQM: downstream transformation of problem constituents

Figure H.6 Surface water models included on the CEAM CD-ROM.

- SWAT (contains the GLEANS pesticide fate model as a component)
- PREWET (predicts fates of pollutants in wetlands)
- GWLF (a simple transport model)
- CREAMS (transport of soluble and sediment-attached chemicals)
- WASP5 (especially the TOXI5 and EUTRO5 components)
- MINTEQA2 (chemical equilibrium model)
- TWQM (in-stream effects of reduced species that may be discharged from dams)
- SMPTOX (toxicant interactions with stream bed sediments)
- WATEQF (chemical equilibrium model)
- VLEACH (chemical fate model)

Geographical Information Systems (GIS)

As indicated above, the use of GIS has become very important when modeling large areas. The main advantages of GIS include an ability to effectively display large amounts of information (relatively easy to incorporate with model output), and in some cases, to organize and automate the data input requirements for the models (requiring a much greater level of integration with a model). It has been especially important when working with nontechnical community groups and when summarizing modeling options. The visual presentation of the massive amounts of output results, or results from monitoring programs, is much more effective for communicating with diverse groups of people.

PUBLIC LAND RECORDS USED IN DIGITAL DATABASE

Data Item	Custodian	Document
Parcel	Dane Co. Land Records & Regulation	Section parcel maps
Soils	U.S.D.A. Soil Conservation Service	Dane County Soil Survey
Slope	U.S.D.A. Soil Conservation Service	Dane County Soil Survey
Slope	U.S. Geological Survey	Quadrangle maps
Wetlands	Wis. Dept. of Natural Resources	Wetlands inventory
Hydrology	U.S. Geological Survey	Quadrangle maps
Farm Tracts & Fields	Dane County, A.S.C.S.	NHAP aerial photo prints
Woodlots	Dane County, A.S.C.S.	NHAP aerial photo prints
Existing Land Uses	Dane County, A.S.C.S.	NHAP aerial photo prints
Planned Land Uses	Dane Co. Regional Planning Comm.	Town of Burke Land Use Maps
Planned Land Uses	Dane Co. Regional Planning Comm.	Hwy 151 Corridor Study
Planned Land Uses	City of Madison, Dept. of Planning	Burke Heights Dev't Plan
Land Use Zoning	Dane Co. Land Records & Regulation	Section zoning maps
Land Use Zoning	City of Sun Prairie, Dept. of Planning	City of Sun Pr. zoning maps
Land Use Zoning	City of Madison, Dept. of Planning	City of Madison zoning maps
Floodplain	Federal Emergency Mgmt. Agency	Flood boundary map
Existing Parks	Dane Co. Land Records & Regulation	Section parcel maps
Planned Parks	Dane County Parks Division	Cherokee Marsh Owner. Map
Existing Sewers	Madison Metro Sewerage District	Sewer. Dist. Interseptor Map
Existing Sewers	City of Sun Prairie, Dept. of Engineer.	City interseptor map
Planned Sewers	Madison Metro Sewerage District	Collection System Design Rep.
Urban Service Areas	Dane Co. Land Records & Regulation	Town of Burke Land Use Plan
Traffic Counts	Wis. Department of Transportation	1987 Highway Traffic maps
Roads/Hwys	U.S. Geological Survey	Quadrangle maps
Farm Tenure	Dane County, A.S.C.S.	Farm operator file
Farm Tenure	Dane Co. Land Records & Regulation	Section Parcel maps
Historic Buildings	Wisconsin Historic Society	Coded quadrangle maps
Archeologic Sites	Wisconsin Historic Society	Coded quadrangle maps
Watershed Boundary	Dane Co. Regional Planning Comm.	Watershed boundary map
Watershed Boundary	U.S. Geological Survey	Quadrangle maps

Figure H.7 0 Availability of data used in early GIS and stormwater modeling studies conducted in Dane County, WI. (From Pickett, S.R., O.G. Thum, and B.J. Hiemann. Using a land information system to integrate nonpoint source pollution modeling and land use development planning. *Land Information and Computer Graphics Facility,* The University of Wisconsin, Madison. 1989.)

GIS has been used for many years, but has recently become much more accessible with improvements in software and significant cost reductions in suitable computer equipment. Various communities in the State of Wisconsin, for example, have used GIS systems integrated with the Source Loading and Management Model (SLAMM) to graphically illustrate development and control options associated with urbanization (Haubner and Joeres 1996; Kim et al. 1993; Kim and Ventura 1993; Ventura and Kim 1993). Figure H.7 (Pickett et al. 1989) shows the availability of data used in some of the early studies conducted in Dane County, WI, while Figure H.8 (Kim and Ventura 1993) shows how the information is integrated with SLAMM to identify critical source areas. Figure H.9 (Pickett et al. 1989) is an example map showing expected changes in suspended solids discharges resulting from development options. SLAMM is currently available from www.winslamm.com.

As noted above, the current development and use of the BASINS model, especially for TMDL evaluations, relies heavily of a GIS framework (Lahlou et al. 1998). Tables H.10 through H.13, from the BASINS *User's Manual* (Lahlou et al. 1998) describe the information contained on the CD-ROMS specific for each EPA region. This wealth of information is available to initial analyses for a specific area, but users are encouraged to incorporate high-resolution information and locally derived data sets for more accurate use. The cartographic data (Table H.10) includes hydrologic boundaries and major roadways, plus census areas and various political boundaries. The environmental data (Table H.11) are to support watershed characterization and environ-

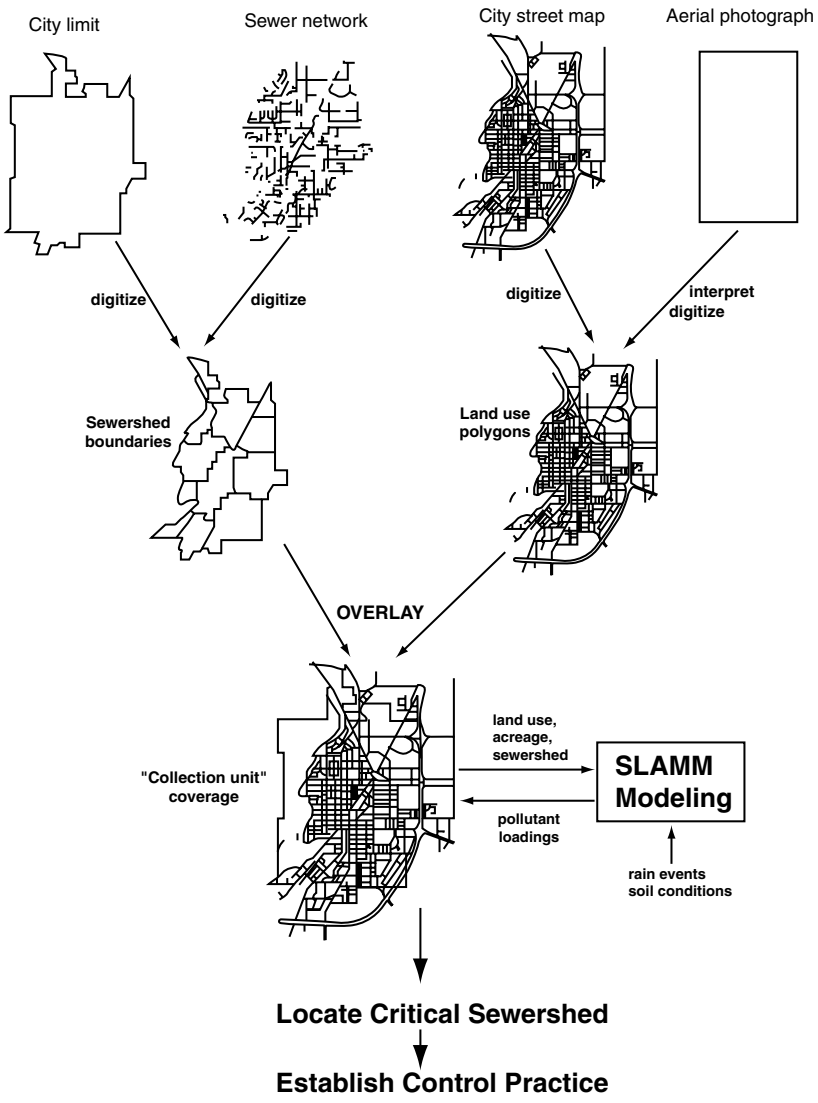

Figure H.80 Integration of information and modeling to identify critical source areas. (From Kim, K. and S. Ventura. Large-scale modeling of urban nonpoint source pollution using a geographical information system. *Photogrammetric Eng. Remote Sensing*, 59(10): 1539–1544. October 1993.)

mental analyses, and include data on soils, topography, land uses, and stream hydrography. This is the most important set of information for modeling local conditions. The environmental monitoring data (Table H.12) include statistical summaries of monitoring results, rainfall records, and limited biological conditions. The point source data (Table H.13) provide information on pollutant loadings from permitted facilities, plus locations of hazardous waste sites. The BASINS assessment tools allow users to make evaluations of water quality, while the available data management utilities delineate watershed boundaries, and can be used to modify the data, or to import new data into the system. The nonpoint source and stream models integrate these data to provide initial evaluations of watershed water quality conditions. BASINS can therefore be a very useful tool to focus specific monitoring efforts to investigate likely water quality problems and use impairments.

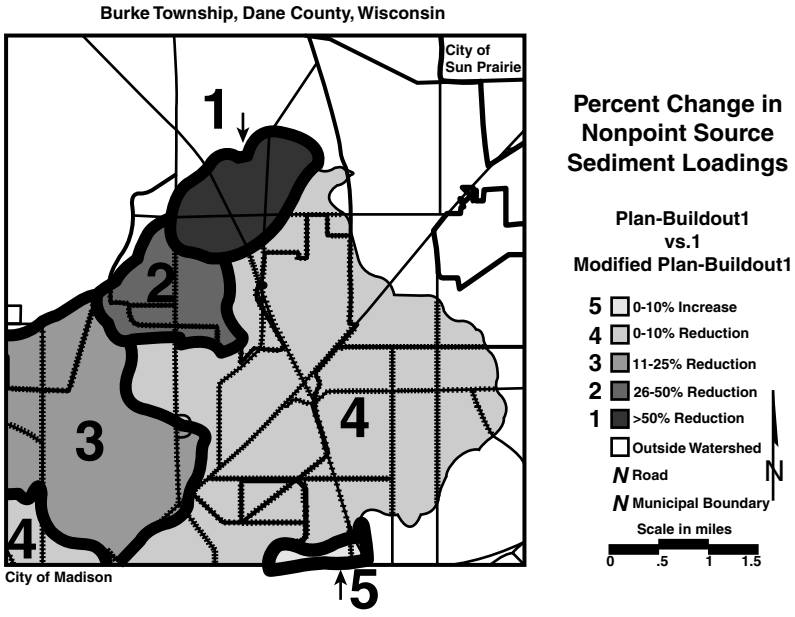

Figure H.90 Example of mapped results showing changes in suspended solids with different development options. (From Pickett, S.R., O.G. Thum, and B.J. Hiemann. Using a land information system to integrate nonpoint source pollution modeling and land use development planning. *Land Information and Computer Graphics Facility,* The University of Wisconsin, Madison. 1989.)

Table H.10 BASINS Base Cartographic Data

BASINS Data Product	Source	Description
Hydrologic unit boundaries	U.S. Geological Survey	Nationally consistent delineations of the hydrographic boundaries associated with major U.S. river basins
Major roads	Federal Highway Administration	Interstate and state highway network
Populated place locations	USGS	Location and names of populated locations
Urbanized areas	Bureau of the Census	Delineations of major urbanized areas used in 1990 Census
State and county boundaries	USGS	Administrative boundaries
EPA regions	USGS	Administrative boundaries

From Lahlou, M., L. Shoemaker, S. Choudhury, R. Elmer, A. Hu, H. Manguerra, and A. Parker. *BASINS, Better Assessment Science Integrating Point and Nonpoint Sources*. Version 2.0. EPA-823-B-98-006. Exposure Assessment Branch, U.S. Environmental Protection Agency. Washington, D.C. November 1998.

SUMMARY

The amount of data required to use these models can be very large. Tables H.14 and H.15 list some of these data needs for watershed-scale models (EPA 1991). Much of the information can be obtained from locally available sources and data summaries, but much will have to be extracted from detailed maps or the basic data to obtain the information in the necessary formats, accuracies, or time scales. In addition, the models need to be calibrated for site-specific conditions (especially pollutant characteristics and rainfall runoff relationships) and verified (comparing monitored outfall quality and quantity with modeled values). Receiving water models also require much local information for efficient use. Besides the watershed-scale information listed in these tables, specific stream processes (such as described in the *Rates,*

Table H.11 BASINS Environmental Background Data

BASINS Data Product	Source	Description
Ecoregions	U.S. Environmental Protection Agency (USEPA)	Ecoregions and associated delineations
National Water Quality Assessment (NAWQA) study unit boundaries	USGS	Delineations of study areas
1996 Clean Water needs survey	USEPA	Results of the wastewater control needs assessment by state
State soil and geographic (STATSGO) database	U.S. Department of Agriculture, Natural Resources Conservation Service (USDA-NRCS)	Soils information including soil component data and soils
Managed area database	University of California, Santa Barbara	Data layer including federal and Indian lands
Reach file version 1 (RF1)	USEPA	Provides stream network for major rivers and supports development of stream routing for modeling purposes (1:500k)
Reach file version 3 (RF3) alpha	USEPA	Alpha version of Reach File 3; provides detailed stream network and supports development of stream routing for modeling purposes (1:100K)
Digital elevation model (DEM)	USGS	Topographic relief mapping; supports watershed delineations and modeling
Land use and land cover	USGS	Boundaries associated with land use classifications including Anderson Level 1 and Level 2

From Lahlou, M. et al., *BASINS, Better Assessment Science Integrating Point and Nonpoint Sources*. Version 2.0. EPA-823-B-98-006. Exposure Assessment Branch, U.S. Environmental Protection Agency. Washington, D.C. November 1998.

Table H.12 BASINS Environmental Monitoring Data

BASINS Data Product	Source	Description
Water quality monitoring stations and data summaries	USEPA	Statistical summaries of water quality monitoring for physical and chemical-related parameters; parameter-specific statistics computed by station for 5-year intervals from 1970 to 1994 and 3-year interval from 1995 to 1997
Bacteria monitoring stations and data summaries	USEPA	Statistical summaries of bacteria monitoring; parameter-specific statistics computed by station for 5-year intervals form 1970 to 1994 and 3-year interval from 1995 to 1997
Water quality stations and observation data	USEPA	Observation-level water quality monitoring data for selected locations and parameters
National sediment inventory (NSI) stations and database	USEPA	Sediment chemistry, tissue residue, and benthic abundance monitoring data for fishing, including type of impairment
Listing of fish and wildlife advisories	USEPA	State reporting of locations with advisories for fishing, including 7Q10 low and monthly mean stream flow
Gauge sites	USGS	Inventory of surface water gaging station data including 7Q10 low and monthly mean stream
Weather station sites	National Oceanic and Atmospheric Administration (NOAA)	Location of selected first-order NOAA weather stations
Drinking water supply (DWS) sites	USEPA	Location of public water supplies, their intakes, and sources of surface water supply
Watershed data stations and database	NOAA	Location of selected meteorologic stations and associated monitoring information used to support modeling
Classified shellfish areas	NOAA	Location and extent of shellfish closure areas

From Lahlou, M. et al., *BASINS, Better Assessment Science Integrating Point and Nonpoint Sources*. Version 2.0. EPA-823-B-98-006. Exposure Assessment Branch, U.S. Environmental Protection Agency. Washington, D.C. November 1998.

Table H.13 BASINS Point Source/Loading Data

BASINS Data Product	Source	Description
Permit compliance system (PCS) sites and computed annual loadings	USEPA	NPDES permit-holding facility information; contains parameter-specific loadings to surface waters computed using the EPA Effluent Decision Support System (EDSS) for 1991–1996
Industrial facilities discharge (IFD)	USPEA	Facility information on industrial point source discharges to surface waters
Toxic release inventory (TRI) sites and pollutant releases data	USEPA	Facility information for 1987–1995 TRI public data; contains Y/N flags for each facility indicating media-specific reported releases
Superfund national priority list site	USEPA	Location of Superfund National Priority List sites from CERCLIS (Comprehensive Environmental Response, Compensation and Liability Information System)
Resource conservation and recovery information system (RCRIS) sites	USEPA	Location of transfer, storage, and disposal facilities for solid and hazardous waste
Minerals availability systems/mineral industry location system (MAS/MILS)	U.S. Bureau of Mines	Location and characteristics of mining sites

From Lahlou, M. et al., *BASINS, Better Assessment Science Integrating Point and Nonpoint Sources*. Version 2.0. EPA-823-B-98-006. Exposure Assessment Branch, U.S. Environmental Protection Agency. Washington, D.C. November 1998.

Table H.14 Typical Input Data Needs for Nonpoint Source Models

1. System parameters
   a. Watershed size
   b. Subdivision of the watershed into homogeneous subareas
   c. Imperviousness of each subarea
   d. Slopes
   e. Fraction of impervious areas directly connected to a channel
   f. Maximum surface storage (depression plus interception storage)
   g. Soil characteristics including texture, permeability, erodibility, and composition
   h. Crop and vegetation cover
   i. Curb density or street gutter length
   j. Sewer system or natural drainage characteristics
   k. Land use
2. State variables
   a. Ambient temperature
   b. Reaction rate coefficients
   c. Adsorption/desorption coefficients
   d. Growth stage of crops
   e. Daily accumulation rates of litter
   f. Traffic density and speed
   g. Potency factors for pollutants (pollutant strength on sediment)
   h. Solar radiation (for some models)
3. Input variables
   a. Precipitation
   b. Atmospheric fallout
   c. Evaporation rates

Adapted from EPA. *Modeling of Nonpoint Source Water Quality in Urban and Non-urban Areas*. Office of Research and Development. U.S. Environmental Protection Agency. Washington, D.C. EPA 600/3-91/039. 1991.

Table H.15 Data Needs for Various Quality Prediction Methods

Method	Data	Potential Source[a]
Unit load	Mass per time per unit tributary area	Derive from constant concentration and runoff, literature values
Constant concentration	Runoff prediction mechanism (simple to complex)	Existing model; runoff coefficient or simple method
	Constant concentration for each constituent	NURP; local monitoring
Spreadsheet	Simple runoff prediction mechanism	e.g., runoff coefficient, perhaps as function of land use
	Constant concentration or concentration range	NURP; local monitoring
	Removal fractions for controls	NURP; Schueler (1987); local and state publications
Statistical	Rainfall statistics	NURP; Driscoll, et.al. (1989); Woodward Clyde (1989); EPA SYNOP model
	Area, imperviousness. Pollutant median and CV	NURP; Driscoll (1986); Driscoll, et al. (1989); local monitoring
	Receiving water characteristics and statistics	Local or generalized data
Regression	Storm rainfall, area, imperviousness, land use	Local data
Rating curve	Measured flow rates/volumes and quality EMCs/loads	NURP; local data
Buildup	Loading rates and rate constants	Literature values
Washoff	Power relationship with runoff	Literature values

[a] Must be calibrated and verified using local monitoring.

From EPA. *Modeling of Nonpoint Source Water Quality in Urban and Non-urban Areas*. Office of Research and Development. U.S. Environmental Protection Agency. Washington, D.C. EPA 600/3-91/039. 1991.

Constants, and Kinetics Formulations in Surface Water Quality Modeling report prepared by Tetra Tech 1985) require calibration and verification. Tables H.14 through H.17 (EPA 1991) list some of the water quality variables modeled and the processes simulated by representative receiving water models. The techniques presented in this book, supplemented by the noted references, will enable the user to effectively collect the needed local data for model calibration and verification. Few models attempt to address in-stream biological process (beyond photosynthesis/respiration for DO evaluations and bacteria die-off). Biological beneficial uses are best compared to actual measurements and comparisons with reference streams. However, models are needed to predict likely future chemical and physical conditions that currently do not exist. The information in this book should enable reasonable evaluations of these predicted conditions for biological use impairments, at least by identifying potential areas of concern. The ability to model biological responses to chemical and physical changes (such as responses to habitat destruction and contaminated sediments that are likely the most serious issues in urban streams) is very uncertain. However, numerous site-specific investigations, especially in the Pacific Northwest and in Canada, are encouraging.

It is therefore important to consider the appropriate uses of models, especially in receiving water investigations. Models are important and critical tools in that they enable us to design experiments and monitoring activities effectively, and to look into the future and examine alternatives. However, there can be substantial error in their predictions, due to incorrectly described processes, lack of data and the natural variability of conditions that simply cannot be adequately explained. This error, coupled with our lack of understanding of cause and effect relationships between the more easily predicted physical/chemical parameters and biological conditions, warrants continued caution. With local experience associated with a commitment to long-term investigations in local waters, our understanding will improve along with our ability to make reasonable conclusions using modeling results.

Table H.16 Non-Toxic Constituents Included in Stream Models

Model Name	DO	CBOD or total BOD	NBOD	SOD	Temp.	Total P	Organic P	PO_4	Total N	Organic N
WQAM	X	X	X	X	X	X		X	X	
DOSAG1	X	X	X	X	X**					
DOSAG3	X	X	X*	X	X**			X		
SNSIM	X	X	X	X	X**					
QUAL-II	X	X	X*	X	X			X		
QUAL-IIe	X	X	X*	X	X			X		
RECEIV-II	X	X	X*	X	X**	X		X	X	X
WASP	X	X	X*	X	X**		X	X		X
AESPO	X	X	X*	X	X**		X	X		X
HSPF	X	X	X*	X	X			X		
HAR03	X	X			X**					
FEDBAK03	X	X			X**					
MIT-DNM	X	X			X**					
EXPLORE-1	X	X	X*	X	X**		X	X		X
WQRRS	X	X	X*	X	X			X		

Model Name	NH_3	NO_2	NO_3	Carbon	Algae or Chl-A	Zooplankton	pH	Alkalinity	TDS	Coliform Bacteria
WQAM									X	X
DOSAG1										
DOSAG3	X	X	X		X				X	X
SNSIM										
QUAL-II	X	X	X		X				X	X
QUAL-IIe	X	X	X		X				X	X
RECEIV-II	X	X	X		X				X	X
WASP	X		X	X	X	X	X	X	X	
AESPO	X	X	X	X	X	X	X	X	X	
HSPF	X	X	X	X	X		X	X	X	X
HAR03										
FEDBAK03										
MIT-DNM										
EXPLORE-1	X	X	X	X	X	X				
WQRRS	X	X	X	X	X	X	X	X	X	X

X* NBOD simulated as nitrification of ammonia.

X** Temperature specified by model users.

From EPA. *Modeling of Nonpoint Source Water Quality in Urban and Non-urban Areas*. Office of Research and Development. U.S. Environmental Protection Agency. Washington, D.C. EPA 600/3-91/039. 1991.

Table H.17 Conventional Pollutants Model Comparison as Used in Waste Load Allocations

Model	Water Quality Temporal Variability	Hydraulic Temporal Variability	Variable Loading Rates	Types of Loads	Spatial Dimensions	Water Body	Water Quality Parameters Modeled	Chemical/Biological Processes Simulated	Physical Processes Simulated
DOSAG-1	Steady-state	Steady-state	No	Multiple point sources	1-D	Stream network	DO, CBOD, NBOD, conservative	1st-order decay of NOBD,CBOD, coupled DO	Dilution, advection, reaeration
SNSIM	Steady-state	Steady-state	No	Multiple point sources and nonpoint sources	1-D	Stream network	DO,CBOD, NBOD, CONSERVATIVE	1ST-ORDER DECAY OF NBOD, CBOD, coupled DO, benthic demand(s), photosynthesis(s)	Dilution, advection, reaeration
QUAL-II	Steady-state or dynamic	Steady-state	No	Multiple point sources and nonpoint sources	1-D	Stream network	DO, CBOD temperature, ammonia, nitrate, nitrite, algae, phosphate, coliforms, nonconservative substances, three conservative substances	1st-order decay of NBOD, CBOD, coupled DO, benthic demand, CBOD setting, nutrient-algal cycle	Dilution, advection, reaeration, heat balance
RECEIV-II	Dynamic	Dynamic	Yes	Multiple point sources	1-D or 2-D	Stream network or well-mixed estuary	DO, CBOD, ammonia, nitrate, nitrite, total nitrogen, phosphate, coliforms, algae, salinity, one metal ion	1st-order decay of CBOD, coupled DO, benthic demand, CBOD settlings, nutrient-algal cycle	Dilution, advection, reaeration

(s) = specified.

From EPA. *Exposure Models Library and Integrated Model Evaluation System*. EPA Office of Research and Development CD-ROM. EPA-600-C-92-002. Revised March 1996.

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APPENDIX I

Glossary

Acclimation. (1) Steady-state compensatory adjustments by an organism to the alteration of environmental conditions. Adjustments can be behavioral, physiological, or biochemical. (2) Referring to the time period prior to the initiation of a toxicity test in which organisms are maintained in untreated, toxicant-free dilution water or soil with physical and chemical characteristics, e.g., temperature, pH, hardness, similar to those to be used during the toxicity test.

Acute. Involving a stimulus severe enough to rapidly induce a response; in toxicity tests, a response observed in 96 hours or less typically is considered an acute one. An acute effect is not always measured in the terms of lethality; it can measure a variety of effects. Note that acute means short, not mortality.

Acute-Chronic Ratio (ACR). The ratio of the acute toxicity (expressed as an LC50) of an effluent or a toxicant to its chronic toxicity (expressed as an NOEL). Used as a factor for estimating chronic toxicity on the basis of acute toxicity data.

Additivity. The characteristic property of a mixture of toxicants that exhibits a cumulative toxic effect equal to the arithmetic sum of the effects of the individual toxicants.

Anoxic. Without oxygen.

Antagonism. The property of a mixture of toxicants that exhibits a less-than-additive cumulative toxic effect.

Aquatic Community. An association of interacting populations of aquatic organisms in a given water body or habitat.

Bioaccumulation. Uptake and retention of environmental substances by an organism from all sources.

Bioavailability. The property of a toxicant that governs its effect on exposed organisms. A reduced bioavailability would have a reduced toxic effect.

Bioconcentration. Uptake and retention of environmental substances by an organism from water. A bioconcentration factor (BCF) can be calculated as the quotient of the concentration of chemical in the tissue (or whole) of an aquatic organism divided by the concentration in the water in which the organism resides.

Biological Assessment. An evaluation of the biological condition of a water body using biological surveys and other direct measurements of resident biota in surface waters.

Biological Criteria (biocriteria). Numerical values of narrative expressions that describe the reference biological integrity of aquatic communities inhabiting waters of a given designated aquatic life use.

Biological Integrity. The condition of the aquatic community inhabiting unimpaired water bodies of a specified habitat as measured by community structure and function.

Biological Monitoring. The use of a biological entity as a detector and its response as a measure to determine environmental conditions. Toxicity tests and biological surveys are common biomonitoring methods.

Biological Survey (biosurvey). Consists of collecting, processing, and analyzing representative portions of a resident aquatic community structure and function.

Chronic. Involving a stimulus that lingers or continues for a relatively long period of time, often 1/10 the life span or more. Chronic should be considered a relative term depending on the life span of an organism. A chronic effect can be lethality, growth, reduced reproduction, etc. Chronic means long term.

Community Component. Any portion of a biological community. The community component may pertain to the taxonomic group (fish, invertebrates, algae), the taxonomic category (phylum, order, family, genus, species), the feeding strategy (herbivore, omnivore, carnivore), or organizational level (individual, population, community association) of a biological entity within the aquatic community.

Conservative Pollutant. A pollutant that is persistent and not subject to decay or transformation.

Control. A treatment in a toxicity test that duplicates all the conditions of the exposure treatments but contains no test material. The control is used to determine the absence of toxicity of basic test conditions, e.g., health of test organisms, quality of dilution water.

Criteria (water quality). An estimate of the concentration of a chemical or other constituent in water which if not exceeded, will protect an organism, an organismal community, or a prescribed water use or quality with an adequate degree of safety.

Criteria Continuous Concentration (CCC). The U.S. EPA national water quality criteria recommendation for the highest in-stream concentration of a toxicant or an effluent to which organisms can be exposed indefinitely without causing unacceptable effect.

Criteria Maximum Concentration (CMC). The U.S. EPA national water quality criteria recommendation for the highest in-stream concentration of a toxicant or an effluent to which organisms can be exposed for a brief period of time without causing mortality.

Critical Life Stage. The period of time in an organism's life span when it is the most susceptible to adverse effect caused by exposure to toxicants, usually during early development (egg, embryo, larvae). Chronic toxicity tests are often run on critical life stages to replace long-duration, life cycle tests since the toxic effect occurs during the critical life stage.

Designated Uses. Those uses specified in water quality standards for each water body or segment whether or not they are being attained.

Dilution of Water. Water used to dilute the test material in an aquatic toxicity test in order to prepare either different concentrations of a test chemical or different percentages of an aqueous sample for the various test treatments. The water (negative) control in a test is prepared with dilution water only.

Disturbance. An event that causes a significant change from the "normal pattern" in an ecological system.

Diversity. The number and abundance of species in a specified location.

Ecological Assessment. An evaluation of the condition of a water body using water quality and physical habitat assessment methods.

Ecotone. A zone of transition between adjacent ecological systems having a set of characteristics uniquely defined by space and time scales and by the strength of interaction between adjacent ecological systems.

Effluent. A complex waste material, e.g., liquid industrial discharge or sewage, which is discharged into the environment.

Elutriate (extract). A sample of water obtained by mixing a solid sample with a specified weight ratio of solvent, usually water, for a specified time and then separating from the solid phase by setting, centrifugation, and/or filtration.

Impact. A change in the chemical, physical, or biological quality or condition of a water body caused by external sources.

Impairment. A detrimental effect on the biological integrity of a water body caused by an impact that prevents attainment of the designated use.

Macroinvertebrates. Large invertebrate organisms, sometimes arbitrarily defined as those retained by sieves with 0.425- to 1.0-mm mesh screens.

Median Lethal Concentration (LC50). The concentration of material to which test organisms are exposed that is estimated to be lethal to 50% of the test organisms. The LC50 is usually expressed as a time-dependent value, e.g., 24-hour or 96-hour LC50; the concentration estimated to be lethal to 50% of the test organisms after 24 or 96 hours of exposure. the LC50 may be derived by observation (50% of the test organisms may be observed to be dead in one test material concentration), by interpolation (mortality of more than 50% of the test organisms occurred at one test concentration and mortality of fewer than 50% of the test organisms died at a lower test concentration; the LC50 is estimated by interpolation between these two data points), or by calculation (the LC50 is statistically derived by analysis of mortality data from all test concentrations).

No Observed Effect Level (NOEL). The highest measured continuous concentration of an effluent or a toxicant which causes no observed effect on a test organism.

Patches (adjacent to ecotones in fluvial systems). Spatial units (e.g., biological communities and ecosystems) determined by patch characteristics and their interactions over various scales. Topography, substrate conditions, organisms, and disturbance influence patch composition, size, location, and shape.

Persistence. That property of a toxicant or an effluent which is a measurement of the duration of its effect. A persistent toxicant or toxicity maintains effects after mixing, degrading slowly. A nonpersistent toxicant or toxicity may have a quickly reduced effect after mixing, as degradation processes such as volatilization, photolysis, etc. transform the chemical.

Population. An aggregate of interbreeding individuals of a biological species within a specified location.

Quality Assurance (QA). A program organized and designed to provide accurate and precise results. Included are selection of proper technical methods, tests, or laboratory procedures; sample collection and preservation; selection of limits; evaluation of data; quality control; and qualifications and training of personnel.

Quality Control (QC). specific actions required to provide information for the quality assurance program. Included are standardizations, calibrations, replicates, and control and check samples suitable for statistical estimates of the confidence of the data.

Reference Controls. Tests using natural water or sediment samples collected from unimpacted areas of the site environs.

Regions of Ecological Similarity. Describe a relatively homogeneous area by similarity of climate, landform, soil, potential natural vegetation, hydrology, or other ecologically relevant variable. Regions of ecological similarity help define the potential for designated use classifications of specific water bodies.

7Q10. The discharge at the 10-year recurrence interval taken from a frequency curve of annual values of the lowest mean discharge for 7 consecutive days.

Static. Describing toxicity tests in which test materials are not renewed.

Sublethal. Involving a stimulus below the level that causes death.

Synergism. The characteristic property of a mixture of toxicants which exhibits a greater than additive cumulative toxic effect.

Total Maximum Daily Load (TMDL). The total allowable pollutant load to a receiving water such that any additional loading will produce a violation of water quality standards.

Toxic Acute Chronic (TC_c). The reciprocal of the effluent dilution that causes no unacceptable effect on the test organisms by the end of the acute exposure period.

Toxic Endpoints. Measurements of an acute or chronic toxicity for toxic substances, including exposure duration, concentration, and observed effects.

Toxic Unit Acute (TU_a). The reciprocal of the effluent dilution that causes 50% of the test organisms to die by the end of the acute exposure period.

Toxicant. An agent or material capable of producing an adverse response (effect) in a biological system, adversely impacting structure or function or producing death.

Toxicity. The inherent potential or capacity of a material to cause adverse effects in a living organism.

Uncertainty Factors. Factors used in the adjustment of toxicity data to account for unknown variations. Where toxicity is measured on only one test species, other species may exhibit more sensitivity to that effluent. An uncertainty factor would adjust measured toxicity upward and downward to cover the sensitivity range of other, potentially more or less sensitive species.

Water Quality Assessment. An evaluation of the condition of a water body using biological surveys, chemical-specific analyses of pollutants in water bodies, and toxicity tests.

APPENDIX J

Vendors of Supplies and Equipment Used in Receiving–Water Monitoring

CONTENTS

GENERAL FIELD AND LABORATORY EQUIPMENT

The following vendors and manufacturers supply a large variety of equipment and supplies typically needed for field environmental investigations:

- Cole-Parmer, 625 East Bunker Court, Vernon Hills, IL 60061-1844, USA. Phone: 800-323-4340, Fax: 847-247-2929. Internet: coleparmer.com

Cole-Parmer is also a comprehensive laboratory supply distributor and carries many field and laboratory items including injection pumps and pump samplers, dredge samplers, and field test kits.

- Cabela's, One Cabela Drive, Sidney, NE 69160. Phone: 800-237-4444, Fax: 800-496-6329. Internet: www.cabelas.com

Cabela's is a comprehensive hunting, fishing, and outdoor gear supplier. It carries low-cost GPS units, other navigation aids, waders, and other general outdoor equipment that is necessary when carrying out a receiving water investigation.

- Fisher Scientific, PO Box 4829, Norcross, GA 30091. Phone: 800-766-7000.

Fisher is a complete scientific equipment and supply distributor and handles a wide variety of laboratory equipment. It also has sample bottles and selected field test kits.

- Forestry Suppliers, 205 W. Rankin Street, Jackson, MS 39201. Phone: 800-647-5368.

Forestry Suppliers carries a selection of field supplies and equipment, including GPS receivers and differential correction units, manual water samplers, pump samplers, depth-integrated samplers, dredge samplers, core samplers, and field test kits.

- Halltech Environmental, Inc., #4-503 Imperial Road N., Guelph, Ontario, CANADA N1H 6T9. Phone: 519-766-4568, Fax: 519-766-0729. E-mail: sales@htex.com; Internet: www.htex.com.

Halltech sells many unique sampling supplies, including depth-integrated samplers and bedload samplers, GIS receivers and satellite telephones, limnology sampling equipment, many types of manual water samplers, cartography and survey equipment, and soil sampling equipment.

- Markson Scientific, Phone: 800-858-2243.

Markson carries a good variety of field equipment, especially its sample splitter and dipper sampler.

- Spectrum Technologies, Inc., 23839 W. Andrew Rd., Plainfield, IL 60544. Phone: 800-248-8873, Fax: 815-436-4460. E-mail: specmeters@aol.com.

Spectrum carries mostly agricultural sampling tools and soil analysis equipment. it carries the excellent line of Horiba dry sensors and the Sentron pH meter, along with inexpensive recording rain gauges, complete recording weather stations, continuous water temperature recorders, and soil moisture and compaction meters, for example.

- Yellow Springs Instruments (YSI), 1700/1725 Brannum Lane, Yellow Springs, OH 45387. Phone: 800-765-4974 or 937-767-7241; Fax: 937-767-1058, E-mail: info@ysi.com; Internet: ysi.com/ysi/envweb.nsf.

YSI is a long-time supplier of rugged field meters, especially for DO and conductivity. Its line of water quality sondes is also very comprehensive and the sondes are capable of long-term deployment and continuous data logging.

- Ben Meadows Company, 3589 Broad St., Atlanta, GA 30341. Phone: 800-241-6401. E-mail: mail@benmeadows.com; Internet: Web: benmeadows.com.

Ben Meadows is a supplier of field research equipment including such items as portable instrumentation and waders.

AUTOMATIC SAMPLERS

The following are selected distributors of automatic water sampling equipment:

- American Sigma (800-635-4567) automatic water samplers
- ISCO (800-228-4373) automatic water samplers
- Campbell Scientific of Logan, UT (801-753-2342) telemetry
- Hazco (800-332-0435) also sells (and rents) pump samplers and many other items
- Vortox Company (909-621-3843) source area samplers

BASIC FIELD TEST KITS

The following are vendors of field test kits and numerous other field equipment and laboratory supplies:

- CHEMetrics, Inc., Route 28, Calverton, VA 20138. Phone: 800-356-3072
- EM Science, 480 S Democrat Road, Gibbstown, NJ 08027. Phone: 800-222-0342
- HACH Company, PO Box 389, Loveland, CO 80539. Phone: 800-227-4224
- La Motte Company, PO Box 329, Chesterfield, MD 21620. Phone: 800-344-3100
- Sentron Integrated Sensor Technology, 33320 1st Way S, Federal Way, WA 98003. Phone: 206-838-7933

SPECIALIZED FIELD TEST KITS

The following vendors supply more specialized field test kits:

- Dexsil (PetroFlag for soil hydrocarbon screening), 1 Hamden Park Drive, Hamden, CT. Phone: 800-4-DEXSIL
- DTECH Environmental Detection Systems (immunoassay test kits), 480 Democrat Road, Gibbstown, NJ 08027. Phone: 800-222-0342
- Strategic Diagnostics, Inc. (SDI) (Water quality testing RaPID Assays test kits), 111 Pencader Dr., Newark, DE 19702-3322. Phone: 800-544-8881; Fax: 302-456-6782, E-mail: techservice@sdix.com; Internet: sdix.com
- Environmental Technologies Group (Metalyzer), 1400 Taylor Avenue, Baltimore, MD 21284. Phone: 800-635-4598
- FCI Environmental Inc. (PetroSense), 1181 Grier Drive, Building B, Las Vegas, NV 89119. Phone: 800-510-3627
- IDEXX (bacteria analysis equipment) 1 IDEXX Drive, Westbrook, MN 04092. Phone: 800-248-2483
- Industrial Municipal Equipment (KoolKount Bacteria Assayer), PO Box 335, Bohemia, NY 11716. Phone: 800-858-4857
- Palintest USA (Palintest metal analyzer) (now distributed by AZUR Environmental), 21 Kenton Lands Road, PO Box 18733, Erlanger, KY 41018. Phone: 800-835-9629
- Tuner Designs (Fluorometers), 845 W. Maude Avenue, Sunnyvale, CA 94086. Phone: 408-749-0994
- Wilks Enterprise, Inc. (Infracal Oil in Water Analyzer), 140 Water Street, Norwalk, CT 06856. Phone: 203-855-9136

PARTS AND SUPPLIES FOR CUSTOM EQUIPMENT

The following sell interesting and hard-to-obtain supplies needed for custom construction of samplers and test units:

- Small Parts (stainless steel and nylon screens of many apertures, polypropylene mesh, etc.), 13980 NW 58th Court, PO Box 4650, Miami Lakes, FL 33014-0650. Phone: 800-220-4242, Fax: 800-423-9009, E-mail: smlparts@smallparts.com, Internet: smallparts.com
- Aquatic Ecosystems, Inc. (culture supplies, e.g., tanks, heaters, food, flowmeters, Lifeguard filters, activated carbon, tanks, pumps, fittings, and pipes made of many materials and sizes), 1767 Benbow Court, Apopka, FL 32703. Phone: 877-347-4788, Fax: 407-886-6787, Internet: aquaticeco.com
- Consolidated Plastics Company, Inc. (*in situ* chamber supplies [e.g., mailing tubing and end caps]), 8181 Darrow Road, Twinsburg, OH 44087. Phone: 800-362-1000, Fax: 330-425-3333, Internet: consolidatedplastics.com

TOXICITY TEST ORGANISMS

The vendors listed below supply toxicity test organisms and culture supplies:

- Aquatic Bio Systems, Inc. (ABS) (Toxicity test organisms), 1300 Blue Spruce Drive, Suite C, Fort Collins, CO 80524. Phone: 800-331-5916 or 970-484-5091, Fax: 970-484-2514, E-mail: absinfo@riverside.com
- Aquatic Research Organisms (ARO), PO Box 1271, Hampton, NH 03842-1271. Phone: 800-927-1650, Fax: 603-926-5278, E-mail: arofish@aol.com, Internet: holidayjunction.com/aro/
- Aquaculture Supply (culture foods and equipment, airstones, Spirulina, etc.), 33418 Old Saint Joe Road, Dade City, FL 33525. Phone: 352-567-8540, Fax: 352-567-3742, E-mail: ASUSA@Aquaculture-Supply.com, Internet: aquaculture-supply.com
- Argent Chemical Laboratories (Nitex mesh for *in situ* chambers, brine shrimp cysts), 8702 152nd Ave. NE, Redmond, WA 98052. Phone: 800-426-6258 or 206-885-3777, Fax: 206-885-2112, E-mail: email@argent-labs.com, Internet: argent-labs.com
- Azur Environmental (Microtox equipment and supplies), 2232 Rutherford Road, Carlsbad, CA 92008-8883. Phone: 760-438-8282, Fax: 760-438-2980, E-mail: maketing@azurenv.com, Internet: azurenv.com
- Pet Warehouse (culture foods and equipment: food, activated carbon, brine shrimp cysts, air pumps, air tubing, etc.) Dept. C93F, PO Box 752138, Dayton, OH 45475. Phone: 800-433-1160 or 937-428-6500, Fax: 800-513-1913 or 937-428-6505, E-mail: service@petwhse.com, Internet: petwhse.com
- Xpedx (Saalfeld Paper) (small, plastic cladocean toxicity testing cups), 4510 Reading Road, Cincinnati, OH 45229. Phone: 800-669-7101 or 513-641-5000, Fax: 800-880-5312 or 513-641-5003, Internet: xpedx.com

LABORATORY CHEMICAL SUPPLIES (AND OTHER EQUIPMENT)

The following vendors supply general laboratory supplies and equipment, plus many field supplies, such as test kits, meters, and sample bottles:

- Fisher Scientific (chemicals, reagents, and laboratory equipment and supplies [e.g., plastic centrifuge tubes]), 2000 Park Lane Dr., Pittsburgh, PA 15275-9952. Phone: 800-766-7000 or 412-490-8300, Fax: 800-926-1166, E-mail: fishersupport@plpit.fishersci.com, Internet: fishersci.com
- Millipore (Milli-Q system supplies and field bacteriological sampling equipment), 80 Ashby Road, Bedford, MA 01730. Phone: 800-645-5476, Fax: 617-275-5550, E-mail: order@millipore.com, Internet: millipore.com
- Supelco (glass, amber vials, standard solutions), PO Box B Bellfonte, PA 16823. Phone: 800-247-6628 or 814-359-3441, Fax: 814-359-5459, E-mail: supelco@sial.com, Internet: sigma-aldrich.com
- Sigma Chemicals (chemicals, dialysis tubing for air lines), PO Box 14508, St. Louis, MO 63178. Phone: 800-325-3010 or 314-771-5765, Fax: 800-325-5052, E-mail: sigma@sial.com. Internet: sigma-aldrich.com
- VWR (glass, amber vials and general equipment and supplies), 1310 Goshen Parkway, West Chester, PA 19380. Orders: 1-800-932-5000. Phone: 800-932-5000 or 610-431-1700, Fax: 610-429-9340, E-mail: solutions@vwrsp.com, Internet: vwrsp.com

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Environmental Toxicology and Chemistry, Vol. 14, No. 7, pp. 1141–1150, 1995
Printed in the USA
0730-7268/95 $9.50 + .00

0730-7268(95)00060-7

CONCENTRATIONS, TRANSPORT AND BIOLOGICAL EFFECTS OF DORMANT SPRAY PESTICIDES IN THE SAN FRANCISCO ESTUARY, CALIFORNIA

KATHRYN M. KUIVILA*† and CHRISTOPHER G. FOE‡
†U.S. Geological Survey, Water Resources Division, Room W-2233, 2800 Cottage Way, Sacramento, California 95825
‡California Regional Water Quality Control Board, 3443 Routier Rd., Sacramento, California 95827

(Received 7 July 1994; Accepted 28 December 1994)

Abstract—The transport and biological effects of dormant spray pesticides were examined in the San Francisco Estuary, California, by measuring dissolved-pesticide concentrations and estimating toxicity using bioassays at a series of sites in January and February 1993. Distinct pulses of pesticides, including diazinon, methidathion, and chlorpyrifos, were detected in the San Joaquin River in January and February and in the Sacramento River in February following rainfall. The higher pesticide loads in the Sacramento River compared with those in the San Joaquin River can be attributed to the greater amount of rainfall in the Sacramento Valley. The use patterns and water solubility of the pesticides can account for the observed temporal and spatial distributions in the two rivers. The pesticide pulses detected at Sacramento were followed through the northern embayment of San Francisco Estuary. In contrast, the pesticide distribution in the Sacramento–San Joaquin Delta changed from distinct pulses to steady increases in concentration over time. Seven-day bioassays indicated that Sacramento River water at Rio Vista was acutely toxic to *Ceriodaphnia dubia* (water flea) for 3 consecutive d and San Joaquin River water at Vernalis for 12 consecutive d. These water samples all had the highest diazinon concentrations. Examination of 96-h LC50 values (lethal concentration that kills 50% of test organisms in 96 H) indicates that measured diazinon concentrations could account for most but not all the observed toxicity. Other pesticides present could contribute to the toxicity.

Keywords—Pesticides San Francisco Estuary Toxicity Diazinon Methidathion

INTRODUCTION

The biological effects of dormant spray pesticides used on orchards in California's Central Valley are of environmental concern; bioassay surveys indicate that San Joaquin River water with elevated concentrations of dormant spray pesticides is often toxic to *Ceriodaphnia dubia* (water flea) [1,2]. Results of previous studies of pesticide concentrations in the Sacramento and San Joaquin rivers in 1991 and 1992 indicate that rainfall is a major mechanism for transporting pesticides from orchards and fields into the river (K. M. Kuivila, unpublished data). Because of the extensive use of dormant spray pesticides in the Central Valley during the wettest times of the year (i.e., winter), there is a need for an understanding of inputs and transport of dormant spray pesticides to the San Francisco Estuary.

Dormant spray pesticides, including diazinon, methidathion, chlorpyrifos, and malathion, are typically applied to stone-fruit orchards in the Central Valley during January and February [3,4]. Diazinon, methidathion, and malathion are relatively hydrophilic with water solubilities ranging from 40 mg/L to 250 mg/L, whereas chlorpyrifos is more hydrophobic with a water solubility of 2.0 mg/L (Table 1). These organophosphate insecticides are acetylcholinesterase inhibitors and are most toxic to zooplankton [5,6].

The objectives of this study were to determine the concentrations, transport, and possible biological effects of dormant spray pesticides in the rivers and estuary following rainfall in January and February 1993. Dissolved-pesticide concentrations were measured in the Sacramento and San Joaquin rivers, and the transport of these pesticides was tracked through the Sacramento–San Joaquin Delta and into San Francisco Bay (Fig. 1). Possible biological effects were estimated using bioassay surveys concurrently with the pesticide sampling. This study was a collaborative effort by the U.S. Geological Survey (USGS) and the California Regional Water Quality Control Board (RWQCB) and is part of a larger research effort by the USGS Toxic Contaminants Hydrology Program to assess quantitatively the transport and fate of pesticides in the San Francisco Estuary.

HYDROLOGIC SETTING AND SAMPLING STRATEGY

Agriculture in the Central Valley of California accounts for 10 percent of the total pesticide usage in the United States. Two major rivers, the Sacramento and San Joaquin, drain this region, converging in a complex delta at the head of San Francisco Estuary (Fig. 1). The average flow of the Sacramento and San Joaquin rivers is 680 and 130 m^3/s, respectively. Within the delta, the flows and flow patterns are controlled extensively by a variety of management strategies. State and federal projects (Fig. 1) export water from the delta to the San Joaquin Valley and the southern part of the state. The entire delta is tidally influenced and the net flow pattern is complex. Water from the Sacramento River primarily flows down the main river channel and out to Suisun Bay, although some of the water is diverted through the delta cross chan-

*To whom correspondence may be addressed.

Table 1. Dormant spray pesticides: Water solubility and amounts applied to orchards in January and February 1990 in the Sacramento and San Joaquin valleys

Pesticide name	Water solubility[b] at given temp. (mg/L)	Amount applied[a] Sacramento Valley (kg)	Amount applied[a] San Joaquin Valley (kg)
Ethyl parathion	24 (25°C)	52,764	37,858
Diazinon	40 (20°C)	21,369	26,906
Methidathion	250 (20°C)	15,544	9,676
Chlorpyrifos	2 (25°C)	3,663	17,524
Malathion	145 (20°C)	4,472	6,130

[a]California Department of Pesticide Regulation, 1990 [3].
[b]Worthing and Walker, 1987 [27].

nel and Georgiana Slough to the state and federal export pumps. The San Joaquin River splits downstream from Mossdale, with some of the water flowing toward the export pumps via lower Old River and Grant Line Canal and the remainder flowing toward Stockton. Northwest from Stockton, the channel deepens and widens, resulting in an increase in water residence time. Water from the San Joaquin River mixes with water from the Mokelumne, Consumes, and Sacramento rivers, and the net flow is toward the pumps via Old and Middle rivers. Little, if any, of the San Joaquin River water gets out into San Francisco Bay. The USGS is currently collecting data on flows and flow patterns to be used to calibrate and validate a hydrodynamic model of the delta [7].

Pesticide concentrations were measured at a series of sites along two major flow paths: the Sacramento River from Sac-

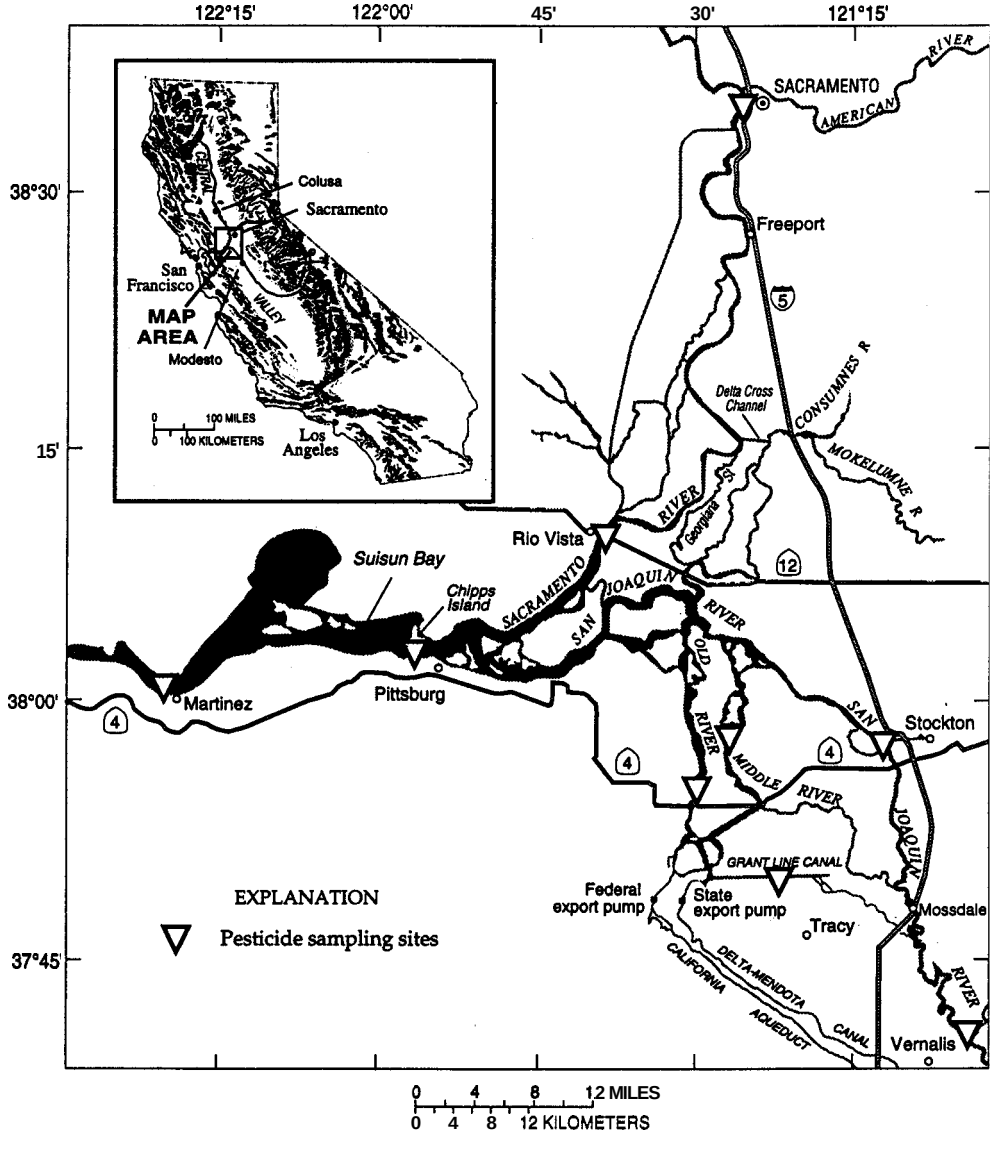

Fig. 1. Location of study area.

ramento to the western boundary of Suisun Bay and the San Joaquin River from Vernalis through Stockton to the export pumps (Fig. 1). Water samples for pesticide analysis were collected daily at all sampling locations (twice a day at Vernalis) using a depth-integrating, discharge-weighted sampler at either one or three verticals, depending on the site. Flow at the Sacramento and Vernalis sites is unidirectional, and sampling studies indicate that the composition and concentrations of dissolved constituents at a single vertical mid-channel are representative of the cross section under most flow conditions (data not shown). Discharge for the Sacramento River was recorded at Freeport (11 river miles downstream from Sacramento) with an ultrasonic velocity meter. Because the site at Freeport is affected by the tide, the discharge was tidally filtered to calculate a daily mean discharge [8]. For the San Joaquin River, discharge also was recorded at Vernalis, a streamflow-gaging station.

In contrast to the Sacramento and Vernalis sites, the flow at the other sites reverses during the tidal cycle. Samples were collected routinely at the tidally affected sites (Fig. 1) during slack after ebbtide, except at the Old and Middle River sites where samples were collected at slack after floodtide. This sampling scheme estimated the most seaward movement of solutes along the flow path through the delta or through Suisun Bay and created a consistency for comparison of daily concentrations.

ANALYTICAL METHODS

Dissolved pesticides were extracted from fiitered 1-L (liter) samples onto C_8 solid-phase-extraction cartridges and eluted with three 2-ml aliquots of hexane:diethyl ether (1:1). The eluant was concentrated and analyzed using a capillary gas chromatograph/ion-trap mass spectrometer in full-scan mode [9,10]. Field blanks using organic-free water were processed every 20 samples; no contamination was detected throughout this study. A minimum of 10% of the samples were collected in duplicate and all analytes agreed within 25% or less. Replicate samples were also routinely sent to the USGS National Water Quality Laboratory for comparison. Although 19 pesticides are included routinely in the analysis, the focus of this study was diazinon, methidathion, chlorpyrifos, and malathion, with method detection limits of 30, 35, 40, and 35 ng/L, respectively. During matrix spike experiments, recovery of these four pesticides in Sacramento and San Joaquin River water was greater than or equal to 83%. For more details on the analytical method and quality-assurance practices, see Crepeau et al. [10].

Seven-day *C. dubia* bioassays [11] were done at Sierra Foothill Laboratory using mortality as an end point. Reproduction was not assessed. Tests were set up in batches 1 to 5 d after water collection with neonates less than 24 h old. All U.S. Environmental Protection Agency (EPA)-recommended water quality parameters, with the exception of alkalinity, were measured and found to be within acceptable limits to support aquatic life. Water from a local spring was used as a control. No mortality within 7 d was ever observed in the control.

RESULTS AND DISCUSSION

The results of this study consist of the following three parts: riverine pesticide concentrations, transport into the estuary, and biological effects of the observed pesticides. Measured concentrations of dissolved pesticides in the Sacramento and San Joaquin rivers were examined in the context of pesticide usage in the valley. Pesticide loads were calculated and the loads of the two rivers compared. Transport of these pesticides was followed through the Sacramento–San Joaquin Delta and into San Francisco Bay. Finally, the biological effects of these pesticides were estimated by using bioassays and by comparing the measured concentrations to regulatory limits.

Pesticide pulses following rainfall

Pulses of diazinon were detected following rainfall in the Sacramento and San Joaquin rivers in previous years (K.M. Kuivila, unpublished data); this phenomenon is similar to the spring flush of herbicides observed in surface-water runoff in the midwestern United States [12]. The riverine pulses of diazinon typically were narrow and well defined; elevated concentrations were measured for only a few days to weeks.

In the Sacramento and San Joaquin valleys, a series of rainstorms (cumulative rainfall greater than 2.5 cm) began on January 6 and continued through January 21 (Figs. 2A and 3A). Dormant spray pesticides were applied either before these rainstorms (late December and early January) or during 2 weeks of dry weather following these rainstorms (late January). Another series of rainstorms began in early February and continued through February 26.

Elevated concentrations of pesticides were detected in the Sacramento River at Sacramento in February but not in January; diazinon and methidathion were the only dormant spray pesticides detected. A few days after the rainfall on February 5, 7, and 8, streamflow at Freeport increased, reaching a maximum on February 14 (Fig. 2A). Similarly, diazinon concentrations increased on February 8 and reached a measured maximum of 393 ng/L on February 12 (Fig. 2B). Distribution of methidathion over time was similar to that of diazinon, but the peak shape was slightly broader, and the maximum concentration was 212 ng/L. It rained again February 17 to 19, and both discharge and pesticide concentrations increased. During this second pulse, the maximum concentrations of diazinon and methidathion were lower and the peaks were more spread out than during the first pulse. The maximum diazinon concentration was 193 ng/L on February 21, whereas methidathion concentration peaked at 71 ng/L on February 22. The discharge also reached a maximum 2 d later (February 24).

In contrast to the Sacramento River, elevated concentrations of pesticides were detected in the San Joaquin River at Vernalis in both January and February. Only diazinon was detected in January, whereas diazinon, methidathion, and chlorpyrifos were detected in February. In the San Joaquin Valley, three periods of rainfall (accumulations of greater than 2.5 cm) occurred, beginning on January 6, 12, and 17. Each rainfall was followed by a corresponding increase in

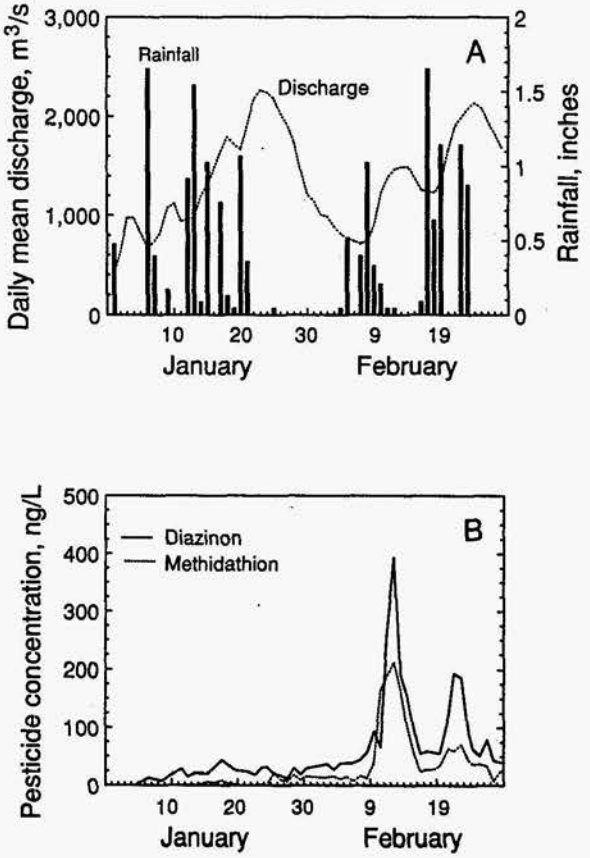

Fig. 2. Sacramento Valley, January and February 1993: (A) daily mean discharge, Sacramento River at Freeport, and rainfall at Colusa; (B) concentrations of diazinon and methidathion, Sacramento River at Sacramento.

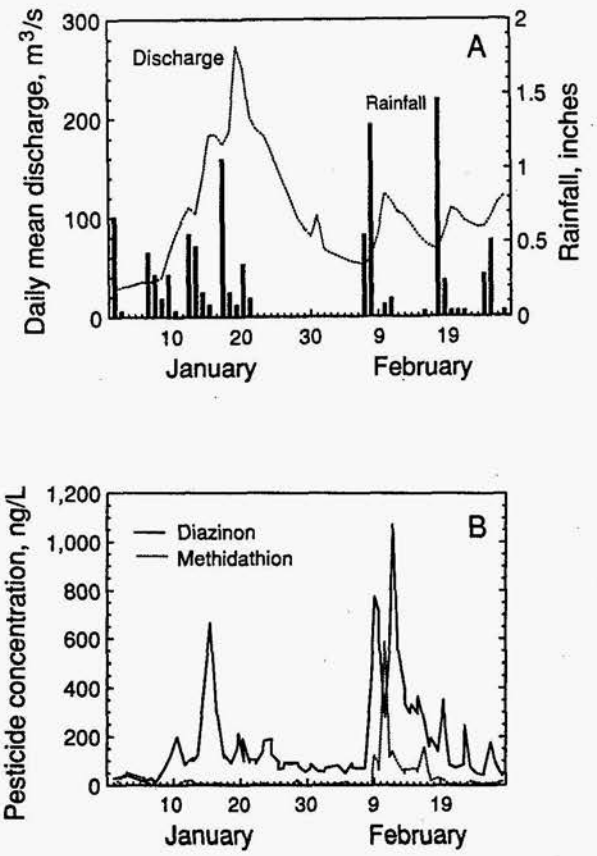

Fig. 3. San Joaquin Valley, January and February 1993: (A) daily mean discharge, San Joaquin River at Vernalis, and rainfall at Modesto; (B) concentrations of diazinon and methidathion, San Joaquin River at Vernalis.

streamflow at Vernalis, with a maximum on January 19 (Fig. 3A). Distribution of diazinon followed a similar pattern with concentration maxima following rainfall but preceding the streamflow maxima (Fig. 3B). Although the amount of rainfall and the corresponding increase in streamflow were similar for each of the three January rainfalls, the maximum diazinon concentrations varied widely (198, 664, and 211 ng/L on January 10, 15, and 19, respectively).

Three series of storms also occurred in February in the San Joaquin Valley, but they were more widely spaced than those in January (Fig. 3A). Streamflow and concentrations of diazinon and methidathion in the San Joaquin River increased at Vernalis on February 8 in response to rainfall on February 7 and 8 (Fig. 3A and B). Two well-defined peaks of diazinon concentration were detected; the first had a maximum concentration of 733 ng/L at 2400 h on February 8, and the second had a maximum concentration of 1,070 ng/L at 1900 h on February 11. Both streamflow and methidathion concentration peaked on February 10, between the two diazinon maxima, with methidathion values reaching 586 ng/L. Two subsequent rainfalls on February 18 and 25 to 26 were followed by corresponding increases in streamflow, a smaller increase in diazinon concentration, and no detectable change in methidathion concentration. Chlorpyrifos was detected only in samples from February 9 to 18 with a maximum concentration of 42 ng/L on February 12 (data not shown).

Pesticide-use patterns

The amount of dormant spray pesticides applied to stonefruit orchards in the Central Valley in 1990 is shown in Table 1. Ethyl parathion was the most commonly used dormant spray before being banned because of human health hazards in 1991. By 1993, the use of ethyl parathion had been replaced by other dormant spray pesticides, but detailed records of 1993 pesticide applications are not yet available. Detection of both diazinon and methidathion in the rivers following rainfall can be attributed to the high water solubilities and high use of these pesticides. The absence of diazinon or methidathion in the Sacramento River in January despite rainfall is probably due to lack of application before the January rains. In the San Joaquin River, detection of only diazinon in January indicates application of diazinon but not methidathion by that date, whereas the detection of both diazinon and methidathion in February suggests application of both pesticides in late January.

Differences in travel times to Vernalis from two geographically separate sources could have resulted in the observed

double pulse of diazinon in February after the February 7 to 8 rainfall period [13]. The concurrent distribution of methidathion showed only a single peak, which occurred between the two diazinon peaks. The relative timing of the methidathion and diazinon peaks indicates that the primary source of methidathion was at a location between the two sources of diazinon.

In 1990, more chlorpyrifos was applied in the San Joaquin Valley than in the Sacramento Valley (Table 1). The low concentrations of chlorpyrifos detected for only a few days in the San Joaquin River, despite a higher use than methidathion, can be explained by the hydrophobic nature of chlorpyrifos. With a water solubility of only 2.0 mg/L, chlorpyrifos has a tendency to sorb onto sediments and will be transported from the orchards primarily via sediment erosion rather than water runoff. In contrast, although malathion has a high water solubility (145 mg/L), it was not detected in any of the water samples during this study. The low use and rapid degradation of malathion in soil [14] can explain the absence of detectable malathion in the two rivers.

Calculation of pesticide loads

Pesticide loads for the Sacramento River were calculated by multiplying the instantaneous measured pesticide concentration by the tidally filtered, daily mean discharge (Fig. 4A). For diazinon, the integrated loads for each peak were 160 kg for February 8 to 16 and 130 kg for February 19 to 25. Although the maximum concentration during the second peak (193 ng/L) was only half that during the first peak (393 ng/L), the loads of diazinon in the river were similar. For methidathion, the integrated loads were 120 and 57 kg, respectively. In contrast to diazinon, the methidathion load was much lower following the second rainfall; the higher water solubility of methidathion as compared with diazinon may account for the higher percentage of the methidathion discharging from the watershed during the first rainfall.

Pesticide loads for the San Joaquin River were calculated by multiplying the instantaneous measured pesticide concentration by the daily mean discharge at Vernalis (Fig. 4B). For diazinon, the integrated load for January 8 to 28 was 48 kg and for February 7 to 28 was 44 kg. Although the diazinon concentrations were much higher in February, the discharge was much lower than in January so that the resulting loads were similar. It is likely that additional diazinon was applied to orchards between the January and February rains, but the lack of detailed data on diazinon application at this time makes it impossible to verify. The load of methidathion was much lower, with only 12 kg of methidathion for February 8 to 19 in the San Joaquin River.

There is a striking contrast between the pesticide loads in the two rivers. The load of diazinon in the Sacramento River for January and February (340 kg) was 3.5 times the diazinon load in the San Joaquin River (98 kg), whereas the difference in methidathion loads (190 and 12 kg, respectively) was a factor of 17. The lack of current pesticide-use data precludes a quantitative comparison of the riverine load to use ratios in the two valleys. In 1990, 1.5 times as much ethyl parathion was applied in the Sacramento Valley as in the San Joaquin Valley and equal amounts of diazinon were applied

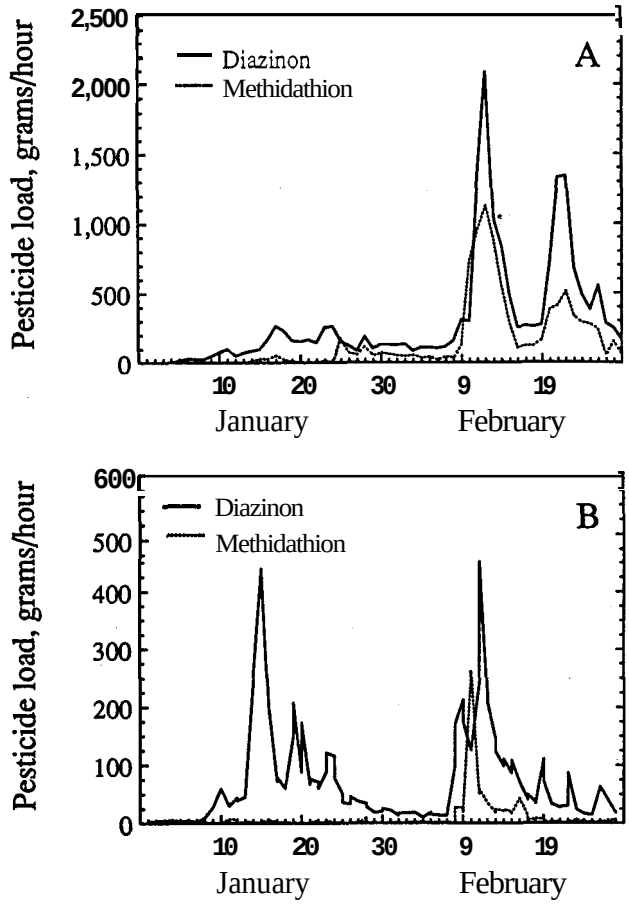

Fig. 4. Pesticide loads: (A) Sacramento River at Sacramento, and (B) San Joaquin River at Vernalis, January and February 1993.

(Table 1). If the entire amount of ethyl parathion used in 1990 was replaced by diazinon (1:1), approximately equal amounts of diazinon would have been applied to the two valleys. In addition, methidathion was probably applied in similar amounts to the two watersheds. These pesticide-use patterns cannot account for the higher load of both diazinon and methidathion in the Sacramento River.

Other factors that influence runoff of pesticides include timing of application relative to rainfall, total amount of rainfall, and saturation of soil due to antecedent conditions. Details are not known about the exact timing of dormant spray application in 1993, but most of the application in both valleys was probably during the dry period at the end of January. The amount of rainfall before and after pesticide application varied greatly between the two valleys. The average rainfall in Sacramento Valley was 16.0 cm in December, 17.9 cm in January, and 18.3 cm in February. In comparison, the San Joaquin Valley was significantly drier with only 5.89, 9.96, and 9.96 cm of rain in December, January, and February, respectively. The differences in the amount of rainfall before and after pesticide application in the two basins could account for the observed differences in pesticide loads.

Pesticide transport into San Francisco Bay

A main flowpath of water down the Sacramento River is along the ship channel past Rio Vista, Chipps Island, and Martinez (Fig. 1). The first pulse of pesticides in the Sacramento River in February was followed from Sacramento through Suisun Bay (the northern embayment of San Francisco Estuary) (Figs. 1 and 5). Initially detected at Sacramento, the diazinon and methidathion concentrations reached maximum values of 393 and 212 ng/L on February 12. The next day, this pulse was detected at Rio Vista, 69.2 river km downstream from Sacramento; the maximum diazinon concentration was 281 ng/L, and the maximum methidathion concentration was 179 ng/L on February 13. At Chipps Island (26.5 river km from Rio Vista), the diazinon concentration reached a maximum of 199 ng/L on February 15 and the methidathion concentration reached a maximum of 123 ng/L on February 14. Finally, diazinon concentrations of 107 and 122 ng/L were detected on February 18 and 20 at Martinez (23.3 river km seaward from Chipps Island). Methidathion concentrations peaked a day earlier at Martinez, with maximum concentrations of 64 and 60 ng/L on February 17 and 18. The approximate travel time was 1 d from Sacramento to Rio Vista, 2 d from Rio Vista to Chipps Island, and 2 to 5 d from Chipps Island to Martinez. As the pesticide pulse moved seaward, the maximum concentration decreased and the pulse dispersed over time, in part because of tidal diffusion.

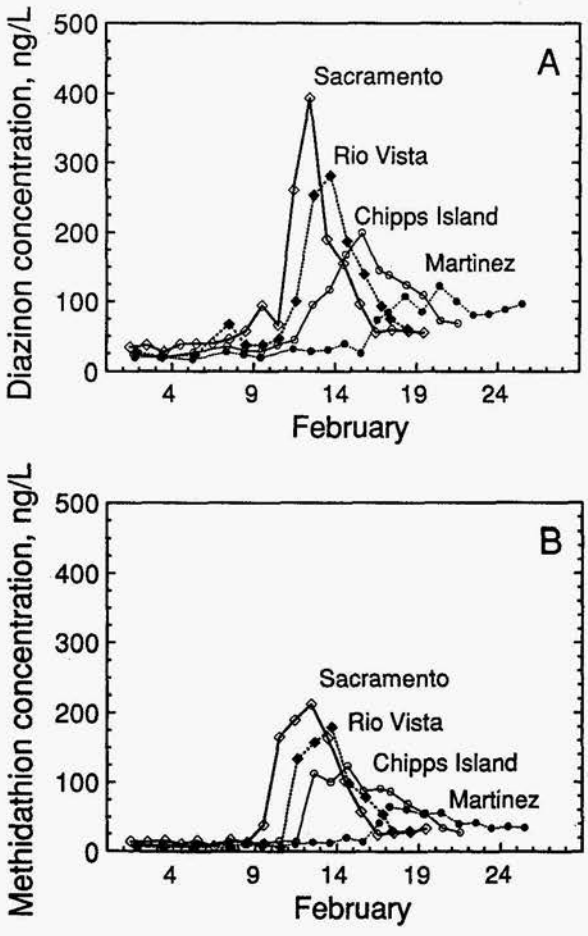

Fig. 5. Sacramento River to San Francisco Bay, February 1993: concentrations of (**A**) diazinon and (**B**) methidathion.

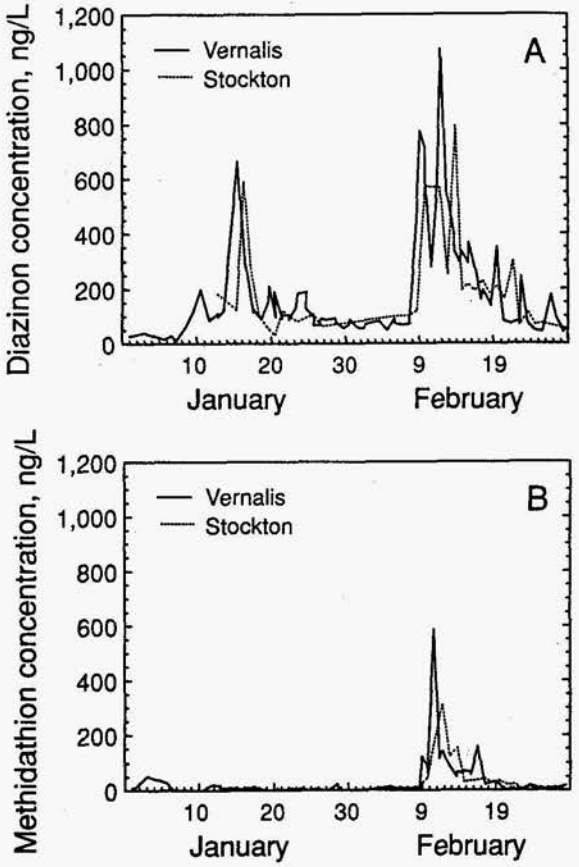

Fig. 6. Vernalis and Stockton, January and February 1993: concentrations of (**A**) diazinon and (**B**) methidathion.

Pesticide transport into the Sacramento–San Joaquin Delta

The pesticide pulse from the San Joaquin River can be followed through the Sacramento–San Joaquin Delta in a similar manner. During high-flow conditions, such as occurred in January and February 1993, there is positive net flow along the San Joaquin River toward Stockton [7]. High concentrations of diazinon, similar to those observed at Vernalis (on January 15 and February 8, 11, and 19), also were detected at Stockton 1 to 2 d later (on January 16 and February 10, 13, and 21) (Fig. 6A). Methidathion peaked at Vernalis on February 10 and at Stockton on February 11 (Fig. 6B).

In the central delta, water from the Sacramento, Mokelumne, and San Joaquin rivers mixes in a series of complex channels and is subjected to tidal-flow reversals [7]. Well-defined pesticide pulses were not observed at the Old and Middle River sites because of the mixing of two separate riverine sources of pesticides and the hydrodynamic complexity

of the delta as described earlier; instead, the concentrations steadily increased through January and February (Fig. 7A and B). The maximum concentration of diazinon was 149 ng/L on February 23 at Middle River and 121 ng/L on February 21 at Old River. Concentrations of methidathion were much lower; the maximum values reached 38 and 42 ng/L at Middle and Old Rivers, respectively.

Biological effects

Bioassays were used to determine potential biological effects of pesticide-contaminated water collected during this study [15]. In February, water samples at Rio Vista and Vernalis were split for pesticide analysis and for use in 7-d *C. dubia* bioassays. In the Sacramento River at Rio Vista, 100% *C. dubia* mortality was observed in water samples collected on February 12, 13, and 14, whereas no mortality was observed in all other February water samples (Table 2). Water samples, which were toxic, also had the highest diazinon concentrations (Table 2 and Fig. 8A). In the San Joaquin River at Vernalis, 100% *C. dubia* mortality was observed in water samples collected for 12 consecutive d (February 8–19) (Table 2). Again, the bioassay mortality corresponded with the highest diazinon concentrations (Fig. 8B and Table 2). Conversely, no toxicity was observed in water collected before (February 5 and 7) or after (February 20–25), the peaks of diazinon concentration.

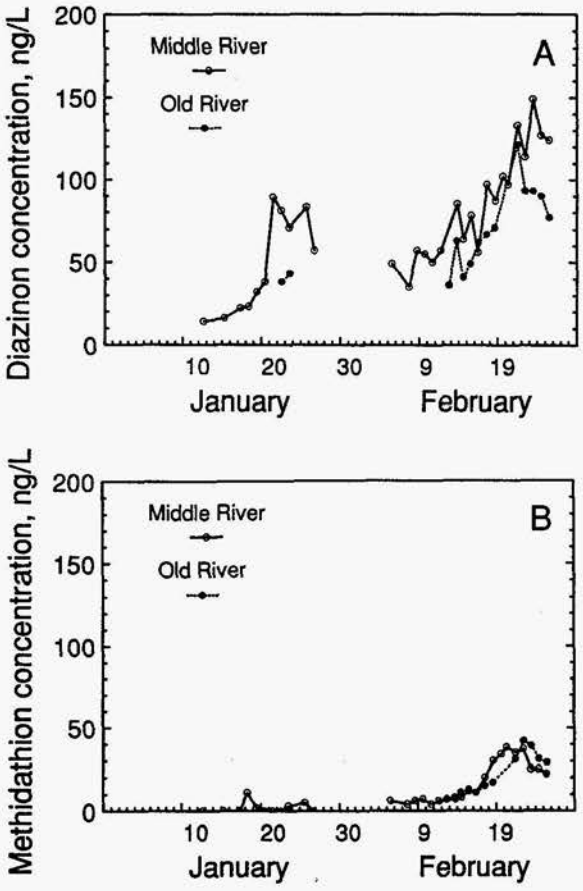

Fig. 7. Middle River and Old River, January through March 1993: concentrations of (A) diazinon and (B) methidathion.

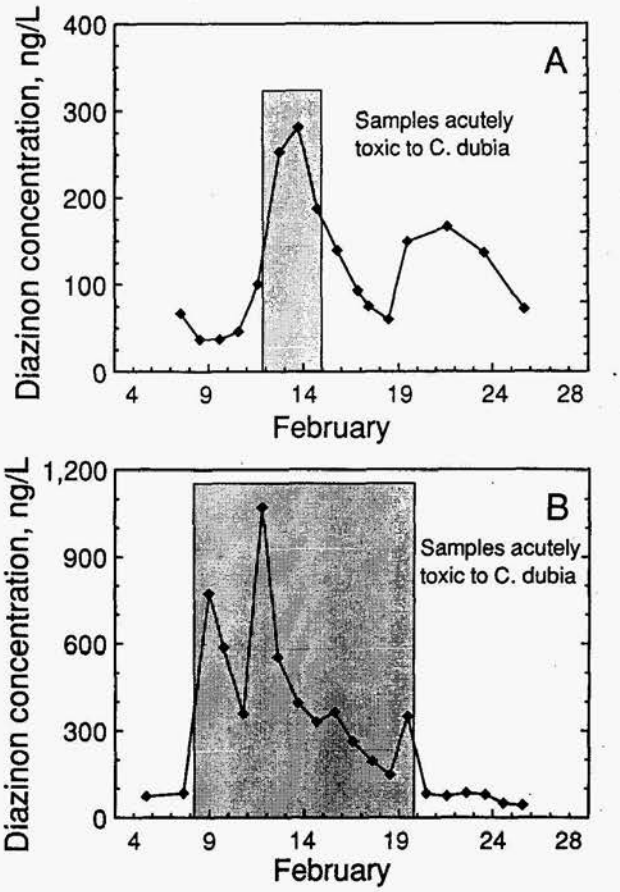

Fig. 8. Diazinon concentrations and water toxicity, February 1993: (A) Sacramento River at Rio Vista; (B) San Joaquin River at Vernalis.

Ambient diazinon concentrations appear sufficiently elevated to explain most of the *C. dubia* mortality. The laboratory 24- to 96-h lethal concentrations for 50% of the exposed population (LC50) for *C. dubia* are between 430 and 550 ng/L [2]. In 7-d tests, concentrations as low as 200 ng/L of diazinon are reported to cause 90 to 100% *C. dubia* mortality, whereas 150 ng/L resulted in no decrease in survival [16]. In the Sacramento River, water samples with diazinon concentrations equal to or greater than 187 ng/L resulted in 100% mortality in 7 d. No apparent toxicity was measured for diazinon concentrations at or below 166 ng/L. In the San Joaquin River, water samples with diazinon concentrations higher than 331 ng/L resulted in 100% mortality in 48 h, whereas samples with diazinon concentrations of 148 to 263 ng/L caused 100% mortality in 7 d. Samples with diazinon concentrations at or below 84 ng/L resulted in no mortality. The toxicity observed in the *C. dubia* bioassays appears to be slightly higher than would be predicted from the diazinon concentrations alone.

Other pesticides, in addition to diazinon, were present in all of the water samples that resulted in a *C. dubia* toxic re-

Table 2. Bioassay results and pesticide concentrations in Sacramento and San Joaquin River water

Sample date	Bioassay results (% mortality)	Dormant spray pesticides (ng/L)			Other pesticides (ng/L)		
		Diazinon	Methidathion	Chlorpyrifos	Atrazine	Carbaryl	Simazine
Sacramento River at Rio Vista							
Feb 7	0	67	tr[a]	nd	tr	nd	71
Feb 8	0	37	12	nd	18	nd	84
Feb 9	0	37	11	nd	nd	nd	65
Feb 10	0	46	tr	nd	30	nd	175
Feb 11	0	100	133	nd	31	nd	309
Feb 12	100[b]	253	157	nd	50	nd	302
Feb 13	100[b]	281	179	nd	45	nd	221
Feb 14	100[b]	187	98	nd	30	nd	106
Feb 15	0	139	78	nd	19	nd	125
Feb 16	10	93	53	nd	tr	nd	90
Feb 17	0	75	29	nd	16	nd	178
Feb 18	0	60	29	nd	tr	nd	96
Feb 19	0	149	55	nd	51	nd	331
Feb 21	0	166	68	nd	37	nd	272
Feb 23	0	136	54	nd	22	nd	157
Feb 25	0	72	42	nd	nd	nd	93
San Joaquin River at Vernalis							
Feb 5	0	73	tr	nd	nd	tr	128
Feb 7	0	84	tr	nd	nd	tr	95
Feb 8	100[c]	773	122	nd	nd	101	103
Feb 9	100[c]	586	36	tr	nd	106	596
Feb 10	100[c]	358	214	tr	nd	62	492
Feb 11	100[c]	1,071	140	31	nd	41	844
Feb 12	100[c]	554	92	42	nd	14	455
Feb 13	100[d]	396	49	32	nd	10	393
Feb 14	100[d]	331	70	tr	nd	nd	247
Feb 15	100[d]	364	56	tr	nd	10	248
Feb 16	100[b]	263	157	tr	nd	nd	180
Feb 17	100[b]	195	22	tr	nd	nd	193
Feb 18	100[b]	148	30	tr	nd	nd	160
Feb 19	100[d]	350	23	nd	nd	nd	360
Feb 20	0	83	tr	nd	nd	nd	187
Feb 21	0	74	nd	nd	nd	tr	238
Feb 23	0	79	17	nd	nd	nd	137
Feb 24	20	49	10	nd	nd	nd	135
Feb 25	0	43	tr	nd	nd	nd	89

nd, not detected.
[a]Trace means compound detected at concentration below method detection limit.
[b]Mortality occurred within 7 d.
[c]Mortality occurred within 24 h.
[d]Mortality occurred within 48 h.

sponse (Table 2). The comparative toxicities of these pesticides are (in order of toxicity) chlorpyrifos, diazinon, methidathion, carbaryl, atrazine, and simazine. Chlorpyrifos was the most toxic pesticide detected; the laboratory 96-h LC50 for C. *dubia* was between 80 and 130 ng/L of chlorpyrifos [2]. The other pesticides detected in these water samples are less toxic than diazinon. For methidathion and carbaryl, the laboratory 96-h LC50s for C. *dubia* were 2,000 and 8,300 ng/L [2,17]. Atrazine and simazine are much less toxic, with reported 48-h LC50s of 6.9×10^6 and 1.0×10^7 ng/L for Duphnia magna (also a water flea) [18,19]. Because the concentrations of methidathion, carbaryl, atrazine, and simazine were an order of magnitude or more lower than their respective LC50s, these compounds probably do not contribute to the observed toxicity. However, the additive or synergistic effects of pesticides are not well understood. In addition, other compounds from agricultural and urban runoff, including trace metals and other organic compounds, could be present and could be contributing to the overall toxicity observed in the bioassay surveys.

Results of this pesticide study are useful to estimate the possible effects of dormant spray pesticides on the ecology of the delta and bay. The National Academy of Sciences and National Academy of Engineering [20] has recommended a guideline of 9 ng/L diazinon as a maximum concentration in surface water for protection of aquatic life, and the International Joint Commission [21] suggests a similar guideline of 8 ng/L diazinon for the Great Lakes. Currently (1995), there is no EPA aquatic-life criterion for diazinon. For chlorpyrifos, the EPA water quality criteria for protection of

freshwater aquatic organisms is 41 and 83 ng/L for chronic and acute exposures, respectively [22]. In all the samples collected during this study, concentrations of diazinon always exceeded the National Academy of Sciences and National Academy of Engineering recommended guidelines, whereas the dissolved concentrations of chlorpyrifos were less than the recommended EPA criteria on all dates except for February 12 on the San Joaquin River.

Bioassay results demonstrate that diazinon and possibly other compounds present in storm runoff were biologically available. Although there is not an extensive toxicological database for diazinon, what is available suggests that other invertebrates are more sensitive to diazinon. For example, the 96-h LC50 for *Daphnia magna, Gammarus faciatus,* and *Chironmomus tentans,* two of which are present in the estuary, are 210,200, and 30 ng/L diazinon, respectively [23–25]. These organisms are 2 to 18 times more sensitive than C. *dubia.* Reproduction was not measured in this study. However, the IC25 for *C. dubia* (concentration that produces a 25% reduction in reproduction) is 125 ng/L diazinon. As with mortality, reproductive impacts for other organisms probably occur at still lower concentrations. Therefore, the pesticide field data suggest that sensitive organisms in the San Francisco Estuary may experience short periods of acutely toxic conditions and longer periods with potentially chronic impacts in the winter.

Ecological effects of pesticides on aquatic biota in the delta have not yet been studied; however, most freshwater zooplankton (copepods, rotifers, and cladocerans) in the delta are in decline [26]; the cause is unknown. More studies need to be conducted to ascertain the impact of pesticides in controlling the abundance and distribution of organisms in the San Francisco Estuary.

SUMMARY AND CONCLUSIONS

Results of this and previous studies indicate that rainfall runoff is an important mechanism for transporting dormant spray pesticides from orchards into rivers. Elevated concentrations of diazinon, methidathion, and chlorpyrifos were detected after rainfall in January and February in the Sacramento and San Joaquin rivers. Timing of pesticide application, amounts of pesticides applied, water solubility, and soil half-life explain most of the observed temporal and geographic differences in riverine pesticide concentrations. Differences in riverine pesticide loads in the Sacramento and San Joaquin rivers are likely due, in part, to variations in amount of rainfall to the basins before and after pesticide application.

Under high-flow conditions in February 1993, diazinon and methidathion were transported in distinct pulses down the Sacramento River and into San Francisco Bay. These pesticides also were transported from the San Joaquin River through the Sacramento–San Joaquin Delta; within the delta, distribution of pesticides was a steady increase in concentration over time, rather than distinct pulses.

Results of 7-d bioassays indicate that Sacramento River water at Rio Vista was acutely toxic to C. *dubia.* for 3 consecutive d and San Joaquin River water at Vernalis for 12 consecutive d (Table 2). Bioassay mortality corresponded with the highest diazinon concentrations at both sites, and diazinon does explain a good deal of the observed *C. dubia* toxicity. In addition, other pesticides were present that could have contributed to the toxicity of the water samples.

Concentrations of diazinon in all water samples collected in this study exceeded the water quality guidelines recommended by NAS/NAE for protection of aquatic life [20]; concentrations of all other pesticides were below any recommended or regulatory limits. More extensive chemical and toxicological testing needs to be done to ascertain the chemicals responsible for causing toxicity, to determine their distribution and fate within the delta, and to evaluate their effect on native organisms.

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Annual Review

Insecticide-caused toxicity to *Ceriodaphnia dubia* (CLADOCERA) in the Sacramento–San Joaquin River delta, California, USA

1. Ingeborg Werner[1,*],
2. Linda A. Deanovic[1],
3. Valerie Connor[2],
4. Victor de Vlaming[3],
5. Howard C. Bailey[1],
6. David E. Hinton[1]

Article first published online: 2 NOV 2009

DOI: 10.1002/etc.5620190126

Issue

Environmental Toxicology and Chemistry

Volume 19, Issue 1, (/doi/10.1002/etc.v19:1/issuetoc) pages 215–227, January 2000

Additional Information

How to Cite

Werner, I., Deanovic, L. A., Connor, V., de Vlaming, V., Bailey, H. C. and Hinton, D. E. (2000), Insecticide-caused toxicity to *Ceriodaphnia dubia* (CLADOCERA) in the Sacramento–San Joaquin River delta, California, USA. Environmental Toxicology and Chemistry, 19: 215–227. doi: 10.1002/etc.5620190126

Author Information

1 Aquatic Toxicology Laboratory, School of Veterinary Medicine, Department of Anatomy, Physiology and Cell Biology, University of California, Davis, California 95616, USA

2 California Regional Water Quality Control Board, Central Valley Region, 3443 Routier Road, Sacramento, California 96827-3098, USA

3 California State Water Resources Control Board, Division of Water Quality, P.O. Box 944213, Sacramento, California 94244-2130, USA

Email: Ingeborg Werner (iwerner@ucdavis.edu)

*Aquatic Toxicology Laboratory, School of Veterinary Medicine, Department of Anatomy, Physiology and Cell Biology, University of California, Davis, California 95616, USA

Publication History

1. Issue published online: 2 NOV 2009
2. Article first published online: 2 NOV 2009
3. Manuscript Accepted: 1 JUL 1999
4. Manuscript Received: 1 DEC 1998

- Abstract
- Article (/doi/10.1002/etc.5620190126/full)
- References (/doi/10.1002/etc.5620190126/references)
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Keywords:

Ceriodaphnia dubia; Toxicity test; Pesticides; Sacramento-San Joaquin Delta

Abstract

In recent years, populations of resident aquatic species in California's Sacramento–San Joaquin Delta, USA, have declined appreciably in numbers. The cause of these declines is not known, but has been attributed to a number of factors including water diversions, loss of habitat, introduced exotic organisms, and toxic compounds. To detect and characterize the spatial extent, severity, frequency, and causes of potential toxicity caused by anthropogenic pollutants, a monitoring study was conducted over a period of two years (1993–1995). Sites were monitored on a monthly basis using the standardized U.S. Environmental Protection Agency freshwater toxicity test with the zooplankton species *Ceriodaphnia dubia*. Twenty-four sites were sampled in 1993 to 1994. During the 1994 to 1995 sampling season, the number of sampling sites was restricted to 20, with special emphasis placed on back sloughs, delta island agricultural drains, and main-stem river sites. Significant mortality or reproductive toxicity in *C. dubia* was detected in 9.8% of 400 water samples tested. Ecologically important back sloughs had the largest percentage of toxic samples. Of 71 and 103 samples collected from back sloughs during 1993 to 1994 and 1994 to 1995, respectively, 14.1% and 19.6% were toxic. To determine the causative chemical(s), toxicity identification evaluations (TIEs) were conducted on 23 toxic samples. These included eight follow-up samples taken to determine whether toxicity at the respective site persisted. Organophosphate (chlorpyrifos, diazinon, malathion) and carbamate (carbofuran, carbaryl) pesticides were identified as primary toxicants. Chlorpyrifos was present at toxic concentrations in 87% of samples tested by TIE. Analysis of data from the follow-up samples suggested that toxicity may have persisted over periods of several days to weeks.

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State of California
California Regional Water Quality Control Board, Los Angeles Region

RESOLUTION NO. R10-005

July 8, 2010

Amendment to the *Water Quality Control Plan for the Los Angeles Region* to Update the Bacteria Objectives for Freshwaters Designated for Water Contact Recreation by Removing the Fecal Coliform Objective

WHEREAS, the California Regional Water Quality Control Board, Los Angeles Region, finds that:

1. On October 25, 2001, the California Regional Water Quality Control Board, Los Angeles Region (Regional Board) adopted, by Resolution No. R01-018, an amendment to the Water Quality Control Plan for the Los Angeles Region (Basin Plan) to update the bacteria objectives for water bodies designated for water contact recreation.

2. The 2001 updated bacteria objectives reflected those established in the California Code of Regulations, title 17, § 7958 "Bacteriological Standards" (Assembly Bill 411, Statutes of 1997) and U.S. EPA's recommended criteria pursuant to Federal Clean Water Act § 304(a), published in "Ambient Water Quality Criteria for Bacteria – 1986" (U.S. EPA, 1986).

3. In "Ambient Water Quality for Bacteria - 1986", the U.S. EPA recommended that *Escherichia coli* (*E. coli*) replace fecal coliform as an indicator of the presence of pathogens in fresh water. This recommendation was based on results of epidemiological studies which found that Enterococcus and *E. coli* (a subset of the fecal coliform group) were the indicators most strongly correlated with illnesses associated with swimming in sewage-contaminated water. These studies found that fecal coliform densities were only weakly correlated with the same illnesses.

4. During the 2001 update, the Regional Board added water quality objectives for *E. coli* in freshwater but did not remove the fecal coliform objective. Rather, the Board allowed a transition period for incorporation of *E. coli* objectives into water quality monitoring programs, and for collection of data on the new objective to establish an adequate monitoring database.

5. EPA sanctions such transition periods as indicated in the "Draft Implementation Guidance for 'Ambient Water Quality Criteria for Bacteria'" (U.S. EPA, 2002). In this guidance document, EPA recommends a single triennial review cycle as the duration of the transition period. However, the Regional Board's transition period has extended through three triennial review cycles.

6. Removal of the fecal coliform objectives for freshwaters designated for water contact recreation is part of the *re-evaluation of the application of bacteria objectives in determining compliance with water quality objectives* that was selected as a project to be addressed during the 2008-10 Triennial Review period per Resolution No. R10-001.

7. This amendment updates the freshwater bacteria objectives in the Basin Plan to maintain consistency with EPA's recommendation that *E. coli* replace fecal coliform as an indicator of the presence of pathogens in fresh water, and removes unnecessary permitting and monitoring requirements that arise from having water quality objectives for both indicators.

8. This amendment will have no impact on public health risk since the *E. coli* objective that is to remain as the sole bacterial indicator for freshwaters designated for water contact recreation was developed based on the same "acceptable" illness rate as the fecal coliform objective, which it replaces. Furthermore, epidemiological studies have shown that concentrations of *E. coli* are more strongly correlated with the incidence of health effects and, specifically, gastroenteritis, than concentrations of fecal coliform. This led EPA to conclude that *E. coli* is a more reliable indicator of risk to public health than fecal coliform.

9. Regional Board staff has prepared a staff report that describes the specific necessity and rationale for this revision to the bacteria objectives. The staff report titled "Proposed Amendment to the Water Quality Control Plan - Los Angeles Region to Update the Bacteria Objectives for Freshwaters Designated for Water Contact Recreation by Removing the Fecal Coliform Objective" is an integral part of this Regional Board action and was reviewed, considered, and accepted by the Regional Board before acting.

10. A CEQA Scoping meeting was conducted on April 19, 2010 at the Junipero Serra Building, 320 W. 4th Street, Los Angeles, California, to solicit input from the public and interested stakeholders in determining the appropriate scope and content of the CEQA analysis for the proposed amendment. This meeting fulfilled the requirements under CEQA (Public Resources Code, § 21083.9). A notice of the CEQA Scoping meeting was sent to interested parties on April 6, 2010.

11. The public has had a reasonable opportunity to participate in the review of the amendment to the Basin Plan. A draft of the Staff Report, the Tentative Resolution and proposed Basin Plan language, and the accompanying environmental checklist was released for public comment on April 22, 2010. A Notice of Hearing and Notice of Filing were published and circulated 45 days preceding Board action; this notice was published in the Los Angeles Times and the Ventura County Star on April 26, 2010. Regional Board staff responded to oral and written comments received from the public.

12. On July 8, 2010, prior to the Board's action on this resolution, a public hearing was held to consider adoption of the proposed revision to the freshwater bacteria objectives. Notice of the hearing was published in accordance with the requirements of Cal. Water Code §13244. This notice was published in the Los Angeles Times and Ventura County Star on April 26, 2010.

13. In amending the Basin Plan to update the freshwater bacteria objectives, the Regional Board considered the requirements set forth in sections 13240, 13241 and 13242 of the California Water Code.

14. The Regional Board has considered the factors set forth in Cal. Water Code §13241. Section 13241 at a minimum requires that water quality objectives ensure reasonable protection of beneficial uses.

    (a) Beneficial uses of the fresh, inland surface waters of the Los Angeles Region are contained in Table 2-1 of the Water Quality Control Plan for the Coastal Watersheds of Los

Angeles and Ventura Counties (Basin Plan). These uses include the designated water contact recreation beneficial uses (REC-1 and LREC-1 uses) of inland surface waters that are protected by the freshwater bacteria objectives being updated.

(b) The environmental characteristics of the inland surface waters in the region are spelled out at length in the Basin Plan and have been considered in developing this amendment. The Los Angeles Region, over which the Regional Board has jurisdiction, includes the coastal drainages between Rincon Point (on the coast of western Ventura County) and the eastern Los Angeles County line, as well as the drainages of five coastal islands (Anacapa, San Nicolas, Santa Barbara, Santa Catalina, and San Clemente). There are ten designated watershed management areas (WMAs) in the Los Angeles Region. These generally encompass a single large watershed within which exist smaller subwatersheds. However, in some cases, the WMA may be a collection of several small, discrete coastal watersheds. The watershed management areas for the Los Angeles Region are listed below:

- Calleguas Creek Watershed
- Channel Islands Watershed Management Area (WMA)
- Dominguez Channel Watershed
- Los Angeles River Watershed
- Los Cerritos Channel Watershed
- Ventura Coastal Streams (WMA)
- San Gabriel River Watershed
- Santa Clara River Watershed
- Santa Monica Bay (WMA)
- Ventura River Watershed

The region covers 4,497 square miles of land overall and roughly 6,084 miles of streams, 17,488 acres of lakes, and 65,304 acres of wetlands. Land use varies considerably. In Ventura County, agriculture and open space exist alongside mixed urban, residential and commercial areas. In northern Los Angeles County, open space is steadily being transformed into residential communities. In southern Los Angeles County, land uses include mixed urban, residential, commercial and industrial.

Currently, 124 water bodies are listed as impaired by high levels of indicator bacteria. Each watershed management area of the Los Angeles Region contains some of these impaired water bodies. The impairments will be addressed by TMDLs that are currently in effect or under development. These TMDLs will be implemented through regulatory mechanisms available to the Regional Board, including but not limited to, NPDES permits, including those for urban runoff and stormwater discharges, waste discharge requirements (WDRs), prohibitions, conditional waivers, enforcement actions or other Regional Board orders. The proposed action will not impose any further requirements in these Regional Board actions.

(c) Water quality conditions that reasonably could be achieved through the coordinated control of all factors which affect water quality in the area have been considered. Implementation of the TMDLs currently in effect and of those under development will ensure that the Los Angeles Region's inland surface waters attain the REC-1 and LREC-1 water quality standards for indicator bacteria. Attainment of the REC-1 and LREC-1 water quality standards through the compliance options identified in the bacteria TMDLs is a reasonably achievable water quality condition for the region. However, to the extent that there would be

any conflict between the consideration of the factor in Water Code § 13241, subdivision (c), and the Federal Clean Water Act, the Clean Water Act would prevail.

(d) Economic considerations were considered. The removal of the fecal coliform objectives for freshwater from the Basin Plan will result in a removal of the associated monitoring and reporting requirements from Regional Board orders, and should therefore result in reduced bacteria water quality monitoring costs to the regulated community.

(e) The need for housing within the region has been considered, but the update to the freshwater bacteria objectives will have no impact on the development of housing in the Los Angeles Region. There are no implementation or compliance requirements associated with this action, and therefore no avenue through which any restrictions to housing development or supply may be imposed.

(f) The update to the freshwater bacteria objectives will have no impact on the need to develop and use recycled water in the Los Angeles Region. Removal of the fecal coliform objective will not impose any requirements for or restrictions on the development and use of recycled water.

15. Under Cal. Water Code § 13242, when adopting water quality objectives in the Basin Plan, a program of implementation for achieving the objectives must be included. This specific action involves the removal of a redundant water quality objective; therefore, a new program of implementation pursuant to § 13242 is not required.

16. Pursuant to Public Resources Code § 21080.5, the Resources Agency has approved the Regional Boards' basin planning process as a "certified regulatory program" that adequately satisfies the California Environmental Quality Act (CEQA) (Public Resources Code, § 21000 et seq.) requirements for preparing environmental documents (14 Cal. Code Regs. § 15251(g); 23 Cal. Code Regs. § 3782). The Regional Board staff has prepared "substitute environmental documents" for this project that contain the required environmental documentation under the State Board's CEQA regulations. (23 Cal. Code Regs. § 3777.) The substitute environmental documents include the staff report, the environmental checklist, the comments and responses to comments, the basin plan amendment language, and this resolution. The amendment is the removal of a redundant objective, which will have no impact on public health risk, while removing an unnecessary regulatory and monitoring requirement for regulated entities. The CEQA checklist and other portions of the substitute environmental documents contain findings related to impacts.

17. The amendment is consistent with the State Antidegradation Policy (State Board Resolution No. 68-16), and the Federal Antidegradation Policy (40 CFR 131.12), in that it does not allow degradation of water quality and ensures that beneficial uses are fully protected.

18. Considering the record as a whole, this Basin Plan amendment will result in no effect, either individually or cumulatively, on wildlife resources.

19. The regulatory action meets the "Necessity" standard of the Administrative Procedures Act, Government Code, § 11353, subdivision (b). Federal law and regulations require that states adopt water quality criteria that protect designated beneficial uses (40 CFR 131.11(a)(1)). Federal regulation further requires that states, in establishing criteria, establish numerical values based on Federal Clean Water Act §304(a) Guidance (40 CFR 131.11(b)(1)). The revised bacteria objectives for freshwaters are based on U.S. EPA's current guidance on

ambient water quality criteria for bacteria published pursuant to the requirements of Federal Clean Water Act §304(a) (U.S. EPA, 1986).

20. The Basin Plan amendment updating the freshwater bacteria objectives for the Los Angeles region must be submitted for review and approval by the State Board, OAL, and U.S. EPA. The Basin Plan amendment will become effective upon approval by OAL and U.S. EPA. A Notice of Decision will be filed following these approvals.

21. If during the State Board's approval process Regional Board staff, the State Board or State Board staff, or OAL determine that minor, non-substantive modifications to the language of the amendment are needed for clarity or consistency, the Executive Officer should make such changes consistent with the Regional Board's intent in adopting this TMDL, and should inform the Board of any such changes.

THEREFORE, be it resolved that:

1. Pursuant to § 13240 of the California Water Code, the Regional Board, after considering the entire record, including oral testimony at the hearing, hereby adopts the amendments to Chapter 3 of the Water Quality Control Plan for the Los Angeles Region to update the bacteria objectives for freshwaters designated as REC-1 and LREC-1 by removing the fecal coliform objectives, as set forth in Attachment A hereto.

2. The Regional Board is taking this action pursuant to Resolution No. R10-001 (Resolved Clause 1(a)), in which the Board identified the re-evaluation of how bacteria water quality objectives should be applied in compliance determination as a basin planning priority to be addressed during the 2008-2010 Triennial Review. Removal of the fecal coliform objectives for freshwaters designated for water contact recreation is one aspect of the re-evaluation.

3. The Regional Board hereby approves and adopts the CEQA substitute environmental documentation, which was prepared in accordance with Public Resources Code § 21159 and California Code of Regulations, title 14, § 15187, and directs the Executive Officer to sign the environmental checklist.

4. The Executive Officer is directed to forward copies of the Basin Plan amendment to the State Board in accordance with the requirements of § 13245 of the California Water Code.

5. The Regional Board requests that the State Board approve the Basin Plan amendment in accordance with the requirements of sections 13245 and 13246 of the California Water Code and forward it to OAL and the USEPA.

6. If during the State Board's approval process, Regional Board staff, the State Board or State Board staff, or OAL determine that minor, non-substantive modifications to the language of the amendment are needed for clarity or consistency, the Executive Officer may make such changes, and shall inform the Board of any such changes.

7. The Executive Officer is authorized to request a "No Effect Determination" from the Department of Fish and Game, or transmit payment of the applicable fee as may be required to the Department of Fish and Game.

I, Samuel Unger, Interim Executive Officer, do hereby certify that the foregoing is a full, true, and correct copy of a resolution adopted by the California Regional Water Quality Control Board, Los Angeles Region, on July 8, 2010.

Samuel Unger, PE
Interim Executive Officer

ATTACHMENT A to Resolution No. R10-005

Amendment to the *Water Quality Control Plan for the Los Angeles Region* to Update the Bacteria Objectives for Freshwaters Designated for Water Contact Recreation by Removing the Fecal Coliform Objective

Amendments:

Delete specific language from the current freshwater bacteria objectives contained in Attachment A to Resolution No. 2001-018, as indicated below:

In Fresh Waters Designated for Water Contact Recreation (REC-1)

1. Geometric Mean Limits
~~a.~~ E. coli density shall not exceed 126/100 ml.
~~b. Fecal coliform density shall not exceed 200/100 ml.~~

2. Single Sample Limits
~~a.~~ E. coli density shall not exceed 235/100 ml.
~~b. Fecal coliform density shall not exceed 400/100 ml.~~

In Fresh Waters Designated for Limited Contact Recreation (LREC-1)

1 Geometric Mean Limits
~~a.~~ E. coli density shall not exceed 126/100 ml.
~~b. Fecal coliform density shall not exceed 200/100 ml.~~

2 Single Sample Limits
~~a.~~ E. coli density shall not exceed 576/100ml.
~~b. Fecal coliform limits shall not apply.~~

State of California
California Regional Water Quality Control Board, Los Angeles Region

RESOLUTION NO. 01-018
October 25, 2001

Amendment to the *Water Quality Control Plan for the Los Angeles Region* to Update the Bacteria Objectives for Water Bodies Designated for Water Contact Recreation

WHEREAS, the California Regional Water Quality Control Board, Los Angeles Region, finds that:

1. The Federal Clean Water Act (CWA) requires the California Regional Water Quality Control Board (Regional Board) to develop water quality objectives which are sufficient to protect beneficial uses designated for each water body found within its region.

2. The proposed amendment to the Water Quality Control Plan for the Los Angeles Region (Basin Plan) was developed in accordance with section 13241 of the Porter-Cologne Water Quality Control Act (California Water Code, Division 1, Chapter 4, Article 3).

3. The current Basin Plan contains total and fecal coliform bacteria objectives to protect waters designated for water contact recreation based on recommendations made by the U.S. EPA in 1976.

4. The amendment proposed for adoption into the Basin Plan will update the current bacteria objectives for waters designated for water contact recreation to include objectives for enterococcus, the ratio of fecal-to-total coliforms, and e. coli in addition to objectives for total and fecal coliform.

5. The amendment will revise Chapter 3 "Water Quality Objectives" of the Basin Plan.

6. The proposed amendment is based on more recent epidemiological studies and research on the most appropriate bacterial indicators.

7. Specifically, in 1983 and 1984, additional epidemiological studies were conducted by the U.S. EPA to determine the most appropriate bacterial indicators and corresponding objectives for waters designated for water contact recreation.

8. Based on these epidemiological studies, in 1986 the U.S. EPA revised its recommended bacteria criteria for waters designated for water contact recreation to include enterococcus for marine waters and enterococcus or e. coli for fresh waters.

9. In 1995, the Santa Monica Bay Restoration Project sponsored a local epidemiological study to determine the most appropriate bacterial indicators and corresponding objectives for marine waters designated for water contact recreation.

10. Based on the Santa Monica Bay epidemiological study and other national studies, the California State Legislature passed a law (Assembly Bill 411 (1997)) requiring the California Department of Health Services (Department) to establish minimum protective bacterial standards for waters adjacent to beaches, which include standards for total coliform, fecal

coliform, and enterococci bacteria, or for other microbiological indicators that the Department determines are appropriate.

11. The Department adopted regulations in 1999 that establish minimum protective bacterial standards for waters adjacent to beaches, including objectives for total coliform, fecal coliform and enterococcus as well as an objective for the ratio of fecal-to-total coliforms.

12. In March 1999, the U.S. EPA made a commitment in its *Action Plan for Beaches and Recreational Waters* that "where a State does not amend its water quality standards to include the 1986 criteria, EPA will act under Section 303(c) of the Clean Water Act to promulgate the criteria with the goal of assuring that the 1986 criteria apply in all states no later than 2003."

13. The U.S. EPA's 1986 bacteria criteria and the bacteria standards contained in the California Code of Regulations, title 17, section 7958 represent the best science available.

14. The Regional Board has considered the costs of implementing the amendment, and finds these costs to be a reasonable burden relative to the environmental benefits.

15. The proposed amendment results in no potential for adverse effect, either individually or cumulatively, on wildlife.

16. The regulatory action proposed meets the "Necessity" standard of the Administrative Procedures Act, Government Code, section 11353, subdivision (b).

17. The amendment is consistent with the State Antidegradation Policy (State Water Resources Control Board (SWRCB) Resolution No. 68-16), in that the changes to water quality objectives (i) consider maximum benefits to the people of the state, (ii) will not unreasonably affect present and anticipated beneficial use of waters, and (iii) will not result in water quality less than that prescribed in policies. Likewise, the amendment is consistent with the federal Antidegradation Policy (40 CFR 131.12).

18. The basin planning process has been certified as 'functionally equivalent' to the California Environmental Quality Act requirements for preparing environmental documents and is, therefore, exempt from those requirements (Public Resources Code, Section 21000 et seq.).

19. Regional Board staff has prepared a staff report dated July 31, 2001, describing the proposed amendment, and sent the staff report to all known interested persons to allow a 45-day public comment period in advance of the public hearing.

20. The Regional Board held a public hearing on October 25, 2001, for the purpose of receiving testimony on the proposed Basin Plan amendment. Notice of the public hearing was sent to all interested persons and published in accordance with California Water Code, section 13244.

21. The Basin Plan amendment must be submitted for review and approval by the SWRCB, Office of Administrative Law (OAL), and U.S. EPA. Once approved by the SWRCB, the amendment is submitted to OAL and U.S. EPA. The Basin Plan amendment will become effective upon approval by OAL and U.S. EPA. A Notice of Decision will be filed.

THEREFORE, be it resolved that

1. Pursuant to sections 13240 and 13241 of the California Water Code, the Regional Board, after considering the entire record, including oral testimony at the hearing, hereby adopts the amendment to the Water Quality Control Plan for the Los Angeles Region as set forth in the attachment.

2. The Executive Officer is directed to forward copies of the Basin Plan amendment to the SWRCB in accordance with the requirements of section 13245 of the California Water Code.

3. The Regional Board requests that the SWRCB approve the Basin Plan amendment in accordance with the requirements of sections 13245 and 13246 of the California Water Code and forward it to OAL and the U.S. EPA.

4. If during its approval process the SWRCB or OAL determines that minor, non-substantive corrections to the language of the amendment are needed for clarity or consistency, the Executive Officer may make such changes, and shall inform the Board of any such changes.

5. The Executive Officer is authorized to sign a Certificate of Fee Exemption.

I, Dennis A. Dickerson, Executive Officer, do hereby certify that the foregoing is a full, true, and correct copy of a resolution adopted by the California Regional Water Quality Control Board, Los Angeles Region, on October 25, 2001.

Dennis A. Dickerson
Executive Officer

October 25, 2001
Date